A STUDY IN RABBITS

OF

IMMUNIZATION TO RED CELL ALLOANTIGENS

AND ITS SUPPRESSION

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ABSTRACT

The main objective of the work contained in this thesis was to investigate in rabbits several aspects of the immune response to allogeneic red cells. Standard serological methods and the survival curves of allogeneic red cells injected i.v. have been used to assess the immune response, and this combined approach has enabled several different categories of response to be recognised. For example, it was found that 14% of rabbits given repeated injections of allogeneic red cells alone failed to produce serologically detectable antibody; in some of these "non-responders" the injected cells survived normally, whereas in others, various patterns of impaired red cell survival were observed. The effect of giving specific IgG antibody with the first injection of red cells was to impair to varying degrees the immune response to that injection; the degree of impairment was dependent upon the amount of antibody given, and over the range of antibody doses studied, no evidence for complete suppression of primary immunization was obtained. By contrast, the i.v. injection of allogeneic red cells treated with N-ethylmaleimide (NEM) induced prolonged immunological unresponsiveness to subsequent injections of untreated cells in 70% of the recipients; NEM-treated red cells were removed rapidly from the circulation and a large proportion accumulated in the bone marrow. The unresponsive state in the small number of rabbits given untreated cells and in the larger number given NEM-treated cells could be terminated by i.m. injections of cells given with FCA.

Results of some preliminary rabbit experiments undertaken to determine physiological normal values essential for the planning and interpretation of the main experiments are also presented; of particular

interest are the data obtained for autologous red cell survival and IgG metabolism. In a small subsidiary study, the *in vitro* and *in vivo* properties of some red cell alloantibodies produced during the course of this work were investigated.

INDEX

Abstract

1. INTRODUCTION

General background 1

Immunization to red cell alloantigens 7

Rabbit blood groups 38

Factors regulating the immune response 44

Antibody-mediated suppression of primary immunization 58

2. METHODS 69

3. SOME OBSERVATIONS ON THE HAEMATOLOGY AND PHYSIOLOGY OF NORMAL SANDY LOP RABBITS

Peripheral blood counts 89

Red cell osmotic fragility 100

Blood volumes 105

Intravascular mixing times 121

The rate of haemodilution following acute blood loss 125

IgG metabolism - kinetic studies 142

- serum IgG levels 172

Survival of autologous red cells - pilot studies with diisopropyl phosphofluoridate ($DF^{32}P$) 176

- studies to determine true red cell survival 184

4. IMMUNIZATION TO RED CELL ALLOANTIGENS IN SANDY LOP RABBITS

The response to red cells given alone 207

The effect of passively-administered IgG antibody on primary immunization 243

The response to red cells treated with N-ethylmaleimide (NEM) 271

5. SOME ADDITIONAL OBSERVATIONS IN SANDY LOP RABBITS ON THE EFFECTS OF ALLOANTIBODIES ON RED CELL SURVIVAL

Effects of naturally-occurring antibodies 296

Experiments with IgG antibodies lytic *in vitro* 303

Experiments with non-complement-binding IgM antibody 315

6. GENERAL CONCLUSIONS

The Rh and Hg blood group systems 331

"Collapse" curves and immunization 333

The effect of NEM-treated red cells and the non-responder 334

Further work 335

REFERENCES 338

PUBLICATIONS

INTRODUCTION

GENERAL BACKGROUND

The discovery of the Rhesus (Rh) blood group system (Landsteiner and Wiener, 1940) at a time when the practice of blood transfusion was expanding rapidly, was clearly of great importance in that it provided a possible explanation for a number of clinical and serological problems which were then being encountered with increasing frequency. Transfusion reactions which could not be explained in terms of ABO incompatibility had been reported by several authors (see review by Wiener and Peters, 1940), and in some cases, immunization to Rh was subsequently shown to have been responsible. The hypotheses which were then being advanced to explain the pathogenesis of erythroblastosis foetalis implied the existence of a blood group system other than ABO, MN or P (Darrow, 1938; Levine and Stetson, 1939), and it was not long before the central role of Rh alloimmunization in this condition was established beyond all reasonable doubt (Levine, Burnham, Katzin and Vogel, 1941).

The discovery was also important in stimulating efforts to improve serological tests for the detection of Rh incompatibility; work in this area led to the rediscovery of the antiglobulin test (Coombs, Mourant and Race, 1945) and to the development of other sensitive tests using albumin (Diamond and Denton, 1945) and enzyme-treated cells (Morton and Pickles, 1947) for the detection of incomplete antibodies. It also raised a number of fundamental questions about the pathogenesis of antibody-mediated red cell destruction in vivo. Wiener (1942) recognised that haemolytic transfusion reactions could occur in the absence of significant

intravascular haemolysis; in particular, he demonstrated by means of the differential agglutination technique (Ashby, 1919) that in such cases, donor red cells may be eliminated from the recipient's circulation within a short time of transfusion. The haemolytic nature of these transfusion reactions was thus firmly established, but the mechanisms involved in destroying the donor cells had still to be explained.

In 1950, Gray and Sterling described the use of ^{51}Cr for the determination of red cell survival in vivo. This method of labelling red cells may be used to study the survival of large or small volumes of autologous or allogeneic cells; moreover, in certain circumstances, the organs in which the cells are destroyed may be identified in living subjects by the use of surface-counting techniques. ^{51}Cr-labelled red cells have been used to study alloimmunization in man in several different types of experiments. For example, the response to primary immunization has been investigated by observing the survival of labelled allogeneic cells injected for the first time into unimmunized subjects; in many cases, after a variable period of time during which the cells survive normally, the survival curve "collapses" (Mollison, 1961) and the cells are removed from the circulation at an accelerated rate before antibody is detectable serologically. In other experiments, attempts have been made to correlate the serological characteristics of red cell alloantibodies with their effects on the pattern of red cell destruction in vivo, and also with the

mechanisms by which the sensitized red cells are removed from the circulation. To this end, the survival of small volumes of labelled allogeneic cells have been followed in subjects who have previously been immunized to a particular antigen on those cells; alternatively, unimmunized subjects have been passively immunized with a purified antibody preparation prior to injection with labelled cells; or again, cells coated with antibody in vitro have been injected.

Experiments of this kind have defined many of the characteristics of antibody-mediated red cell destruction in vivo, but many other aspects of immunization to red cell alloantigens in man have yet to be explained. For example, it is not known what factors determine the overall immunogenicity of an antigen, nor is there any satisfactory explanation for the fact that antibodies of a given blood group specificity tend to be of a particular immunoglobulin class and to have fairly predictable serological characteristics; the structure of the antigenic determinants and their carriers are doubtless of great importance, but in the case of most red cell antigens, very little information is available on these points. The significance of "collapse" curves is far from clear, particularly in those cases in which serologically detectable antibody is not subsequently found, or in which further injections of the same donor cells survive normally; it is possible that factors determining these partial responses may also be important in determining the failure of certain individuals to manifest any detectable response to the i.v. injection of allogeneic red cells of a particular type.

The value of passively-administered antibody in preventing primary alloimmunization has been firmly established in the case of Rh, but the mechanism by which this is achieved is not known. As already indicated, these problems have been investigated by means of observations made in the course of clinical practice and also by means of experiments in volunteers; in addition, some experiments in laboratory animals, using xenogeneic antigen-antibody systems, have provided plausible explanations for several of the findings in man (Brown, Lachmann & Dacie, 1970; Schreiber & Frank, 1972 a & b).

The rabbit has been recognised for many years as a useful animal in which to study immunization to red cell alloantigens (see, e.g. Levine and Landsteiner, 1929). Largely as a result of the work of Carl Cohen (see, e.g. Cohen, 1969) the characteristics of several blood group systems in the rabbit have now been well defined. One system in particular, Hg in Cohen's terminology (see later), has been found to be especially suitable for the investigation of immune responses to red cell alloantigens, and has been used extensively in the present work. In the main part of the thesis, the immune response of the rabbit to repeated injections of allogeneic red cells has been assessed by standard serological tests and also in terms of the survival curves of the injected cells. This combined approach has revealed a wider range of responses; it has indicated, for example, that those rabbits which fail to produce serologically-detectable antibody may be further

subdivided according to the characteristics of the survival curves of the injected cells. The "normal" patterns of response having been established, the effects of injecting allogeneic red cells under various other conditions have been investigated; in particular, suppression of primary immunization by passively-administered antibody has been studied, as well as the induction of tolerance by injecting red cells treated with N-ethylmaleimide. In a small number of additional experiments, the effects in vivo of several red cell alloantibodies with different serological characteristics have been investigated. In order to interpret the results of the main experiments, it was necessary to establish a number of haematological and physiological "normal" values in these rabbits; investigations of this kind resulted in some additional interesting observations and are reported in a separate section.

Before proceeding to the experimental part of this thesis, an account will be given of some relevant work in man and animals. Four topics will be discussed:

1. Immunization to red cell alloantigens.
2. Rabbit blood groups.
3. Factors regulating the immune response.
4. Antibody-mediated suppression of primary immunization.

INTRODUCTION

IMMUNIZATION TO RED CELL ALLOANTIGENS

Optimal dose of red cells for immunization

Timing of the immune responses

"Collapse" curves

(i) "Collapse" followed by the appearance of serologically-detectable antibody

(ii) "Collapse", but no detectable antibody

(iii) "Collapse" followed by normal survival

(iv) Normal survival of repeated injections

Serological characteristics of red cell alloantibodies

Biological effects of the interaction between red cells and antibodies

(i) Complement activation - observations in vitro

(ii) Consequences of complement activation in vivo

(a) Intravascular lysis

(b) Extravascular destruction

(iii) The effect in vivo of antibodies which do not bind complement

(iv) Interactions between sensitized red cells and the mononuclear phagocyte system (MPS).

Optimal dose of red cells for immunization

Most of the experiments in man have involved the i.v. administration of D-positive red cells to D-negative individuals and immunization has been assessed serologically. The minimum dose of cells required to induce primary immunization has not been definitely established. Jacobowicz, Williams and Silberman (1972) gave repeated i.v. injections of 0.01 ml R_2r blood to previously untransfused D-negative volunteers every two weeks; two male subjects developed anti-D, one after 6 and one after 10 injections, indicating that about 0.03 and 0.05 ml cells respectively had been a sufficient stimulus for primary immunization. In this series, 5 out of 8 subjects had produced antibody at three months, having received doses of red cells varying between 0.03 and 0.11 ml; the remaining 3 individuals were thought to be "non-responders" (see later) .

It has been claimed that, following a single i.v. injection of allogeneic red cells, the proportion of recipients responding is lower, the smaller the volume of red cells injected. For example, Pollack, Ascari, Crispen, O'Connor and Ho (1971) found anti-D in 80% of D-negative subjects 6 months after transfusion with 1 unit (500 ml) R_1r blood; following 40 ml D-positive cells, Davey, Campbell and James (1969) found anti-D in 33% of cases; and in a series in which only 1 ml R_1r red cells was injected, Mollison, Hughes-Jones, Lindsay and Wessely (1969) found anti-D in 14% of cases. On the other hand, it has been pointed out that observations such as these are not strictly

comparable in that red cells of different phenotypes had in some cases been used for injection and the responses had been assessed using serological tests of different sensitivities (Gunson, Stratton, Cooper and Rawlinson, 1970). These latter authors found anti-D in 4 out of 5 volunteers given 0.5 ml R_2 R_2 red cells and in 5 out of 6 given 5 ml of the same cells. With regard to the question of phenotype of the cells used for immunization, it is interesting to note that in a later series from Mollison's laboratory (Samson and Mollison, 1975) R_1 R_2 cells were used in experiments otherwise similar to the ones quoted above and on this occasion a larger proportion of subjects (42%) produced anti-D after the first injection of cells; however in these two series only 11 subjects were given R_1r cells and 12 subjects R_1 R_2 cells so that the difference in response is not statistically significant (p=0.091, Fisher's exact method). Mollison's two series clearly illustrate the point raised by Gunson et al. (1970) about the sensitivity of the tests used for detecting immunization; in the earlier experiments, the survival of a second injection of cells was subnormal at 11 days in an additional 6 (out of 14) subjects in whom antibody was not detectable serologically, suggesting that the overall response to the first injection may have been as high as 8 out of 14, or 57%; by similar reasoning, in the second series, a response to the first injection may have occurred in 9 out of 12 cases (75%).

In accordance with observations made in experimental animals, there is evidence to suggest that in man, an optimal secondary immune response to red cell alloantigens may be induced by a dose of cells

smaller than that required to elicit primary immunization, although there is little quantitative data on this point. In pregnancy, it is widely recognised that in the majority of cases, the volume of cells gaining access to the maternal circulation as a result of transplacental haemorrhage (TPH) is insufficient, except at the time of delivery, to cause primary immunization; on the other hand, in a woman already immunized, it is usual for the titres of allo-antibody to rise during the course of pregnancy. Observations relating to the re-stimulation of D-negative volunteers for the production of anti-D immunoglobulin indicate that a single injection of 0.2 ml R_1r red cells i.v. is sufficient to produce a maximum increase in antibody titre in the majority of immunized subjects (Holburn, Cleghorn and Hughes-Jones, 1970).

Timing of the immune response

In attempting to assess the time taken for an individual to manifest a primary immune response to red cell alloantigens, account must once again be taken of the tests used to detect immunization. For example, in the case of Rh immunization, it has been shown that antibody responses or impaired red cell survival suggestive of a secondary response may sometimes be seen following a second or subsequent injection of D positive red cells when no serolocially detectable antibody was found previously (Mollison et al., 1969; Samson and Mollison, 1975). Consequently, while it may be true to say that readily detectable antibody is usually found only after several weeks or months have elapsed following the first immunizing dose of cells, it is possible that in many cases evidence of immunization

would be obtained earlier by the use of more sensitive tests. In the series of Pollack et al. (1971) in which 22 D-negative volunteers were transfused with 500 ml R_1r blood, anti-D was found in the serum of 9 subjects at 2 months, in another 7 at 3 months, in 1 at 4 months and in 1 more at 5 months. Following the injection of smaller volumes of R_2 R_2 cells, essentially similar results were obtained in the small series reported by Gunson et al. (1970); in one subject injected with 5 ml cells anti-D was first detected at 37 days; in another 4 subjects given 5 ml cells and in 4 others given 0.5 ml cells, antibody was first detected between 63 and 130 days following injection; in all of the subjects maximum titres of anti-D were attained 2-3 weeks after the antibody was first detected. If "collapse" curves are interpreted as evidence of an immune response, then in the series of Samson and Mollison (1975) referred to above, immunization had occurred by 14 days in 1 subject, by 28 days in 4 subjects and by 42 days in another 2 subjects; in all 7 of these subjects antibody was eventually detected serologically (without further stimulation in 5 cases) but there was no correlation between the time of "collapse" and the appearance of antibody in the serum.

"Collapse" curves

In some cases it has been observed that ABO compatible allogeneic red cells injected into previously unstimulated recipients may survive normally for about two weeks and then be removed from the circulation at an increased rate. A curve of this type has been

called a "collapse" curve (Mollison, 1961b). It might reasonably be assumed that accelerated destruction of the injected cells indicates an immune response, and indeed, in many of these cases, serologically detectable antibody may be demonstrated subsequently. In other cases, however, no serological evidence of immunization is found; furthermore, injections of the same cells given at a later date may survive normally. Although "collapse" curves are more frequently seen following the injection of small volumes of red cells, they have also been reported following transfusion with therapeutic volumes of blood. In fact, the phenomenon was first recorded in a study of the survival of stored red cells (Loutit, Mollison and Young, 1943) in which survival was determined using the differential agglutination technique. In 3 cases out of 35, the curves were essentially normal for 7-21 days but thereafter survival was subnormal and all the remaining cells were eliminated by 40-60 days. In one of these cases, a second transfusion of cells from the original donor again showed impaired survival, whereas, in a different recipient, cells from this same donor survived normally (Mollison, 1951); no antibody could be detected serologically.

Following a first injection of a small volume of ABO compatible allogeneic red cells labelled with ^{51}Cr, four patterns of response have been recorded:

(i) <u>"Collapse" followed by the appearance of serologically-detectable antibody.</u> The timing of the antibody response in relation to "collapse" has been very variable, and in some cases, antibody has been found only after further injections of red cells

have been given (see, for example, Samson and Mollison, 1975). For the sake of completeness, it should be mentioned that normal survival of a first injection of cells, followed some time after their complete elimination, by the appearance of antibody in the serum, may be considered as a variant of this response; in these cases subsequent injections of cells are usually cleared rapidly.

Following the injection of ABO compatible, D compatible cells into 15 volunteers, Adner and Sjolin (1957) found two examples of this pattern of response; one subject produced anti-E 8 weeks after the first injection, and the other produced anti-Fy^a 12 days after the second injection. In a paper containing additional, similar experiments (Adner, Foconi and Sjolin, 1963), this pattern of response was also seen in 2 out of 14 K-negative subjects given K-positive cells; one formed anti-K only after a second injection of cells, the first injection having survived normally; the other subject formed anti-E, again after two injections, both of which had shown impaired survival. Attempts at deliberate immunization with the D antigen presumably result in a similar pattern in many cases, although there is little published work directly relating to this point. In one recent series (Samson and Mollison, 1975), 8 out of 12 subjects injected with 1 ml D-positive cells responded as follows: in 3 cases antibody was found after "collapse" of the first survival curve, in 2 others antibody appeared after the first injection had survived normally (at least to 42 days), and in each of the remaining 3 cases, two injections of cells showed impaired survival before antibody could be detected. An additional, special case, in which antibody was detected transiently, will be described later.

(ii) "Collapse", but no detectable antibody. This group comprises those cases in which survival of a first injection of cells "collapses" and subsequent injections show severely curtailed survival without antibody being detectable at any time. Clearly, a total inability to respond by the production of antibody can only be presumed; many examples are on record of antibody being undetectable following decreased survival of 1, 2 or sometimes 3 consecutive injections of cells (Adner and Sjolin, 1957; Brown and Smith, 1958; Adner et al., 1963), but it is open to speculation whether antibody would not eventually have been found following further injections. In this category might be included certain cases of haemolysis associated with blood transfusions in which no antibody could be demonstrated (e.g. Jandl and Greenberg, 1957; Stewart and Mollison, 1959; Mollison, 1962).

(iii) "Collapse", followed by normal survival. A very remarkable observation in many of the series already quoted is the occurrence of normal survival of allogeneic red cells in certain subjects in whom previous injections of cells from the same donor had been cleared rapidly. In general, the first injection "collapses" as already described; subsequent injections of the same cells may show some increase in the rate of destruction initially or may survive entirely normally (Adner and Sjolin, 1957); alternatively, there may be gradual decrease in the rate of destruction of a series of injections (Goudsmit and Krijnen, 1957; Adner et al., 1963). With one exception, antibody has not been detectable serologically in these cases.

Three special cases will be mentioned. In experiments in which 12 D-negative volunteers were given 1 ml D-positive red cells at the same time as a small dose of IgM anti-D, the rate of destruction injected cells was initially increased in all but 1 subject but then became normal (as expected - see later); in 5 cases the curves then "collapsed", but in 2 of these cases, a second injection of cells showed only slightly increased destruction, and a third injection survived normally (Holburn, Frame, Hughes-Jones and Mollison, 1971). Because of the small number involved, it is clearly impossible to draw any firm conclusions from these observations but it is interesting to speculate whether the passively-administered IgM antibody might not have played a part in inducing this type of response in these two cases. The other special case was described by Samson and Mollison (1975). The first injection of D-positive red cells in a D-negative recipient showed slightly subnormal survival at 42 days; following a second injection, survival was subnormal by 21 days and by 35 days, when only 5% ^{51}Cr activity remained, anti-D was found transiently; a third injection of cells survived normally and no antibody was subsequently detectable. The antibody, which had been found on 3 occasions over a period of 2 weeks, was present in low concentration and appeared to be IgG.

(iv) Normal survival of repeated injections. In a number of cases, neither antibody production nor impaired red cell survival can be detected following several injections of allogeneic cells. In the series of K-negative receipients given 2 or 3 injections of K-positive blood (Adner et al., 1963), 12 subjects did not form antibody and 9 of these had completely normal survival curves; in 2 of the remaining

3 cases, survival of subsequent injections of cells improved. Following repeated injections of D-positive cells into 34 D-negative subjects (Mollison, Frame and Ross, 1970), 9 failed to make antibody; in 8 of these, normal survival curves were seen with the first injection of cells and on all other occasions on which labelled cells were given (some of the intervening injections were of unlabelled cells); these subjects received from 2 to 6 injections. Again in relation to immunization to the D antigen (Samson and Mollison, 1975), 3 out of 12 control subjects failed to make anti-D following 2 injections of cells, and in all 3 cases survival of the injected cells was normal on both occasions. It would seem therefore, that whereas impaired survial of allogeneic cells might occur transiently, or in some cases repeatedly, in the absence of detectable antibody, on the contrary, alloantibody has not been found in association with repeatedly normal red cell survival in any of these experiments. However, some examples are known of alloantibodies which are demonstrable in vitro, yet which do not seem to cause accelerated destruction of the corresponding red cells in vivo; these include anti-Xg^a (Sausais, Krevens and Townes, 1964), anti-Yt^a (Dobbs, Prutting, Adebahr, Allen and Alter, 1968), and possibly anti-Chido (personal observations). Whether this phenomenon is dependent upon the properties of the antibody, the antigen or both is not known.

Serological characteristics of red cell alloantibodies

The fundamental differences between the effects observed with red cell antibodies of different specificities reside first in the properties of the particular immunoglobulin class or sub-class to which the antibody belongs; secondly, in the kinetics of the antigen-

antibody reaction; and thirdly, in the ability of the red cell-antibody combination to produce secondary reactions, such as the activation of complement. Some of the characteristics of antibodies and their effects on red cells may be determined by serological tests; it may thus be possible to identify the immunoglobulin class to which the antibody belongs, to define the conditions under which it combines with red cells, and to detect the consequences of the combination, e.g. red cell agglutination, fixation of complement components, red cell lysis. This information may then be correlated with the biological effects observed in various circumstances. In this section, the serological characteristics of some human red cell alloantibodies will be described.

In general, antibodies to human red cells are predominantly IgG or IgM. IgA blood group antibodies are not uncommon as naturally-occurring anti-A and anti-B (Adinolfi, Mollison, Polley and Rose, 1966), but are usually present only in low titre and have not been found in the absence of IgM or IgG antibodies of the same specificity (Mollison, 1972 p.237); they occur infrequently as immune antibodies. In those subjects in whom naturally-occurring antibodies (partly or wholly IgM) are found, the injection of red cells or blood group specific substances may produce an increase mainly in the IgM component, e.g. anti-A in group B persons (Abelson and Rawson,1961), anti-Le^a and anti-Le^b in Le(a-b-) persons (Mollison, Polley and Crome, 1963); on the other hand, a substantial rise in the IgG component may predominate, as in the case of group O persons given injections of A or B red cells (Abelson and Rawson, 1961).

By analogy with animal experiments primary immunization to human red cell alloantigens might be expected to result in an initial IgM response of low titre which then decreases in favour of a more prolonged production of IgG antibody. In fact, the dura tion and intensity of the IgM response in man varies considerably and is frequently not detectable. Mollison (1972 p.280) has suggested that the failure to detect IgM may, in some cases at least, be due to the insensitivity of the tests used to distinguish between IgG and IgM when they are present in low concentrations. In the case of primary Rh immunization in volunteers, the earliest response has been found by most investigators to be partly or wholly IgG; in some series, IgM has also been found in about half of the cases (Gunson et al., 1970), whereas in others, IgA has accompanied IgG before the appearance of IgM (Davey et al., 1969). On the other hand, Jacobowicz et al. (1972) found that the earliest antibody which they could detect using enzyme-treated cells was 2 mercaptoethanol sensitive, suggesting that it was IgM. In the majority of cases however, it is agreed that the IgM is of low titre and may subsequently become undetectable; nevertheless, on restimulation, a large proportion of immunized subjects may again exhibit an IgM response which is transient in some but persistent in others (Holburn et al., 1970).

Tests using enzyme-treated cells have been shown to be more sensitive than other routine methods of detecting both IgM anti-D (Holburn et al., 1971) and IgG anti-D (W.J. Jenkins cited by Mollison, 1972 p.434); such methods clearly do not permit any conclusions to be drawn directly about the class of antibody detected in a serum.

Anti-D, detectable only by enzyme methods, may occasionally be found in the serum of a woman during, or shortly after her first Rh incompatible pregnancy (see, e.g. Woodrow and Donohoe, 1968); positive results using these methods may also be the first serological indication of primary immunization in volunteer experiments (see, e.g. Gunson et al., 1970). In view of the doubts which have been expressed about the class(es) of antibody involved in these tests, some observations of Weiner and Wingham (1966) are of interest; "enzyme-only" anti-D was found in eluates of cord-blood cells from 2 out of 5 infants whose mothers also had serum antibodies detectable only by an enzyme method, thus indicating that these particular antibodies were capable of crossing the placenta.

With only one exception, examples of anti-D, whether IgG or IgM, do not bind complement. The exception was an antibody ("Ripley") which was found to cause lysis of D-positive red cells in vitro (Waller and Lawler, 1962) and which was subsequently shown to be IgG and to bind complement components to the red cell (Harboe, Muller-Eberhard, Fudenberg, Polley and Mollison, 1963).

The subclass of IgG to which an antibody belongs may well be relevant to its biological activity (see p. 21). Unfortunately, reliable antisera for typing IgG subclasses are not generally available, so that information on this topic is limited. It would appear that in the case of anti-D, most examples of this alloantibody are predominantly IgG_1 and IgG_3 (Natvig and Kunkel, 1968; Morrell, Skvarill and Rufener, 1973); indeed, a recent study revealed only IgG_1 and IgG_3 anti-D, whether formed as a result of pregnancy or by immunization of volunteers (Devey and Voak, 1974). However, it has

also been claimed that about 1 in 10 examples contain significant amounts of IgG_4 (Frame, Mollison and Terry, 1970), and in a few cases, IgG_2 has also been identified (Engelfreit, cited by Mollison, 1972, p.281).

Antibodies of several other blood group systems have also been investigated extensively particularly the naturally-occurring and immune antibodies of the ABO and Lewis systems, which provide many examples of IgM and IgG complement-binding antibodies. Other antibodies which bind complement include all examples of anti-Jk^a (mostly IgG, some IgM; Polley, Mollison and Soothill, 1962), some examples of anti-K (usually IgG, some IgM; Polley, 1964), and some of anti-Fy^a (usually IgG; Mollison, 1972, p.327). Like anti-D, Rh antibodies of other specificities do not bind complement, nor do most examples of anti-K and anti-Fy^a. Further reference will be made to the characteristics of some of these antibodies in the next section.

Biological effects of the interaction between red cells and antibodies

With few exceptions, the presence of antibody on the surface of a red cell does not directly induce any major metabolic changes in that cell. Antibody-mediated red cell destruction occurs therefore, as a result of secondary interactions between the altered red cell membrane and its environment. The mechanisms involved in these interactions will now be considered.

(i) Complement activation - observations in vitro. The attachment of certain antibodies to the red cell membrane leads to the activation of complement. If complement activation proceeds to completion, red cell lysis will occur (Muller-Eberhard, 1969), probably as a result of the generation of phospholipase-C activity (Lachmann and Thompson, 1970). On the other hand, the only manifestation of complement activation may be the attachment to the red cell membrane of the early components of the sequence (C3, C4 and their products) detected by specific reagents in the antiglobulin test. The ability of an immunoglobulin molecule to activate complement is a function of its Fc region, and there is evidence to suggest that two or more Fc regions bound to the red cell membrane in close juxtaposition are required (Borsos and Rapp, 1965). Therefore, by virtue of its pentameric structure, a single IgM molecule which has reacted with more than one antigenic determinant may activate complement (Ishizaka, Tada and Ishizaka, 1968) and, in the case of red cell IgM antibodies, a single molecule, thus bound, can cause lysis (Humphrey and Dourmashkin, 1965). By contrast, many IgG molecules must be bound before, by chance, two adjacent antigen sites are occupied with the necessary approximation of two Fc regions (Humphrey and Dourmashkin, 1965).

IgG subclasses differ in their ability to activate complement; IgG_1 and IgG_3 do so readily, whereas IgG_2 is only slightly active and IgG_4 not at all (Ishizaka, Ishizaka, Salmon and Fudenberg, 1967). That this is not the only factor determining the behaviour of an antibody is, however, exemplified by the case of anti-D; all examples examined (except "Ripley") have failed to activate complement in spite

of the fact that most of them are predominantly IgG_1 and IgG_3. It has been suggested that the relative positions of the two Fc regions required for complement activation may be critical, in which case the number and spatial arrangement of antigen sites on the red cell would be important. Again in the case of D, there is evidence which indicates that the antigenic determinants may be situated in crypts on the red cell surface (Pollack, 1965), an arrangement which would impair the appropriate juxtaposition of active Fc regions. Clearly, this cannot be the explanation in the case of certain other alloantibodies (e.g. anti-K, anti-Fy^a, anti-S) in which different examples of a particular specificity may or may not bind complement.

With regard to IgM antibodies, it has been shown in experiments in rabbits that heterogeneity with respect to complement-binding capacity can exist in a single antiserum of a given specifity (Hoyer, Borsos, Rapp and Vanier, 1967), an observation in some ways analagous to that seen with the IgG subclasses. Also, the arrangement of antigenic sites on the red cell membrane has been invoked to explain the failure of IgM anti-D antibodies to bind complement (Pollack, 1965). More recently, Holburn, Cartron, Economidou, Gardner and Hughes-Jones (1971) have presented evidence to suggest that D sites may occur in clusters on the red cell membrane, another (or an additional) way in which steric hinderance of antibody attachment may occur.

Finally, there are several reasons for believing that the reaction between complement and IgG differs from that between complement and IgM. For example, the optimum temperature for the reaction of complement with IgG is 0°C whereas that for the reaction with IgM is 37°C (Colten, Borsos and Rapp, 1967). Some observations *in vivo* emphasising this difference will be discussed later.

(ii) Consequences of complement activation *in vivo*.

Following the activation of complement-binding by red cell antibodies *in vivo*, red cells may either be destroyed in the circulation as a result of complement-mediated lysis, or they may be removed from the circulation (mainly in the liver and spleen) by interacting with cells of the mononuclear phagocyte system (MPS).

(a) *Intravascular lysis.* This type of red cell destruction occurs most frequently with antibodies of the ABO blood group system. Jandl, Jones and Castle (1957) injected ^{51}Cr-labelled group A red cells from 30-40 ml whole blood into each of two group B recipients whose sera contained strongly lytic anti-A. In both cases, there was rapid lysis of the injected cells, with haemoglobinaemia; 90% of the injected radioactivity appeared in the plasma within 60 seconds of injection, and relatively little activity was detected by surface counting over the liver and spleen. By contrast, in two subjects whose sera contained anti-B which caused agglutination but no lysis *in vitro*, the half-disappearance time of group B red cells injected i.v. was about 4 minutes, and the maximum

^{51}Cr activity found in the plasma at any time was equivalent to only 10% of that injected. These authors also compared the level of haemoglobin appearing in the plasma following the i.v. injection into normal subjects of equal amounts of haemoglobin in the form of either haemoglobin solution, ABO incompatible cells or D incompatible cells. A maximum of about 85% of the injected haemoglobin solution was detected in the plasma by 15 minutes, and the haemoglobin was thereafter cleared from the circulation with a $T\frac{1}{2}$ of about 100 minutes. In the case of the ABO incompatible cells, a maximum of 40% of the injected haemoglobin appeared in the plasma within 5-10 minutes, and after a "mixing period" of about 35 minutes, during which the level stayed fairly constant, exponential removal with a $T\frac{1}{2}$ of 90 minutes was seen; the "mixing period" presumably represents a balance between haemoglobin clearance on the one hand, and, on the other hand, further haemoglobin release resulting from the continuing disruption of sequestered red cells. With the D incompatible cells, despite their rapid clearance from the circulation, the amount of haemoglobin appearing in the plasma increased only gradually during the first hour after injection to reach a maximum of 10%, and then very slowly declined. Thus, even with a non-complement-binding antibody, a small amount of haemoglobin appeared in the plasma, presumably as a result of red cell fragmentation during phagocytosis.

Similar results with anti-A and anti-B antibodies were reported by Cutbush and Mollison (1958) following the injection of small volumes of ^{51}Cr-labelled incompatible red cells. In addition, these authors

showed that in the case of anti-A, even when this antibody causes no visible haemolysis *in vitro* and is of low agglutinin titre a substantial proportion of injected A-positive red cells may undergo rapid intravascular lysis.

As mentioned previously a number of cases have also been described in which rapid haemolysis and haemoglobinuria have followed transfusion with "compatible" blood; in the case described by Stewart and Mollison (1959), all available *in vitro* tests, including an indirect antiglobulin test using enzyme-treated donor cells, were negative. The mechanism of haemolysis in these cases has not been satisfactorily explained.

(b) *Extravascular destruction.* It now seems likely that the extravascular destruction of red cells by complement-binding antibodies follows different patterns which are dependent, not only upon quantitative aspects of complement binding, but also upon the class of immunoglobulin involved. First, the mechanism involved in the clearance of complement-coated red cells may be affected to some extent by the presence on the cell surface of the immunoglobulin molecule itself; secondly, the kinetics of complement binding (as mentioned above), and the biological properties of the bound complement appear to differ depending upon whether IgG or IgM antibody initiate the binding process. Some observations on the actions of complement-binding antibodies in man will now be described followed by a discussion of some recent work in animals relating to the mechanisms involved.

Selwyn and Hackett (1949) first observed that red cells coated with IgM cold agglutinin in vitro and injected into normal subjects may take several hours to equilibrate in the circulation before being removed at a normal rate. Jandl, Jones and Castle (1957) and Lewis, Dacie and Szur (1960) found that a proportion of red cells so treated were initially sequestered in the liver, and were then released back into the circulation, apparently undamaged. When a small volume of untreated red cells is injected into a subject whose serum contains a corresponding IgM complement-binding antibody which does not cause intravascular lysis, the pattern of clearance is somewhat different. In the presence of a potent antibody, clearance of the cells may follow a single exponential pattern (e.g. anti-Le^a: Mollison and Cutbush, 1955). Alternatively, a weaker example of an IgM complement-binding antibody may result in a two-component curve, in which initially, a proportion of the injected cells is removed at an accelerated rate, and the remainder are then cleared more slowly (e.g. anti-A_1, anti-Le^a: Mollison and Cutbush, 1955; anti-HI: Mollison, 1959).

The temporary sequestration of red cells treated in vitro with complement-binding IgG antibodies has not been documented; multi-component curves are usually found under these conditions (e.g. anti-Fy^a: Mollison, Crome, Hughes-Jones and Rochna, 1965). A similar pattern of clearance is also often seen when a small volume of unsensitized cells is injected into a subject whose serum contains an antibody with these characteristics (e.g. anti-Fy^a, anti-K: Mollison and Cutbush, 1955; anti-Fy^a: Cutbush and Mollison, 1958).

In other cases, clearance may follow a single exponential pattern (e.g. anti-Jk^a: Cutbush and Mollison, 1958). With this latter example, the importance of the number of antigen sites on the red cell was also demonstrated in that the half-disappearance time for Jk^aJk^a cells was about 4 minutes whereas that for Jk^aJk^b cells was about $18\frac{1}{2}$ minutes; unfortunately, in this case survival of the Jk^aJk^b cells was followed only until about 90% had been cleared, so that it is uncertain whether or not a multi-component curve would have been found.

Some of these observations may be interpreted in the light of recent animal experiments. Brown, Lachmann and Dacie (1970) used a human IgM cold antibody (anti-I) to initiate complement binding to rabbit red cells *in vitro* and *in vivo*. In the normal rabbit, injections of this antibody cause mainly intravascular lysis, although a small degree of extravascular destruction is also seen (Brown, 1970). On the other hand, following the injection of antibody into rabbits congenitally deficient in C6, red cells (presumably in the form EAC1423(5)) were temporarily sequestered in the liver and there was evidence of only a minor degree of intravascular lysis. In these cases it was shown that, shortly after the injection of antibody, red cells could be agglutinated *in vitro* by anti-C3 antiglobulin and also by immunoconglutinin and conglutinin; however, there was a progressive loss of reactivity with anti-C3 so that by 3-5 hours after injection, at a time when the sequestered red cells had returned to the circulation, agglutination

was barely detectable. Using the same cold antibody and serum from C6-deficient rabbits, it was possible to prepare in vitro ^{51}Cr-labelled red cells in the form EAC43(5). Following injection into C6-deficient (and normal) rabbits, these cells were cleared from the circulation with a $T\frac{1}{2}$ of about 4 minutes and a similar pattern of temporary sequestration in the liver was found; furthermore, histological examination of the liver showed, during the phase of sequestration, many red cells adherent to, and partially phagocytosed by, Kupffer cells. It was found that as the number of treated cells injected was increased from 0.4×10^{10} to 5×10^{10}, a smaller proportion of the cells was removed from the circulation, although in absolute terms, the number removed increased up to a maximum of about 2×10^{10}. With maximum sequestration, the majority of the cells had reappeared in the circulation by 2-3 hours, whereas sequestration of smaller numbers of cells was associated with a reduced return to the circulation, until, with the smallest dose (0.4×10^{10}), none had returned by $3\frac{1}{2}$ hours. In contrast to these findings in which complement activation could proceed to C5, the injection of cold antibody into rabbits depleted of C3 and the later components of the complement sequence (by means of cobra venom factor) produced no increase in red cell destruction; similarly, red cells in the form EC4, injected into C3-depleted (and normal) rabbits, survived normally.

These experiments suggest that in the case of this particular IgM antibody, bound C3 is essential for the interaction between red cells and cells of the mononuclear phagocyte system; that the degree

of interaction may vary, depending upon the number of cells involved, from adherence and separation, with only minor membrane changes (large dose), to complete erythrophagocytosis (small dose); that separation of red cells from phagocytes is temporally related to the inactivation of bound C3, possibly by conglutinogen activating factor (Lachmann and Muller-Eberhard, 1968); and that these changes may occur whether or not antibody remains attached to the red cell membrane.

Using a different experimental system, Schreiber and Frank (1972 a and b) have made observations with complement-binding antibodies of both IgM and IgG classes. They have performed experiments in guinea-pigs using rabbit anti-guinea-pig red cell antibodies to determine in vitro quantitative aspects of complement fixation to red cells by these two classes of antibody, and to relate these characteristics to the effects observed on the survival of sensitized red cells in vivo. For IgM, they found that the attachment of 1 antibody molecule to the red cell surface could initiate complement binding, and that when 60 or more such complement-binding sites were present on each red cell, survival in vivo was impaired. Following the injection of such IgM-coated red cells into normal guinea-pigs, impaired survival was associated with rapid sequestration of a proportion of the cells in the liver, but after 1-2 hours, the sequestered cells, still with IgM on their surface, but now resistant to lysis by complement, returned to the circulation and survived normally.

Completely normal survival without temporary sequestration was observed when cells treated with similar amounts of IgM were injected into C3-depleted (cobra venom factor-treated) or C4-deficient (congenital) guinea-pigs, and also if IgM-treated cells were incubated with fresh guinea-pig serum before injection into normal animals. In the case of IgG, some 2000 antibody molecules were required before a single complement-binding site was formed on a red cell, but only 1.4 complement-binding sites (average for population of cells studied) were required before impaired survival in vivo was seen. The survival curve under these circumstances showed at least two components. For example, with 17 IgG complement-fixing sites per cell, about 90% of the red cells were cleared in 2 hours mainly by the spleen, and the survival curve, plotted on semi-log paper, was very slightly curvilinear; most of the remaining cells were cleared over the next 4 days, and the last 1% or 2% appeared to survive normally. In C3-depleted or C4-deficient guinea-pigs, IgG-sensitized red cells were still cleared at an accelerated rate, but not as quickly as in normal animals; e.g. with 17 complement-fixing sites per cell, about 50% of the injected cells were cleared in 2 hours and the remainder survived normally. Similarly, incubation of IgG treated cells with fresh guinea-pig serum before injection into normal animals resulted in some improvement in survival of the cells, although survival was still markedly subnormal.

In these experiments, the results with the IgM antibody agree substantially with those of Brown, Lachmann and Dacie (described above). In addition, they demonstrate that the mechanism of destruction of red

cells by these complement-binding antibodies differs quantitatively and qualitatively, depending upon whether IgG or IgM antibody is involved. A molecular basis for the interaction will be considered later.

(iii) The effect in vivo of antibodies which do not bind complement. Apart from the one exception already mentioned, Rh antibodies, whether IgG or IgM, do not bind complement. Anti-D is the antibody which has been most extensively studied because of its importance in HDN and because it is the most frequently encountered immune alloantibody in man. Following the injection of a small volume of D-positive red cells into a subject whose serum contain IgG anti-D, the red cells are cleared from the circulation exponentially and are destroyed mainly in the spleen (Mollison and Cutbush, 1955; Jandl, Jones and Castle, 1957; Hughes-Jones, Mollison and Veall, 1957). Even with very small amounts of antibody on the red cells, there is some evidence to suggest that an exponential pattern of removal is seen. For example, in the case of a D-negative subject given an i.m. injection of 1 µg anti-D 24 hours before an i.v. injection of 0.3 ml D-positive cells, survival of the injected cells was normal for about 5 days and then they were removed with a half-disappearance time of about 4-5 days (Mollison and Hughes-Jones, 1967). An estimate of the rate at which a small volume of D-positive red cells coated in vitro with different amounts of a particular example of anti-D were removed from the circulation was obtained by Mollison, Crome, Hughes-Jones and Rochna (1965); the survival curves were slightly curvilinear on a semi-log plot (presumably because of the relatively rapid elution of the antibody in vivo) and

the rates of removal of the cells were therefore determined from the initial slopes of the curves. Surface counting was also done. The rate of removal ($T\frac{1}{2}$) varied from more than 24 hours when the amount of antibody was 1.6 µg per ml red cells, to about 20 minutes with about 30 µg antibody per ml cells, and sequestration occurred mainly in the spleen. Amounts of antibody in excess of 40 µg per ml red cells resulted in clearance with a $T\frac{1}{2}$ of about 10 minutes and greatly increased hepatic sequestration. A small number of observations were made using other examples of anti- D and similar initial clearance rates were seen. Little information has been published about the effects in vivo of other IgG non-complement-binding antibodies (such as the other Rh antibodies, and some examples of anti-K, anti-S and anti-Fy^a), but in the case of anti-E (Cutbush and Mollison, 1958) and anti-K (Mollison and Cutbush, 1955; Hughes-Jones, Mollison and Veal, 1957), the available data, albeit limited, is consistent with a similar pattern of destruction.

With regard to IgM antibodies which do not bind complement, the pattern of removal and rate of destruction of incompatible red cells may be related to the amount of antibody on the red cell. For example, in a subject whose serum contained a potent anti-c, a small volume of cc cells was removed from the circulation expoenentially with a $T\frac{1}{2}$ of 2-3 minutes, whereas, cC cells were removed with a $T\frac{1}{2}$ of 6-8 minutes; furthermore, in the case of the cC cells, there was some indication of a decrease in the clearance rate after 95% of the cells had been removed (Cutbush and Mollison, 1958). Two-component curves are

frequently seen following the injection of red cells into subjects whose serum contains weak IgM antibodies. The half-disappearance time of the rapid component may be measured in minutes (e.g. anti-Lu^b: Cutbush and Mollison, 1958) or, at the other extreme, in days (e.g. anti-M: Mollison, 1972, p.478); similarly, the second component may also show markedly impaired survival, or survival may be almost normal in some cases. In experiments in which amounts of purified IgM anti-D varying from 30-150 μg were injected into D-negative volunteers at the same time as 1 ml D-positive red cells, a rapid phase of clearance lasting about 24 hours was seen in 13 out of 14 cases; the rate of destruction then decreased progressively until, by 5-10 days after injection, the rate of destruction was normal (Holburn, Frame, Hughes-Jones and Mollison, 1971).

The red cell survival curves with more than one component which are sometimes seen with complement-binding antibodies are explicable, at least in theory, in terms of the animal experiments already described, i.e. the change in rate of destruction may be related to the inactivation of bound complement. On the other hand, there is as yet no completely satisfactory explanation for the multi-component survival curves which are sometimes seen with IgM antibodies which do not bind complement. Mollison (1972, p.485) considers four possible explanations,

1. Uneven distribution of antigen with a population of red cells.
2. Varying susceptibility to destruction within a red cell population depending, for example, on age.

3. Exhaustion of the supply of antibody.

4. Elution of antigen from red cells following transfusion.

To these might be added,

5. "Inactivation" of bound antibody.

6. Reduction in the numbers of sensitized cells below a critical concentration required for intravascular agglutination.

1 and 2 may be considered together. Evidence for the uneven distribution of the D-antigen on the red cell surface has been mentioned (Holburn, Cartron, Economidou, Gardner and Hughes-Jones, 1971) and it was suggested that some of the D sites were consequently inaccessible to IgM antibody. By an extension of this argument, it is conceivable that part of a population of D-positive cells might have sites almost totally inaccessible to IgM, thereby rendering them resistant to destruction by this antibody (cf. the different rates of destruction of Lu^bLu^b and Lu^aLu^b cells in the presence of anti-Lu^b: Cutbush and Mollison, 1958). Certainly, in the case of the rabbit red cell alloantibody, anti-Hg^A, there is some evidence for heterogeneity of the red cells with regard to destruction by IgM antibody (Burton and Mollison, 1968); in a rabbit injected with IgM anti-Hg^A and HgA positive red cells, at a time when the rapid phase of red cell destruction was over, a second injection of cells alone was destroyed initially at a greater rate than the residual cells from the first injection.

3. In the same rabbit experiments (Burton amd Mollison, 1968), it was shown that after the initial phase of rapid destruction was over, a further injection of IgM anti-Hg^A brought about the rapid clearance of a proportion of the remaining cells. However, in many of the experiments relating to IgM-induced red cell destruction in man, exhaustion of antibody would seem to be an unlikely cause of impaired red cell destruction in view of the small volumes of cells injected.

4. With the exception of the Lewis substances, no significant degree of elution of red cell antigens has been found to occur in man. Even in the case of Lewis, the rate of elution of the antigens is such that this cannot be held a plausible explanation for the pattern of red cell destruction observed with IgM anti-Lewis antibodies (which are in any case complement-binding).

5. This concept is entirely hypothetical and is analagous to the inactivation of bound complement components. However, "inactivated" bound antibody, if it exists, clearly does not prevent the attachment of additional "active" antibody to the red cells (see, e.g. Burton and Mollison, 1968).

6. The agglutination and entrapment of sensitized red cells within the circulation has been postulated as a mechanism for antibody-mediated red cell destruction (Wasastjerna, 1951; Jandl, Jones and Castle, 1957). Alternative explanations now seem more likely in many instances (see next section), but in certain circumstances, this

mechanism remains a possibility (see e.g. Romano and Mollison, 1975). If this mechanism does operate the opportunities for close contact in the circulation would doubtless be limited when only small numbers of cells were involved, and it seems likely that for any given level of sensitization, a minimum number of cells might therefore be required. Consequently, although a sufficient number might be present initially to permit intravascular agglutination, as the agglutinated cells were removed, so the remaining cells would be less likely to come into adequate contact with each other; when this situation prevailed, increasing the amount of circulating antibody might nevertheless lead to stable agglutination under conditions which previously would have been ineffective in this respect.

(iv) Interactions between sensitized red cells and the mononuclear phagocyte system (MPS). Explanations at the cellular level for the mechanisms of antibody-mediated red cell destruction have been put forward on the basis of some recent in vitro observations. Red cells coated with IgG (either as specific anti-red cell antibody, or as IgG molecules bound non-specifically by chromic chloride) will attach to human monocytes, macrophages and some large lymphocytes in tissue culture, and will subsequently sphere and fragment, or be ingested. This phenomenon is inhibited by free IgG and free Fc fragments of IgG, except when the haematocrit of the incubation mixture is greater than 0.7, a condition comparable to that prevailing in the splenic circulation. Attachment of sensitized cells in also inhibited by exposure of the phagocytic cells to sulphydryl blocking agents, such as N-ethylmaleimide (Lo Buglio, Cotran and Jandl, 1967).

Furthermore, it has been shown that in the case of human monocytes, IgM coating alone is ineffective in causing red cell attachment, and that the IgG-mediated process is specifically inhibited by free IgG_1 and IgG_3 subclasses, but not by IgG_2 and IgG_4 (Huber and Fudenberg, 1968). Further experiments by these latter authors (see e.g. Huber and Fudenberg, 1970) indicate that human monocytes and macrophages have surface receptors for C3. Red cells coated with C3 (with or without other complement components and IgM antibody) will bind to the phagocytes but are not ingested; on the other hand, C3 plus IgG on the red cells leads to their adherence and ingestion, even in the presence of free IgG. These observations are entirely consistent with the findings in the animal experiments described above, and also with the data which has been accumulated on antibody-mediated red cell destruction in man. On the other hand, they do not provide any explanation for the characteristic way in which non-complement-binding IgM antibodies bring about red cell destruction in vivo; certainly, in these experiments there was no evidence for interaction between red cells coated with IgM alone and the MPS.

INTRODUCTION

RABBIT BLOOD GROUPS

Rabbit blood group systems

Red cell antibodies in the rabbit

Impaired survival of incompatible red cells as an indicator of immunization to red cell alloantigens

Table 1. Nomenclature used for one blood group system in the rabbit (Cohen, 1962 and 1969).

	Blood types					
Hg genotype	Cohen	Castle	Kellner	Anderson	Joysey	Heard
Hg^A/Hg^A	APR	H_2	g	A	B	Y
Hg^D/Hg^D	DJKNR	O	-	O	C	W
Hg^F/Hg^F	FKP	H_1	G	B	A	Z
Hg^A/Hg^F	AFJKPR	H_1H_2	Gg	AB	AB	YZ
Hg^A/Hg^D	ADIJKNPR	H_2	-	A	BC	YW
Hg^D/Hg^F	DFJKNPR	H_1	-	B	AC	WZ

Rabbit blood-group systems

Cohen (1969) has described 6 genetic loci controlling rabbit blood-group systems. The system designated Hg by Cohen is the one which has been most extensively used in serological work in rabbits, but prior to 1955, several terminologies were in use. Cohen suggested that a uniform nomenclature be adopted, and made specific proposals compatible with contemporary genetic usage and blood-group terminology. Throughout this work, Cohen's terminology is used but for ease of reference, Cohen's Hg system is compared in table 1 with five terminologies used by other authors quoted in this work.

The systems Hb, Hc, He, Hh and Hq (see table 2) each have one frequent and one infrequent allele; the relative gene frequencies of these alleles and the poor antigenicity of the factors they control means that in practice, alloimmunization resulting from incompatibility at these loci is rarely observed and for the present purposes may be disregarded. On the other hand, variations in the remaining system, Hg frequently gives rise to blood-group incompatibility between individual rabbits, and injections of such incompatible red cells often results in alloimmunization.

Blood groups of the rabbits used in the present studies were determined using antisera detecting the three antigens A, D and F. It was intended that investigations of alloimmunization should always involve the injection of cells from donors of genotype Hg^A/Hg^A or Hg^A/Hg^F into recipients of genotype Hg^F/Hg^F. It has already been

Table 2. Rabbit blood groups according to the terminology of Cohen (1969).

Locus	Typing sera	Alleles	Genotypes	Blood types
Hg	Anti-A	Hg^A	Hg^A/Hg^A	APR
	Anti-D	Hg^D	Hg^D/Hg^D	DJKNR
	Anti-F	Hg^F	Hg^F/Hg^F	FKP
	Anti-I	Hg^N	Hg^N/Hg^N	JKNR
	Anti-J		Hg^A/Hg^D	ADIJKNPR
	Anti-K		Hg^A/Hg^F	AFJKPR
	Anti-N		Hg^A/Hg^N	AIJKNPR
	Anti-P		Hg^D/Hg^F	DFJKNPR
	Anti-R		Hg^D/Hg^N	DJKNR
			Hg^F/Hg^N	FJKNPR
Hb	Anti-B	Hb^B	Hb^B/Hb^B	B
	Anti-M	Hb^M	Hb^M/Hb^M	M
			Hb^B/Hb^M	BM
Hc	Anti-C	Hc^C	Hc^C/Hc^C	C
	Anti-L	Hc^L	Hc^L/Hc^L	L
			Hc^C/Hc^L	CL
He	Anti-E	He	He/-	E
		he	he/he	-
Hh	Anti-H	Hh	Hh/-	H
		hh	hh/hh	-
Hq	Anti-Q	Hq^Q	Hq^Q/Hq^Q	Q
	Anti-S	Hq^S	Hq^S/Hq^S	S
			Hq^Q/Hq^S	QS

pointed out that because serologically-detectable antibody was not always found following the accelerated clearance of injected red cells, it was sometimes impossible to confirm the specificity of the immune response inferred from the red cell survival studies. Nevertheless, it can be shown that a response to known rabbit red cell antigens other than A was most unlikely under the experimental conditions prevailing. Using blood group antisera identifying only the first 3 out of the 4 possible alleles Hg^A, Hg^D, Hg^F and Hg^N, it might be argued that some animals grouped as AA were in fact AN. However, this mistake seems unlikely for the following reasons. The strength of serological reactions was always graded and it was found that the heterozygotes Hg^A Hg^F and Hg^A Hg^D always gave weaker reactions with anti-Hg^A (anti-A) than the presumed homozygotes Hg^A Hg^A (see also Cohen, 1958a); it might reasonably be expected that the heterozygote Hg^AHg^N would also have given weak reactions with anti-A, but no weak reactions with anti-A were found in isolation. By the same argument it is possible that the recipient might have been of the genotype Hg^F/Hg^N, but this would only serve to reduce the number of foreign antigens introduced in certain cases.

The most likely red cell antigenic constitution of the animals selected for the present experiments must therefore have been as indicated in table 3. If, under these circumstances, cells bearing the A antigen are injected into rabbits of genotype FF, alloantigens of the Hg locus other than A which might also be transferred are as follows: R (from AA), R and J (from AF) and R, N, J and I (from AN). The infrequency

Table 3. Red cell antigenic constitution (with respect to the Hg locus) of donor and recipient rabbits used in the present experiments.

		RECIPIENT	POTENTIAL DONORS		
		Hg^F/Hg^F	Hg^A/Hg^A	Hg^A/Hg^F	(Hg^A/Hg^N)
Hg ANTIGENS	A		+	+	+
	D				
	*F	+		+	
	I				+
	J			+	+
	*K	+		+	+
	N				+
	*P	+	+	+	+
	R		+	+	+

Based on Cohen (1969) with amendments (personal communication).

* Antigens present on recipient's cells.

() Unlikely donor (see text).

with which antibodies of these specificities have been encountered argues against their being a significant cause of confusion in the present experiments. With regard to the antigen J, for example, Cohen (1960b) found that in spite of many attempts to raise anti-J antisera in J-negative rabbits, only immunizations involving J-positive D-positive donors and J-negative D-negative recipients were effective.

Red cell antibodies in the rabbit

During the course of this work, naturally-occurring antibodies to rabbit red cells were found in a few cases. Some of the characteristics of these antibodies are described later; suffice it to say that in the one case in which in vivo effects were studied, the antibody did not cause any significant increase in the rate of destruction of "incompatible" donor red cells (see p. 299). No examples of naturally-occurring antibodies analagous to the antithetical anti-A and anti-B of the human ABO blood group system have been described in rabbits.

It is known that for a given strain of rabbits, the frequency of successful immunization against red cell alloantigens varies, depending upon the particular antigen being studied. With regard to the Hg locus, Cohen (1962) found that A was the "strongest" antigen; using a standard immunization procedure, with adjuvant, the injection of red cells bearing the A antigen into FF or FD recipients was the only donor-recipient combination resulting in 100% success of immunization as judged by the production of serologically-detectable antibody.

On the other hand, following i.v. injection of A-positive red cells into A-negative rabbits, the frequency with which antibody is found seems to vary from laboratory to laboratory (see p.228). There is evidence to suggest that the ability to respond to red cell alloantigens is genetically determined. For example, it has been shown that the incidence of immunization may be modified to a considerable extent by the presence or absence on the donor red cells of alloantigens other than those against which immunization is intentionally directed (Cohen, 1960a; Woodrow, Elson and Donohoe, 1973).

Impaired survival of incompatible red cells as an indicator of immunization to red cell alloantigens

By comparison with the experience of other workers using different strains of rabbits, the Sandy lops used in the present studies were poor antibody producers. Whereas originally it had been intended that serological results should be supplemented with data from red cell survival studies in the interpretation of the investigations described in this work, in practice, great reliance has had to be placed on the survival curves alone in many of the experiments.

In several instances, impaired survival of a small dose of allogeneic red cells in an animal passively immunized with the corresponding antibody has been shown to be one of the most sensitive methods currently available for detecting that antibody in the recipient's circulation (Mollison and Hughes-Jones, 1967; Burton and Mollison, 1968; Frame and Mollison, 1972). However, when the injected cells are used

both as immunogen and as detector of active immunization, the specificity of any immunological response can only be determined with certainty by using additional tests (see p.77). In the present investigations, the specificity of the accelerated red cell clearance response was assumed to be the same as that of antibody which was later demonstrated serologically in the majority of cases. With rare exceptions, antibody of only a single specificity could be identified, namely anti-A, but such antibody frequently could only be detected following more than one injection of red cells.

INTRODUCTION

FACTORS REGULATING THE IMMUNE RESPONSE

Cells involved in the immune response

(i) T-cells and B-cells

(ii) T-cell regulation of B-cell activity

(iii) The role of the macrophage

(iv) Some observations in the rabbit

(a) The antigen-reactive cell (ARC)

(b) The antibody-forming cell (AFC)

The induction of tolerance

Genetics of the immune response - "responders" and "non-responders"

The experiments presented in this thesis attempt to define the immune responses of rabbits to allogeneic red cells. The different types of response observed presumably reflect variations in the cellular and humoral events which constitute the immune responses in a given animal under given conditions. A full discussion of these events is beyond the scope of this work, but in the brief account which follows, some of the factors regulating the immune response are described, particular emphasis being given to those aspects which seem to be especially relevant to the present work.

Cells involved in the immune response

(i) T-cells and B-cells. That two cell types are involved in the normal response to foreign antigens was first clearly shown in chickens by Cooper, Peterson and Good (1965). They described two populations of lymphocytes, one dependent upon the presence of the thymus gland (T-cells), and the other on the bursa of Fabricius (B-cells). In mammals, two populations of lymphocytes have also been characterized, T-cells, whose development is likewise dependent upon the thymus, and B-cells (bone-marrow or burse dependent) for which no single tissue influencing their development in a way analagous to the bursa in chickens has been identified (Miller and Mitchell, 1968). Functionally, T-cells are the mediators of cellular immunity, whereas B-cells are the effectors of humoral antibody production, but the production of specific antibody in response to most antigens requires the cooperation of both T-cells and B-cells (Claman, Chaperon and Triplett, 1966; Miller and Mitchell, 1967). However, a number of

antigens, such as pneumococcal polysaccharide (PPS) and polymerized flagellin from S. adelaide (POL), which are relatively simple molecules with linear repetitive structures, have been shown to induce a response independently of T-cell function (Humphrey, Parrott and East, 1964; Armstrong, Diener and Shellam, 1969); furthermore, in the absence of T-cells, these antigens usually elicit the formation of IgM antibody only, and in slightly increased doses will induce immunological unresponsiveness (Feldman, 1971).

(ii) T-cell regulation of B-cell activity. Several ways in which T-cells may regulate B-cell activity have been proposed. There is evidence to suggest that cooperation between T- and B-cells in the production of specific anti-haptene antibody following stimulation by haptene-carrier complexes is induced in each cell type by different, but specific determinants on the antigen. Thus, in the primary response, carrier molecules given alone will activate T-cells which in turn, enhance the primary anti-haptene response of the B-cells on challenge with haptene-carrier complex; challenge with haptene attached to a different carrier results in a normal primary anti-haptene response (Mitchison, 1970; Katz, Paul, Goidl and Benacerraf, 1970). In addition, it has been shown that the carrier molecule has distinct determinant sites which are recognized independently by T- and B-cells (Hamaoku, Takatsu and Kitzagawa, 1973). Haptene determinants may contribute to the specificity of molecules stimulating T-cells, but they are themselves usually very poor carriers for other haptenes. On the basis of experiments of this kind, it is postulated that T-cells respond to a different range of antigen specificities from B-cells,

that their receptors are of low affinity and therefore require an array of determinants for activation, and that they will only process antigens with particular arrangements of determinants.

T-cells activated by specific antigens have been shown to release humoral factors which have a non-specific effect on B-cells and stimulate them to synthesize antibody in response to their own specific antigenic determinants. Thus, although primary stimulation of T- and B-cells occurs in response to appropriate specific determinants on a given antigen, it would appear that the enhancement of the B-cell primary or secondary response is not necessarily dependent upon the stimulation of the same T-cell population (Playfair and Marshall-Clarke, 1973). Similarly, the use of non-specific T-cell stimulation by Pokeweed mitogen (PWM) or Phytohaemagglutinin (PHA) can be shown to induce B-cell activity. Non-specific T-cell stimulation, the "allogeneic effect", has also recently been described in graft-versus-host reactions (Katz, Goidl, Paul and Benacerraf, 1971).

While T-cells do not determine the class of antibody produced by any particular B-cell (Shearer and Cudkowicz, 1969), there is a considerable body of evidence to suggest that T-cell activity causes selective recruitment of B-cells committed to produce IgG (Taylor and Wortis, 1968; Miller, Basten, Sprent and Cheers, 1971). T-cells may have a direct inhibitory effect on IgM producing B-cells; in experiments in which T-cell depletion was brought about by the use of

anti-lymphocyte serum (ALS), IgG production was depressed whereas IgM levels or the number of direct plague-forming cells (PFC) in the spleen were increased (Baum, Liebermann and Frenkel, 1969; Baker, Barth, Stashak and Amsbaugh, 1970). Alternatively, enhancement of the IgG response may secondarily depress IgM production (Moller and Wigzell, 1965).

(iii) The role of the macrophage. In addition to T- and B-lymphocytes, the macrophage is known to play an important role in the immune response, particularly in relation to the processing of particulate antigen. Mosier (1967) has shown *in vitro* that the PFC response of mouse-spleen cells to sheep red blood cells (SRBC) is dependent upon the presence of a population of surface-adherent cells. There is also evidence to suggest that the unresponsiveness of newborn animals to certain antigens is a reflection, at least in part, of the immaturity of the macrophage system; responsiveness may be induced by the transfer of syngeneic adult macrophages (Martin, 1966; Argyris, 1968). However, it is also known that T-cells of newborn mice are relatively ineffective in reconstitution experiments (Claman, Chaperon and Triplett, 1966).

As indicated earlier (see p. 36) monocytes and macrophages have been shown to possess surface receptors for immunoglobulins and complement components. It has been suggested that complex antigenic structures may be attached to these receptors on the macrophage membrane through the mediation of naturally-occurring antibody; the F(ab) portion

of the antibody selects the antigenic determinant(s) which will be protected when the whole antigen is taken into phagolysosomes for digestion, and it is these determinants or their close neighbours which will subsequently react with lymphocytes to induce an immune response (Bamford and Black, 1972). Feldman and Basten (1972) have postulated that the source of the naturally-occurring antibody in the above reaction is the T-cell. The normal sequence of events leading to humoral antibody production would then involve antigen-T-cell interaction, processing of the resulting antigen-antibody complex by macrophages, and finally, presentation of suitably prepared antigenic determinants to the B-cells.

(iv) Some observations in the rabbit. The anatomical arrangement of functionally distinct immunocompetent cells varies in different species of laboratory animals and has been studied most extensively in mice and rabbits. The results of some observations made in rabbits will now be reviewed because of their possible relevance to some of the findings to be presented later.

Richter and his colleagues recognise at least two distinct cell types (in addition to macrophages) which are essential for the induction of a primary humoral immune response in rabbits. These are the antigen-reactive cell (ARC), which is mainly, if not exclusively, located in the bone-marrow, and the antibody-forming cell (AFC), which is found in spleen and lymph nodes but which is absent from the bone marrow (Richter and Abdou, 1969; Richter, Rose and Abdou, 1970).

(a) The antigen-reactive cell (ARC). Following the exposure of bone-marrow cells to antigen in vitro, ARC undergo transformation into blast cells. This response does not occur when the cells are taken either from a rabbit that has been exposed to 800r irradiation or from one that has been injected with the test antigen 24 hours previously. In the latter circumstance, blast transformation in response to other antigens still occurs (Singhal and Richter, 1968). It would seem, therefore, that following exposure to a specific antigen, the stimulated ARC migrate from the bone-marrow, and that none of the cells remaining is capable of reacting with that antigen.

A rabbit irradiated with 800r will not exhibit a primary humoral immune response to antigen as manifested either by the production of specific plaque-forming cells in the spleen or by the production of specific circulating antibody. The capacity to respond to antigen may be restored by reconstituting the irradiated animal with ARC using bone-marrow cells from a normal rabbit. However, if the donor rabbit is immunized against the test antigen 24 hours before the bone-marrow cells are taken, as might be predicted from in vitro experiments mentioned above, the recipient will remain unresponsive to this antigen and yet will be capable of responding to other antigens. Similarly, the tolerant state induced in neonatal rabbits by the injection of human serum albumin (HSA) or bovine gamma-globulin (BGG) may be broken by the injection of bone-marrow cells from normal adult animals; once again, cells from donors immunized to the test antigen do not restore responsiveness to that antigen (Abdou and Richter, 1969a).

Another property of the ARC which has been exploited in reconstitution experiments of this kind is that of their adherence to specific antigen. If bone-marrow cells from a normal rabbit are passed through a column of glass beads coated with antigen (e.g. sonicated SRBC), the effluent cells are incapable of restoring the capacity of irradiated rabbits to respond to that antigen while remaining effective with respect to other antigens. Furthermore, elution of the retained cells from the column provides a cell population capable of restoring responsiveness specifically to the column antigen in irradiated rabbits (Abdou and Richter, 1969b).

Cells in different compartments of bone-marrow of a single normal rabbit may restore immunological responsiveness to SRBC (or horse red blood cells - HRBC - or both) in more than one irradiated recipient rabbit. This suggests, at first sight, a multiclonal origin of ARC specific for SRBC, but it is clearly possible that these results reflect the immune responses to a complex antigen bearing multiple antigenic determinants (Richter and Abdou, 1971).

(b) The antibody-forming cell (AFC). Following immunological reconstitution of an irradiated rabbit with bone-marrow cells from a normal donor, it has been shown, using allotypic markers, that the antibody produced in response to subsequent immunization with SRBC is of recipient origin (Richter and Abdou, 1969). In this experimental system, the antibody response was not noticeably different from that of a normal rabbit. It appears therefore that AFC are resistant to 800r

irradiation and are incapable of manifesting PFC activity in the spleens of irradiated animals without the cooperation of ARC. In the rabbit AFC are found in lymph-nodes and spleen but are absent from the bone-marrow; whether precursors of AFC are localized in, or dependent on, any particular lymphoid tissues has not been established, nor whether, following antigenic stimulation, they migrate from their tissue of origin to other sites.

Cells performing analogous functions to those described above for the rabbit have been identified in mice. The essential features may be summarized as follows. Viable thymus- or thoracic duct-lymphocytes (ARC) are essential for the development of a normal or near normal 19S haemolysin-forming cell response in the spleens of neonatally thymectomized mice immunized with SRBC; the antibody forming cells are of host allotype (Miller and Mitchell, 1968). ARC constitute the larger proportion of thoracic duct lymphocytes, are also found in small numbers in the thymus gland, but are absent from the bone-marrow, whereas AFC precursors are found in the bone-marrow and in the thoracic duct lymph (Nossal, Cunningham, Mitchell and Miller, 1968). Many of the functions and inter-relationships of these cells in the mouse have been described in earlier sections. Of particular relevance to the present discussion, however, is the work of Taylor (1968). This author found that the antibody response of heavily irradiated mice to bovine serum albumin (BSA) could be restored by reconstitution with bone-marrow and thymus cells from normal mice; on the other hand, when the donor of the thymus cells had been immunized with BSA,

immunocompetence with respect to this antigen was not restored in the recipient. It was suggested that stimulated thymus cells vacate the gland. More recently, Diener, Kraft and Armstrong (1973) have described the properties of antigen-binding cells in mice which, on stimulation with antigen, differentiate into larger, immunologically active cells.

The induction of tolerance

Many factors affect the outcome of exposure of a normal animal to antigen. The nature of the antigen, its complexity, whether in soluble or particulate form, the amount given, and the route of administration are all important in this respect. Also, the species or strain of the recipient animal and its previous experience of the same or similar antigens can modify the immunological response. These factors presumably influence the way in which antigen is presented to the immunocompetent cells and the response of these cells to antigen and their interaction with one another. In contrast to the usual response to antigen characterised by specific humoral antibody production or cell-mediated immunity, specific immunological unresponsiveness, or tolerance, may result.

Studies by Chiller, Habicht and Weigle (1971) have clearly demonstrated that the unresponsive state induced in adult mice by the injection of de-aggregated human gamma-globulin (HGG) may involve both T- and B-cell suppression. The development of B-cell unresponsiveness was shown to take longer, to be of shorter duration, and to require higher doses of tolerogen than was the case for T-cells. The overall

pattern of tolerance observed in these circumstances therefore reflected T-cell unresponsiveness. On the other hand, both partial tolerance induction to bovine serum albumin (BSA) and specific antibody-mediated immunosuppression in response to this antigen in adult rabbits have been shown to result from B-cell suppression (Heller and Siskind, 1973). Reference has already been made to the ease with which T-cell independent antigens may induce tolerance and this therefore provides another example of B-cell tolerance.

Chiller, Romball and Weigle (1973) have shown that the mechanism of tolerance induction may differ in neonatal and adult animals of the same species; in adult rabbits, tolerance to BSA was preceded by a transient exhibition of antibody-producing cells suggesting exhaustive differentiation of a precommitted B-cell line, whereas in the neonate, no such response was observed. This difference of response may be interpreted in terms of the immaturity of the macrophages and/or T-cells in the neonatal rabbit so that no initial antigen processing can take place. That neonatal rabbits possess potentially functional B-cells is evident from the observation that BSA given with Freund's complete adjuvant (FCA) results in antibody formation. A similar combination of T-cell immaturity and normal B-cell potential with respect to SRBC has been demonstrated in neonatal mice (Claman, Chaperon and Triplett, 1966; Chiscon and Golub, 1972).

Genetics of the immune response: "responders" and "non-responders".

The intensity of an immune response following the injection of certain antigens into laboratory animals seems to be related to the species or strain of animal used. Since the development of simple synthetic polypeptides for use as antigens or haptene carriers in immunological studies, it has become possible to investigate variations in the immune response to simple, well-defined antigenic determinants. Levine, Ojeda and Benaccerraf (1963) showed that the development both of cell-mediated and humoral immunity in Hartley strain guinea-pigs in response to dinitrophenyl conjugates of poly-L-lysine (DNP-PLL) are dependent upon the inheritance of a simple Mendelian dominant characteristic. In this particular case, the specificity of the genetically-determined response related to the PLL carrier, because DNP responses could be elicited by other DNP-carrier conjugates. The first demonstration of the genetic control of the response to a specific antigenic determinant was provided by McDevitt and Sela (1965). These authors used a branched, multichain, synthetic polypeptide, poly (tyrosine, glutamic acid) -poly-DL-alanine-polylysine ((TG)-A-L) and showed that C57 mice were good responders compared with CBA mice; at least a 10-fold difference in antigen-binding capacity of serum from the two strains of immunized mice was observed. Immunization with (HG)-A-L, in which histidine replaces tyrosine in the former polypeptide, resulted in the converse situation, the CBA mice responding much better than the C57 mice. The ability to respond appeared to be inherited as a Mendelian dominant characteristic.

Some of the features of the inherited inability to respond to simple antigens suggest that the failure occurs at the T-cell level. Guinea-pigs that do not respond to DNP-PLL exhibit neither humoral nor cell-mediated immunity: if DNP-PLL is conjugated to another carrier, BSA, antibodies to DNP and PLL are produced in previously unresponsive animals, but delayed hypersensitivity to PLL is still lacking (Green, Paul and Benacerraf, 1966). Both responder and non-responder mice given a first, small injection of (TG)-A-L show evidence of B-cell stimulation early in the response by the formation of specifically reacting direct PFC in the spleen; this can also be demonstrated using neonatally thymectomized progeny of "responder" mice. On further antigenic stimulation with (TG)-A-L only the non-thymectomized mice produce an IgG antibody response (Mitchell, Mishell and Herzenberg, 1971).

The situation with regard to more complex antigens is less well defined. The importance of genetic control of immune responses to red cell alloantigens was discussed by Wiener (1946) in relation to the variable responses to the Rh antigen observed in man. It has now been established that only about 70% of Rh(D)-positive individuals are capable of responding to the i.v. injection of Rh(D)-positive cells with the production of anti-D (Wiener, 1949; Krevens, Woodrow, Nosenzo and Finn, 1964; Mollison, Frame and Ross, 1970). It is likely that the inherited ability to respond to such antigens is controlled by multigeneic determinants, a possibility supported by observations made with regard to the red cell alloantigen Hg^A in rabbits (Woodrow, Elson and Donohoe, 1973). However, analysis of the problem

is complicated by the presence of multiple antigenic determinants on red cells and their possible mutually cooperative effect in bringing out facilitation of immunization (Issit, 1965; Archer, Cook, Mitchell and Parry, 1969; McBride and Schierman, 1971; Issit, McKeever, Moore and Wilkinson, 1973). Nevertheless, in rabbits, the concentration and affinity of anti-DNP produced in response to immunization with the relatively simple antigen, DNP conjugated to bovine gamma-globulin (DNP-BGG) has been shown to be related to allotype (Werblin, Kim, Rose, Benacerraf and Siskind, 1973).

INTRODUCTION

ANTIBODY-MEDIATED SUPPRESSION OF PRIMARY IMMUNIZATION

Hypotheses

(i) Antigen destruction or deviation

(ii) Competition for antigen

(iii) Direct inhibition of immunocompetent cells

Some other observations on the effect of different classes and sub-classes of passive antibody on the immune response

Hypotheses

Three main ways have been suggested in which the administration of passive antibody may interfere with the sequence of events leading to primary immunization.

(i) Alteration of the antigenic particle or molecule in such a way that specific determinant structures are destroyed and catabolised before they can be presented to immunocompetent cells.

(ii) Competition between passively administered antibody and immunoglobulin-like receptors present on immunocompetent cells for available antigen - the so-called "peripheral" mechanism.

(iii) Direct action of the antibody on the ability of immunocompetent cells to process antigen or to produce antibody of that particular specificity - the "central" effect.

These mechanisms are not mutually exclusive and there is evidence to suggest that under different circumstances one or other may predominate. Factors which are likely to be important in determining the mode of immunosuppression include: the characteristics of the passively-administered antibody, its avidity, immunoglobulin class, complement-binding properties; the nature of the antigen involved, whether soluble or particular, "strong" or "weak", route of administration, with or without

adjuvant; the immunocompetence of the recipient animal, neonatal or adult, pregnant or non-pregnant. The following brief, and necessarily incomplete summary of the situation relates particularly to suppression of immunization to red cell antigens by passively administered IgG antibody.

(i) Antigen destruction or deviation. The observations which led to the use of anti-Rh for prophylaxis against HDN were dependent upon non-specific antibody-mediated immunosuppression. It was suggested that the protective effect of ABO incompatibility on immunization to Rh might be due to the rapid elimination of foetal red cells from the maternal circulation before Rh immunization could take place (Race and Sanger, 1950). This type of immunosuppression has been investigated experimentally in man by giving Rh-positive ABO-incompatible red cells to Rh-negative individuals (Stern, Davidsohn and Masaitis, 1956; Stern, Goodman and Berger, 1961), and in rabbits, using human red cells and rabbit anti-human red cell antibody (Hager, 1967; Pollack, Gorman, Hager, Freda and Tripodi, 1968). In both series of experiments, the antibodies involved were complement-binding; in the human experiments, the ABO-incompatible cells were probably removed from the circulation within a few minutes of injection as a result of intravascular lysis (see, e.g. Jandl, Jones and Castle, 1957; Cutbush and Mollison, 1958); in the rabbit experiments, the injected cells were removed rather more slowly, with a half-disappearance time of about 0.4 days, and there was evidence of increased hepatic sequestration. It is known that red cell stroma or sonicated red cells are less antigenic than intact red cells (Schneider and Preisler, 1966; Hager, 1967) and it may well be that

complement-binding, which leads to disruption of the red cell membrane, is important in suppressing immunization in the above experiments. Alternatively, the localization of antigen in different tissues has been shown to be related to the immunological response observed (Cruchaud, 1968; Souhami, 1972) and to the immunological status of the recipient (Nossal, Ada, Austin and Pye, 1965; Lang and Ada, 1967), so that deviation of potential antigen from responsive to unresponsive sites may bring about suppression of a primary immune response. However, it seems that effective suppression of the immune response to SRBC in different strains of mice by specific antibody is associated with very variable tissue distribution of the injected cells (Koros and Hamill, 1973).

(ii) Competition for antigen. In a number of experimental systems, suppression of primary immunization by passively-administered antibody cannot be explained in terms of deviation of the antigen from immunoreactive sites. When different antigenic determinants are present on the same molecule or red cell, suppression of the immunological response is usually limited to that determinant against which the passive antibody is directed (Brody, Walker and Siskind, 1967; Pollack, Gorman, Hager, Freda and Tripodi, 1968; Elson, Woodrow and Donohoe, 1971); furthermore, the response to other determinants on the same antigen may even be enhanced (Pearlman, 1967; Schierman, Leckband and McBride, 1969). On the other hand, Woodrow (1974) has produced evidence which suggests that in man, suppression of immunization to red cell alloantigens by passive antibody is non-specific. He gave

i.v. injections of 1 ml K-positive, D-positive red cells to 62 K-negative, D-negative volunteers; 31 of these subjects were also given 16 μg anti-K i.m. at the same time and in these individuals the allogeneic cells were cleared from the circulation by 24 hours. Whereas 11 out of 31 of the control group formed anti-D after 2 injections of cells, only 1 of the 31 subjects given anti-K formed anti-D ($p = 0.0013$).

That passively administered antibody might compete with potentially responsive cells for antigen was suggested by studies relating to the affinity of antibody produced during the course of an immune response (Siskind, Dunn and Walker, 1968). Using a soluble haptene-protein conjugate (DNP-BGG) in mice it was found that under normal circumstances, the affinity of the humoral antibody produced increased with time, that with very low doses of antigen the peak antibody affinity was somewhat reduced, and that high doses of antigen delayed the production of high-affinity antibody. Furthermore, partial suppression of the immune response with passive antibody resulted in the active formation of reduced amounts of high-affinity antibody, and it was shown that the effectiveness of immunosuppression was related to the affinity of the passively-administered antibody (Walker and Siskind, 1968). Similar conclusions were reached by Moller (1969) in relation to the suppression of the response to SRBC in mice. Animals were injected i.v. with either a high or low dose of SRBC and, after 1-2 days, with an identical amount of mouse hyper-immune anti-SRBC antibody. The immune response was assessed by observing PFC production in the spleens of the injected animals.

It was found that, compared with control animals given SRBC alone, passive antibody was more efficient in suppressing immunization in the high dose group, so that, in absolute terms, more PFC were produced in response to the low dose of cells under these circumstances. This was taken to indicate that with a lower antigen dose, a greater proportion of high-affinity antibody-forming cells are stimulated, and that passive antibody suppresses low-in preference to high-affinity cells (relative to its own affinity). Work in rabbits suggests that the same selective mechanism based on the affinity of antibody or antibody-forming cells may be operative in partial antibody-mediated suppression of the immune response to soluble antigen (Heller and Siskind, 1973) and particulate antigen (Pearlman, 1967).

These experiments emphasise the importance of competition between circulating antibody and immunocompetent cells for antigen in regulating an established immune response (see also Lees and Sinclair, 1973, and preceeding papers from the same laboratory). While it is possible that many examples of "suppression" of the immune response by passive antibody are in fact examples of a regulated, low level response, there is evidence to suggest that, at least in certain cases. alternative mechanisms are operative in preventing any measurable response (see later).

An important argument against the direct competition of passive antibody for unprocessed antigen playing a significant role in suppressing primary immunization is the observation that, in several situations in which suppression appears to be complete, only a small proportion of the antigen can possibly have been bound by antibody.

For example, Rh immunization in man may be suppressed when as little as 5-10% of the D-sites on the red cells are bound by antibody (Hughes-Jones and Mollison, 1968). It is true, however, that only a minute proportion of the antigen injected in to the circulation is involved in eliciting a primary immune response, and it is therefore possible that passive antibody might in some way be concentrated at the relevant sites and there compete more favourably for potentially immunogenic antigen. That macrophages might perform this function was suggested by the observations of Ryder and Schwartz, (1969). These authors injected anti-SRBC antibody in to newborn mice and six weeks later, at a time when no passive antibody could be detected serologically, injected thioglycollate i.p. followed shortly afterwards by SRBC. Macrophages were harvested from the peritoneal cavity and contained substantial numbers of SRBC. This preparation injected in to normal mice produced no antibody response, in contrast to a similar preparation from normal mice, which evoked a brisk response. On the other hand, spleen cells from antibody-treated mice were unaffected in their ability to restore immunocompetence to SRBC in irradiated recipients.

(iii) Direct inhibition of immunocompetent cells. It might be argued that the macrophage effect described in the last paragraph rightly belongs under this heading, especially if, as postulated by Feldman and Basten (1972) the sequence of cellular reactions with antigen is T-cell, macrophage, B-cell. However, the argument is largely semantic and the "central" effect hypothesis is generally taken to imply T- and B-cell (or ARC and AFC) inhibition.

Rowley and Fitch (1964) found that spleen cells from adult rats, exposed to anti-SRBC antibody *in vitro*, or obtained from passively immunized animals, were ineffective in restoring immunological responsiveness to irradiated animals on challenge with SRBC. They postulated a direct inhibitory effect of passive antibody on AFC in the spleen to account for this observation, but it now seems more likely that their spleen-cell preparation contained a variety of cell types, including macrophages,and that the latter were responsible for the effects observed. Certainly, Moller (1964), Ryder and Schwartz (1969) and others have been unable to show such an effect in other experimental systems. Furthermore, using *in vitro* techniques, Pierce (1969) has since found that exposure of rat lymphoid cells to antibody did not impair the immune response in a mixed-cell culture system, whereas macrophages, similarly treated, did so.

Some other observations on the effect of different classes and sub-classes of passive antibody on the immune response.

Experiments in rabbits in which Hg^A-positive red cells coated with agglutinating amounts of antibody were injected in to Hg^A-negative recipients suggested that the incidence of primary immunization was higher, and the appearance of circulating antibody more rapid than in control animals given untreated cells (Cohen and Allton, 1962).

Whether IgM antibody may more readily bring about augmentation of the immune response than IgG has not yet been established in relation to Rh. The original observations of Clarke, Donohoe, McConnell, Woodrow, Finn, Kreevans, Kulke, Lehane and Sheppard (1963) have been re-evaluated in the

light of follow-up studies and provide no firm evidence of augmentation (see Mollison, 1972, p.317). Similarly, in a small number of experiments in which Rh-negative male volunteers were given 1 ml Rh-positive red cells and 39-90 µg IgM anti-Rh i.v., no evidence of augmentation was apparent (Holburn, Frame, Hughes-Jones and Mollison, 1971).

Some evidence suggesting that small doses of specific IgG antibody might augment the response to Rh-positive cells was reported by Pollack, Gorman, Hager, Freda and Tripodi (1968). They gave 5 ml Rh-positive blood to Rh-negative subjects followed, three days later, by amounts of IgG anti-Rh ranging from 1 to 40 µg. A larger proportion of the recipients given 10 µg passive antibody were immunized at 3 months than those in any other dosage group, and a larger proportion of the responders formed IgM antibody.

It has been established in animals that under certain circumstances immune responses may be augmented by small doses of specific antibody of both IgG and IgM classes (Pearlman, 1967). Recent work on the regulation of the immune response by IgG antibody may provide an explanation for many of the effects of passively administered IgG and also suggest ways of investigating the action of IgM. It has been shown that the Fc portion of IgG is essential for terminating an established immune response to SRBC in mice (Chan and Sinclair, 1973). Whereas intact IgG possesses this potential for suppression, its effect can be blocked by $F(ab')_2$ fragments of the same antibody. $F(ab')_2$ given alone is ineffective in suppressing an immune response.

Table 4. The effect of passively administered antibody of different sub-classes, and directed against different parts of the immunizing complex, on the IgG anti-haptene responses in guinea-pigs immunized with haptene-carrier conjugates. Compiled from the data of Vuagnat, Neuveu and Voisin (1973 a and b).

Passively administered antibody		Anti-haptene response			
		IgG_1		IgG_2	
Specificity	Sub-class	1	2	1	2
Anti-haptene	IgG_1	↓(+)	↑(++)	↓(++)	↓(+)
	IgG_2	↓(++)	N.	↓(++)	↓(++)
	$F(ab')_2$	↓(++)	N.D.	↓(++)	N.D.
Anti-carrier	IgG_1	↓(++)	N.	↓(++)	↓(+)
	IgG_2	Normal		↓(+)	N.

1. Primary immunization with antigen-antibody complexes

2. Challenge with antigen alone

N. Normal response

↓(+) Moderate depression

↓(++) Marked depression

↑(++) Marked increase

N.D. Not done

It has been suggested that this effect is mediated by selective deviation of antigen to immunocompetent cells (including macrophages) which bear appropriate Fc surface receptors. Vuagnat, Neveu and Voisin (1973a and b) have studied the effects of passively administered IgG of different subclasses and different specificities on the immune response of guinea-pigs to haptene-carrier conjugates. The sub-classes of antibody used for passive immunization were IgG_1 (responsible for passive cutaneous anaphylaxis in guinea-pigs) and IgG_2; antibodies directed specifically against haptene or against carrier were used. The responses of the guinea-pigs following administration of antigen-antibody complexes were assessed in terms of the active production of IgG_1 and IgG_2 anti-haptene antibody and are summarized in table 4. The important points to note from the table are:

(i) The IgG_2 antibody response following passive antibody of either sub-class or either specificity is uniformly depressed.

(ii) The IgG_1 antibody response following passive IgG_2 anti-haptene antibody is depressed and following IgG_2 anti-carrier antibody is normal.

(iii) The IgG_1 antibody response following IgG_1 anti-haptene antibody is slightly depressed initially and is subsequently augmented in response to haptene-carrier challenge; on the other hand, following IgG_1 anti-carrier antibody, the IgG_1 response is depressed throughout.

These results are interpreted on the basis of the following observations.

(a) Production of IgG_1 is dependent on T-cell function, at least in mice (Taylor and Wortis, 1968; Torrigiani, 1972)

(b) Again in mice, B-cells bear surface receptors for IgG_1 (Basten, Miller, Sprent and Pye, 1972; Basten, Warner and Mandel, 1972).

(c) Guinea-pig macrophages bear surface receptors for IgG_2 (Berken and Benacerraf, 1966).

It is concluded that immunization with IgG_2-antigen complexes leads to removal of antigen by macrophages and that this would be expected to impair humoral antibody production. This is in fact so, except for the normal response of IgG_1 production following injection of IgG_2 anti-carrier antibody. No explanation for this is offered but it is noted that previous workers have found that passively administered anti-carrier antibodies either have no effect or depress the anti-haptene response (Benacerraf and Gell, 1959; Mitchison, 1971; Katz, Paul, Goidl and Benacerraf, 1970; Hamaoku, Takatsu and Kitagawa, 1971). IgG_1-antigen complexes, on the other hand, would be expected to persist in immuno-responsive sites. Anti-haptene IgG_1 antibody will mask the haptene determinants but will permit T-cell priming by the carrier so that on being challenged with haptene-carrier complex, the anti-haptene IgG_1 response will be greatly augmented. By contrast, IgG_1 anti-carrier antibodies would not permit carrier stimulation of T-cells and so would depress the anti-haptene response.

METHODS

Animals

Venepuncture procedures

Immunization using Freund's complete adjuvant (FCA)

- Cells with FCA
- Boosting injections of cells alone

Plasmapheresis

Serology

- Blood grouping antisera
- Preparation of red cells for use in agglutination tests
- Agglutination tests
- Antibody detection
- Controls

Radioactive isotopes

- Measurement of ^{51}Cr, ^{125}I and ^{131}I
- Measurement of ^{32}P
- Accuracy of counting

Labelling of red cells with ^{51}Cr *in vitro*

Labelling of red cells with ^{32}P *in vitro*

Labelling of red cells with diisopropylphosphofluoridate ($DF^{32}P$) *in vivo*

Preparation of IgG from whole rabbit serum

Trace labelling of IgG with radioactive iodine

Immunoelectrophoresis

Estimation of IgG in pure solution

Estimation of IgG in whole serum

Haemoglobin concentration

Packed cell volume

To avoid repetition, some of the methods used in several different parts of the work have been brought together in this section. This arrangement has made it possible to refer only briefly to methods in some sections; in fact, precise details have been given in other sections only when such details constitute the essence of the work as, for example, in the section on the relation between ^{51}Cr-labelling procedure and Cr red cell survial (p.184).

Animals

Most of the rabbits used in the following experiments were Sandy lops obtained from two colonies in which preliminary blood group screening tests had revealed a high incidence of Hg^A(A)-negative animals. The majority of the rabbits tested had red cells of the phenotype Hg (A-D-F+) and were of the presumed genotype $\underline{Hg^F Hg^F}$. Occasionally the phenotypes Hg (A+D-F-) and Hg (A+D-F+) were found. Rabbits lacking the antigen Hg^A are usually referred to in this work as Hg^A(A)-negative. A number of black Havana rabbits of appropriate blood groups were also used as red cell donors and for raising specific blood group antisera. Full lops were supplied by the Radiobiological Research Unit, Harwell and half lops and Havanas by Hylyne Commercial Rabbits, Cheshire. A small number of rabbits were also bred from the above-mentioned stock in the animal house at St. Mary's Hospital Medical School.

Animals of about six months of age or more and of both sexes were used. They were maintained throughout on a standard animal house diet of Oxoid SG1 with the addition of fresh green vegetables and hay when available. Free access to water was provided. Where minor alterations to this regimen were made, such as the addition of Lugol's iodine to drinking water during metabolic studies using protein labelled with radioactive iodine, this will be indicated in the appropriate section.

Venepuncture procedures

With few exceptions, the marginal veins of the ears were used both when giving intravenous injections and when taking blood samples. In order to facilitate venepuncture, the selected vein was often occluded proximally by means of a small clip. When a timed sample was being taken, the clip was applied immediately before the intended time of sampling. In addition it was sometimes necessary to dilate the blood vessels of the ear prior to venepuncture by applying xylol locally as a rubifacient.

Blood samples that were to be anticoagulated were taken with a syringe and a 19 or 21 gauge needle. When dilution of the sample was not contraindicated a drop of liquid heparin was taken into the sampling syringe and also dispensed into the sample container; when a solid anticoagulant was used, samples were taken into a dry syringe and transferred immediately to pots containing EDTA (1-2 mg per ml whole blood). In those experiments in which dilution of

materials injected into the circulation was being measured, a period of at least 3 minutes was allowed to elapse before sampling in order to permit thorough mixing (see p.123). Furthermore, in these circumstances, early samples were always taken from a vein other than the one used for injection, usually from one in the other ear.

Blood samples for serum were taken by inserting a 21 guage needle into a dilated vein and allowing about 5 ml blood to flow through the needle into an empty container. Sometimes petroleum jelly was applied to the ear and the vein wall was incised with the cutting edge of a 21 gauge needle or a scalpel blade; blood was then allowed to drip directly into a container. These samples without anti-coagulant were allowed to clot at room temperature; the clot was detached from the wall of the container with a wooden applicator and the sample was left overnight at $4^{\circ}C$ before being centrifuged and the serum separated.

Immunization using Freund's complete adjuvant (FCA)

An attempt was made to raise high titre alloantibody by injecting certain rabbits with allogeneic red cells and FCA i.m.; after an interval of about two weeks, further injections of cells alone were given i.v. on several occasions. In other rabbits, in which the survival of allogeneic red cells given i.v. had repeatedly been normal, cells and FCA were given i.m. at the same time as further i.v. injections of ^{51}Cr-labelled cells.

Cells with FCA. Donor red cells were washed twice in phosphate-buffered isotonic saline and a 50% suspension in saline was prepared. Equal volumes of this suspension and FCA (Difco Laboratories) were mixed by repeatedly drawing the material into, and expelling it from, a syringe through a 21 gauge needle. A total of 1 ml of this preparation was given to each animal in divided doses into the muscles of both hind quarters and the loose s.c. tissue at the back of the neck; occasionally, four equal injections were given into the muscles of each limb.

Boosting injections of cells alone. When attempting to produce high titre alloantibody, red cells alone were injected i.v. about 2 weeks after the injection of cells with FCA. The cells were first washed twice and resuspended in saline. Between 0.1 and 0.5 ml red cells were usually given repeatedly at about 2 week intervals and as soon as the antibody titre was sufficiently high, regular plasmapheresis was undertaken.

Plasmapheresis

In order to obtain large volumes of plasma from any particular rabbit, a plasmapheresis technique was used which enabled the collection of about 150-200 ml plasma per week. A 19 or 20 gauge Butterfly infusion set was inserted into the central vein of one ear, and through this it was possible to withdraw blood at the rate of about 20-30 ml per minute, at least initially. In this way, 60-80 ml blood could be obtained in a few minutes. While the blood (anticoagulated with ACD) was being separated, the i.v. line was kept open with a slow

infusion of heparin-saline from a constant-rate infusion pump. The separated red cells, suspended in saline, were then reinfused. The ACD plasma was recalcified by the addition of 0.4 ml M calcium chloride solution for every 6 ml ACD solution used, and was allowed to stand at 4°C overnight before the fibrin clot was removed. The whole procedure could be completed within ½ an hour and could be repeated frequently (twice in one day on occasions) with no apparent ill effect on the donors (see p.126). Usually, two or three donations a week were taken. Antibody titres were measured in several rabbits during the course of such a bleeding programme and were generally found to be unaltered over the period of 1-2 weeks studied.

Serology

In all of the serological tests described below, 3.3% magnesium sulphate solution was used for washing red cells, for making red cell suspensions and for diluting sera. The use of magnesium sulphate solution avoids non-specific agglutination which is sometimes observed when red cells are suspended in isotonic saline (Heard, Hinde and Mynors, 1949).

Blood grouping antisera. Antisera detecting the red cell antigens Hg^A, Hg^D and Hg^F were kindly provided by Mrs. Valerie Joysey and Dr. Carl Cohen. Details of these antisera and the way in which they were used is shown below.

Source	Specificity	Dilution	
V. Joysey	Anti Hg^A (5)	1 in 10	Used with 1% suspension of washed red cells
	Anti Hg^F (1275)	1 in 10	
	Anti Hg^D (2855)	1 in 10	
C. Cohen	Anti Hg^A	1 in 20	Used with 1% suspension
	Anti Hg^F	1 in 25	of washed red cells
	Anti Hg^D	1 in 25	treated with ficin

Preparation of red cells for use in agglutination tests.

Blood samples were anticoagulated with liquid heparin or ACD solution and the red cells were washed three times in 3.3% magnesium sulphate solution.

1. For use in tests with untreated cells, 1% or 5% suspensions of the washed cells were prepared in 3.3% magnesium sulphate solution.

2. Red cells were treated with papain (British Drug Houses) by Low's method (1955). A 5% suspension of treated cells in 3.3% magnesium sulphate solution was used in the agglutination tests.

3. Ficin-treated red cells were prepared as follows: 0.25 g ficin (Koch-Light) was stirred with 25 ml phosphate buffer, pH 7.4 (Hendry, 1948) for 10 minutes at room temperature. The solution was centrifuged and filtered. For use, this stock solution was diluted 1 in 10 with 3.3% magnesium sulphate solution. 2 volumes of

dilute ficin solution were added to 5 volumes of a 5% suspension of red cells, and the mixture was incubated at 37°C for 15 minutes. The cells were washed twice in magnesium sulphate solution at 37°C and a 5% suspension was prepared for use in the agglutination tests.

Agglutination tests. When reading agglutination tests, the results were graded as recommended by Dunsford and Bowley (1955, p.91).

1. For tests with antibodies capable of agglutinating untreated red cells, equal volumes of serum or diluted serum and a 1% suspension of cells were incubated at 37°C for 1 hour. The results were read microscopically.

2. For incomplete antibodies, an indirect antiglobulin test was used with a specific sheep anti-rabbit IgG reagent (H 139). This antiserum, provided by Dr. R.S. Lane, gave only a single line on immunoelectropheresis against whole rabbit serum; it was used in a dilution of 1 in 40 in the antiglobulin tests. One volume of test serum or diluted test serum and 1 volume of a 5% suspension of red cells were incubated at 37°C for 1½ hours. The red cells were washed three times in magnesium sulphate solution and the results were read by a standard tile technique.

3. When enzyme-treated cells were used in agglutination tests, one volume of serum or diluted serum was incubated at 37°C for 5 minutes and one volume of a 5% suspension of the treated cells, also pre-warmed to 37°C, was then added. Incubation was continued for a further 1 hour and results were read microscopically on heated slides.

Antibody detection. Sera were screened for the presence of antibodies directed against donor red cells by means of the following tests:

1. Agglutination of untreated cells suspended in magnesium sulphate solution.
2. Indirect antiglobulin test.
3. Tests with enzyme-treated cells.

Whenever positive results were obtained, the specificity of the antibody was sought by repeating the appropriate test(s) using no less than four examples of cells positive for the antigen and no less than five examples of cells lacking it.

Controls. Known positive and negative cells and sera as appropriate were processed in parallel with each batch of serological tests to act as reagent controls and also to detect non-specific agglutination. In addition, when serum from a rabbit given injections of allogeneic red cells was tested for antibody, serum taken from the same rabbits prior to injection with donor cells was also set up in parallel in most cases.

Radioactive isotopes

Details of the four isotopes used during the course of this work are given below.

Isotope	Half Life	Type of Emission Measured	Emission Energy MeV	Compounds used
^{51}Cr	27.8 d	γ	0.323	Sodium chromate
^{125}I	60 d	γ	0.035	Sodium iodide
^{131}I	8 d	γ	0.364	Sodium iodide
^{32}P	14.3 d	β	1.71	Diisopropyl phosphorofluoridate Sodium orthophosphate

Measurement of ^{51}Cr, ^{125}I and ^{131}I. These isotopes were measured by virtue of their γ-ray emissions in a standard, two-channel, well-type scintillation counter (Packard Instruments Ltd.). Appropriate corrections to observed counting rates had to be applied when either of the two isotopes was detected on both channels at the selected settings. In these cases, correction factors were determined empirically by counting samples of each isotope alone on both channels; "stray" counts on the inappropriate channel could then be expressed in terms of the true counting rate for that isotope measured on the correct channel. The combination of isotopes used in these experiments was such that only one of the pair gave rise to "stray" counts.

Measurement of ^{32}P. A liquid sample Geiger Muller Tube (Type MCH 20th Century Electronics Ltd., Surrey) was used for measuring this isotope. The operating voltage used was that recommended by the manufacturer and checked in this laboratory. This tube requires a sample of at least 10.5 ml. To meet this requirement, rabbit blood samples obtained in the following experiments had to be diluted at least 1 in 11 for determination of ^{32}P activity. Since the samples were diluted in water the internal quenching effect of the samples was negligible and no correction for this factor had to be applied. Counts were recorded using a Panax scaler, with a "dead time" setting of 400 μs. Corrections for "dead time" were applied according to a standard formula (see Belcher and Vetter, 1971, p.64).

Accuracy of counting. When N = total number of counts observed for a given sample:

$$\text{Standard deviation (S.D.)} = \sqrt{N}$$

$$\text{Coefficient of variation}(\sigma) = \frac{\sqrt{N}}{N} \times 100$$

In the experiments to be described, most of the samples were sufficiently radioactive to enable more than 10,000 counts to be recorded in a reasonable period of time. However, towards the end of red cell survival experiments and in certain other situations where samples of low activity had to be counted, a coefficient of variation of up to 3 or 4% had to be accepted.

Labelling of red cells with ^{51}Cr *in vitro*

In most of the experiments in which ^{51}Cr-labelled red cells were used, a citrate-wash method of labelling was employed (Mollison, 1961; Garby and Mollison, 1971). In one series of experiments, the results of red cell survival studies obtained with this method were compared with those in which an ACD method of labelling was used (see p.184).

Sodium chromate (^{51}Cr) solution B.P. (CJS.1P) was obtained from the Radiochemical Centre, Amersham. For use, this solution was diluted with a sterile isotonic saline so that the required activity was contained in a volume not less than 0.2 ml. Methods for the preparation of citrate-phosphate-dextrose (CPD) and acid-citrate-dextrose (ACD) solutions are given by Mollison (1961a)and Loutit and Mollison (1943) respectively.

Labelling of red cells with ^{32}P *in vitro*

In a few experiments red cell volume was determined using autologous red cells labelled with ^{32}P (see Mollison, 1972, p.691) Phosphorus-32 (PBS.1) was obtained from the Radiochemical Centre, Amersham.

Labelling of red cells with diisopropyl phosphofluoridate ($DF^{32}P$) *in vivo*

$DF^{32}P$ was used as an *in vivo* red cell label to study autologous red cell survival in the rabbit. The effect of injecting different doses of $DF^{32}P$ on the amount bound by the red cells and also the effect of dose on the initial rate of elution of the label were also investigated. The method of labelling was the same in all of these experiments.

Reagents. DFP (^{32}P) injection (PB. 117P/118 P. Radiochemical Centre, Amersham). Sterile solution of diisopropyl phosphorofluoridate (^{32}P) in propylene glycol. Specific activity 200-400 μCi/mg DFP. For use, this solution was diluted immediately before injection with sterile isotonic saline.

Procedure. A 1 ml syringe was filled with freshly diluted DF^{32}P solution and was connected to a 19 gauge Butterfly infusion set. Air was expelled from the system and the volume was adjusted to the 1 ml mark on the syringe. The primed apparatus was connected to a variable rate infusion pump, set to deliver 1 ml in about 10 minutes. The Butterfly needle was inserted into the central vein of one ear, following which the pump was started. On completion of the infusion, the needle was removed from the vein and gentle pressure applied to the puncture site for a few minutes to prevent leakage. One hour was allowed to elapse before taking blood samples from the other ear.

Preparation of IgG from whole rabbit serum

Pure IgG was prepared from whole rabbit serum by precipitation with ammonium sulphate followed by DEAE chromatography. Serum or recalcified plasma obtained by plasmapheresis was used as starting material.

19 volumes saturated ammonium sulphate solution were added to 31 volumes serum and the mixture was stirred continuously for ½ hour at room temperature. The precipitate was separated by centrifugation

and was redissolved in a volume of distilled water about half that of the starting serum sample. The solution was concentrated to about 10 ml by pressure dialysis and left in equilibrium with 0.02 M Tris buffer overnight.

A column of DEAE (DE52) was set up as follows. 100 g DE52 was washed first in 1 litre 0.2 M Tris buffer pH 7.9, then in ten separate 1 litre volumes of 0.02 M Tris buffer. A column (100 x 2 cms) was packed with the final suspension and 0.02 M Tris buffer was run through overnight. The protein solution was applied to the top of the column and eluted with 0.04 M Tris buffer. Fractions were collected using an LKB Uvicord fraction collector, the protein concentration being monitored by spectrophotometry at 280 nm.

IgG was eluted in the first peak although subsidiary peaks were sometimes observed which also contained IgG alone. The nature of the protein was demonstrated by immuno-electrophoresis. Fractions from peaks containing only IgG were pooled and concentrated by pressure dialysis.

Sometimes, phosphate buffer pH 6.5 was used, in which case the eluting buffer concentration was 0.0175 M. The method used for preparation of IgG for metabolic studies was different from that given above and is described in detail in the appropriate section.

Trace labelling of IgG with radioactive iodine

The procedure used in these experiments is a modiciation of the iodine monochloride method for protein labelling described by McFarlane (1958). In principle, carrier- and reducing agent-free radioactive iodine (as sodium iodide) is added to a solution of iodine monochloride. When the pH is raised to about 9, hypoiodite is formed. Under alkaline conditions the hypoiodite is available for iodine exchange with the radioactive iodide present, and also for substitution in tyrosine rings of the protein.

Reagents

1. Molar glycine in 0.25 M sodium chloride solution.
2. Molar sodium hydroxide solution.
3. Iodine monochloride (ICl) solution containing 0.421 mg I per ml.
4. Sodium iodide (^{125}I) for protein iodination, (IMS 30, Radiochemical Centre, Amersham). Sodium iodide (^{125}I) in NaOH solution, pH 8-11, free from reducing agent. Specific activity 14 mCi/µg iodine.
5. Sodium iodide (^{131}I) for protein iodination (IBS. 3. Radiochemical Centre, Amersham). Sodium iodide (^{131}I) in NaOH solution, pH 8-11, free from reducing agent. Specific activity 7-26 mCi/µg iodine.
6. IgG solution - approx. 10 mg protein per ml in isotonic saline.

Procedure. Glycine buffer was prepared by mixing 17 parts reagent 1 with 3 parts reagent 2; the final pH was adjusted to 9.1 by the dropwise addition of M NaOH solution. 1 ml of this buffer was added to the protein solution in a universal container and throughout the labelling procedure the mixture was stirred rapidly by means of a magnetic

stirrer, care being taken to avoid frothing. In order to label about 10 mg protein, 0.2 ml ICl solution (reagent 3) was pipetted into a 10 x 70 mm test tube and mixed with an appropriate volume of radioactive sodium iodide solution. To this was added 1 ml glycine buffer; the contents of the tube were mixed rapidly using a Pasteur pipette and were transferred by dropwise addition as quickly as possible to the stirred buffered protein solution. The resulting solution was dialysed against several changes of buffered isotonic saline, pH 7.4, to the first litre of which 0.5 ml 20% potassium iodide had been added. The volumes of the pooled dialysis fluid and the dialysed protein solution were recorded and their radioactivity was measured in order to determine the percentage uptake of iodine, the molar ratio of iodine to protein and the specific activity of the labelled preparation.

Calculations

a) Percentage uptake

Let V_D = volume of pooled dialysis fluid

C_D = its activity (ct/min per ml), also

V_P = volume of labelled protein solution and

C_P = its activity

Then total counts in dialysis fluid = $V_D \times C_D$

and total counts in protein solution = $V_P \times C_P$

Therefore, the percentage, Y, of the added radioactive iodine taken up by the protein is given by:

$$Y = \frac{V_P \times C_P}{(V_P \times C_P) + (V_D \times C_D)} \times 100$$

b) Molar ratio of iodine to protein

Let P = amount of protein labelled (mg)

I = amount of iodine (mg) added in the ICl solution

and Y = percentage uptake of iodine, as calculated in (a) above.

Expressed as moles, the amount of protein labelled

$$= \frac{P \times 10^{-3}}{1.6 \times 10^{5}}$$, where the relative molecular mass of IgG = 1.6×10^{5}

and the amount of iodine bound

$$= \frac{I \times 10^{-3}}{127} \times \frac{Y}{100}$$, where the relative molecular mass of I = 127

The iodine to protein molar ratio in the labelled preparation is thus

$$\frac{I \times 1.6 \times 10^{3} \times Y}{127 \times P}$$

c) Specific activity

This is derived from the mass of protein labelled and its radioactivity (μCi). It is expressed as μCi per μg protein.

Immunoelectrophoresis

Electrophoretic separation of serum proteins in agar gel followed by identification of the separated components with appropriate antisera by immunodiffusion was carried out according to the method of Grabar and Williams (1953). The following antisera were used during the course of the work:

Hyland goat anti-rabbit whole serum 8233 H002A1.

Hoechst anti-rabbit whole serum 1428R.

Sheep anti-rabbit whole serum SKO IPI.

Goat anti-rabbit IgG GK 16-I-PI.

Goat anti-rabbit IgM GK 15-2-PI.

Estimation of IgG in pure solution

The concentration of a protein in solution may be determined by measuring the optical density at 280 nm. Absorption of light at this wavelength is a property of certain amino acids with aromatic side chains (such as tyrosine) and for any given protein species is therefore directly related to the concentration of the protein present.

Observations by Dr. N.C. Hughes-Jones in this laboratory have shown that a solution containing 100 µg human IgG per ml (as determined by the Kjeldahl method), when measured at 280 nm with a 1 cm light path, has an optical density of 0.135. This value was used in the present work for estimating the protein concentration of solutions containing IgG alone.

Procedure. Dilutions of the test solution of IgG were made in phosphate-buffered isotonic saline pH 7.4 so that the optical density observed at 280 mµ, using silica cells with a 1 cm light path, was between 0.1 and 0.2. From the above-mentioned relationship, the concentration in the undiluted sample could be deduced.

Estimation of IgG in whole serum

A quantitative single-phase radial-diffusion technique with antibody to IgG incorporated in an agar gel was used (Fahey and McKelvey, 1965).

Sheep anti-rabbit antiserum (H 139) was kindly provided by Dr. R.S. Lane. The appropriate dilution of antiserum for incorporation into the agar gel was determined empirically on the basis of ring size and edge definition over the range of IgG concentrations studied, and the optimal dilution was found to be 1 in 5. A pure solution of IgG was prepared as described on p.81 . Its concentration was determined as described above, and appropriate dilutions extending over the concentration range of the samples to be tested were used to prepare a standard curve. Test sera and standards were added to wells in the agar gel in volumes of 2 µl. The precipitin rings were allowed to develop for 24 hours at 4°C. Two measurements of the diameter of each ring were made at right-angles using a magnified micro-scale.

Haemoglobin concentration

Except in the section dealing with normal rabbit haematology (p. 89) in which blood counts were carried out on whole blood samples using a Coulter Counter Model "S" (Coulter Electronics), haemoglobin (Hb) estimations were made using either the cyanmethaemoglobin or the oxyhaemoglobin method (see Dacie and Lewis, 1968, pp. 37 and 39).

Packed cell volume

Packed cell volume (PCV) was measured either directly using Wintrobe's method or a microhaematocrit method, or it was deduced from the Hb concentration of the sample. In conformity with recommended current practice, PCV is expressed as a proportion of unity rather than as a percentage (e.g. 0.362, not 36.2%)

Wintrobe's method. As a relatively large volume of blood is required for this method it was used only rarely. Blood was spun in Wintrobe Haematocrit tubes in a centrifuge of radius 15 cms for 55 minutes. A correction for trapped plasma was applied (Chaplin and Mollison, 1952).

Microhaematocrit method. Blood was centrifuged in capillary tubes in a high speed microhaematocrit centrifuge for 5 minutes and the PCV was measured directly with a microhaematocrit reader (Hawkesley Ltd., England). No correction was applied to the observed results for two reasons: first, estimates of the amount of plasma trapped using this method vary from 1 to 3% (see Mollison, 1972, p.111), and secondly, the estimates referred to were made using human blood which has a higher mean corpuscular volume (MCV) than that of rabbits (see p.95), a factor which is probably important in determining the amount of plasma trapped.

PCV deduced from Hb concentration. In some cases, packed cell volume was deduced from the Hb concentration of the sample. Assuming a mean corpuscular haemoglobin concentration (MCHC) of 34.5 g per dl packed cells (see p.96) the PCV is given by: $\frac{\text{Hb (g per dl)}}{34.5}$

SOME OBSERVATIONS ON THE HAEMATOLOGY AND PHYSIOLOGY OF NORMAL SANDY-LOP RABBITS

PERIPHERAL BLOOD COUNTS

Procedure

Results

Discussion

Blood counts were determined on the peripheral blood of a number of the rabbits used during the course of this work in order to establish normal values for this laboratory. Details of these investigations are given here.

Procedure

Approximately 4 ml venous blood was taken into pots containing EDTA (1-2 mg per ml blood). All samples were processed within an hour of collection.

A full blood count comprised estimations of haemoglobin (Hb), haematocrit (Hct), mean cell volume (MCV), mean corpuscular haemoglobin concentration (MCHC), red cell count (RBC) and white cell count (WBC), measured with a Coulter Electronic Counter, Model S. The instrument was standardized against the manufacturer's 4C reference control. Differential leukocyte counts were performed on blood films stained with May Grunwald-Giemsa, according to the method described by Dacie and Lewis (1968, p.62); 200 cells were counted. Reticulocyte counts were estimated on supravitally stained preparations using brilliant cresyl blue and 1000 cells were counted (see Dacie and Lewis, 1968, p.30).

Results

The results have been divided into two groups based upon the age and the previous bleeding history of the rabbits tested.

Group I. All the animals in this group were aged about 5 months, had not been used in any experiments and had had only about 5 ml blood taken for blood grouping tests several weeks before sampling for the present investigations. Ten full blood counts, 11 total and 9 differential white blood counts, and 9 reticulocyte counts were done.

Group II. Twenty animals tested were aged between 1 and 2 years and had been involved in experiments in which 1 ml blood samples had been taken with varying frequency over a period of about six months. The total volume of blood removed varied between about 10 and 30 ml. None had been bled for at least two weeks preceding these examinations. Full blood counts were performed.

Individual Coulter results for the two groups are shown in Tables 5 and 6, and are compared in Table 7 using Student's "t" test. Results for reticulocyte and differential leukocyte counts are shown in Tables 8 and 9 respectively.

Discussion

Archer (1965) suggests that veterinary "normal values" are best determined locally, first because various methods have been used to establish published figures, and secondly because the values are likely to be affected to some extent by prevailing environmental factors. Certainly, in the case of rabbits, diet has been shown to influence blood counts (Courtice and Gunton, 1949) and, possibly related to diet, seasonal variations in counts have also been described (Pearce and Casey, 1930a). The work of Armin, Grant, Pels and Reeve (1952) indicates that

[illegible]	Wintrobe	5.06	12.2	35.5	70	24	34	..	..	54.0	5.0	0	41.0	..		
[illegible]	Wintrobe[3]	6.52	10.9	36.0	55	17	30	..	..	32.0	15.0	0	53.0	..	..	1
[illegible]	Wintrobe[3]	15.00	14.9	36.9	25	10	40	..	..	47.5	1.5	6	50.0	1.0	..	2
	Knoll[5,6]	..	..	..	..	..	..	..	..	66.0	4.8	4.0	21.9	3.3	..	4
	Ponder *et al.*[8]	15.35	..	..	..	..	..	7.4 x 4.0	11.2	57.0	7.4	24.0	7.4	4.3		
[illegible]key	Hall[16]	4.94	..	..	..	..	..	..	14.2	42.6	2.8	0.3	52.1	1.2	..	8
	Ponder *et al.*[8]	5.50	..	..	..	..	..	7.2	8.4	71.5	2.5	2.5	22.0	1.5	..	3
	Shukers *et al.*[45]	5.20	12.2	40.0	77	24	31	..	15.1	36.0	..	..	59.0	..	475	19
		(4.6–5.8)	(10.9–13.5)	(36–44)					(9.7–20.5)	(20–52)			(44–74)			
	Wintrobe[3]	5.44	13.2	43.5	84	26	31	..	..	23.0	3.0	1.5	70.5	2.0	..	4
[illegible]ea pig	Petri[15]	7.81	..	38.7	48	..	..	..	6.6	28.0	..	..	65.0	7.0	698	44
	Drastisch[10]	9.24	14.3	47.1	51	16	30	..	..	..	..	..	..	..	..	7
	Scarborough[12]	9.70 ± 1.70	..	..	..	..	..	6.1	8.5	26.2	2.0	0.3	67.8	7.5	..	60 ±
	Wintrobe[3]	8.88	15.1	43.0	49	17	35	..	11.0	..	..	..	..	..	270	1
	Kamenoff[25]	9.55	..	..	..	..	..	..	..	..	..	..	..	..	..	118
[illegible]opotamus	Wintrobe[21]	4.00	10.1	31.9	79	26	32	..	12.0	39.0	4.7	1.0	46.0	9.3	250	4
	Jordan[4]	5.90	10.0	35.0	..	..	..	..	15.3	26.0	3.3	1.0	62.8	7.0		
g	Wintrobe[3,46]	7.63	15.0	46.3	58	19	33	5.5	7.0–20.0	..	..	..	..	..	..	300
	Götze[43]	7.09 ♂	13.0	43.2	60	18	30									
		6.90 ♀	11.8	38.9	57	17	30									
	Scarborough[12]	6.74	..	..	..	..	..	6.1	8.0–20.0	39.0	4.5	1.2	52.1	3.3	..	215
[illegible]bbit	Wintrobe *et al.*[20]	6.29 ± 0.60	13.0 ± 1.5	39.8 ± 4.3	64 ± 4	21 ± 1.6	33 ± 1.7	..	..	..	..	..	..	..	..	61
	Casey *et al.*[47]	5.37	11.9 (?)	(males only)		..	..	..	7.7	49.4*	1.5	6.7	32.9	9.5	500	180
	Scarborough[12]	5.62 ± 1.20	..	..	..	..	..	6.7	7.9	43.4*	2.0	4.3	41.8	9.0	500	900 ±
[illegible]t	Wintrobe *et al.*[20]	6.60 ± 0.76	13.0 ± 1.2	39.4 ± 3.6	61 ± 4	20 ± 2.1	33 ± 2.3	..	8.5	4.0	0.5	0	90.0	..	330	73
	Scarborough[12]	8.50 ± 1.50	..	..	..	..	..	6.3	8.0–15.0	27.0	2.1	0.8	67.9	5.3	800	200 ±
	Wills and Mehta[44]	9.55	14.0	..	..	..	..	5.9								
	Cameron and Watson[49]	8.50	14.6	43.5	52	17	30	5.98	21.4	8–24	0–4	..	70–89	1–6	673	..
		8.70	13.8	41.8	49	16	34	6.14	20.4	..	..	..	..	..	532	..
[illegible]ep	Wintrobe[3]	10.50	12.9	35.7	35	13	35	..	5.0	28.0	8.0	0	62.0	2.0	350	4
	Drastisch[10]	10.90	11.8	38.5	35	11	31	..	..	..	..	..	..	..	..	1
[illegible]unk	Wintrobe[3]	10.00	15.1	51.4	54	16	30	..	16.0	48.0	7.0	0	42.0	3.0	540	2
[illegible]ock[illegible]uck	Jordan[4]	7.33	13.9	48.0	66	19	30	7.4	15.7	70.2	1.7	0.4	26.2	0.7	..	3

R.B.C., red cell count in millions per c.mm.; Hgb., hemoglobin in grams per 100 cc. blood; Ht., volume of packed red cells in cc. per 100 cc.; M.C.V., mean corpuscular volume in cubic microns; M.C.H., mean corpuscular hemoglobin in micromicrograms; M.C.H.C., mean corpuscular hemoglobin concentration in per cent; Diam., mean erythrocyte diameter (in dry smears) in microns; W.B.C., leukocyte count in thousands per c.mm.; N., neutrophils; E., eosinophils; B., basophils; L., lymphocytes; Mo., monocytes, all per cent; Pl., platelets in thousands per c.mm. Values following ± in most instances represent 1 standard deviation.

*In rabbits and in guinea pigs the cells listed under "N" are "pseudo-eosinophils," "amphophils" or "heterophils." The granules are eosinophilic for the most part, rounded and of fairly uniform size but are not as densely packed as in true eosinophils. In these animals the granules in the true eosinophils are very abundant, large and ovoid or bluntly uniform.

different strains of rabbits may have different "normal values". Likewise, within a given strain, certain measurements such as haemoglobin and PCV have been found to be related to body weight (and, by inference to maturity) of the individual rabbit (Bocci and Viti, 1966).

Because of differences in methodology, little is to be gained by a close comparison of published data with the present findings; however, when information is available certain similarities and differences are worthy of brief comments.

Packed cell volume has been measured in a reliable way by several authors and some examples of mean values from published work are shown below, together with the mean haematocrit values for groups I & II in the present work. The differences are unremarkable in view of what has already been said about expected variation between animals.

Author	Rabbits		PCV or Hct	Method
Armin et al., 1952	100	"Albino"	0.353	Wintrobe
	9	"Brown"	0.336	Wintrobe
Zizza and Reeve, 1958	5	?	0.403	Wintrobe
Mott, 1965	5	?	0.396	*Microhaematocrit
Chalmers, Corner and White, 1967	10	NZW	0.368	Wintrobe
Knorpp and Pixberg, 1970	39	NZW	0.367	Microhaematocrit
Present work; Groups I and II, 1974	30	S. Lop	0.383	Coulter "S"

* Not corrected for trapped plasma

While there seems to be good agreement about the figure for total white cell count in normal rabbit blood (means of about 10 x 10^9 per l), several authors find the major cell population to be lymphocytes (Scott and Simon, 1924; Bushnell and Bangs, 1926), whereas others report a predominance of neutrophils (Cunningham, Sabin, Sugiyama and Kindwall, 1925; Pearce and Casey, 1930b). In the present work lymphocytes invariably outnumbered neutrophils, usually to a considerable extent (Table 9). This variation may be related to the strain of rabbit under investigation but the age of the rabbits tested may also be important. Although differential counts were measured only in young animals (taken from Group I), total white cell counts for older rabbits in Group II were of similar magnitude, so that any decrease in the proportion of lymphocytes would have been associated with a corresponding increase in neutrophils. However, single observations in two somewhat older animals did show slightly reduced total counts with a small predominance of neutrophils.

Reticulocyte counts were of an order of magnitude to be expected from consideration of the red cell life-span (see p.195) assuming that reticulocytes retain their characteristic staining properties for about 2 days after leaving the bone marrow. Values ranging from 1-2.7% have been reported by Card & Valberg (1967) in New Zealand White rabbits.

Comparison of Groups I and II (Table 7) shows that in Group II, red cell indices were slightly lower with the exception of MCV which was increased. Though minor, the differences in Hb, MCV, MCHC and RBC were significant at the 1% level. No characteristic haematological picture emerged from these observed minor changes which are possibly

related to a number of factors, such as increasing age of the rabbits maintenance under specific laboratory conditions and repeated blood loss by sampling. In this connection, Valberg, Card, Paulson and Szirek (1967) were unable to show any significant difference between MCV in 16 New Zealand White rabbits made anaemic by daily bleeding for one month (73 $\pm$ 4.4 fl; range 66-76) and 12 control animals (71 $\pm$ 3.4 fl; range 67-80). The red cells were suspended in Eagle's medium for determination of MCV on a Model B Coulter Counter. It may be relevant that iron stores of the chronically bled rabbits were supplemented by i.m. injections of iron before bleeding was started.

With regard to morphology, rabbit peripheral blood films are similar to those of man with the following qualifications. Red cells are smaller (human MCV 82-92 fl). Neutrophil granules are larger and more eosinophilic (called pseudo-eosinophils by some authors - Pearce and Casey, 1930c) although these cells remain easily distinguishable from eosinophils when stained with May Grunwald-Giemsa stain. About 8% of white cells were disrupted during preparation of films, thereby obscuring their identity; this was an invariable finding.

Table 5. Haematological values in the normal rabbit (Group I)

Rabbit Number	Hb (g/dl)	Hct	MCV (fl)	MCH (pg)	MCHC (g/dl)	RBC/pl	WBC/nl
R 15	14.0	0.392	62	22.2	35.8	6.31	
16	13.3	0.373	61	22.0	35.9	6.07	
17	13.6	0.383	64	22.9	35.6	5.93	
18	13.2	0.365	64	23.0	36.2	5.70	
19	15.1	0.429	64	22.5	35.4	6.72	
20	14.3	0.398	63	22.6	36.0	6.32	
21	14.3	0.397	63	22.6	36.0	6.29	
22	14.0	0.384	63	23.0	36.6	6.08	
23	13.7	0.368	60	22.3	37.3	6.12	
24	15.2	0.414	60	22.2	36.7	6.83	
Mean (10)	14.1	0.390	62.4	22.5	36.1	6.24	(21) 11.8
S.d.	0.7	0.020	2.5	0.3	0.6	0.34	2.0
S.e.	0.2	0.006	0.5	0.1	0.2	0.1	0.4

Values ranging from 8.5-15.0 in 21 normal rabbits (R1-9, 11, 12, 15-24)

Table 6. Haematological values in the normal rabbit (Group II)

Rabbit Number	Hb (g/dl)	Hct	MCV(fl)	MCH(pg)	MCHC(g/dl)	RBC/pl	WBC/nl
S 65	13.8	0.407	65	22.0	34.2	6.24	11.6
66	12.7	0.366	63	21.6	35.0	5.83	9.9
68	13.6	0.398	64	21.7	34.5	6.25	10.4
70	13.2	0.385	66	22.7	34.6	5.75	9.3
72	12.2	0.360	65	21.9	34.2	5.53	13.5
73	11.6	0.351	66	21.8	33.5	5.30	8.4
76	11.4	0.338	62	21.0	33.9	5.37	11.0
77	12.8	0.376	64	21.9	34.4	5.82	13.4
79	11.9	0.357	64	21.5	33.7	5.51	11.9
80	12.6	0.368	66	22.7	34.5	5.52	15.0
83	12.7	0.369	64	22.0	34.7	5.72	11.2
85	13.2	0.379	64	22.2	34.9	5.88	9.1
87	13.7	0.393	68	23.7	35.2	5.76	16.6
88	13.4	0.392	68	23.3	34.5	5.71	15.1
89	13.0	0.373	64	22.4	35.5	5.77	15.0
90	13.5	0.397	66	22.5	34.4	5.97	11.9
91	13.5	0.406	72	23.8	33.5	5.61	12.3
92	14.8	0.429	67	23.0	34.7	6.39	13.1
95	12.8	0.368	65	22.7	35.0	5.60	11.3
98	13.5	0.393	65	22.1	34.6	6.05	13.4
Mean (20)	13.0	0.380	65	22.3	34.5	5.78	12.2
S.d.	0.8	0.022	2	0.7	0.5	0.29	2.2
S.e.	0.2	0.005	0.5	0.2	0.1	0.1	0.5

Table 7. Haematological values in the normal rabbit. Comparison of results from groups I and II

	Hb (g/dl)	Hct	MCV (fl)	MCH (pg)	MCHC (g/dl)	RBC/pl	WBC/nl
Group I	12.7-15.5	0.350-0.430	58-66	21.9-23.1	34.9-37.3	5.56-6.92	7.8-15.8
Group II	11.4-14.6	0.336-0.424	61-69	20.9-23.7	33.5-35.5	5.20-6.36	7.8-16.6
"t"	3.61	1.21	-3.86	0.84	8.01	3.88	-0.612
DF	28	28	28	28	28	28	39

Results are shown as a range determined by the mean ± 2 S.d.

When p = 0.01 critical value of t = 2.8 (DF 28) and 2.7 (DF 39)

Table 8. Reticulocyte counts in the normal rabbit

R 5	2.0%
12	4.1
15	2.8
16	1.9
17	1.9
18	2.9
20	1.8
22	1.6
24	3.4
Mean (9)	2.5
S.d.	0.9
S.e.	0.3

Table 9. Differential white cell counts in the normal rabbit

Rabbit Number	TWC per µl	Neutrophils		Lymphocytes		Monocytes		Eosinophils		Basophils		Smears	
		Abs	%	Abs	%	Abs	%	Abs	%	Abs	%	Abs	%
R 1	13,700	5,800	42.0	6,700	49	400	3.0	0	0	250	2.0	550	4
2	12,900	2,500	19.5	8,800	68	400	3.0	50	0.5	250	2.0	900	7
3	9,000	3,100	34.5	5,050	56	50	0.5	50	0.5	50	0.5	700	8
4	9,700	3,900	40.5	4,400	45	300	3.5	0	0	50	1.0	1,050	11
5	8,500	3,100	36.5	4,850	57	80	1.0	80	1.0	40	0.5	350	4
6	15,000	5,200	34.5	8,250	55	250	1.5	100	1.0	0	0	1,200	8
7	10,700	4,250	39.5	4,850	45	75	1.0	150	1.5	75	1.0	1,300	12
8	12,500	4,450	35.5	6,500	52	120	1.0	120	1.0	60	0.5	1,250	10
9	9,200	1,950	21.0	6,550	71	40	0.5	40	0.5	70	1.0	550	6
Mean (9)	11,200	3,800		6,200		190		65		95		870	
S.d.	2,300	1,260		1,600		150		50		90		350	
S.e.	800	400		500		50		17		30		120	

SOME OBSERVATIONS ON THE HAEMATOLOGY AND PHYSIOLOGY OF NORMAL SANDY-LOP RABBITS

RED CELL OSMOTIC FRAGILITY

Procedure

Results

Discussion

While studying the effect of different concentrations of N-ethylmaleimide (NEM) on the osmotic fragility of rabbit red cells, it was noted that cells which had been washed in dextrose-saline alone were more fragile than human red cells. It seemed relevant therefore to determine whether unwashed rabbit red cells were also more osmotically fragile than human cells.

Procedure

A stock solution of 1 g/dl phosphate buffered saline, pH 7.4, was prepared using a dry powder supplied for osmotic fragility testing by British Drug Houses Limited. Appropriate dilutions of this solution were made in distilled water. Solutions were occasionally kept overnight at 4°C but were generally used only on the day of preparation. The pH of various dilutions was checked from time to time.

Blood samples from eight rabbits were tested in three batches. Three rabbits had not been used in any previous experiment and the remainder had had only small volumes of blood removed up to three weeks before being tested. With each batch, a sample of human blood was tested in parallel. Venous blood was taken through a 21 gauge needle into a dry plastic syringe and was transferred to polystyrene pots containing dry heparin. The samples were mixed gently until the blood appeared well oxygenated. An auto-diluter was used for adding precise volumes (approximately 0.05 ml) of blood to 5 ml of the NaCl solution; the diluter was charged with a given concentration of NaCl and all blood

samples were diluted in duplicate; NaCl solution was then cleared from the machine by flushing through with air, and the next concentration was used to wash out the system several times before proceeding. The cell-saline suspensions were allowed to stand at room temperature for 30 minutes, were then mixed gently and centrifuged at 1,500 g for five minutes. The optical density of the supernatant in each sample was read in duplicate on a Gilford 300 spectrophotometer at 540 nm. Results for each supernatant were expressed as a percentage of the optical density of the cells-distilled water supernatant.

Results

In all animals tested, the red cells were shown to be more osmotically fragile than human cells. Median corpuscular fragility (MCF) of human cells (from one donor) determined on three occasions ranged from 0.420-0.425 (g/dl NaCl). In samples from eight different rabbits, the range of MCF was 0.460-0.490. Details of each test are shown in Table 10.

Discussion

Because these tests were undertaken primarily to provide a baseline for comparison with NEM treated cells, certain refinements such as the preparation of many intermediate saline dilutions and strict temperature control by use of a water bath were not adopted. However, the results with the human samples suggest that adequate precision for the present purposes was attained.

The MCF of normal rabbit red cells which had been washed and incubated with dextrose saline for 2 hours had been found to be 0.495 (see p.294). Results of the 8 tests reported here were somewhat lower (0.460-0.490, mean 0.477) but still indicate a definite increase in fragility compared with human red cells (normal range 0.400-0.445; Dacie and Lewis, 1968, p.13). Card and Valberg (1967) gave values for MCF of 0.44 for normocytes and 0.45 for macrocytes (produced in response to phenylhydrazine induced haemolysis) in New Zealand White rabbits. No detailed work concerning rabbit red cell osmotic fragility appears to have been published.

The present findings of increased osmotic fragility of rabbit red cells compared with human red cells were not entirely unexpected. An important factor influencing the osmotic fragility of red cells is the ratio between their surface area and their volume (Murphy, 1967) and it has been shown (p.95) that red cells are smaller in the rabbit than in man. That this ratio is not the sole factor involved is, however, well illustrated by comparison of camel and sheep red cells (see Ponder, 1948, p.347). The ratio of surface area (in μm^2) to volume (in fl) for camel red cells (55/29) is not very different from that for sheep red cells (67/32) whereas the resistance of the former to lysis in hypotonic NaCl is very much greater than that of the latter. Metabolic factors in red cells must therefore also be of considerable importance in determining fragility characteristics.

Table 10. Percentage haemolysis of rabbit and human red cells exposed to different concentrations of sodium chloride solution. The median corpuscular fragility (MCF) is also shown.

NaCl g/dl	R1	R2	R4	R6	Human	R21	R22	Human	S55	S56	Human
1.0	0	0	0	0	0	0	0	0	0	0	0
0.8	0	0	0	0	0	0	0	0	-	-	-
0.7	0	0	0	0	0	0	0	0	1	1	0
0.6	1	1	1	1	0	0	0	0	3	4	0
0.5	23	11	23	17	0	21	42	1	32	32	4
0,45	79	53	80	72	8	80	90	13	-	-	-
0,4	92	92	96	93	63	91	93	73	92	91	72
0,3	95	95	97	98	85	95	97	98	94	93	95
H_2O	100	100	100	100	100	100	100	100	100	100	100
MCF	0.480	0,460	0,475	0,470	0,420	0.480	0.490	0.420	0.480	0.485	0.425

SOME OBSERVATIONS ON THE HAEMATOLOGY AND PHYSIOLOGY OF NORMAL SANDY-LOP RABBITS

BLOOD VOLUMES

Procedure

Calculations

Results

Discussion

While investigating IgG metabolism in the normal rabbit using ^{125}I-labelled IgG, simultaneous determinations of red cell volume (RCV) and plasma volume (PV) were made in these animals. It was thus possible to derive a value for the relationship between whole body haematocrit (H_B) and venous haematocrit (H_V) which could then be used in other experiments to give an estimate of total blood volume (TBV) and plasma volume when only the circulating volume of red cells had been measured. Details of 30 experiments in 27 rabbits are described in this section.

Procedure

Adult animals of both sexes and varying ages were used. Autologous red cells were labelled either with ^{51}Cr or ^{32}P using a citrate-wash method. ^{125}I-labelled allogeneic IgG which had been "screened" in another rabbit (see p.147) was used for plasma volume estimations. In most experiments, venous haematocrit was measured either by the Wintrobe method or using a microhaematocrit technique; in the remainder, Hb concentration of venous samples was determined and PCV estimated from this value (see p.88).

The method used in individual cases is indicated in the tables of results. Blood samples were anticoagulated with solid EDTA.

Three groups of rabbits are described below in which certain differences in procedure were adopted. In addition, it should be emphasized that all of the rabbits, apart from four in Group 1 (R22, R32, R34, R36) had been involved in other experiments before blood volume

estimations were made. However, those in Groups 1 and 2 were considered "normal" in that they had been bled only intermittently of small volumes of blood totalling no more than 10-20 ml; some had not been bled for several months before the present experiments and none had been bled during the preceding month. Animals in Group 3 had a more varied bleeding history and for this reason have been kept separate from the main analyses. Furthermore, no weights were recorded for the rabbits in Group 3 on the days of the blood volume estimations.

Group 1. Plasma and red cell volumes were determined independently in six rabbits. In two animals (S23, S24), 1 ml serum containing ^{125}I-IgG was injected i.v.; four blood samples, each of about 1.3 ml, were taken during the following hour, the first about 5 minutes after injection. Red cell volumes was then measured by injecting 2 ml of a suspension of ^{51}Cr-labelled red cells. In the other four rabbits the protocol was similar to that already given, except that red cell volume was measured first; furthermore, for the measurement of plasma volume in these 4 rabbits, a different batch of serum containing ^{125}I-IgG was used.

For plasma volume estimation, plasma was separated by centrifugation of the blood samples and ^{125}I activity in 0.5 ml aliquots was measured. Duplicate dilutions of the injection material in alkaline potassium iodide solution (see Dacie and Lewis, 1968, p.357) were also counted. For the estimation of red cell volume the PCV of the sample taken following injection of the labelled cells was measured. Each whole

blood sample was then lysed with saponin, and 0.5 ml aliquots were counted for ^{51}Cr activity. Radioactivity of duplicate dilutions of the injection suspensions in 0.04% (v/v) ammonia water was also determined.

Group 2. In this group of sixteen rabbits, red cell volume alone was measured. Either 1 or 2 ml of a suspension of ^{51}Cr-labelled red cells was injected i.v. and a single sample of about 1.3 ml was taken between 3 and 10 minutes after injection of the labelled cells. The procedure was then as described for red cell volume determination in Group 1.

Group 3. Red cell volumes alone were measured in this group of rabbits. In three animals (S24, S43, S46) ^{51}Cr-labelled red cells were used as already described; in the other five, ^{32}P was used as a red cell label. In principle, measurement of red cell volume using ^{32}P-labelled cells is essentially similar to that for ^{51}Cr, with the exception that a correction has to be applied to the activity determined in blood samples to allow for loss of label due to elution. The precise time of sampling was therefore noted. The elution rate of ^{32}P from rabbit red cells was not determined in these experiments.

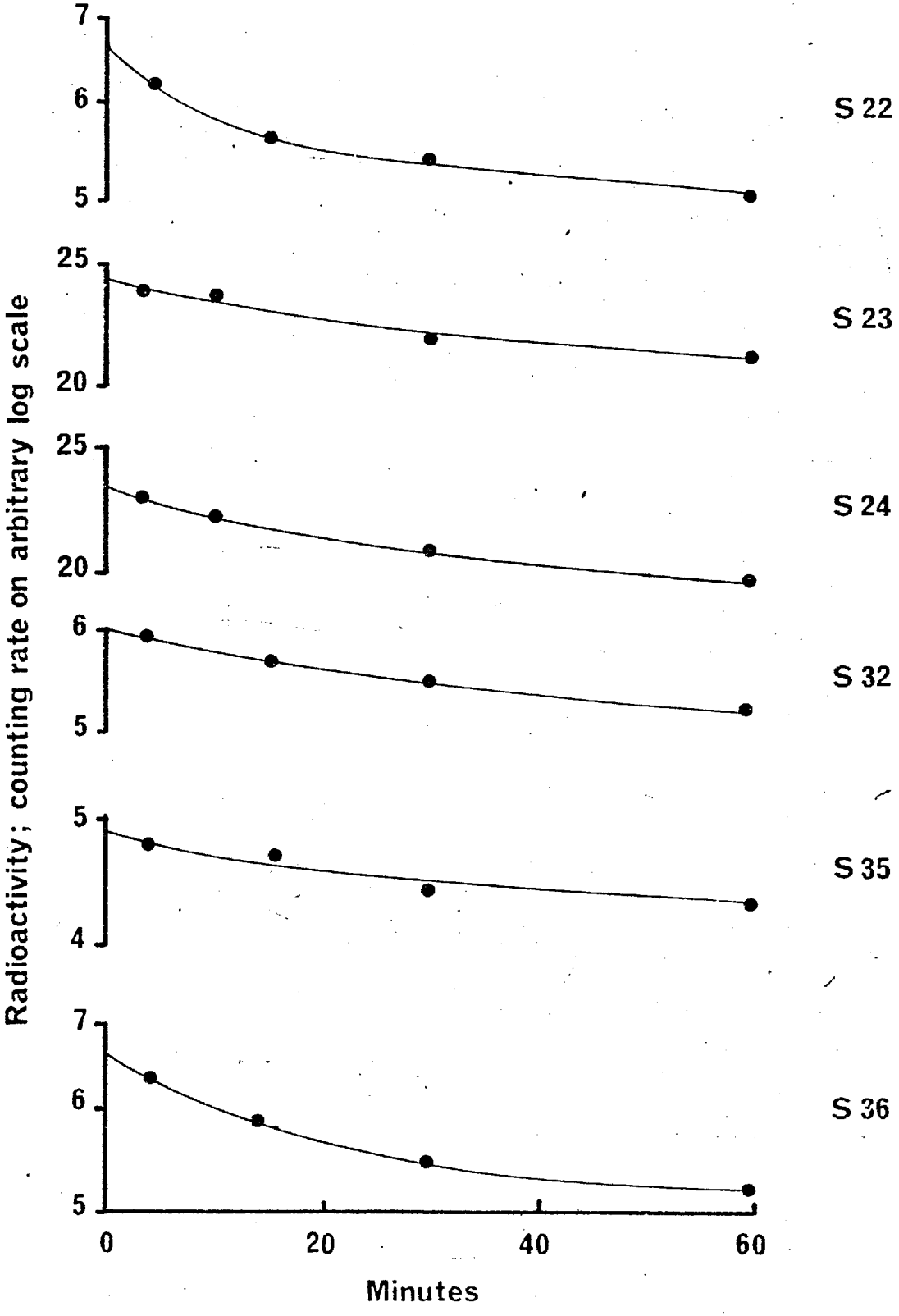
Calculations

a) Plasma volume (^{125}I) is given by:

$$\frac{\text{Radioactivity of standard} \times \text{Dilution of standard} \times \text{Volume injected}}{\text{Radioactivity of plasma sample at zero time}}$$

(NB. Radioactivity = ct/min per unit volume).

Figure 1. ^{125}I activity in the plasma of six rabbits at various times after the injection of ^{125}I-labelled IgG. The activity at zero time was determined by retrograde extrapolation, as shown.


Radioactivity; counting rate on arbitrary log scale
7
6
5
S 22
25
20
S 23
25
20
S 24
6
5
S 32
5
4
S 35
7
6
5
S 36
0
20
40
60
Minutes

The value of the denominator was determined by plotting the serial observations for each case on semilogarithmic paper and extrapolating to zero time. Examples of these plots are shown in Fig.1.

b) Red cell volumes (^{51}Cr) is given by:

$$\frac{\text{Radioactivity of standard} \times \text{Dilution of standard} \times \text{Volume injected}}{\text{Radioactivity of red cells at zero time}}$$

In practice, radioactivity in lysed whole blood was determined and multiplied by 1/PCV to convert to red cell activity. The PCV was corrected, where relevant, for trapped plasma (see p.88). A correction for elution of ^{32}P was applied when this isotope was used for labelling red cells on the assumption that the label is lost at a constant rate amounting to 10% during the first 60 minutes following injection (see Mollison, 1972, p.103-104).

c) In Groups 2 and 3, total blood volume was derived from red cell volume thus:

$$TBV = \frac{RCV}{PCV \times 0.843}$$

where 0.843 was the mean value for H_B/H_V determined by experiment in Group 1 rabbits. Plasma volume in these animals was then obtained by subtraction:

$$PV = TBV - RCV$$

Results

The mean value for the ratio H_B/H_V determined in six rabbits was 0.843 with a range of 0.803 to 0.890 (see Table 11).

Individual and mean values for the measured and derived volumes obtained in Groups 1 and 2 are shown in Tables 11, 12, 13 and 14. Individual results for animals in Group 3 are shown in Table 15 and have not been analysed further for the reasons already given.

Discussion

Several values for the ratio H_B/H_V in the normal adult rabbit have been reported. Armin et al. (1952) measured red cell volume and plasma volume using ^{32}P-labelled red cells and T 1824 respectively in 29 albino rabbits. They obtained a mean value for H_B/H_V of 0.85 (s.d. 0.034) with individual observations ranging from 0.80-0.93. Concentrations of the markers in the blood at zero time were deduced from measurements made on serial samples taken during the first 30 minutes after injection. The use of T 1824 for measurement of plasma volume has been criticized by Zizza and Reeve (1958) on the basis of estimations made in 6 rabbits using ^{131}I-labelled rabbit albumin and the dye simultaneously. They found that T 1824 always gave a larger volume than ^{131}I-albumin even though both zero time concentrations were derived in exactly the same way by retrograde extrapolation. It was suggested that free T 1824 leaked rapidly from the circulation and that any delay in binding to protein would thus account for the observed discrepancy in estimates of plasma volume; in addition the binding of T 1824 to

albumin is reversible which also contributes to its high rate of loss from the plasma (27.4% in the first hour) compared with that of ^{131}I-albumin (15.5%). Estimates of H_B/H_V made in 5 rabbits by these authors using ^{32}P to measure red cell volume and T 1824 (with appropriate corrections) for plasma volume, gave a mean value of 0.895. Chalmers et al. (1967) were unable to confirm the early loss of T 1824 from the plasma. They made simultaneous determinations of plasma volume in 3 rabbits using the dye and ^{131}I-human serum albumin. No precise details of the investigations were given but the authors concluded that both techniques gave equally good results; using T 1824 and ^{51}Cr-labelled red cells, they found the ratio between whole body haematocrit and arterial (aorta) haematocrit to be 0.86 (s.e. of mean 0.017). Although 3 of their rabbits had been adrenalectomised and treated with guanethidine and 3 others had undergone section of carotid sinuses and aortic nerves, the results did not differ significantly from those in 3 normal animals, so that all 9 were treated as a homogeneous "normal" group for analysis.

An entirely different approach using a rapid viviperfusion technique has given a remarkably similar value for H_B/H_V (Mott, 1968). Adult rabbits, anaesthetized with sodium pentobarbitone were simultaneously bled and perfused with Locke's solution. The total amount of haemoglobin and plasma protein recovered from each animal was related to the concentrations in samples of venous blood taken before perfusion to give an estimate of red cell and plasma volumes. The ratio H_B/H_V was 0.87 (s.d. 0.06).

The figure of 0.843 for H_B/H_V found in the present experiments in 6 Sandy lop rabbits is slightly lower than the other quoted values. It may be that the ratio is truly variable in different strains of rabbits. No work has been reported relating specifically to this question, but plasma and red cell volumes in rabbits have been shown to be dependent upon a number of factors. Armin et al. (1952) found a difference (p = approximately 0.08) between red cell volumes in 71 " albino" (17.15 ml/kg; s.d. 1.74 ml) and 9 "brown" rabbits (18.35 ml/kg; s.d. 2.44 ml) suggesting that breed might be important in predicting red cell volume. The two groups were certainly not well matched with regard to sex of the animals but analysis of the figures for the "albino rabbits" shows no significant difference in red cell volumes between 31 male (17.3 ml/kg; s.d. 1.5 ml) and 38 female animals (17.1 ml/kg; s.d. 2.0 ml). Similarly, Knorpp and Pixberg (1970) using New Zealand White rabbits, reported red cell volumes of 19.3 ml/kg (s.d. 2.4 ml) for 10 males and 18.6 ml/kg (s.d. 2.4 ml) for 29 females; the results are not significantly different (p = 0.5). Bocci and Viti (1966) used "screened" ^{131}I-labelled rabbit plasma protein to determine plasma volumes in 35 male rabbits (breed unspecified) of various weights and demonstrated an inverse correlation between plasma volume per unit body weight and total body weight. Finally, it has been pointed out that the nature of the diet may influence blood volumes in rabbits (Courtice and Gunton, 1949).

The ratio H_B/H_V may be falsely reduced if

a) plasma volume is overestimated

b) red cell volume is underestimated

c) venous haematocrit is overestimated

or any combination of these three.

a) The use of iodine-labelled IgG for measurement of plasma volume suffers from the same drawback as the use of labelled albumin, namely, gradual distribution in a space larger than the plasma compartment. Because the rate of loss of both proteins from the plasma is relatively slow compared with the initial mixing rate in the plasma, it is possible to estimate the dilution volume at the time of injection by taking serial samples and extrapolating the measured radioactivity in each sample back to zero time. The accuracy of such an extrapolation procedure will vary with certain characteristics of the injected material and will be greatest when the rate of loss of the marker from the plasma compartment is both slow and steady. In the present studies the average loss of labelled IgG from the plasma during the first hour following injection was about 16.5% which is similar to the loss rate of 15.5% for iodine labelled rabbit albumin found by Zizza and Reeve (1958). The use of "screened" IgG in the present study removes the possibility of very rapid early loss of label which might otherwise occur if denatured protein or free radioactive iodide were present in the injection material. Movement of IgG between intravascular and extravascular compartments is dealt with in greater detail in a later section (see p.142); suffice it to say that a curvilinear plot on semi-log paper of the type shown in Fig.1 is to be expected during the period of these observations. Andersen (1962) found that ^{131}I-labelled IgG gave lower values for plasma volume than ^{131}I-albumin in humans and it has been suggested that over-estimation of plasma volume by T 1824 and by ^{131}I-albumin has been responsible for part of the difference observed between whole body and venous haematocrit (Andersen and Gabuzda, 1964).

It is concluded therefore that the lower values for H_B/H_V obtained in the present series of experiments compared with other published results are unlikely to have arisen from overestimation of plasma volumes.

b) Loss of ^{51}Cr from labelled red cells either before or after injection would lead to overestimation of red cell volume and consequently to an increase in H_B/H_V . Delayed mixing of the injected red cells could in theory give false low values for red cell volume, but is most unlikely to have occurred in the present experiments as mixing of intravenously injected cells in Sandy lop rabbits has been shown to be complete within about 1 minute (see p.123). It is also most unlikely that authors using ^{32}P as a red cell label overestimated cell volume in their rabbits as they used a retrograde extrapolation method for determining the initial ^{32}P level immediately following injection (Armin et al., 1952; Zizza and Reeve, 1958).

c) In this work venous haematocrits determined by Wintrobe's method have been corrected for trapped plasma on the basis of observations made with human blood (Chaplin and Mollison, 1956). Estimates of plasma trapping in microhaematocrit tubes, again using human blood, have varied between 1% and 3% (Garby and Vuille, 1961; England, Walford and Waters, 1972) and are almost certainly directly related to the observed PCV. In the present work, no correction has been applied to the observed PCV determined in this way. Any error involved would therefore tend to overestimate PCV and consequently underestimate H_B/H_V. However, in only 2 of the 6 rabbits in Group 1 was this method used so that the application of a correction does not greatly alter the overall mean value for H_B/H_V.

Table 11. Blood volumes in 6 rabbits (group 1) determined by independent measurement of red cell volume (^{51}Cr-labelled red cells) and plasma volume (^{125}I-labelled IgG); the ratio of whole body haematocrit, H_B, to venous haematocrit, H_V, was calculated from these results.

Rabbit Number	Weight (kg)	Red cell volume		Plasma volume		Total blood volume		H_B	H_V*	H_B/H_V
		(ml)	(ml/kg)	(ml)	(ml/kg)	(ml)	(ml/kg)			
S23	3.25	47.9	14.7	108.6	33.4	156.5	48.1	0.306	0.344 (M)	0.889
S24	3.32	54.0	16.3	112.3	33.8	166.3	50.1	0.325	0.365 (M)	0.890
R22	3.55	50.2	14.1	103.3	29.1	153.5	43.2	0.334	0.392 (W)	0.835
R32	3.37	54.3	16.1	110.0	32.7	164.3	48.7	0.330	0.411 (W)	0.803
R35	3.67	59.0	16.1	134.8	36.7	193.8	51.5	0.304	0.376 (W)	0.809
R36	3.25	48.4	14.9	100.1	30.8	148.5	45.7	0.326	0.400 (W)	0.815
Mean (6)	3.40	52.3	15.4	111.5	32.7	163.3	47.9	0.321	0.381	0.843
S.d.	0.17	4.3	0.9	12.2	2.6	16.6	3.0	0.013	0.025	0.039
S.e. of mean	0.07	1.7	0.4	5.0	1.1	6.8	1.2	0.005	0.009	0.012

* The method used for determining packed cell volume is shown thus: (M) microhaematocrit
(W) Wintrobe

Table 12. Red cell volumes (^{51}Cr-labelled red cells) determined in 16 rabbits (group 2). The whole body haematocrit, H_B, was deduced from the ratio $H_B/H_V = 0.843$.

Rabbit number	Weight (kg)	Red cell volume		PCV (H_V)*	Deduced H_B
		(ml)	(ml/kg)		
S39	2.75	51.7	18.8	0.342 (M)	0.288
S43	2.97	49.1	16.5	0.342 (M)	0.288
S53	2.87	47.7	16.6	0.356 (Hb)	0.300
S54	2.85	43.4	15.3	0.322 (Hb)	0.271
S55	3.40	57.5	16.9	0.348 (Hb)	0.293
S56	3.08	48.5	15.7	0.319 (Hb)	0.269
S57	3.21	53.3	16.6	0.339 (Hb)	0.286
S65	3.43	56.6	16.5	0.372 (M)	0.313
S66	3.46	53.9	15.6	0.388 (M)	0.327
S70	2.75	43.9	16.0	0.372 (M)	0.313
S72	3.28	46.9	14.3	0.362 (M)	0.305
S77	3.07	46.6	15.2	0.379 (M)	0.319
S92	3.85	54.9	14.5	0.375 (M)	0.316
S95	3.87	55.0	14.2	0.352 (M)	0.297
S96	3.51	48.1	13.7	0.374 (M)	0.315
R23	3.90	56.9	14.6	0.359 (W)	0.303
Mean (16)	3.26	50.9	15.7	0.356	0.300
S.d.	0.39	4.7	1.3	0.020	0.017
S.e. of mean	0.10	1.2	0.3	0.005	0.004

* The method used for determining packed cell volume (PCV) is shown thus: (M) microhaematocrit

(Hb) Hb (g/dl) ÷ 34.5

(W) Wintrobe

Table 13. Total blood volumes and plasma volumes in 16 rabbits (group 2) derived from measurement of red cell volumes (^{51}Cr-labelled red cells) and assuming a value of 0.843 for the ratio H_B/H_V.

Rabbit number	Weight (kg)	Total blood volume		Plasma volume	
		(ml)	(ml/kg)	(ml)	(ml/kg)
S39	2.75	179.3	64.5	127.6	45.9
S43	2.97	170.4	56.2	121.3	40.0
S53	2.87	159.0	54.1	111.3	37.8
S54	2.85	160.1	56.0	116.7	40.8
S55	3.40	196.2	56.9	138.7	40.2
S56	3.08	180.3	57.7	131.8	42.2
S57	3.21	186.4	57.8	133.1	41.3
S65	3.43	180.8	52.4	124.2	36.0
S66	3.46	164.8	46.1	110.9	31.0
S70	2.75	140.2	50.5	96.3	34.7
S72	3.28	153.8	46.1	106.9	32.1
S77	3.07	146.1	46.7	99.5	31.8
S92	3.85	173.7	43.4	118.8	29.7
S95	3.87	185.2	46.3	130.2	32.5
S96	3.51	152.7	42.7	104.6	29.3
R23	3.90	187.8	46.9	130.9	32.7
Mean (16)	3.26	169.8	51.5	118.9	36.1
S.d.	0.39	16.5	6.3	12.9	5.1
S.e. of mean	0.10	4.1	1.6	3.2	1.3

Table 14. Mean values for blood volume results in 22 rabbits shown in Tables 11, 12 and 13.

	Wt (kg)	Red cell volume		Plasma volume		Total blood volume	
		(ml)	(ml/kg)	(ml)	(ml/kg)	(ml)	(ml/kg)
Mean	3.30	51.3	15.6	116.9	35.2	168.0	50.5
S.d.	0.34	4.5	1.2	12.9	4.8	16.4	5.8
S.e. of mean	0.07	1.0	0.2	2.7	1.0	3.5	1.2

Table 15. Red cell volumes (^{51}Cr- or ^{32}P-labelled red cells) determined in 8 rabbits (group 3). The whole body haematocrit, H_B, was deduced from the ratio H_B/H_V = 0.843 in order to derive values for total blood volume and plasma volume.

Method	No.	Red cell vol (ml)	H_V*	H_B	Total blood vol (ml)	Plasma vol (ml)
^{51}Cr	S24	53.9	0.336 (Hb)	0.283	190.4	136.5
	S43	50.5	0.355 (Hb)	0.299	168.9	118.4
	S46	35.9	0.289 (Hb)	0.244	147.1	111.2
^{32}P	S32	36.7	0.306 (Hb)	0.258	142.2	105.5
	S43	39.6	0.290 (Hb)	0.244	162.2	122.7
	S50	40.9	0.304 (Hb)	0.256	159.8	118.9
	S51	48.2	0.376 (W)	0.317	152.0	103.8
	S52	36.4	0.270 (Hb)	0.228	159.6	123.2

* The method used for determining packed cell volume is shown thus:

(Hb) Hb (g/dl) - 34.5

(W) Wintrobe

SOME OBSERVATIONS ON THE HAEMATOLOGY AND PHYSIOLOGY OF THE NORMAL SANDY-LOP RABBIT

INTRAVASCULAR MIXING TIME

Procedure

Results

Comment

Some of the experiments to be described in this work involved the determination of dilution factors of material injected intravenously. It seemed appropriate therefore to investigate the time taken following injection, for complete mixing to occur within the circulation of the normal rabbit as a guide to the length of time that should be allowed to elapse before sampling in such circumstances.

Procedure

Seven rabbits were studied. Autologous red cells were labelled with ^{51}Cr and 2 ml of the prepared suspension was injected i.v. over as short a time as possible. A stop watch was started at the beginning of the injection. Samples were taken by syringe, the first as soon as practicable after injection of the labelled cells (usually within one minute). In four animals three further samples were then taken at approximately 2, 3 and 5 minutes, and in the other three animals only one further sample was taken, between 4 and 11 minutes after injection. Radioactivity was measured in duplicate 0.5 ml aliquots of each sample.

Results

In each case, radioactivity in the last sample (taken at 4-11 minutes) was assumed to represent complete mixing and the differences in counting rates between the last and the other samples were expressed as a percentage of the fully mixed value. Individual results are shown in Table 16.

Comment

Most of the results show no substantial difference between early and late samples. The error in pipetting 0.5 ml aliquots of lysed blood is of the order of ± 3%. Only two results fall outside these limits. Both samples were taken at 30 seconds after injection; one shows slight excess activity (+4.7%) and the other a rather larger deficit (-12.2%). Two other very early samples (25 and 40 seconds) did not show a significant difference from their corresponding 5 minute samples (-2.0% and +2.6% respectively). It is noted that in each individual set of observations differences from the reference sample were always positive or always negative. The significance, if any, of this observation is not immediately obvious.

Based on these findings, it is concluded that after i.v. injection of red cells, thorough mixing within the circulation is virtually complete in about 1 minute; in other experiments, a minimum time of 3 minutes has been allowed to elapse between the completion of i.v. injections and occlusion of vessels for sampling.

In addition, in those experiments in which the injected material leaves the circulation to equilibrate with the extravascular space (e.g. IgG), serial samples were taken from 3 minutes onwards in order to determine the initial mixing volume by retrograde extrapolation.

Table 16. Radioactivity in samples of venous blood taken at various times after i.v. injection of ^{51}Cr labelled autologous red cells into seven rabbits.

Rabbit No	Sample time	Activity ct/min per 0.5 ml	Difference as % of last sample
65	0min 30s	1548	-12.2
	4 10	1764	ZERO
70	1 00	3867	-0.09
	2 00	3872	-0.08
	3 30	3838	-1.7
	5 00	3903	ZERO
72	1 15	3021	+1.9
	2 15	2999	+1.1
	3 30	2999	+1.1
	5 00	2966	ZERO
77	1 00	4225	-2.0
	2 00	4205	-2.5
	3 15	4255	-1.3
	5 00	4313	ZERO
92	0 40	1647	+2.6
	1 40	1615	+0.6
	3 00	1607	+0.1
	5 00	1605	ZERO
95	0 30	2920	+4.7
	11 00	2790	ZERO
96	0 25	2775	-2.0
	5 00	2831	ZERO

SOME OBSERVATIONS ON THE HAEMATOLOGY AND PHYSIOLOGY OF NORMAL SANDY-LOP RABBITS

THE RATE OF HAEMODILUTION FOLLOWING ACUTE BLOOD LOSS

Procedure

Calculation

Results

Discussion

In a number of rabbits plasmapheresis was used in order to obtain large amounts of plasma while maintaining the donors in good health. During each procedure, 60-80 ml whole blood was withdrawn over 5-10 minutes, and the red cells, resuspended in saline, were returned within half an hour. While the bleeding appeared to cause the animals no distress, it was noticed that towards the end of the saline-red cells infusion some rabbits became rather restless and began to cough and sneeze. It has been shown that haemodilution occurs rapidly following blood loss in the rabbit (Chalmers et al., 1967; Mott, 1968) and it therefore seemed possible that circulatory overload might account, at least in part, for the observations in the infused animals. In the present experiments the rate of haemodilution after blood loss was investigated in a small number of Sandy lop rabbits.

Procedure

(a) Detailed investigation was carried out in one rabbit (R31) in which red cell volume and plasma volume were measured independently using ^{51}Cr-labelled red cells and biologically "screened" ^{125}I-labelled IgG (see p.147).

Autologous red cells were labelled with ^{51}Cr and 2 ml of a saline suspension of these cells was injected i.v. A blood sample (1 ml) was taken after 5 minutes for estimation of red cell volume. About 2 hours later 5 ml whole serum containing ^{125}I-labelled IgG was injected i.v. Blood samples (1 ml) were taken at 3, 8, 15 and 30 minutes

following the serum injection. The 30 minute sample was taken through a Butterfly needle inserted into the median vein of one ear and as soon as this sample had been taken, the syringe was detached leaving the needle in the vein. Immediately, a large syringe containing a drop of heparin was attached and 66 ml blood was withdrawn during the next 5 minutes. On completion of the "bleed", a dry sampling syringe was again attached to the needle and a 1 ml sample was taken. The Butterfly needle was then withdrawn. Further 1 ml blood samples were taken at the following times after completion of the "bleed": 6, 15, 30, 90, 180 minutes and finally at 24 hours.

(b) Similar experiments were performed in 2 other rabbits (S65, S96) but using labelled red cells only. Briefly, ^{51}Cr-labelled autologous red cells were injected i.v., and a blood sample was taken for estimation of total blood volume. Each animal was then bled of 60 ml venous blood in 3-4 minutes. Further samples were taken at about 5, 15, 30, 90 and 180 minutes after completion of the "bleed" and again at 24 hours.

In all of these experiments, 1 ml blood samples were taken into dry plastic syringes and transferred to pots containing dry EDTA. Each rabbit had free access to food and water except for a period of about 45 minutes, from just before the "bleed" until after the 30 minute sample had been taken. Packed cell volume (PCV) was measured in duplicate using a microhaematocrit technique; no correction for trapped plasma was applied (see p.88). In some cases, ^{125}I activity was measured in 0.5 ml aliquots of separated plasma; in the other cases, ^{125}I and/or

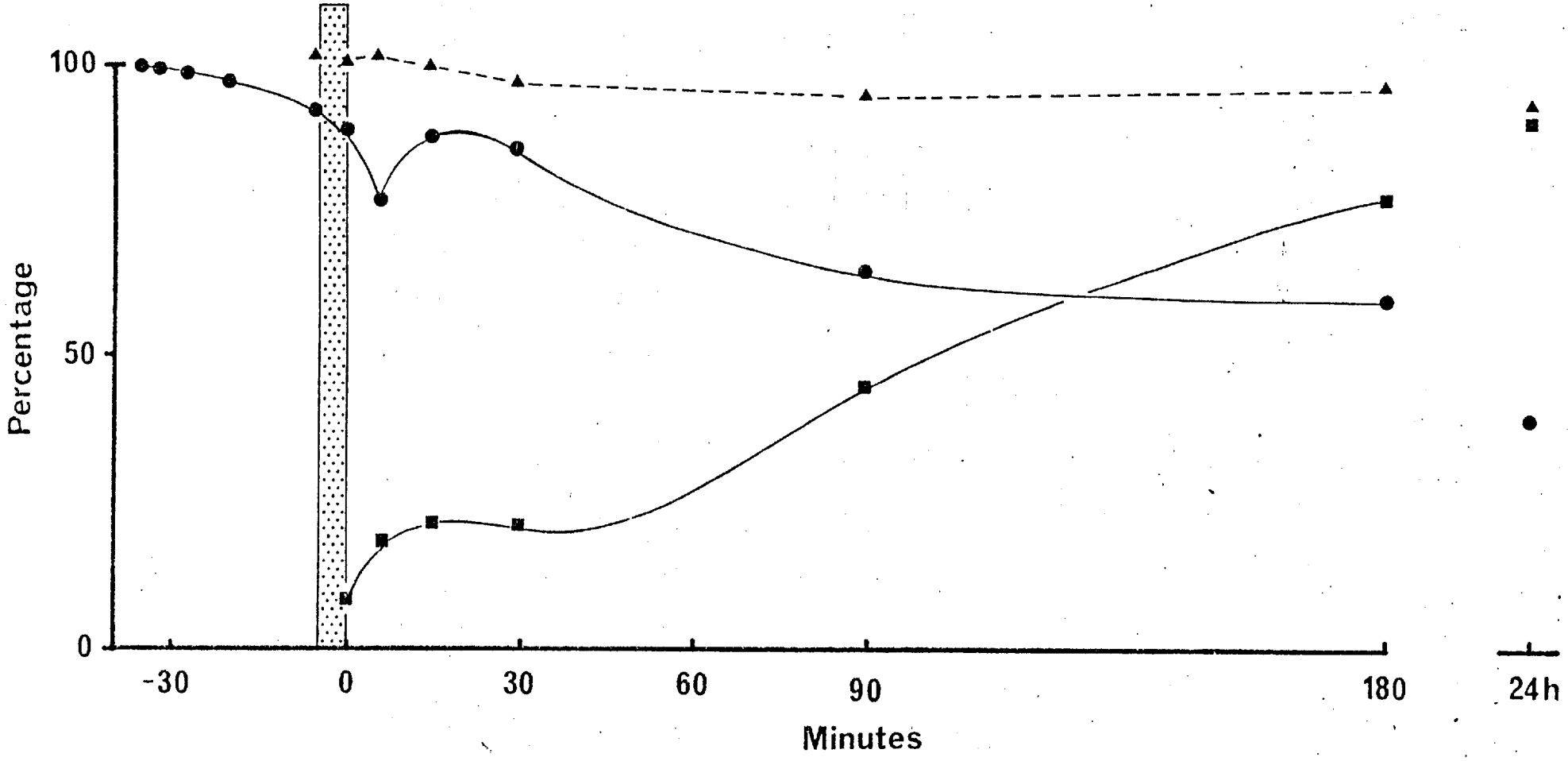
^{51}Cr activity were measured in 0.5 ml aliquots of lysed whole blood and, where appropriate, corrections were applied to ^{125}I results to allow for counts due to ^{51}Cr. When the PCV of the sample was known, ^{125}I and ^{51}Cr activity could be expressed as ct/min per ml plasma or red cells respectively. Radioactivity in aliquots of known dilutions of the injection materials was measured and thus the amount of activity injected could be calculated.

Calculations

(a) For the rabbit given both ^{51}Cr-labelled red cells and ^{125}I-labelled IgG, the initial red cell volume (RCV) and plasma volume (PV) were calculated as described on p. . From these values total blood volume (TBV), body haematocrit (H_B) and the ratio between H_B and venous haematocrit (H_V) were determined.

The total amount (ct/min) of each isotope in the circulation immediately before the "bleed" was calculated as the product of the RCV (or PV) and ct/min per ml red cells (or plasma) measured in the sample taken immediately preceding the "bleed" (RCV and PV were corrected for losses due to sampling before the "bleed"). The total activity removed from the circulation by the "bleed" was calculated as the product of the volume of blood removed and its activity (ct/min per ml); the volumes of red cells and plasma removed were also known from its PCV. It was assumed that there was no significant loss of activity by any other route (see Discussion) during the brief period of rapid blood loss so that the activity remaining in the circulation immediately afterwards was obtained simply by difference.

Figure 2. The effect of blood loss in rabbit R31. During the period indicated by the stippled area 66 ml venous blood was removed.. Red cell ^{51}Cr activity (▲) and plasma ^{125}I activity (●) are shown, together with the increases in total blood volume (■), expressed as a percentage of the volume of the "bleed".


100
50
0
Percentage
-30
0
30
60
90
180
24h
Minutes
129

The RCV and PV immediately after the "bleed" were then calculated from the estimate of total ^{51}Cr and ^{125}I activity present in the circulation and the respective ct/min per ml red cells and plasma measured at that time. Once again TBV, H_B and the ratio H_B/H_V were determined. As there was no substantial difference in the ratio H_B/H_V before and after the "bleed" (see Results), the mean value of the observed ratios was used to calculate TBV at various times thereafter from the ^{51}Cr data according to the relationship

$$TBV = \frac{^{51}Cr \text{ ct/min present in circulation}}{^{51}Cr \text{ ct/min per ml whole blood} \times H_B/H_V}$$

Corrections were made for losses of ^{51}Cr activity due to sampling and also for decrease in specific activity (sp. act.) of the red cells.

b) In the 2 rabbits given labelled red cells alone, three additional assumptions were made in calculating the results. First, a value of 0.843 was used throughout for H_B/H_V (see p.111). Secondly, the amount of radioactivity (ct/min per ml whole blood) in the "bleed" was not measured; by analogy with the preceding experiment it was derived as 0.954 x the activity of the sample taken immediately before rapid blood loss. Finally, no corrections for changes in sp. act. could be applied except to the 3 and 24 hour samples, as PCVs were not done on the other samples.

Results

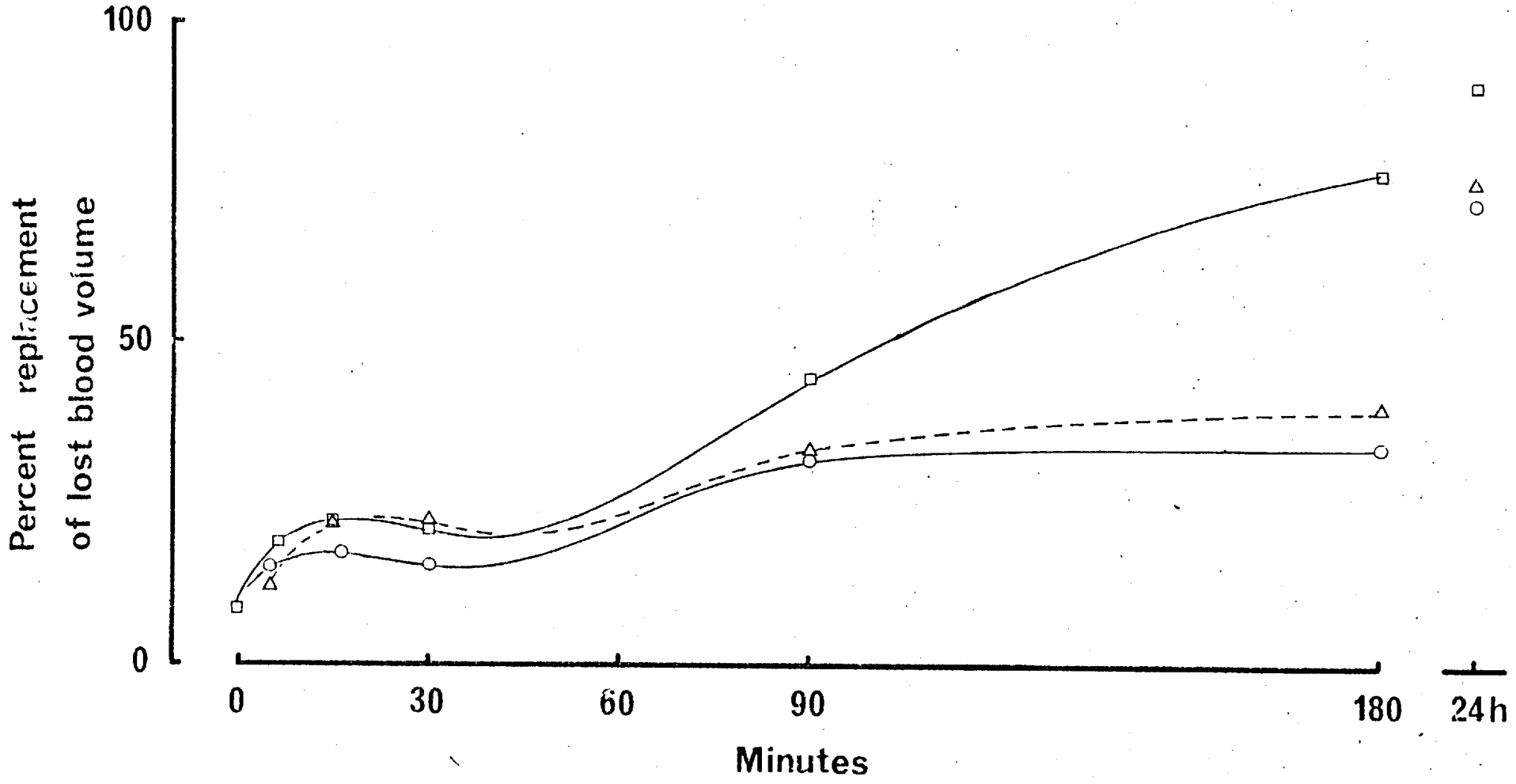
Data obtained in the experiment with R31 are summarized in Tables 17, 18 and 19 and in Fig.2. The important findings in this rabbit were as follows.

^{51}Cr specific activity of red cells. The sp. act. of the circulating red cells fell very gradually during the first $3\frac{1}{2}$ hours following injection of the labelled cells; by 24 hours above 93% of the initial sp. act. was present.

^{125}I specific activity of plasma. Initially there was a gradual decline in sp. act. of the plasma following injection of the labelled IgG so that immediately before the "bleed" approximately 10% of the activity had left the plasma. Samples taken immediately after the "bleed" and again 5 minutes later showed a sharp decrease in plasma sp. act. but by 15 minutes, sp. act. had risen again to a level almost as great as that prevailing just before the "bleed". In fact, if it is assumed that the rate of loss of labelled IgG from the plasma observed initially were to be maintained throughout the period under discussion, then the peak sp. act. after the "bleed" was greater than would have been expected if the rabbit had not been bled. Between 15 and 30 minutes following the "bleed", the initial slow rate of fall in sp. act. was again observed and by 24 hours only about 40% of the initial value was found.

Effect of blood loss on the ratio H_B/H_V. Calculated values for the ratio H_B/H_V in R31 before and immediately after the rapid removal of about 30% of the circulating blood volume were 0.844 and 0.829 respectively. There was therefore no evidence of any substantial change induced by severe acute blood loss.

Figure 3. Changes in total blood volume in three rabbits following the rapid removal of about 30% of the circulating blood volume; the increases in blood volume are expressed as a percentage of the volume of blood removed. R31 (□), S65 (△), S96 (O).


100
50
0
Percent replacement of lost blood volume
0
30
60
90
180
24h
Minutes

In all three rabbits, the difference between the predicted blood volume immediately after the "bleed" (initial TBV - volume of blood withdrawn) and each subsequent measurement of TBV was expressed as a percentage of the volume of blood removed. The values found at various times after the "bleed" in each of the three rabbits are shown in Fig.3 and Table 20. It can be seen that there was an initial phase of rapid haemodilution lasting 5-15 minutes during which 15-20% of the lost volume was replaced. TBV then remained essentially unaltered up to the time of the 30 minute sample. Samples taken at $1\frac{1}{2}$, 3 and 24 hours indicated a gradual return of TBV towards the original values, but at 24 hours there was still a deficit of between 8 and 28% of the volume removed.

Discussion

The early, minor changes in ^{51}Cr sp. act. of the circulating red cells observed in one rabbit during the first 2 or 3 hours following the injection of labelled cells (and after bleeding) argue against any significant number of unlabelled red cells being delivered abruptly to the circulation as an acute response to blood loss. By 24 hours, however, changes in sp. act. may be considerable, and in the present work decreases of between 7.0 and 10.6% of the initial values were observed. A decrease of this magnitude can be explained by the combined action of two well recognised factors, namely, elution of ^{51}Cr from the labelled cells and also the proportional loss of labelled and unlabelled cells (by normal destructive processes) from the circulation and their replacement with unlabelled cells from the erythropoietic tissues. This decrease in

sp. act. must be taken into account when calculating total blood volume from the later ^{51}Cr data; failure to apply appropriate corrections leads to overestimation of blood volume (see Table 18) and may be one reason why Chalmers et al. (1967) found that 24 hours following the acute loss of 26% of the blood volume in rabbits, the TBV was about 10% greater than before the "bleed".

The overall decrease in plasma ^{125}I sp. act. (about 40% remaining after 24 hours) was expected; it is known that IgG injected into the plasma is distributed into a volume more than twice that of the plasma (see p.169). The accelerated decrease in sp. act. beginning during the "bleed" was also expected and represents the rapid dilution of labelled intravascular IgG by extravascular fluid. However, the ensuing increase in plasma sp. act. to levels almost as great as those prevailing before the "bleed", in the face of an increasing TBV and stable RCV (see Fig.2), must indicate that the concentration of ^{125}I (presumably as ^{125}I-labelled IgG) in the plasma was increasing during this phase. The overall pattern of the plasma sp. act. curve suggests that, following the "bleed"

(a) the initial reduction in ^{125}I activity occurred as a result of dilution with extravascular fluid containing little or no labelled IgG.

(b) the normal efflux of IgG from the circulation slowed down or ceased.

(c) the labelled IgG which had left the circulation during the 30 minutes preceding the "bleed", returned 5-15 minutes after bleeding, in a concentration greater than that present in the plasma.

Mott (1968) has demonstrated in rabbits that during haemodilution following stepwise bleeding, the fluid entering the circulation has a higher albumin:globulin ratio than both plasma and lymph, an observation which is consistent with the initial rapid fall in plasma ^{125}I sp. act, in the present experiments; in addition, even if the labelled IgG which had left the plasma were to have equilibrated within the extravascular space, its concentration would necessarily have been much lower than that in the plasma. Item (a) is thus readily explained. However, in order to explain both of items (b) and (c), one must postulate a "limited" extravascular space which contains those IgG molecules which have most recently left the circulation and which are present in a concentration greater than that in the plasma. Although mere speculation, the relevant moves of the labelled IgG molecules injected in the present experiments might be as follows. IgG leaving the plasma moves first into a "limited" compartment (say, for argument's sake, into endothelial cells) where the concentration of IgG is greater than in the plasma; after a short sojourn it would normally pass into the "general" extravascular space. Following acute blood loss, the deficit in TBV is partly compensated by the rapid transfer of fluid from the "general" extravascular space (in the present experiments, virtually free of labelled IgG); when the transfer of fluid slows down, the "limited" compartment makes a contribution to the plasma and also returns concentrated IgG (including labelled IgG, in the present experiments). The data presented here is clearly very limited, but some other observations on this "limited" extravascular compartment are discussed in the next section of this work (see p. 160).

Whatever the explanation of the plasma sp. act. changes may be, one consequence is that labelled IgG (and possibly other proteins) cannot readily be used to assess plasma volume accurately during, and shortly after haemorrhage. In the present work, one determination of plasma volume was made immediately following the "bleed" assuming a constant plasma sp. act.; the error involved in this assumption was probably no more than 1 or 2% and the effect of correcting this deficit would have been to make the H_B/H_V ratio slightly closer to that observed before the "bleed". All other relevant blood volumes were calculated from the ^{51}Cr data.

Observations in rabbits of the ratio H_B/H_V before and immediately after acute blood loss have not previously been reported. In dogs, measurements have been made 15 minutes or more after the completion of bleeding when no substantial change in the ratio was observed (Reeve, Gregersen, Allen, Sear and Walcott, 1953 (a) and (b).

The triphasic increase in blood volume seen in the present experiments (see Fig.3) probably represents an initial redistribution of fluid from the extravascular space (replacing about 20% of the deficit) and a final more gradual replacement of total body fluid by conservation of dietary and metabolic water. The intervening plateau may be related to the fact that in these experiments access to food and water was witheld until after a blood sample had been taken 30 minutes after the completion of the "bleed".

In conclusion, it is probable that under the conditions for plasmapheresis of rabbits described in the introduction to this section, some 20% of the total volume removed from the circulation had been restored by the time the separated cells were ready for infusion. It seems unlikely therefore that infusion of a volume equal to that of the venesection should give rise to any serious problems of circulatory overload. Nevertheless, since these observations were made, the volume of the suspension of red cells for infusion has been reduced accordingly.

Table 17. ^{51}Cr data for R31 showing changes in specific activity of the red cells.

Sample	Time (min)	(a) ct/min per ml whole blood	(a) corrected for sampling	PCV	Specific activity	
					ct/min per ml red cells	%
1 (red cell volume)	0	6644	-	0.340	19541	100.0
2 (pre "bleed")	192	6267	-	0.315	19890	101.8
3 ("bleed")	-	5924	-	0.307	19265	98.6
4 (post "bleed" 0 min)	197	5690	5690	0.290	19621	100.4
5 (" " 6 ")	203	5440	5467	0.275	19783	101.2
6 (" " 15 ")	212	5234	5286	0.270	19384	99.2
7 (" " 30 ")	227	5096	5172	0.270	18875	96.6
8 (" " 90 ")	287	4523	4613	0.245	18462	94.5
9 (" " 180 ")	377	4060	4161	0.217	18667	95.5
10 (" " 24 h)	1637	3770	3883	0.207	18171	93.0

Table 18. Data giving rate of haemodilution following blood loss in R31.

Sample	H_B/H_V	TBV (ml)		(b) - 148.4*	Increase in blood volume as % of volume lost
		(a)	(b)		
Pre "bleed"	0.844	214.4+	214.4	66.0	-
Post "bleed" (0 min)	0.829	156.3+	154.1	5.7	8.6
" " (6 ")	-	161.5	160.5	12.1	18.3
" " (15 ")	-	167.0	162.7	14.3	21.7
" " (30 ")	-	170.7	162.0	13.6	20.6
" " (90 ")	-	191.4	177.7	29.3	44.4
" " (180 ")	-	212.2	199.1	50.7	76.8
" " (1440 ")	-	227.4	207.7	59.3	89.8

(a) uncorrected for ^{51}Cr specific activity of red cells.

(b) corrected " " " " " " "

* 148.4 = predicted blood volume after removal of 66 ml blood.

\+ sum of RCV and PV measured independently with ^{51}Cr and ^{125}I.

Table 19. ^{125}I data for R31 showing changes in specific activity of the plasma. Estimates of plasma volume are also given.

Sample	Time (min)	ct/min per ml whole blood	PCV	Specific activity		Plasma volume (ml)
				ct/min per ml plasma	%	
(0)	(0)	-	-	(730)	100.0	160.3 (a)
1	3	-	0.320	727	99.6	-
2	8	-	0.315	719	98.4	-
3	15	-	0.315	712	97.6	-
4 (pre "bleed")	30	461	0.315	674	92.3	157.6 (a)
5 ("bleed")	-	448	0.307	647	88.6	-
6 (post "bleed" 0 min)	35	458	0.290	645	88.4	118.7 (a)
7 (" " 6 ")	41	406	0.275	562	76.9	122.9 (b)
8 (" " 15 ")	50	467	0.270	640	87.6	125.1 (b)
9 (" " 30 ")	65	454	0.270	622	85.3	124.4 (b)
10 (" " 90 ")	125	353	0.245	468	64.1	140.1 (b)
11 (" " 180 ")	215	339	0.215	433	59.3	161.5 (b)
12 (" " 24 h)	1475	227	0.207	286	39.2	170.1 (b)

(a) ^{125}I estimation

(b) ^{51}Cr estimation, $H_B/H_V = 0.836$

Table 20. Difference between TBV at various times after bleeding and TBV predicted immediately after bleeding, expressed as a percentage of the volume of blood removed.

Time after bleeding (min)	R31	S65	S96
0	8.6	-	-
5	18.3 (6)*	11.2	14.5
15	21.7	21.2	16.5 (16)*
30	20.6	21.8	14.8
90	44.4	32.7	31.3
180	76.8	40.0	33.8
1440	89.8	75.0	72.0

* Actual sampling time

SOME OBSERVATIONS ON THE HAEMATOLOGY AND PHYSIOLOGY OF NORMAL SANDY-LOP RABBITS

IgG METABOLISM - KINETIC STUDIES

Methods

IgG preparation

Labelling of IgG with ^{125}I and ^{131}I

Biological "screening" of labelled IgG

Animals used for kinetic studies

Injection protocol

Samples

Measurement of radioactivity

Estimation of initial plasma volume

Analysis of results

Correction for losses of radioactivity due to sampling

Estimation of catabolic rate of IgG and distribution of IgG between plasma and extravascular space

Uptake of IgG into plasma following i.m. injection

Distribution of IgG within the extravascular space

Results

Blood volume, the ratio H_B/H_V and the IgG space

Plasma activity following i.m. and i.v. injection

Uptake of IgG from i.m. injection sites

Catabolic rate and distribution ratio of IgG

Extravascular compartments and intercompartmental flow rates

Discussion

IgG METABOLISM - SERUM IgG LEVELS

Methods

Preparation of standard solutions of IgG

Test sera

Measurement of IgG concentration in sera

Results

In experiments to be described later (see p. 245), the immunological response of A-negative rabbits to intravenously administered A-positive red cells given at the same time as passively injected IgG anti-A was assessed by following the survival of a subsequent i.v. injection of A-positive red cells given alone. It was essential therefore that sufficient time should elapse between these injections of red cells for the concentration of residual passive antibody to fall to levels which would not impair survival of the second injection. In the present experiments, metabolic studies were undertaken using IgG labelled with radio-iodine primarily to determine the rate of disappearance from the plasma of IgG given by i.v. injection; in addition, the opportunity was taken of investigating other aspects of IgG metabolism in the rabbit. Serum concentrations of IgG were also determined in a number of normal rabbits and are reported in this section.

IgG KINETIC STUDIES

Methods

IgG preparation. Rabbit IgG was prepared from a pool of whole serum by sequential anion exchange and gel filtration chromatography. Tris-HCl buffer, pH 8.0, was used in conjunction with diethylamino-ethyl cellulose (DE 52, Pharmacia); isotonic saline was used with G200 Sephadex.

Pooled serum from 5 Sandy Lop rabbits was dialysed against 20 m M tris-HCl buffer and was run onto a column of DE 52 which had been equilibrated with the same buffer. A concentration gradient of eluting

buffer was established by pumping 200 m M buffer into a closed mixing flask containing 20 m M buffer and thence to the column. The presence of protein in the eluate was detected by monitoring its optical density at 280 nm and fractions were collected using an LKB Uvicord fraction collector. The first major protein peak to be eluted contained IgG and a substantial proportion of high molecular weight lipoprotein; fractions from this peak were pooled and concentrated by pressure dialysis. The material was then put through a column of G200 Sephadex. On the first passage, most of the lipoprotein was separated in the first peak but there was some carry-over to the second peak which contained the IgG. The fractions comprising the second peak were pooled, concentrated and recycled through G200, and on this passage there was only a small lipoprotein peak which was well separated from that of the IgG. The IgG fractions were pooled, concentrated and equilibrated against 20 m M tris-HCl buffer and the material was run onto a column of DE 52 equilibrated with the same buffer. Elution with 40 m M buffer produced 4 overlapping protein peaks. The pooled, concentrated fractions corresponding to each of these peaks were subjected to immunoelectrophoresis; three of the pools (I, II & IV) contained only IgG and the other one (III), comprising about one-sixth of the IgG recovered, showed in addition a trace of another protein, probably IgA (see Fig.4). The amount of IgG in each fraction was estimated by measuring the optical density of appropriate dilutions at 280 nm (see p.86). It was concluded that the contaminating protein in fraction III was present in amounts too small substantially to affect the proposed studies, whereas separation of the particular species of IgG molecules in this fraction from the protein to be labelled would possibly have prejudiced the results (see Cohen and Freeman, 1960). The four fractions were therefore pooled to give a solution containing 5.5 mg IgG per ml.

Figure 4. Immoelectrophoresis of IgG fractions I-IV and normal rabbit serum (N.S.) using two antisera: (a) Hyland goat anti-rabbit serum, 8233 H002 A1, (b) sheep anti-rabbit serum SKO-1P1.

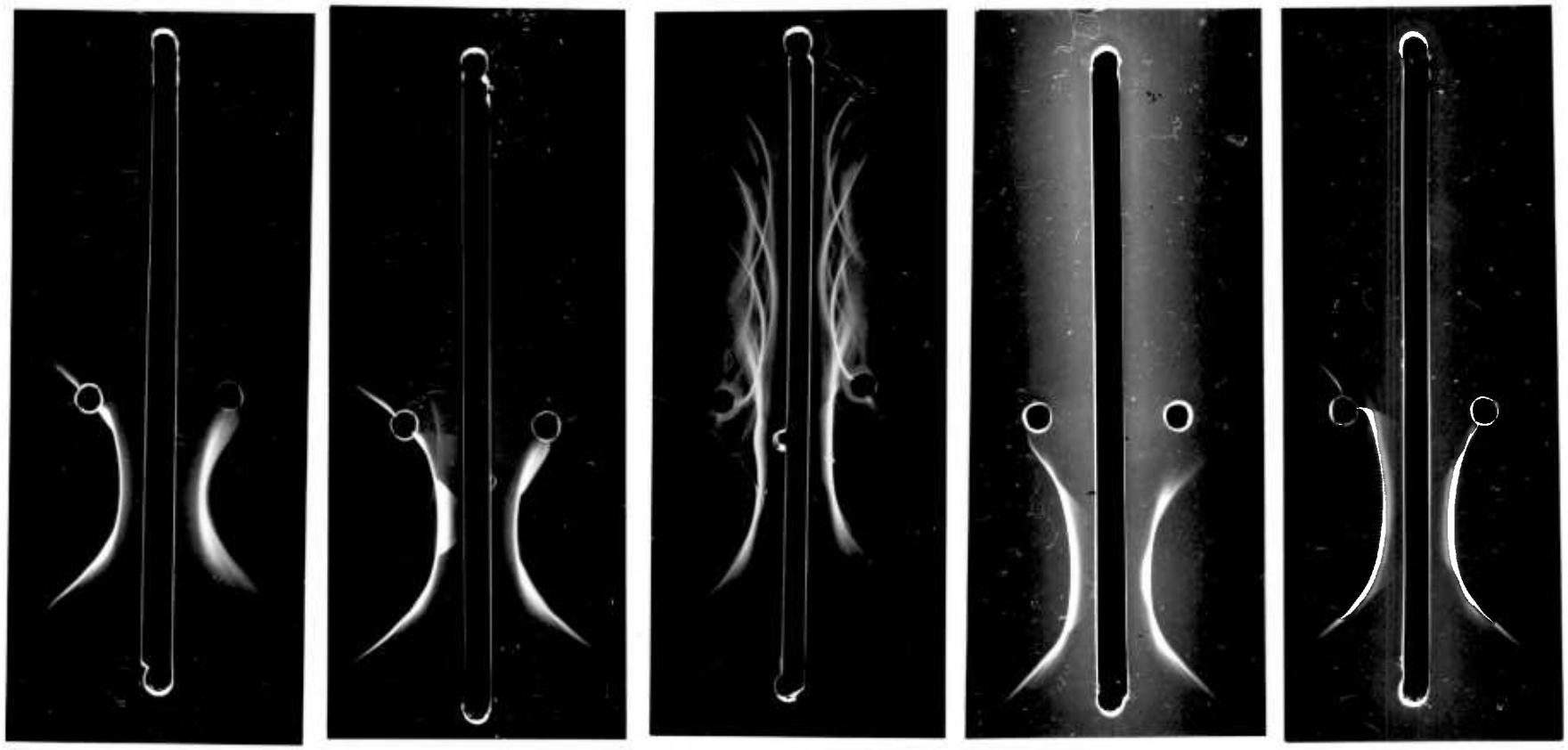


Figure 4

Labelling of IgG with ^{125}I or ^{131}I. Two 6 ml portions of the IgG preparation, each containing 33 mg IgG, were labelled with radioactive iodine by the iodine monochloride method (see p.83). For one portion, 318 μCi ^{125}I was used, of which 65% was bound to the protein, indicating an I:protein molar ratio of 0.8 under the conditions of labelling. For the other portion, 432 μCi ^{131}I was used; 68% was bound and the iodine:protein molar ratio was 0.9.

Biological "screening" of labelled IgG. Following overnight dialysis of the labelled protein against phosphate-buffered saline, pH 7.4, the ^{125}I-labelled preparation was injected i.v. into one Sandy Lop rabbit, and the ^{131}I-labelled preparation into another, in order to ensure the removal of rapidly catabolised radioactive iodine present either as free iodide or released from any protein which might have been denatured by the preparative procedures (McFarlane, 1956). The animals used for screening the protein had been prepared by giving Lugol's iodine in the drinking water (20 drops per litre) for 3 days before injection. After about 16 hours, serum containing IgG labelled either with ^{125}I or with ^{131}I was obtained from these animals, the activity being approximately 1.5-2 μCi per ml. Radioactivity, confined to the IgG precipitin lines, was demonstrated by immunoelectrophoresis of each of the sera, followed by autoradiography as shown in Fig.5. These sera were injected into the experimental animals within 4 hours of removal from the "screening" rabbits.

Figure 5(a) Immunoelectrophoresis of serum taken from R31 and R18 16 h after injection with ^{131}I-labelled IgG and ^{125}I-labelled IgG respectively. Electrophoresis of the injection materials is also shown.

Figure 5(b) Auto-radiographs of the immunoelectrophoresis plate shown in Figure 5(a); only the IgG precipitin lines show evidence of radioactivity.



Figure 5(a)

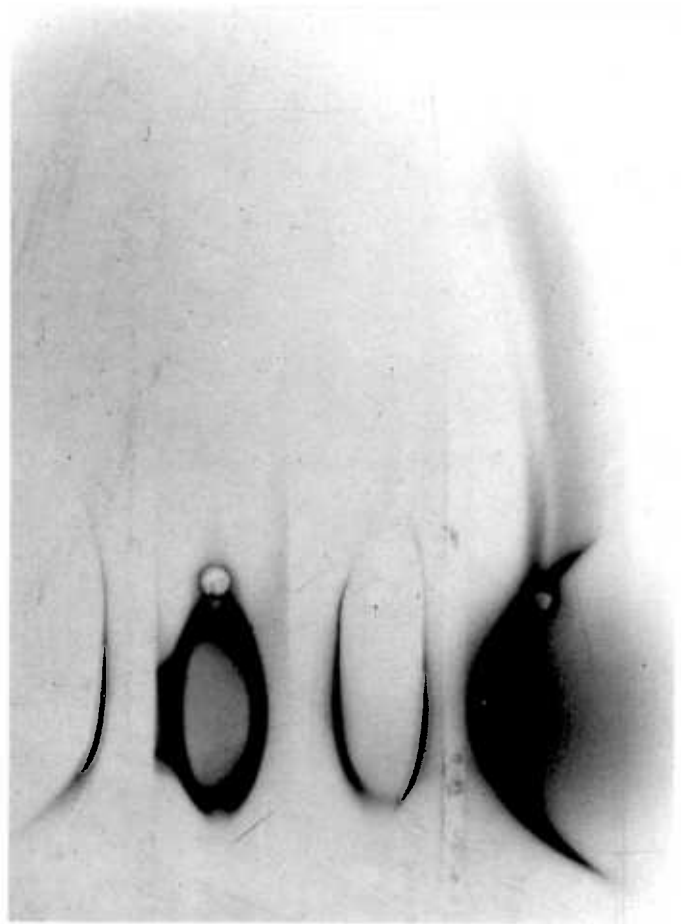


Figure 5(b)

Animals used for kinetic studies. Four adult Sandy Lop rabbits weighing between 3.25 and 3.67 kg were used. For 4 days before the injection of labelled IgG and for the whole period during which samples were taken, they were given drinking water containing 20 drops Lugol's iodine per litre. Except for the first 2 hours following injection, when blood samples were being taken frequently, the rabbits were allowed free access to a standard animal house diet and iodine-containing water.

Injection protocol. About 2 hours before injection of the labelled IgG, red cell volume was estimated in each of the recipients using autologous red cells labelled with ^{51}Cr by the citrate-wash method (see p.80). Estimation of red cell volume enabled a value for the ratio H_B/H_V to be determined in each case and thereby served as a check on the completeness of the i.v. injection of labelled IgG.

For the IgG studies, each rabbit was given 1 ml ^{131}I-labelled IgG in 2 equal doses of 0.5 ml by deep injection into the muscles of each thigh. This procedure was followed immediately by the injection of 1 ml ^{125}I-labelled IgG into a marginal vein of one ear. Both i.m. and i.v. injections of labelled IgG into any one rabbit were completed within 2-3 minutes and for timing the early samples, a stop-watch was started at the end of the i.v. injection.

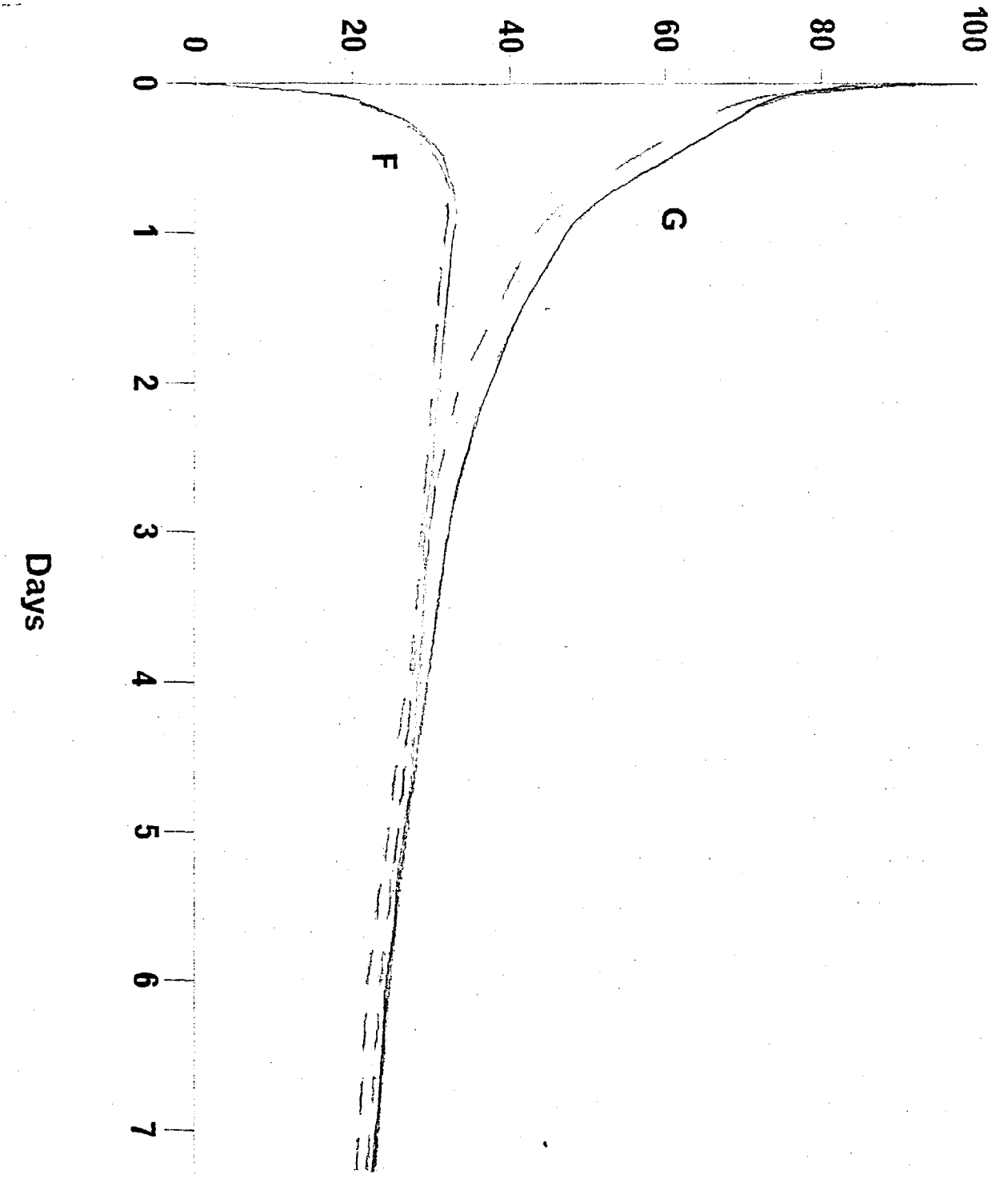
Samples. Whole blood samples of about 1.3 ml were taken from a vein in the ear contralateral to that used for injection; they were taken into a dry plastic syringe and transferred to pots containing

approximately 2.5 mg EDTA. As soon as possible after collection (always within 2 hours of sampling), samples were centrifuged and the plasma removed and stored at 4°C. Samples were taken at approximately the following times, the exact time of sampling being noted in each case: 5, 15, 30 minutes, 1, 1½, 3, 4½, 6, 9, 12, 18, 24 hours, then daily for seven days and twice weekly for a further two weeks.

Measurement of radioactivity. 0.5 ml volumes of each plasma sample were counted in a standard gamma counter. Samples from individual rabbits were counted in batches as soon as practicable before there was any significant loss of activity from low counting rate specimens. Known dilutions of both sera used for injection were made in alkaline KI (see Dacie and Lewis, 1968, p.357) and aliquots of these were counted with each batch of samples to allow correction of the results in individual rabbits for radioactive decay. For most samples, the counting error (coefficient of variation) was less than 1% but in some low activity samples (e.g. early observations following i.m. injections) the error was about 3%. Simultaneous measurement of ^{125}I and ^{131}I activity were made and, under the selected counting conditions, appropriate cross-corrections had to be applied to the ^{125}I results for counts due to ^{131}I (see p.78). These values are referred to as raw counts.

Estimation of initial plasma volume. Plasma volume at the time of i.v. injection of ^{125}I IgG was determined as follows for each rabbit. Corrected ^{125}I counts in the first four samples were plotted on semi-logarithmic paper and the slightly curvilinear plot, fitted by eye, was extrapolated to zero time. From the number of counts injected and the zero time value for plasma activity obtained in this way, plasma volume was calculated using a simple dilution prinicple (see p.108).

Figure 6. Computer tracing showing the average values (R22, R32, R36) for the proportion of IgG in the plasma at various times after i.m. (F) and i.v. (G) injection of the "screened", labelled IgG preparations. The broken lines represent the analyses of the observed plasma radioactivity; the continuous lines show the effect of correcting for losses due to sampling (see text).



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Analysis of results

The data relating to IgG kinetics obtained in these experiments were analysed by Dr. Denis Mollison at King's College, Cambridge. A brief description of the principles involved is given below; a fuller mathematical treatment of the analysis is given in a paper by Smith and Mollison (1975).

Correction for losses of radioactivity due to sampling. Obtaining frequent early measurements of plasma radioactivity necessarily involved removing a significant amount of the injected dose of labelled IgG (about 5% and 10% respectively following i.m. and i.v. injection). Allowance was made for each sample removed on the assumption that if it had been left in the animal it would have behaved like a dose of equal size injected i.v. at that time. On this basis, the corrected curve for the proportion of IgG in plasma following i.v. injection (G) could be calculated proceeding inductively from one sample time to the next; the corrected curve for the i.m. injection (F) could then be calculated directly (see below). These corrected curves are compared with the sample curves for the average data in 3 cases in Fig.6.

Estimation of catabolic rate of IgG and distribution of IgG between plasma and extravascular space. The apparent catabolic rate, α, was estimated by measuring the slope of the corrected plasma curves between days 10 and 25. During this period of observation, each curve was well described by a single exponential function which was taken to indicate that equilibration factors were no longer playing a significant part.

By extrapolation of this line beyond day 25, the area under the curve, and hence the true catabolic rate, β (1/area under the curve), could be determined. This extrapolation procedure introduces a potential source of error, but as the deduced area under the curve, from day 25 onwards, constitutes only about 10% of the total area, the error is unlikely to be substantial. From these two estimates of catabolic rate, the distribution of IgG between the plasma and extravascular space is given by

$$DR = \frac{\beta - \alpha}{\alpha}$$

assuming that only IgG within the plasma compartment is subject to degradation and removal from the body. Alternatively, an estimate of DR may be obtained from the occupancy function curve, E (see below), and is approximately equal to the area under this curve.

Uptake of IgG into plasma following i.m. injection. On the single assumption that IgG reaching the plasma following i.m. injection behaves exactly as though it had been injected i.v. at that moment, the contents of the i.m. injection site (U) can be calculated directly by deconvoluting the i.v. curve (G) from the i.m. curve (F). This calculation has been made in the present analysis by numerical sequence approximation, treating successive 15-minute intervals discretely; between sample times F was approximated by linear interpolation and G by exponential interpolation.

Distribution of IgG within the extravascular space. If it is assumed that the loss rate of IgG from the plasma to the extravascular space is proportional to the amount of IgG in the plasma, the occupancy

function, E, for the extravascular space can be calculated by a process of "autodeconvolution". Thus it is assumed that between times s and s + ds, a proportion $\gamma G(s)ds$ of IgG is lost to the extravascular space (also, a proportion $\beta G(s)ds$ is catabolised); similarly, between times t and t + dt, an amount $G(s)e(t - s)dtds$ (integrated over all times s earlier than t) is returned to the plasma. In terms of compartmental analysis, this is equivalent to assuming that there exists a flow rate (or rates) to the extravascular space, but makes no assumption about the return flow from the extravascular space. Thus, if the extravascular space in fact consists of a number of well-defined compartments, the function E should turn out to be a sum of exponentials, one for each compartment. Estimates of the rate constants, γ , were based on observations made during the first 30 minutes following injection, that is, before any substantial amount of IgG had re-entered the plasma from the extravascular space.

Results

Of the 4 rabbits used in these experiments, one requires special consideration. On the day of the IgG injection, R35 became pregnant. The gestation time in the rabbit is about 28 days, so that during the course of the present observations, significant changes in the fluid compartments of this rabbit undoubtedly occurred with, almost certainly, concommitant effects on the distribution and metabolism of IgG. Where relevant, the results in this rabbit will therefore be dealt with separately.

Figure 7. Computer tracing showing the proportion of IgG which has not been accounted for in the IgG space at various times following i.m. injection. Average of 3 cases (R22, R32, R36).

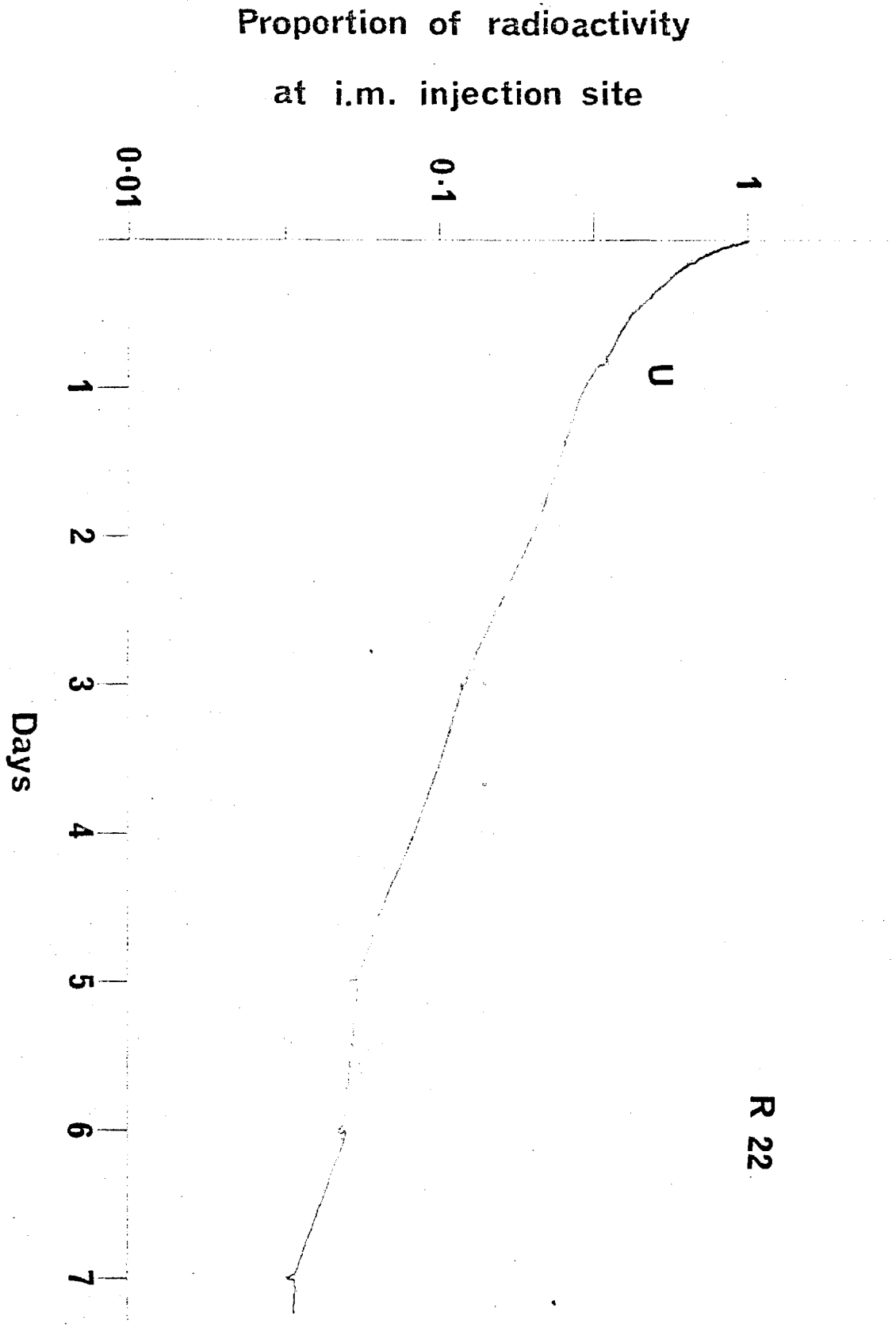
Proportion of radioactivity
at i.m. injection site
1
0·1
0·01
U
0
1
2
3
4
5
6
7
Days

Blood volume, the ratio H_B/H_V and the IgG space. Details of initial red cell volume, plasma volume and venous haematocrit, together with calculated values of H_B/H_V are shown in Table 21. In addition, values for IgG space have been calculated from plasma volume and the distribution ratio of IgG between extravascular and intravascular space (see later).

Plasma activity following i.m. and i.v. injection. Sequential raw plasma counts following i.m. and i.v. injections into the 4 rabbits are shown in Table 22. Following i.m. injection, plasma levels rose gradually to a maximum of about 40% of the levels that would have been achieved instantaneously had the IgG been given by the i.v. route; peak levels were reached within 1-2 days after which the levels gradually decreased. By contrast, the i.v. curve fell steeply during the first few hours following injection, the rate of fall gradually decreasing from the outset. By about day 7, the rates of fall of both the i.m. and the i.v. curves were similar and from this time onwards the observations were well fitted by a single exponential function (see Fig.6).

Uptake of IgG from i.m. injection sites. As shown in Fig.7, the rate of loss of IgG from i.m. injection sites was not constant, being somewhat faster initially and continuing to decrease, at least over the first 7 days. R22 showed the fastest rate of uptake so that 70% of the injected dose had left the i.m. site by 1 day; in this rabbit 10% had still not been accounted for in the plasma by 3.5 days. At the other extreme, R32 required 2.1 days for 70% of the injected dose to leave the i.m. site. In all but R22, 10% had still not beenaccounted for in the

Figure 8. Computer tracing showing the proportion of IgG which has not been accounted for in the IgG space at various times following i.m. injection into R22; rapid clearance from i.m. site.



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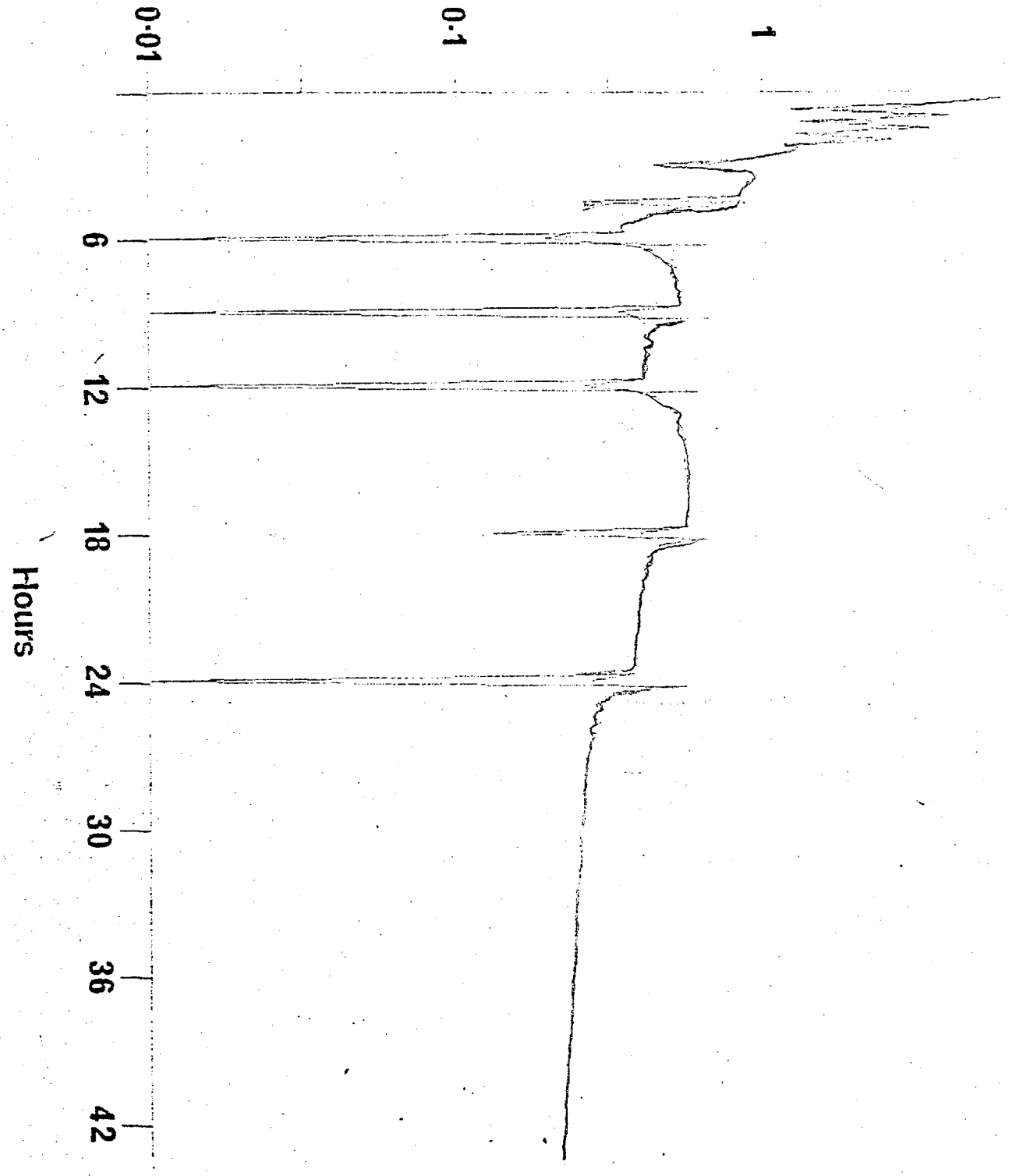
Figure 9. Computer tracing showing the proportion of IgG which has not been accounted for in the IgG space at various times following i.m. injection into R32; slow clearance from i.m. site.

R 32
Proportion of radioactivity
at i.m. injection site
1
0·1
0·01
U
1
2
3
4
5
6
7
Days

Figure 10. Computer tracing showing the occupancy function (E) of extravascular space by the injected IgG (see text).

Occupancy function (E)

of e.v.s. by IgG



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plasma at 7 days. Rate constants for early and late uptake in R22 and R32 are compared in Table 23 and the uptake curves, U, for these two rabbits are shown in Figs. 8 and 9.

Catabolic rate and distribution ratio of IgG. Individual values for the rate constants determined in these experiments and also the distribution ratios of IgG between extravascular space and plasma are shown in Table 24. The average apparent catabolic rate, α, observed in the 3 non-pregnant rabbits was approximately 0.08 $days^{-1}$, corresponding to a $t_{\frac{1}{2}}$ of about 8.6 days; the mean value for the true catabolic rate β was 0.20 $days^{-1}$. In the pregnant rabbits, R35, values for α and β were approximately 0.10 and 0.16 $days^{-1}$ respectively. For the 3 non-pregnant animals, the amount of IgG in the extravascular space was greater than that in the plasma by a factor of about 1.3; this ratio for the pregnant rabbit was 0.9.

Extravascular compartments and intercompartmental flow rates. Subject to magnification of sample errors, the occupancy function of the extravascular space (E) may be described as consisting of 2 components as shown in Fig.10. Thus, although most of the IgG molecules enter the extravascular space within a few hours of arriving in the plasma, most of them return to the plasma very quickly (80-90% within 6 hours). On the other hand, a smaller proportion of the plasma IgG molecules enters the extravascular space for a relatively long period of time, during which they are unavailable for the brief visits. In terms of actual occupancy of the extravascular space (the area under the E curve) the slow component, i.e. visit longer than about 6 hours, accounts for about 90% of the time spent in the extravascular space. In summary, the average IgG molecule

passes from the plasma into the extravascular space several times a day (given by the rate constant γ in Table 24) returning rapidly on 80-90% of occasions; on the remaining visits it has a half-life in the e.v.s. of 1-2 days.

Discussion

The radioiodine-labelled IgG used in this experiment fulfils the requirements set out by Schultze and Hermans (1966, p.451) for proteins suitable for use in metabolic studies. The method used for isolation of the IgG is a comparatively mild form of protein fractionation (Cohen, 1958) and permits the preparation of IgG in a high degree of purity with no significant denaturation. Iodination of proteins by the iodine monochloride method has now been investigated extensively (see e.g. Cohen, Holloway, Matthews and McFarlane, 1956; Campbell, Cuthbertson, Matthews and McFarlane, 1956; McFarlane, 1957; Freeman, 1966), and there is no reason to believe that the conditions of labelling used in the present experiments should lead to any significant denaturation of the IgG preparation. Furthermore, the material used in the metabolic studies had been biologically screened so that any protein denaturated either during purification or iodination would have been removed (McFarlane, 1956). No practical test exists to determine whether such a labelled preparation has had a subspecies of IgG removed during processing which might thereby alter its overall metabolic characteristics, a possibility raised by the observations of Cohen and Freeman (1960) on the metabolism of human γ-globulin. The precaution was taken in the present experiment of including all of the fractions containing exclusively or predominantly IgG following chromatography but the loss of a portion of IgG with particular metabolic properties may nevertheless have occurred.

No urinary iodine excretion measurements were made in this experiment. From what has already been said about the preparation of the labelled IgG and from consideration of the results, it is unlikely that any significant increase in catabolism due to the presence of denaturated protein would have been detected by such measurements. One area in which iodine excretion may have helped is in relation to the 10% of the i.m. injection which was still unaccounted for in the plasma 7 days after injection. A possible explanation for this observation is that following i.m. administration, about 10% of the injected material is catabolised locally. If this were to occur fairly rapidly, it might have been apparent from urinary iodine excretion measurements. Alternatively, the use of a surface counter over the injection site could be used in suitable experiments in an attempt to resolve this problem.

Many different methods have been used in the past to study IgG metabolism in the rabbit. Observation of the fall in titre of passively administered antibody provides a relatively simple method for determining the half-life of IgG and requires no in vitro protein isolation procedure; however, the measurements lack accuracy and there is always the possibility of antibody of more than one immunoglobulin class being administered, though this problem should be relatively easy to resolve (Taliafero and Talmage, 1956). Most workers have used IgG isolated from whole serum by precipitation with neutral salts (e.g. Taliafero and Talmage, 1956; McFarlane, 1956; Cohen et al., 1956) or by alcohol fractionation (Dixon, Talmage, Maurer and Deichmiller, 1952). All of these methods result in preparations containing small amounts of several species of protein molecules in addition to IgG. In some studies,

biosynthetically labelled (^{14}C, ^{35}S) proteins were isolated in this way; in others the isolated proteins were subsequently labelled with radio iodine in vitro. The greatest value for the half-life of IgG obtained using these methods was 7.0 days. McFarlane's maxim that the longer a labelled protein persists in the blood, the nearer it approximates to the animal's own unlabelled protein (McFarlane, 1956) should not be adhered to too rigidly in view of what has been said above in relation to selection of subspecies of protein with different metabolic properties, but it suggests that the results obtained in the present experiments are at least as reliable as those previously reported.

The immediate practical implications of the present findings relate to the disappearance from the plasma of passively administered IgG anti-Hg^A given in experiments to be described later (see p.243). Although the IgG antibody was prepared by a similar method to that used for the metabolic studies, it is still possible that the specific antibody molecules represent a metabolically distinct sub-population of IgG. Certainly, it has been shown that certain specific antibodies in human serum may behave differently from the bulk of IgG on chromatography (see e.g. Frame and Mollison, 1969). The validity of applying the present findings for IgG to predicting the behaviour of anti-Hg^A derives some support from observations made with anti-Hg^A. In several experiments in which a large i.v. dose of anti-Hg^A (300 µg/kg body weight) was given at the same time as a small dose of Hg^A-positive red cells (0.02 ml/kg body weight) to Hg^A-negative rabbits, the titre of antibody in serum taken at various times after injection was determined using an indirect antiglobulin test. In all cases, the clearance half-time of the injected red cells was in the order of a few minutes and no evidence of active immunization as a result of this injection

was obtained; furthermore initial titres were ignored to allow for dilution of the injected antibody in the total IgG space. Observations were too few to enable precise estimations to be made but values for the half-disappearance time of anti-Hg^A were found to vary between 5 and 11 days (see Table 25).

Without making any compartmental assumptions, the mathematical treatment of the data obtained in these experiments reveals two exponential functions relating to the transfer of IgG between plasma and the extravascular space (see E curves). This means that the extravascular space may be considered as comprising two compartments, one having fairly rapid exchange of IgG with the plasma, the other having a relatively slow exchange rate but nevertheless accounting for the major occupancy of the e.v.s. by IgG. Although this tells one nothing about the anatomical nature of these compartments, explanations in terms of two well-defined transfer processes for protein may be entertained. There is now abundant evidence that pinocytosis may play an important part in the passive transfer of extracellular fluid and its contents across epithelial and endothelial surfaces. The pinocytic vesicles are believed to move passively by Brownian movement (Casley-Smith, 1963) and implicit in this hypothesis is their movement both ways across, say, the endothelial cell. Transport of ^{125}I-labelled gamma-globulin from tissue to blood has been demonstrated in the rabbit, using isolated, perfused gastrocnemius preparations (Perry and Garlick, 1973) and the rates at which this occurred (range 0.005 - 0.06 min^{-1}) were of the same order as the rate constants for the rapid component of the E curve in the present experiments. One possible concept of the rapidly exchanging IgG

compartment is that of the pinocytic vesicles and the extravascular space in the immediate vicinity of the endothelial cells; this would allow IgG molecules to be removed from the plasma, a proportion either being retained temporarily in the pinocytic vesicles, or being only transiently released to the e.v.s., and then recaptured for return to the plasma. A similar concept may be invoked to explain the rapid return to the plasma of recently lost IgG following acute haemorrhage (see p.135). Any IgG molecules delivered to the e.v.s. and not returned to the blood by this mechanism would presumably distribute themselves in the extravascular space and be returned to the plasma by other, less direct means, e.g. via the lymphatics, possibly corresponding to the slow component of the E curve in the present analysis.

The values for the rate constants α, β and γ obtained in the pregnant rabbit (R35) were similar to those in some of the other rabbits. On the other hand, the distribution of IgG between extravascular and plasma compartments was clearly very different. However, the validity of these calculations in this rabbit is open to question in view of the fact that during the period of observation, a steady state was presumably never achieved.

Table 21. Initial values for red cell volume (^{51}Cr) and plasma volume (^{125}I-IgG) in the 4 experimental rabbits. The initial venous haematocrit (H_V), the ratio H_B/H_V and the IgG space are also given.

Rabbit number	Weight (kg)	Red cell volume (ml)	Plasma volume (ml)	Venous haematocrit (H_V)	H_B/H_V	IgG space	
						(ml)	(ml/kg)
R22	3.55	50.2	103.3	0.392	0.835	236.0	66.5
R32	3.37	54.3	110.0	0.411	0.803	254.2	75.4
R36	3.25	48.4	100.1	0.400	0.815	227.7	69.9
R35	3.67	59.0	134.8	0.376	0.809	254.9	69.4

Table 22. Plasma ^{131}I- and ^{125}I-activity in 4 rabbits at different times after simultaneous i.m. and i.v. injection of IgG labelled with the respective isotopes of iodine.

Rabbit number	R22		R32		R36		R35	
Weight (kg)	3.55		3.37		3.25		3.67	
Time	i.m.	i.v.	i.m.	i.v.	i.m.	i.v.	i.m.	i.v.
5 min	17	6125	15	5950	15	6331	19	4793
15	57	5607	23	5659	71	5920	43	4695
30	112	5404	55	5509	182	5415	82	4428
1 h	226	5013	135	5077	335	5181	193	4335
$1\frac{1}{2}$	380	4812	199	4815	428	4775	265	4127
2	467	4655	327	4422	515	4655	349	3930
3	561	4492	250	4300	539	4454	362	3837
$4\frac{1}{2}$	607	4160	295	3980	607	4397	427	3802
6	659	4135	375	4012	618	4090	454	3677
9	742	3845	415	3585	679	3900	518	3290
12	818	3755	484	3410	664	3495	563	3230
18	828	3200	528	2898	685	2980	578	2728
1 d	820	2880	547	2571	666	2715	645	2678
2	700	2075	603	2128	633	2160	676	2374
3	668	1870	586	1888	594	1764	580	1775
4	616	1678	596	1808	558	1589	563	1640
5	595	1600	553	1580	515	1420	551	1534
6	534	1428	509	1410	479	1291	491	1338
7	512	1355	489	1400	431	1186	483	1283
10	404	1029	463	1186	365	974	449	1134
14	304	726	354	904	263	679	324	794
20	191	456	234	611	166	397	179	415
25	114	258	153	442	76	270	89	218

Injections. ^{131}I (i.m.) 224,000 ct/min

^{125}I (i.v.) 660,600 ct/min

Table 23. Extreme values for the rates of uptake of IgG from i.m. sites in the rabbits studied.

	Uptake rates ($days^{-1}$)	
Observation period	0-1 day	4-7 days
R32 (minimum)	1.25	0.12
R22 (maximum)	4.60	0.30

Table 24. Values for rate constants α, β & γ and the ratio of extravascular to intravascular IgG compartments (DR) determined in 4 rabbits. The values of α and β have been calculated from data obtained with i.v. and i.m. injections of IgG.

Rabbit number	Apparent catabolic rate α ($days^{-1}$)		True catabolic rate β ($days^{-1}$)		γ ($days^{-1}$)	DR
	i.v.	i.m.	i.v.	i.m.		
R22	0.08986	0.08192	0.2153	0.1981	6.24	1.2845
R32	0.06467	0.06635	0.1646	0.1700	3.99	1.3106
R36	0.08409	0.09371	0.2246	0.2324	7.81	1.2746
Mean	0.07954	0.08066	0.2015	0.2001		1.2899
R35	0.10042	0.09535	0.1674	0.1649	4.22	0.8908

Table 25. Anti-Hg^A titre (IAT) in Hg^A-negative rabbits at various times after the i.v. injection of an IgG preparation of anti-Hg^A. 300 ug antibody and 0,02 ml Hg^A-positive red cells were injected per kg body weight into each recipient.

		Anti-Hg^A titre (IAT)				
Rabbit number		A57	A65	A65	A86	A86
Days after injection	0	128	-	-	128	-
	9	-	-	16	-	16
	15	-	-	8	-	8
	21	16	8	-	-	-
	23	-	-	4	-	-
	29	-	-	4	16	4
	34	-	4	-	-	-
	37	4	-	-	-	-
	40	-	-	2	8	-
	41	-	-	-	-	4
	42	-	4	-	2	-
	48	0	4	-	-	-
	49	-	-	0	-	1
	64	-	0	-	-	-
$t_{\frac{1}{2}}$ (days)		8	11	9	5	11

Figure 11. Immunoelectrophoresis of IgG fractions I and II and of normal rabbit serum (N.S.) using Hyland goat anti-rabbit serum 8233 H002 A1.



Figure 11

SERUM IgG LEVELS IN RABBITS INJECTED WITH ALLOGENEIC RED CELLS

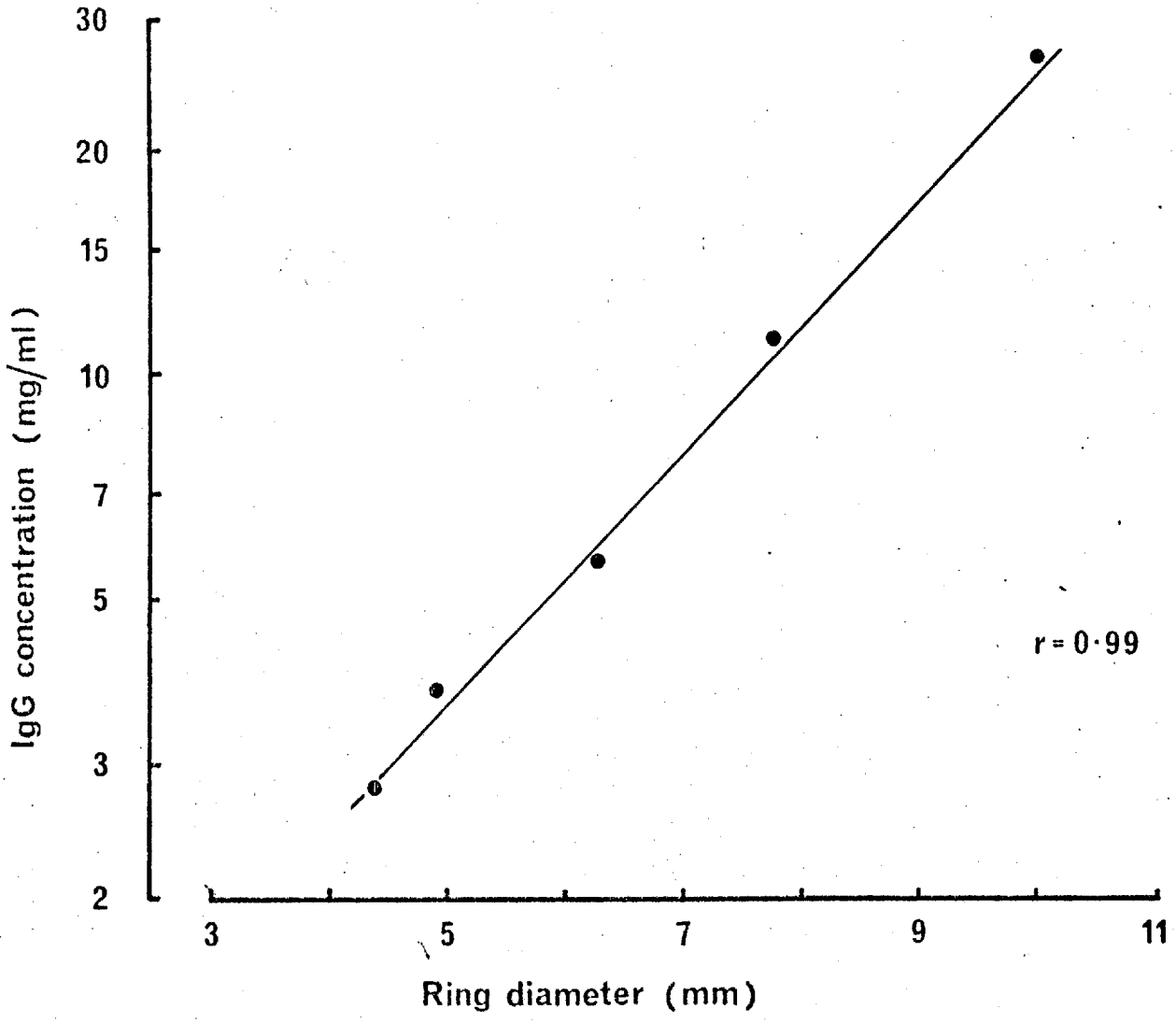
Methods

A quantitative, single phase, gel diffusion technique, in which sheep anti-rabbit-IgG was incorporated in agar plates, was used to measure concentrations of IgG in whole serum (see p.87).

Preparation of standard solutions of IgG. A solution of IgG was prepared from pooled rabbit serum by ammonium sulphate precipitation and anion exchange chromatography (see p.81). Two protein peaks, both of which were shown by immunoelectrophoresis to contain only IgG, were eluted from the DEAE column (see Fig.11). These two samples were pooled, and from the resulting solution four standards were prepared containing 2.80, 3.75, 5.60 and 11.20 mg IgG per ml in phosphate-buffered isotonic saline, pH 7.4. Concentrations of IgG were determined by measuring optical densities of the solutions at 280 nm (see p.86).

Test sera. Sera were obtained from 18 A-negative rabbits selected on the basis of their responses to i.v. injections of A-positive red cells (see p.227and p.286). In group 1 are 8 rabbits that had responded by showing progressive impairment of survival of injected cells; in group 2 are 4 rabbits that showed normal or almost normal survival of repeated injections of cells; and in group 3 are 6 rabbits given a first injection of allogeneic red cells treated with N-ethylmaleimide, following which normal or almost normal survival of subsequent injections of untreated cells was observed. A summary of the injections each rabbit had received prior to testing in the present experiment, together with the type of response observed is shown in Table 26.

Figure 12. Standard plot of IgG concentration against diffusion ring diameter obtained with known concentrations of a purified IgG preparation.


30
20
15
10
7
5
3
2
IgG concentration (mg/ml)
3
5
7
9
11
Ring diameter (mm)
r = 0·99

Measurement of IgG concentrations in sera. Test sera and standards were added to wells in the agar plates in 2 µl volumes; each sample was tested in duplicate. In addition, two wells in each plate were used for a sample of pooled whole serum from 10 rabbits; these served as a control on each plate. The ring diameters of the standard solutions were plotted against the logarithm of their concentrations and a straight line relationship was obtained (see Fig.12). This standard plot was then used to deduce the IgG concentrations of the serum samples from the ring diameters observed.

Results

The values for IgG concentration (mg/ml) observed in the 18 rabbits tested are shown in Table 26. There is no significant difference between the mean values shown for the 3 groups so that an overall mean value is also given.

These results are discussed in later sections (see pp. 227 & 286)

Table 26. Serum IgG concentrations in 18 Hg^A-negative rabbits. The animals in group 1 showed progressive shortening of survival of Hg^A-positive red cells injected i.v. on several occasions, whereas those in groups 2 and 3 showed normal or almost normal survival of repeated injections of Hg^A-positive red cells.

	Rabbit number	IgG (mg/ml serum)	Summary of injections of Hg^A-positive red cells
Group 1	S73	12.4	3 i.v. injections of cells alone
	S76	11.8	-do.-
	S79	13.1	-do.-
	S72	11.3	3 i.v. injections of cells, the first with
	S77	12.9	-do.- (passive antibody
	S89	9.7	-do,-
	S70	13.0	3 i.v. injections, the first of NEM-treated cells
	S97	13.8	4 i.v. injections, the first of NEM-treated cells
	n=8	$\bar{x}$=12.2 (s.d.=1.3)	

Table 26 (cont'd).

	Rabbit number	IgG (mg/ml serum)	Summary of injections of Hg^A-positive red cells
Group 2	S41	13.1	3 i.v. injections of cells alone
	S45	14.0	-do.-
	S94	10.3	4 i.v. injections of cells alone
	S67	12.4	3 i.v. injections of cells alone and 1 i.m. with FCA
	n=4	$\bar{x}$=12.4 (s.d.=1.7)	
Group 3	S69	12.4	3 i.v. injections of cells, the first of NEM-treated cells
	S75	12.6	-do.-
	S78	12.4	-do.-
	S81	11.7	-do.-
	S84	10.4	-do.-
	S93	11.3	-do.-
	n=6	$\bar{x}$=11.8 (s.d.=0.8)	
Groups 1-3	n=18	$\bar{x}$=12.1 (s.d.=1.2)	

SOME OBSERVATIONS ON THE HAEMATOLOGY AND PHYSIOLOGY OF THE NORMAL SANDY LOP RABBIT

SURVIVAL OF AUTOLOGOUS RED CELLS - PILOT EXPERIMENTS WITH $DF^{32}P$

Procedure

- Red cell volume determination
- Labelling of red cells with $DF^{32}P$
- Handling of blood samples
- Measurement of ^{32}P activity

Results

Discussion

SURVIVAL OF AUTOLOGOUS RED CELLS - STUDIES TO DETERMINE TRUE RED CELL SURVIVAL

Procedure

- Labelling of red cells
- Blood samples
- Measurement of radioactivity
- Expression of results
- Estimation of red cell volume and total blood volume
- Calculation of Cr elution rate

Results

Blood volumes

Uptake of DFP by red cells

^{51}Cr survival curves

$DF^{32}P$ survival curves

Comparison of ^{51}Cr and $DF^{32}P$ survival curves

Calculation of true red cell survival

Discussion

One of the methods used in the present work for quantitating the immune response to red cell alloantigens in rabbits was to determine the time taken for the survival of intravenously injected allogeneic red cells labelled with ^{51}Cr to become subnormal (see p.210). It was therefore necessary to define normal red cell survival in rabbits as estimated with ^{51}Cr-labelled autologous red cells. Preliminary observations indicated that the $T_{50}Cr$ determined in this laboratory was longer than had been reported by previous authors, possibly because with the citrate-wash method of labelling used in these experiments, the rate of Cr elution from the reinjected red cells was relatively slow. An attempt to estimate directly the rate of Cr elution was therefore undertaken by labelling red cells simultaenously with ^{51}Cr and $DF^{32}P$. In a small subsidiary study, a comparison was made between survival in different rabbits of autologous red cells labelled with ^{51}Cr either by the citrate-wash method or by an ACD method. Pilot experiments to determine the amount of DFP taken up by rabbit red cells *in vivo* following i.v. injection of DFP will be described first.

PILOT EXPERIMENTS WITH $DF^{32}P$

Experiments were performed in 3 rabbits in which different doses of DFP were injected i.v. The amount of DFP present on the red cells was measured approximately 1 hour after injection and again 5 days later.

Procedure

Red cell volume determination. In each case, 1 ml blood from a marginal ear vein was taken into a syringe containing a drop of liquid

heparin. The heparinized blood was labelled with 12 μCi ^{51}Cr using the citrate-wash method (see p.80). The washed, labelled red cells were resuspended in isotonic saline to a volume of about 1.5 ml of which 1 ml was injected i.v. into the donor rabbit. About 15-20 minutes after injection of the labelled red cells, a blood sample of about 1.5 ml was taken into a syringe containing a drop of liquid heparin. The sample was lysed with saponin and 1 ml was counted for ^{51}Cr activity in a standard gamma counter. 1 ml of a 1 in 100 dilution of the injection suspension was also counted. The haemoglobin concentration of the lysed sample was measured using the cyanmethaemoglobin method and from this value, the venous packed cell volume (H_V) was deduced assuming an MCHC of 34.5 g/dl (see p.88). It was then possible to calculate the circulating red cell volume (RCV). The total blood volume (TBV) was deduced from the RCV and H_V using the following relationship (see p.110):

$$TBV = \frac{RCV}{H_V \times 0.843}$$

Hence the plasma volume could be derived.

Labelling of red cells with $DF^{32}P$. Within 2 hours of injecting the ^{51}Cr-labelled red cells, an i.v. infusion of $DF^{32}P$, freshly diluted in isotonic saline was started; 1 ml solution containing approximately 20, 40 or 80 μg DFP (^{32}P activity 3.4, 6.8 and 13.6 μCi respectively) was given over about 10 minutes from a constant rate infusion pump (see p.81). Blood samples were taken about 1 hour after completing the infusion of DFP and again 5 days later; on each occasion about 1.5 ml blood was taken into a dry syringe and was immediately transferred to a pot containing dry EDTA. Known dilutions of the injection solutions were prepared and saved for analysis.

Handling of blood samples. Each anticoagulated whole blood sample was processed immediately it had been taken. 1 ml was washed into a tube containing 1 g/dl phosphate-buffered saline and the volume was adjusted to 11 ml with saline. After thorough, gentle mixing, the specimen was centrifuged; the supernatant was removed as completely as possible without disturbing the cell button and was retained for radioactive counting. Two further washes were carried out in this way, the supernatants being discarded. The washed packed cells were lysed with 0.04% (v/v) ammonia water and the final volume of the lysate was adjusted to 11 ml. The haemoglobin concentration in the remnant of the whole blood sample was estimated and the H_v was deduced as described above. The lysate and first supernatant from the 1 hour sample and the diluted injection solution were stored at 4°C until the end of the experiment when samples relating to each individual rabbit were counted within a short time of one another.

Measurement of ^{32}P activity. The supernatants and lysed blood samples, prepared as described above, and also the diluted injection solutions were each transferred to a liquid sample Geiger-Muller tube for estimation of ^{32}P activity. The counting error was less than 1% in all samples except those of the plasma dilutions from day 5 samples in which, because of low counting rates, the error was about 5%. Corrections to the observed ^{32}P counting rates were applied for counts due to ^{51}Cr in the lysed blood samples. The amount of radioactivity (ct/min) injected was calculated as the product of the ct/min of the diluted material and the dilution factor. Similarly, the counting rate per ml red cells (or plasma) was given by:

$$\text{ct/min in lysate (or supernatant)} \times \frac{11}{H_v \text{(or "plasmatocrit")}}$$

For the purposes of calculating the amount of DFP associated with red cells and plasma on day 5, it was assumed that the RCV remained constant and that any change in H_V reflected proportional alteration in H_B and hence in plasma volume.

Results

The amount of $DF^{32}P$ injected varied from about 0.4-1.5 µg/ml red cells. Over the dose range studied, there was an inverse relationship between the amount of DFP injected and the proportion taken up by the red cells, although in absolute terms, the amount on the red cells increased from about 0.04-0.11 µg/ml rbc as the dose increased. The amount found on the red cells after 5 days was around 67% of that present 1 hour after injection, irrespective of the absolute amount bound initially. The concentration of DFP in the plasma 1 hour after injection was at least 50% of that on the red cells; after 5 days the value ranged from 4-8%. The results of these experiments are given in detail in Table 27.

Discussion

The initial uptake of $DF^{32}P$ by rabbit red cells observed in these experiments is generally much greater than values reported by Heimpel, Hoffman and Keiderling (1964) following injection of comparable amounts of $DF^{32}P$ into rabbits by the i.m. route. However, inconsistencies in the data published by these authors make precise comparison difficult; for example, in their Table 1, the values given for the amount of DFP injected (mg), the amount present on the red cells (µg/g Hb) and the percentage incorporation indicate that 4 out of the 5 rabbits illustrated

had red cell volumes 2 or even 3 times that found in the rabbits used in the present experiments. On the other hand, the figures recorded by these authors for percentage incorporation of DFP into the red cells following i.v. injection of approximately 10 μg $DF^{32}P$/kg body weight range from about 20% when the dose was given in a few seconds to 30% when it was infused over about half an hour, (compared with the value of about 9% in the present experiments). In another paper from the same laboratory (Shirakura, Heimpel and Keiderling, 1964) the maximum uptake of $DF^{32}P$ by rabbit erythrocytes labelled in vitro was found to lie between 0.1 and 0.15 μg/ml red cells; similar values for the maximum amount of DFP which could be irreversibly bound to human red cells were found by Bratteby and Wadman, 1968, and Matsuda, 1969.

From the present experiments, it was concluded that the amount of DFP attached to red cells following the i.v. injection of about 1.5 μg/ml circulating red cells would be suitable for the proposed red cell survival studies; the amount of DFP bound would thus enable the incorporation of sufficient ^{32}P activity to give adequate counting rates in blood samples taken during the survival experiments and yet would be well within the range found by others to be satisfactory for labelling red cells.

Table 27. Amounts of DF^{32}P associated with red cells and plasma 1 hour and 5 days after i.v. infusion of different amounts of DFP into 3 rabbits.

Rabbit number	Red cell volume (ml)	DFP injected		At 1 hour				At 5 days			
				DFP on red cells		DFP in plasma		DFP on red cells		DFP in plasma	
		µg	µg/ml RBC	%	µg/ml RBC	%	µg/ml plasma	%	µg/ml RBC	%	µg/ml plasma
S43	50.4	20.2	0.4018	9.6	0.0387	14.2	0.0242	6.5	0.0263	0.9	0.0018
S46	35.8	41.3	1.1536	9.2	0.1062	15.1	0.0561	6.0	0.0697	0.7	0.0031
S24	53.9	82.3	1.5300	7.4	0.1130	10.4	0.0628	4.9	0.0754	0.7	0.0060

RED CELL SURVIVAL STUDIES

Red cell survival studies were carried out in two groups of animals.

(a) In 5 rabbits, autologous red cells were labelled simultaneously with ^{51}Cr *in vitro* and with DF^{32}P *in vivo* in order to determine the rate of ^{51}Cr elution and to obtain an estimate of mean red cell life span.

(b) Red cell survival curves observed in 2 rabbits after labelling autologous red cells with ^{51}Cr by the citrate-wash method were compared with those in two other rabbits whose red cells had been labelled with ^{51}Cr by an ACD method.

Procedure

Labelling of red cells. (a) Autologous red cells were labelled with ^{51}Cr and DF^{32}P in 5 rabbits (S53-57). In each case, approximately 1 ml venous blood was taken from a marginal ear vein into a dry plastic syringe. The blood was immediately mixed with 10 ml citrate-phosphate-dextrose (CPD) solution, pH 6.9 and centrifuged. After discarding the supernatant, approximately 50 µCi ^{51}Cr as sodium chromate was added; the amount of chromate added contained the equivalent of 0.15 µg Cr, i.e. about 0.45 µg Cr/ml red cells. After incubating at 37°C for 10 minutes, the cells were washed twice with isotonic saline. The washed red cells were resuspended in isotonic saline to a volume of about 1.5 ml, of which 1 ml was injected i.v. into the donor animal. Five minutes after injection, a 1 ml blood sample was taken from a different vein for estimation of red cell volume. Within 10 minutes of this sample

being taken, a slow continuous i.v. infusion of DF^{32}P, freshly diluted in isotonic saline was started (see p.81); a total of 77 µg DFP (28.6 µCi ^{32}P) in 1 ml solution was injected over about 10 minutes into the central vein of one ear.

(b) Survival of autologous red cells labelled with ^{51}Cr alone was studied in 4 rabbits. In each case, 2 ml blood from a marginal ear vein was drawn into a syringe containing a drop of liquid heparin. The heparinized blood was mixed with 0.5 ml ACD in 2 cases (A90, S21), and with 10 ml CPD solution in 2 further cases (A92, A99). Each sample was centrifuged and the supernatant discarded. A solution of sodium chromate containing approximately 50 µCi ^{51}Cr and the equivalent of 0.75 µg Cr (just less than 1 µg Cr/ml red cells) was added to the packed cells and mixed gently. After incubating at 37°C for 10 minutes, the cells were washed twice with isotonic saline. The washed cells were resuspended in isotonic saline to a volume of about 6 ml, of which 5 ml was injected i.v. into the donor animal.

Blood samples. (a) In the experiment using ^{51}Cr and DF^{32}P, 1 ml samples were taken 1 hour after completion of the DFP infusion and subsequently at increasing intervals for about 6 weeks, as indicated in the graphs. Samples were taken into dry plastic syringes, transferred immediately into tubes containing 2.5 mg dry EDTA and mixed gently. Within a few minutes of collection of each sample, 0.5 ml of the anticoagulated blood was washed into a tube containing approximately 20 volumes of 1 g/dl phosphate-buffered saline. After mixing, the samples were centrifuged; the supernatant was removed as completely as possible

without disturbing the cell button. Two further washes were carried out in this way. The packed cells were then lysed with 0.04% (v/v) ammonia water containing 10 mg sodium azide/dl, the final volume being adjusted to 10.5 ml.

(b) In the experiments using ^{51}Cr alone, 1 ml samples were taken between 5 and 10 minutes after injection of the labelled cells and at increasing intervals for about 8 weeks. Blood was taken into syringes containing a drop of liquid heparin. Before counting, the samples were lysed with saponin.

All samples, prepared as described above in (a) and (b), were stored at $4^{o}C$ until the end of each survival study. Then samples from each individual rabbit were counted together in batches within a few hours of each other.

Measurement of radioactivity. (a) Samples containing both ^{32}P and ^{51}Cr. Each complete 10.5 ml lysed sample, prepared as described above, was first transferred to a liquid sample Geiger-Muller tube for measurement of ^{32}P activity. Initially, the counting error was less than 2%, but in some of the later samples, the error was about 3% because of the lower counting rates. Corrections were later applied to the observed counting rates for counts due to ^{51}Cr.

After the ^{32}P activity had been measured, 2 ml of each lysed sample was counted in a standard gamma counter. Corrections were applied for counts due to ^{32}P.

(b) Samples containing ^{51}Cr alone. 0.5 ml amounts of the lysed whole blood samples were counted in a gamma counter, sufficient counts being recorded to keep the counting error at less than 1%.

Expression of results. The haemoglobin concentration of each sample was determined by the cyanmethaemoglobin method and results were expressed as ct/min per g Hb.

Observed results were corrected for cumulative blood loss due to sampling as suggested by Mollison (1972 pp.31-32), either using derived values for total blood volume based on estimates of red cell volume (see below), or assuming total blood volume to be 150 ml.

Corrected values were expressed as percentages of the value of the sample taken on the day of injection.

Estimation of red cell volume and total blood volume. In the 5 rabbits in which both ^{51}Cr and DF^{32}P were used, red cell volume was deduced from measurements of ^{51}Cr activity in blood samples taken 5 minutes after injection of a measured amount of ^{51}Cr-labelled red cells. In practice, the haemoglobin concentration of the 5 minute blood sample was estimated and H_V deduced, assuming the MCHC of normal rabbit red cells to be 34.5 g/dl (see p.88). Total blood volume was derived from red cell volume and true body haematocrit, the latter being calculated as $0.843 \times H_V$ (see p.116).

Calculation of Cr elution rate. It was assumed that from day 7 onwards (day 10 in the case of S55), there was no significant elution of $DF^{32}P$ from the red cells whereas ^{51}Cr continued to elute at a constant rate (see later). The observed counting rates for ^{32}P and ^{51}Cr (uncorrected for sampling) were expressed as a percentage of the values observed on day 7 (day 10 for S55); then, for any day T, measured from the 100% reference point

$$\frac{\text{Observed } ^{51}Cr\ (\%)}{\text{Observed } DF^{32}P\ (\%)} = e^{-KT}$$

where K ($days^{-1}$) is a first order rate constant. K was calculated from each pair of observations after day 7 (or day 10) in each rabbit and mean values for the elution rates were obtained; these mean values for K were used to correct each set of ^{51}Cr survival data (corrected for sampling losses) as follows. On any day t, measured from the time of injection,

$$\text{actual Cr}(\%) = \frac{\text{observed Cr } (\%)}{e^{-Kt}}$$

Results

Blood volumes. In the five rabbits injected with both $DF^{32}P$ and ^{51}Cr, red cell volumes varied from 43.4 to 57.3 ml and deduced total blood volume varied from 159.1 to 195.9 ml. Individual results are given in Table 28.

Uptake of DFP by red cells. From the amount of $DF^{32}P$ injected and the red cell volume estimates, it was calculated that the uptake of $DF^{32}P$ by the circulating red cells at 1 hour ranged from 6.8-9.0%

Figure 13. ^{51}Cr autologous red cell survival curves corrected for sampling losses. x: 5 cases in which the citrate-wash method was used and in which survival was also estimated with $DF^{32}P$. △, O: 2 further cases in which the citrate-wash method was used for comparison with 2 cases (▲, ●) in which an ACD method was used.

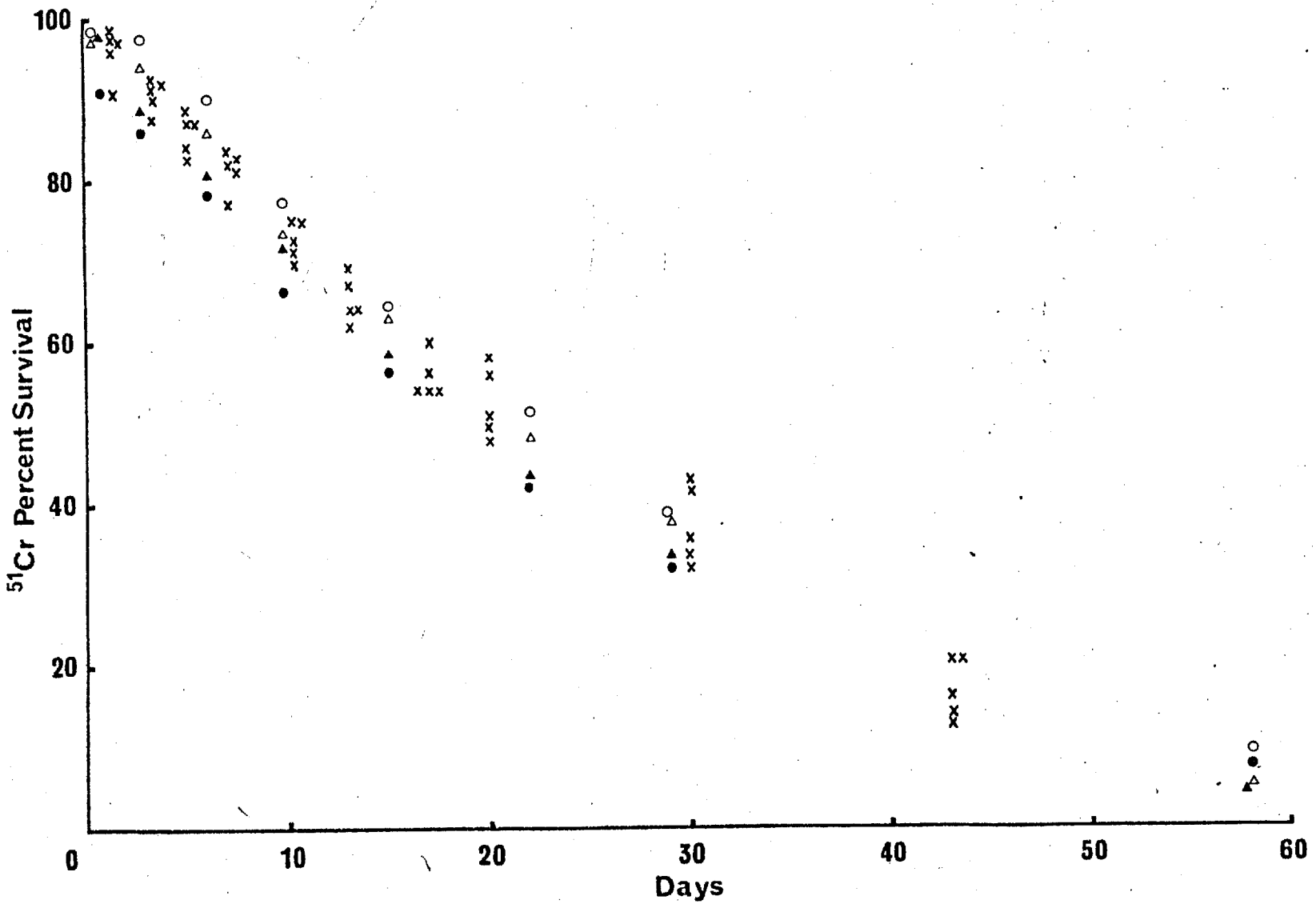
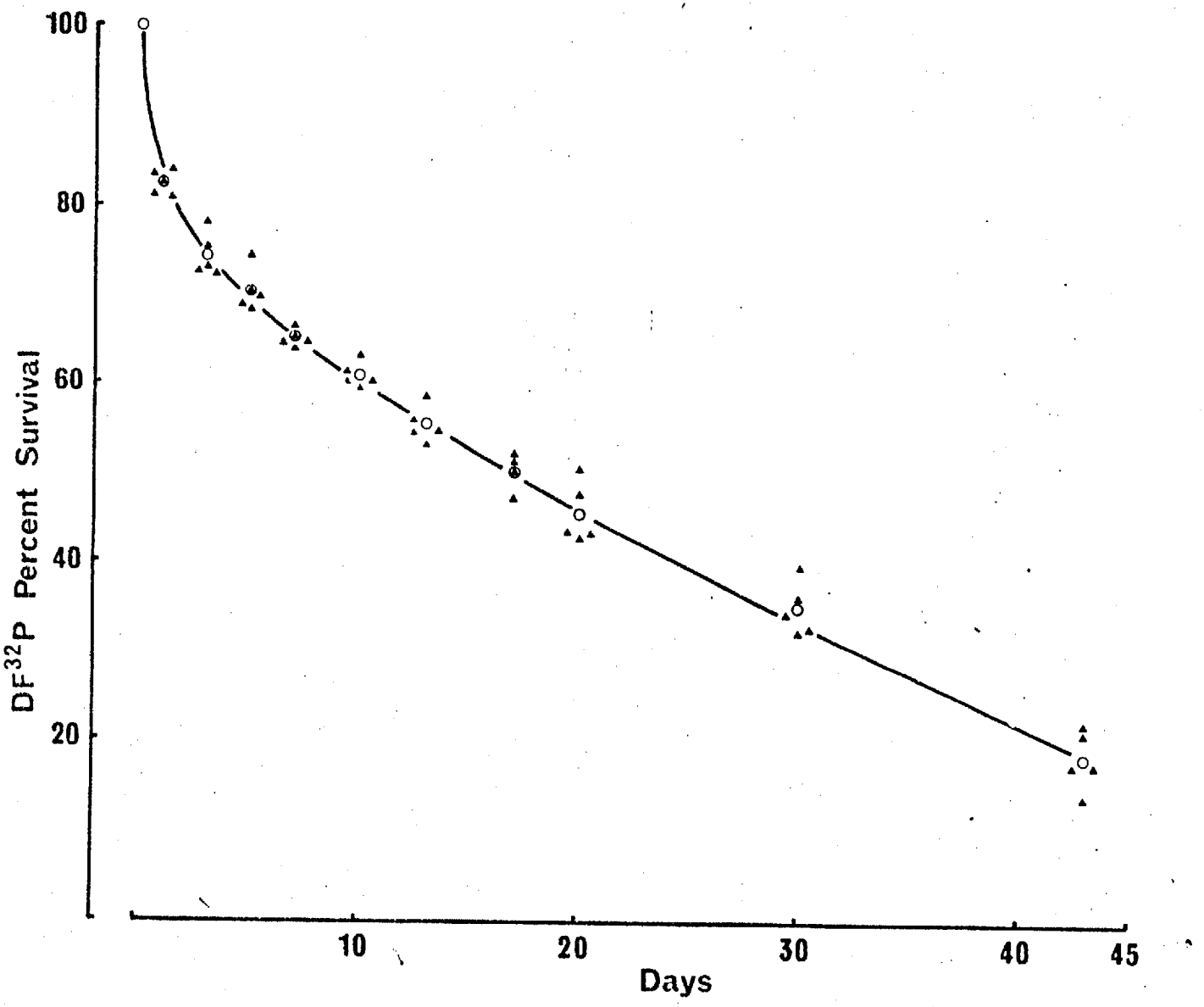

100
80
60
40
20
0
10
20
30
40
50
60
Days
^{51}Cr Percent Survival

Figure 14. $DF^{32}P$ autologous red cell survival curve corrected for sampling losses. O : mean values; ▲ : individual values in 5 rabbits. The curve has been fitted by eye.


100
80
60
40
20
DF32P Percent Survival
10
20
30
40
45
Days

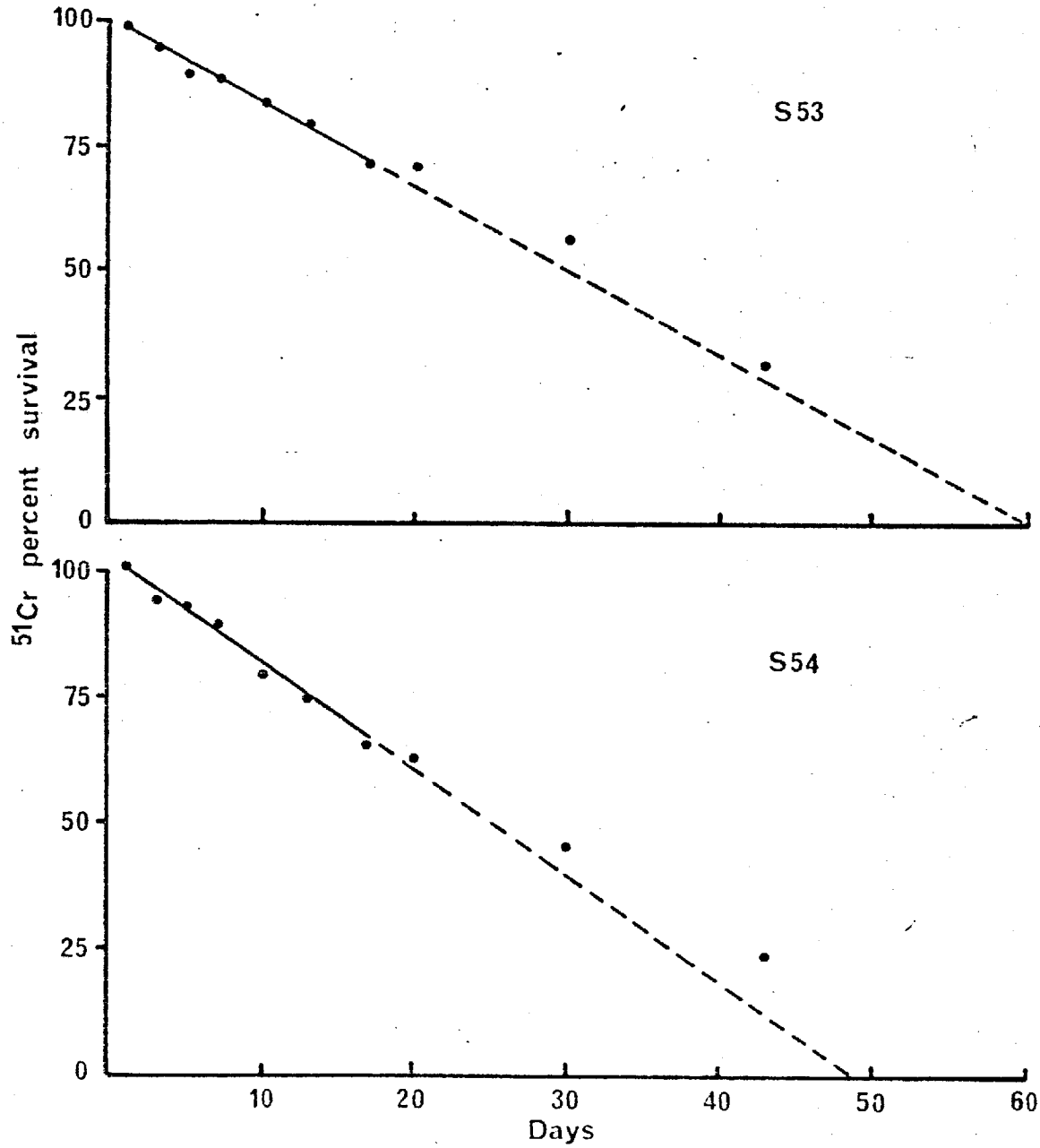
(mean 8.0%) of the injected dose, corresponding to 0.116-0.130 μg DFP/ml red cells (mean 0.123 μg). Individual results are shown in Table 29.

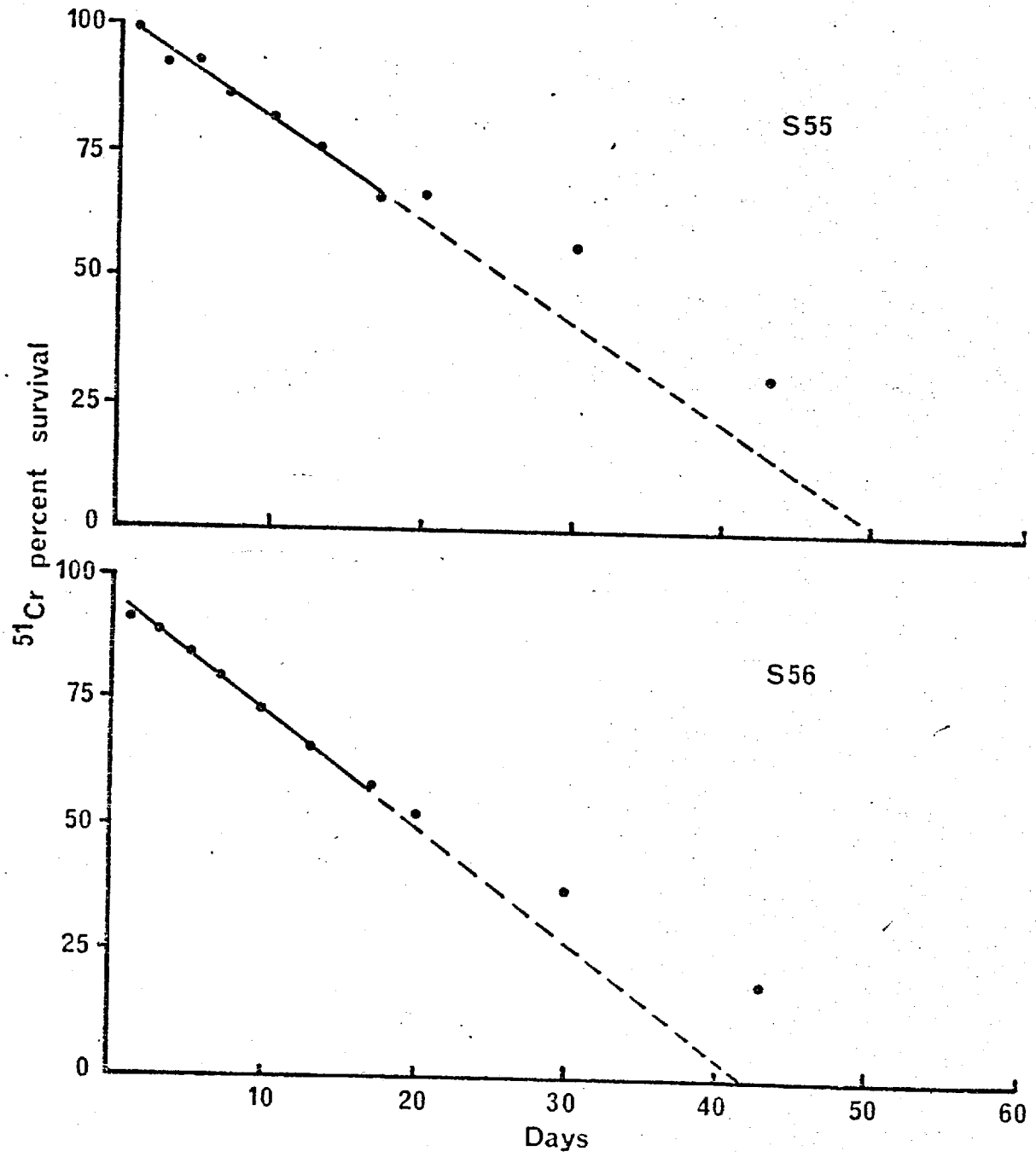
^{51}Cr survival curves. Fig.13 shows ^{51}Cr survival results (corrected for cumulative sampling losses) in 9 rabbits. With the citrate wash method, values for $T_{50}Cr$ were as follows: 24.2, 19.5, 23.0, 18.8 and 20.1 days for the 5 rabbits in which $DF^{32}P$ was also injected (S53-57), and 21.5 and 22.2 days for the 2 rabbits in which only ^{51}Cr was injected (A92, A99); in the two cases in which an ACD method was used to label the red cells (A90, S21), $T_{50}Cr$ was 17.9 and 18.9 days. Although the mean T_{50} for the seven cases in which the citrate-wash method was used is 21.3 days and that for the two cases in which an ACD method was used is 18.4 days, the difference is not statistically significant (p = approximately 0.09, "t" test).

$DF^{32}P$ survival curves. As shown in Fig.14, about 20% of the injected $DF^{32}P$ was lost from the red cells in the first 24 hours and the survival curve approached linearity only after about the first 7 days (see later). From the observations made on days 30 and 43, a further decrease in the rate of fall of $DF^{32}P$ activity was apparent, leading to slight curvilinearity of this part of the survival curve.

Comparison of ^{51}Cr and $DF^{32}P$ survival curves. In the five cases in which both labels were used, the ratio Cr/DFP rose slightly over the first few days. However, from day 7 onwards the ratio fell progressively in all cases except S55, in which a decrease was not observed until day 10.

Figure 15. True red cell survival curves in 5 rabbits obtained by correcting observed Cr survival for sampling losses and for Cr elution. The continuous line was fitted by a "least squares" method to the data obtained over the first 17d. Extrapolation of this line to the time axis gives an estimate of mean red cell life-span.


100
75
50
25
0
S53
100
75
50
25
0
S54
51Cr percent survival
10
20
30
40
50
60
Days


100
75
50
25
0
S55
^{51}Cr percent survival
100
75
50
25
0
S56
10
20
30
40
50
60
Days

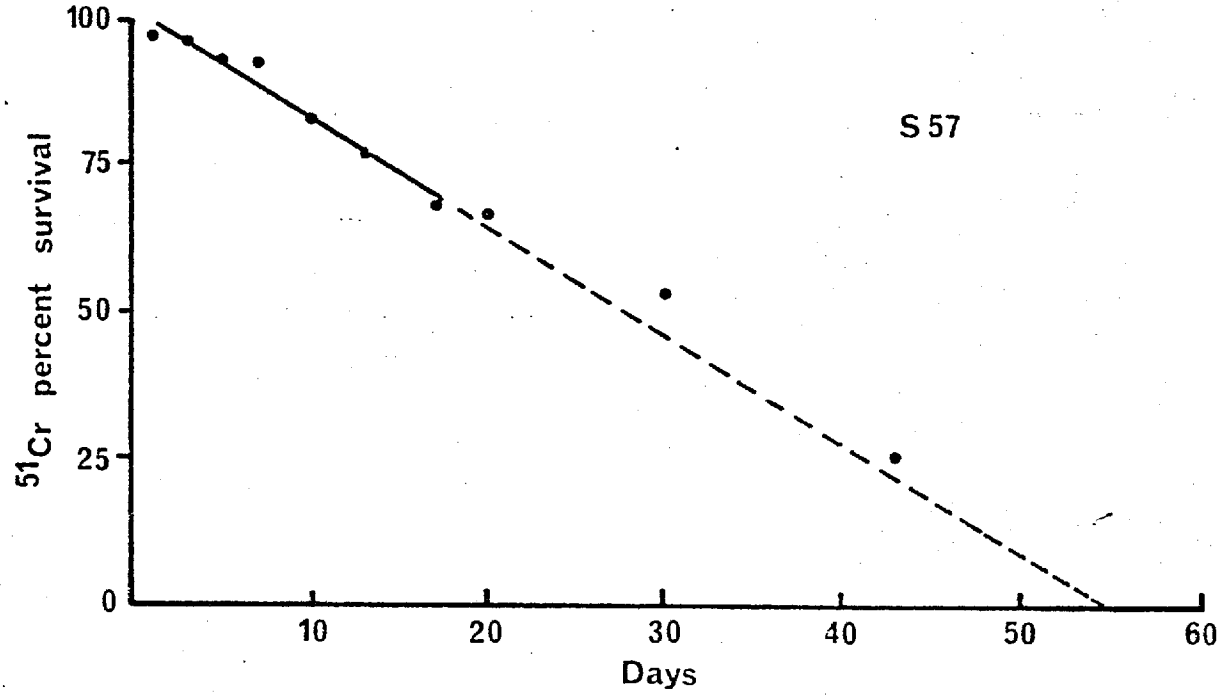
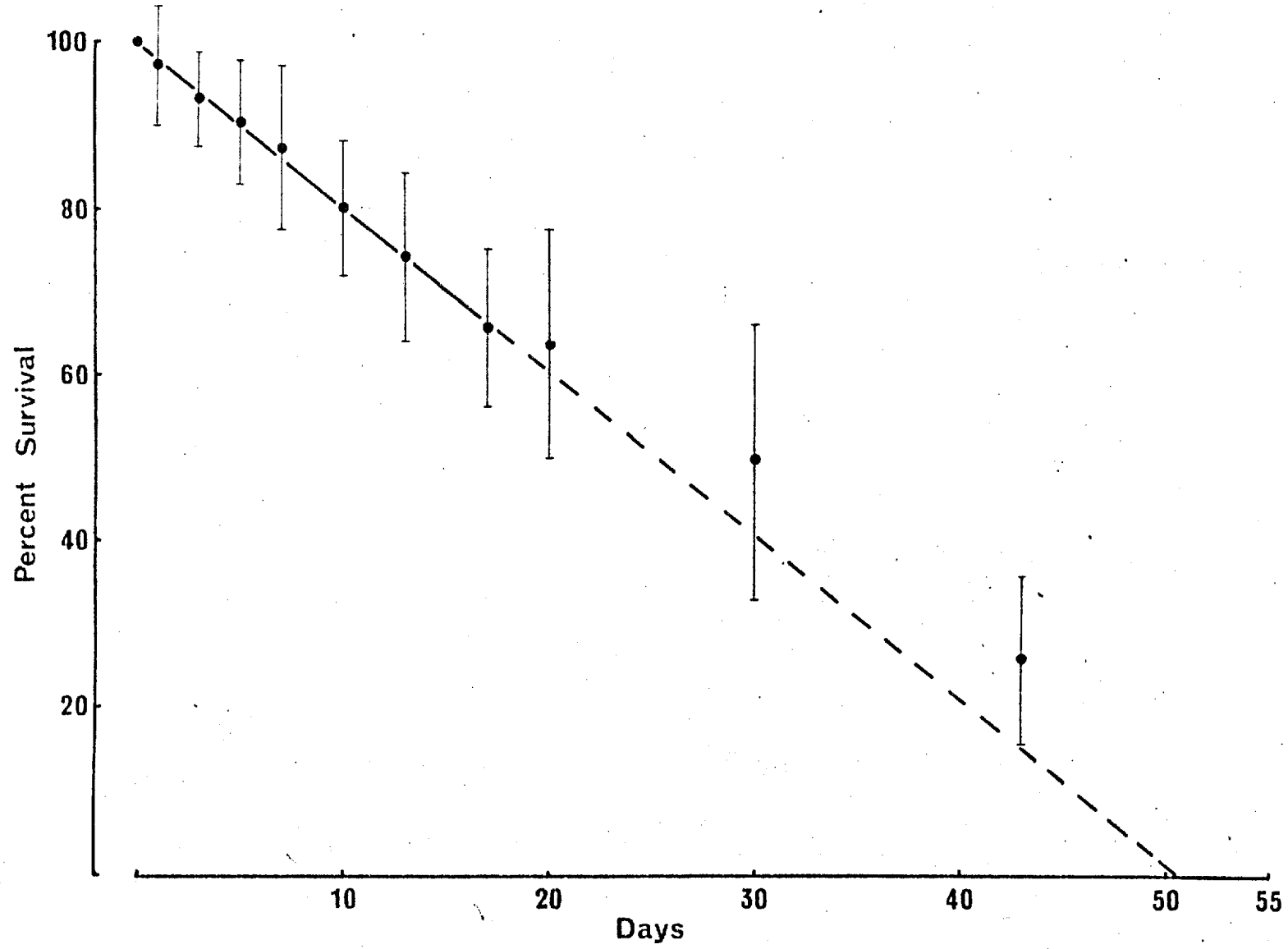

100
75
50
25
0
^{51}Cr percent survival
S 57
10
20
30
40
50
60
Days

Figure 16. True red cell survival curve. Each point shows the mean (± 2 s.d.) of observations made in the 5 rabbits illustrated in Figure 15. The continuous line was fitted by a "least squares" method to the mean values over the first 17d ($r = 0.99$).


100
80
60
40
20
Percent Survival
10
20
30
40
50
55
Days

These results indicate that, during the first 7 days or so, elution of DFP from the red cells occurs more quickly than elution of Cr and that, thereafter, the converse applies. From a consideration of the survival data, the most likely explanation of this observation is that there is rapid elution of DFP initially but negligible elution after about day 7 and that by contrast, the rate of Cr elution remains relatively constant throughout (except for a slightly increased loss noted in the first 24 hours). Accordingly, Cr/DFP ratios from day 7 onwards (day 10 in the case of S55) were used to determine the rate of Cr elution as already described and it was then assumed that the rate of Cr elution was in fact constant from day 1 onwards. Values for Cr elution, based on measurements from day 7 onwards (day 10 in S55) are shown in Table 30 and ranged from 0.49-1.40% per day in different rabbits (mean 1.0%).

Calculation of true red cell survival. Using the Cr data corrected for sampling losses and Cr elution as described above, true red cell survival was calculated in the 5 cases in which both Cr and DFP were injected. The corrected Cr survival curves for each of the 5 cases are shown in Fig.15 and a composite curve derived from the mean survival value on each sampling day is shown in Fig.16. As the figures show, the data for the first 17 days are well fitted by a straight line; the method of least squares was used to fit the lines and the correlation coefficient in each case was greater than 0.98. Mean cell life was deduced by extrapolating the fitted line to the time axis and varied from 41.6 to 59.9 days, with a mean value of 50.4 days. The corrected Cr survival data are given in Table 31.

Discussion

The $T_{50}Cr$ observed in the present series following labelling by the citrate-wash method averaged 21.3 days for 7 cases (range 18.8-24.2 days). This is slightly longer than the figures of 19 days (6 cases) reported by Marvin and Lucy (1957), 17 days (5 cases) reported by Heimpel et al. (1964), and of 16-17 days (31 cases) reported by Miescher (1956), and considerably longer than the figure of 12 days (17 cases) reported by Gardener et al. (1961). It is possible that the method of treating the red cells before incubation with ^{51}Cr affects the subsequent rate of Cr elution (Mollison, 1961). In the present series, there was in fact a suggestion that $T_{50}Cr$ was shorter when ACD blood was labelled than when the citrate-wash method was used although the difference was not significant (p = approximately 0.09); observations in a larger number of rabbits are needed to settle this question.

Simple inspection of the Cr survival data (Fig.13) shows that over about the first 20 days, decrease in Cr activity is only very slightly curvilinear; thereafter, the curvilinearity increases. This is in marked contrast to the DF^{32}P disappearance curve (Fig.14) which shows maximal curvilinearity initially, is substantially linear between days 7 and 20, and thereafter shows slight curvilinearity to the final observations on day 43.

Analysis of these data indicates that after injection of the labels, the rate of loss of $DF^{32}P$ is greater than that of ^{51}Cr for about the first 7-10 days and that from this time onwards, the rate of loss of ^{51}Cr exceeds that of $DF^{32}P$ by approximately 1% per day. Bove and Ebaugh (1955) gave i.m. injections of $DF^{32}P$ to normal human subjects in doses ranging from 500-900 µg (about 7-14 µg/kg body weight) and observed an initial period of 5-10 days during which the specific activity of the red cells decreased at a faster rate than subsequently; from their reported data it can be calculated that the amount of DFP attached to the red cells of these subjects shortly after labelling lay between about 0.07 and 0.17 µg/ml cells. Because of similar observations on the early decrease in specific activity of red cells following in vivo labelling with $DF^{32}P$ in patients with no evidence of a haemolytic state, Eernisse and van Rood (1961) used only DFP data from 7 days onwards for calculating "normal" red cell survival in these cases; the dose of DFP which was given by i.m. injection varied between 370 and 1120 µg (about 5-16 µg/kg body weight). Thus there is no evidence to suggest that varying the dose of DFP used for in vivo labelling of red cells substantially affects the duration of the initial phase of label elution.

The extent of loss of $DF^{32}P$ from the circulating red cells in the first 24 hours, namely 20%, in the present series is similar to that of 25% found in rabbits by Heimpel et al. (1964). Although it has been shown that after labelling rabbit red cells in vitro with DFP, the extent of the early loss is positively related to the amount of DFP

used in the incubation mixture (Shirakura et al., 1964) there is no decisive evidence for any similar relationship following in vivo labelling of red cells either in man (Eernisse and van Rood, 1961) or in the rabbit (see p.182), when the doses of DFP used are in the range 5-30 µg/kg body weight. Nevertheless, Hjort, Paputchis and Cheney (1960) found that when a very large dose of DFP, namely 120 µg/kg body weight, was given i.m. to rats, about 70% of the activity initially associated with the red cells was lost during the ensuing 2 days. A similar increase in early losses was reported by Cline and Berlin (1962) when the dose of DFP given i.v. to dogs was varied between 30 and 260 µg/kg body weight. At the other extreme, Wadman (1972) has shown that when very low doses of DFP in the range 0.2-3.0 µg/kg body weight were used in man, losses of red cell activity during the first 24 hours were reduced to 8%. It is interesting to note that, even with the optimal labelling conditions used by Wadman, some loss due to elution could be detected as late as 4 days after injection.

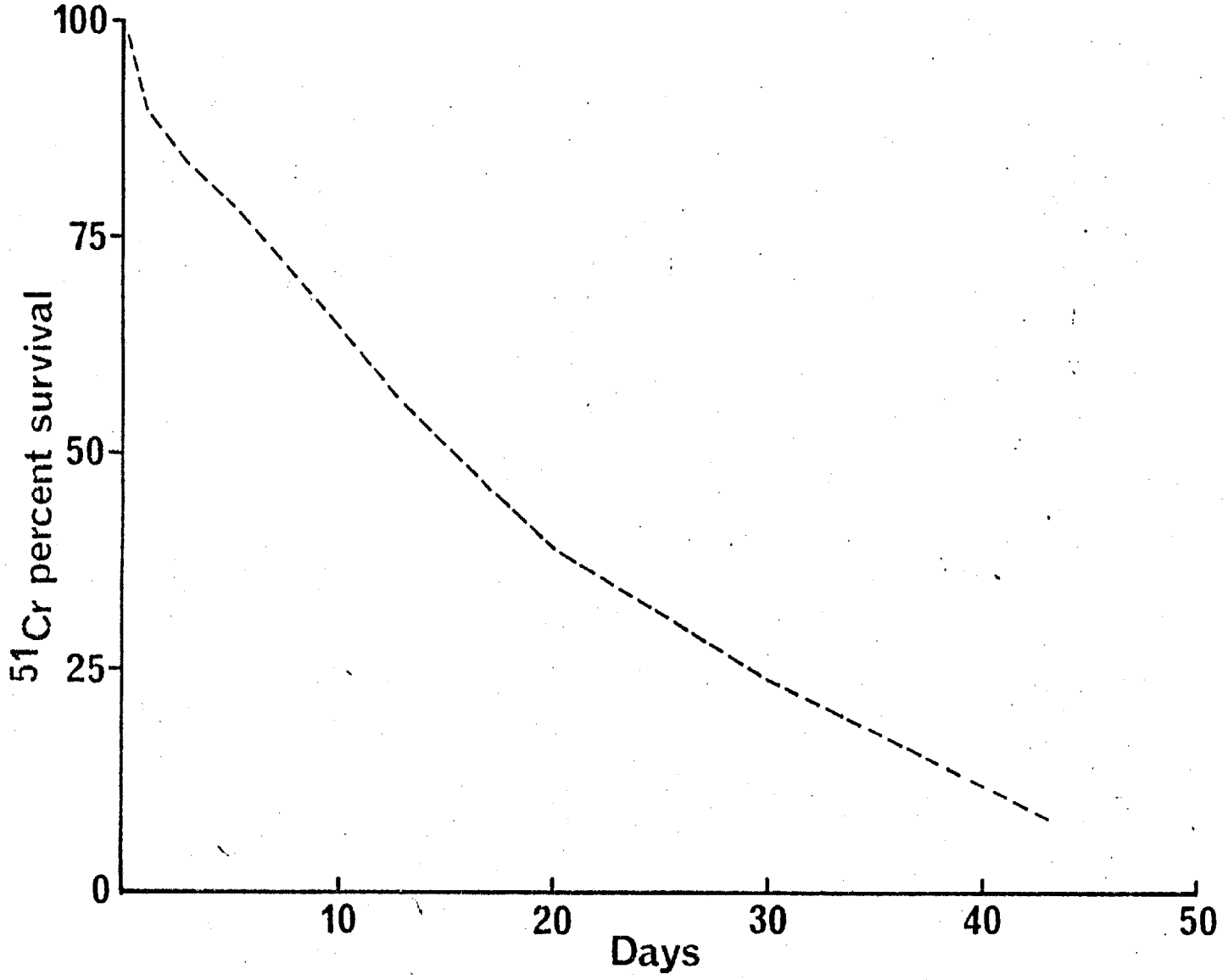
Heimpel et al. (1964) in their experiments in rabbits assumed that there was no further elution of $DF^{32}P$ after 24 hours and, taking the 24-hour values as 100%, found T_{50} to be 19-21 days in five rabbits. By the same procedure Karle (1968a) found $T_{50}DF^{32}P$ to be about 21 days in one rabbit. If the same assumptions are applied to the present data, T_{50} is approximately 22 days. However, as elution of DFP continues for about 7 days, the T_{50} DFP calculated in this way does not correctly indicate the T_{50} of true red cell survival.

In the present series, the rate of Cr elution calculated from the $^{51}Cr/DF^{32}P$ ratios from day 7 onwards was 0.49-1.40% (average 1.00%) per day. This rate is slightly less than that observed in humans, namely 0.6-2.0% (average 1.19%) per day, also using the citrate-wash method of ^{51}Cr labelling (Garby and Mollison, 1971).

In calculating true red cell survival from the present results, it has been assumed that the rate of elution of Cr was constant from day 1 onwards. As shown in Fig.16, the mean corrected Cr data between days 1 and 17 are well fitted by a straight line (correlation coefficient 0.99); the three subsequent observations exceed by increasing amounts the values predicted by extrapolation of the earlier data. Survival curves in which the slope is initially linear on an arithmetical plot, thereafter becoming curvilinear, are to be expected when red cell destruction is due predominantly to senescence but with a large variation in red cell life-span (Dornhorst, 1951). In the past it has been generally assumed that the curvilinear slopes of red cell survival observed in rabbits either with random labelling or with cohort labelling (Neuberger and Niven, 1951; Brown and Eadie, 1953; Card and Valberg, 1967) are due to random red cell destruction superimposed on red cell death from senescence, but this interpretation now seems less likely. So far as the cohort-labelling experiments are concerned, analysis of the data is rendered difficult by the fact that when methods depending upon incorporation of a metabolic label such as ^{15}N-glycine or ^{59}Fe are used, labelling continues for a period of many days. An additional complication arises when $DF^{32}P$ is used as a cohort label, because of the prolonged initial period of elution (see Card and Valberg, 1967).

In principle, it should be possible to confirm that the initial slope of red cell survival is linear on an arithmetical plot by determining survival by differential agglutination using compatible red cells (Ashby, 1919). However, a suitable blood group system combining poor antigeneity and potent agglutinating alloantibody have not yet been identified in the rabbit. Cross-transfusion experiments have been performed by Wright et al. (1955); these authors raised red cell grouping and agglutinating antisera by random cross immunization within their rabbit colony, but did not identify the blood groups system(s) involved. Their findings in a group of 21 rabbits of a mean T_{50} for red cell survival of about 12 days and a maximum survival time which varied in different rabbits between 30 and 64 days strongly suggests that alloimmunization of the recipient rabbits had been induced in some cases by the donor cells. Against this possibility, it was argued that in none of the recipients could antibody to the donor cells be detected during the survival study. But it has been shown in rabbits, using differences within the Hg blood group system that curtailed survival of intravenously injected allogeneic red cells may occur on several occasions, apparently as a result of immunization, before specific humoral antibody can be detected (see p.214). Moreover, in the cross-transfusion experiments of Wright et al (1955), 7 out of the 21 receipient rabbits were challenged with a further injection of donor cells and in 1 rabbit, antibody against the donor cells was subsequently detected. No other reports of experiments using the differential agglutination technique to determine true red cell survival in rabbits appear to have been published.

Figure 17. Lower limits (mean - 2 s.d.) of ^{51}Cr survival curve for autologous red cells labelled by the citrate-wash method. Data from 7 rabbits not corrected for sampling or Cr elution (see text).


100
75
50
25
0
^{51}Cr percent survival
10
20
30
40
50
Days

In some of the experiments to be described later, in which allogeneic red cells labelled with ^{51}Cr by the citrate-wash method were injected i.v., comparisons between red cell survival data have frequently been made on the basis of the T_{sn}, i.e., the time taken for survival to become definitely "subnormal" (see p.210). "Normal" lower limits have been established from data obtained in the present experiments in the 7 rabbits whose own red cells were labelled with ^{51}Cr using the citrate-wash method. As a similar sampling schedule was used in all of the experiments, the "normal" lower limits of red cell survival for these purposes were derived from the raw data (not corrected for elution or sampling) by taking the mean minus 2 standard deviations of the percent survival at various times after injection (see Fig.17).

Table 28. Blood volumes in five rabbits S53-57.

Rabbit number	Red cell volume (ml)	Total blood volume (ml)*
S53	47.7	159.1
S54	43.4	160.2
S55	57.5	195.9
S56	48.5	180.6
S57	53.3	186.7

* Derived from red cell volume and an assumed H_B/H_V ratio of 0.843

Table 29. Uptake of $DF^{32}P$ into circulating red cells one hour after injection.

Rabbit number	Dose of $DF^{32}P$ injected (μg/ml RBC)	$DF^{32}P$ Uptake	
		μg/ml RBC	% of injected dose
S53	1.61	0.1300	8.05
S54	1.77	0.1208	6.81
S55	1.34	0.1211	9.04
S56	1.59	0.1299	8.19
S57	1.44	0.1157	8.01
Mean		0.1235	8.02

Table 30. Values of rate constants, K(days^{-1}), for Cr elution, calculated from Cr/DPF ratios in 5 rabbits at various times after DFP elution had decreased to negligible amounts.

		S53			S54			S55		
Day	'T'	^{51}Cr (%)	$DF^{32}P$ (%)	K	^{51}Cr (%)	$DF^{32}P$ (%)	K	^{51}Cr (%)	$DF^{32}P$ (%)	K
7	0	100	100	-	100	100	-	100	100	-
10	3	90.5	94.4	0.0141	85.7	89.8	0.0156	90.7	91.4	0.0026
13	6	83.1	89.6	0.0125	76.6	81.4	0.0101	79.1	80.6	0.0031
17	10	71.6	79.2	0.0101	63.2	75.7	0.0180	68.1	70.6	0.0036
20	13	68.7	75.6	0.0074	57.9	62.7	0.0061	59.9	64.2	0.0035
30	23	49.0	58.8	0.0079	37.1	46.9	0.0102	40.7	47.8	0.0070
43	36	23.9	32.8	0.0088	16.5	25.2	0.0118	19.2	25.4	0.0078
Mean K				0.0101			0.0120			0.0049
Elution rate % per day				1.004			1.192			0.488

Table 30 (continued)

	S57				S56			
Day	'T'	$^{51}Cr(\%)$	$DF^{32}P(\%)$	K	'T'	$^{51}Cr(\%)$	$DF^{32}P(\%)$	K
7	0	100	100	-	-	-	-	-
10	3	84.8	91.9	0.0268	0	100	100	-
13	6	74.7	82.7	0.0169	3	88.8	92.6	0.0139
17	10	62.3	-	-	7	74.2	81.8	0.0140
20	13	57.8	65.0	0.0090	10	72.9	78.3	0.0071
30	23	40.5	50.8	0.0098	20	55.8	58.7	0.0025
43	36	15.2	20.3	0.0080	33	26.4	34.1	0.0078
Mean K				0.0141				0.0091
Elution rate % per day				1.400				0.905

Table 31. ^{51}Cr red cell survival data (corrected for sampling losses and Cr elution) from 5 rabbits in which Cr elution rates were determined using ^{51}Cr and DF^{32}P. Correlation coefficient, "r", and mean cell life (MCL) estimates are based upon data from day 0-17.

Day	S53	S54	S55	S56	S57	Mean	S.d.
0	100.0	100.0	100.0	100.0	100.0	100.0	-
1	98.4	100.5	99.2	91.1	97.2	97.3	3.65
3	94.6	94.0	92.5	88.7	96.2	93.2	2.84
5	88.7	92.7	92.8	84.6	93.2	90.4	3.72
7	88.0	89.8	86.6	79.6	92.8	87.4	4.91
10	82.7	79.9	81.9	73.1	83.0	80.1	4.10
13	78.6	74.8	75.3	65.5	76.9	74.2	5.09
17	71.0	65.6	65.5	58.0	68.1	65.6	4.82
20	70.7	62.8	66.7	52.3	66.7	63.8	7.02
30	56.2	45.1	56.0	37.1	53.7	49.6	8.33
43	31.3	23.3	30.2	19.0	24.6	25.7	5.08
"r"	0.9948	0.9925	0.9934	0.9910	0.9822	0.9982	
MCL (days)	59.7	48.9	50.5	41.6	54.7	50.4	

IMMUNIZATION BY RED CELL ALLOANTIGENS IN SANDY LOP RABBITS

THE RESPONSE TO RED CELLS GIVEN ALONE

Procedure

- Red cell survival
- Injection schedules
- Description of red cell survival curves
- Serological tests for antibody
- Estimation of IgG concentration
- Sites of sequestration of red cells undergoing accelerated clearance

Results

- Survival of allogeneic red cells
  - (i) Normal responders
  - (ii) Non-responders or very poor responders
- Comparison of responses to different doses of red cells
- Comparison of responses to AA and AF red cells
- Relationship between accelerated clearance and antibody detection
- Comparison of serum IgG concentrations in responders and non-responders
- Sequestration sites of red cells undergoing accelerated clearance

Discussion

Following a small i.v. injection of allogeneic red cells, primary immunization in the rabbit is declared by the production of serologically detectable antibody in only a minority of cases. On the other hand, presumptive evidence of immunization may be obtained by observing the survival of a small dose of intravenously-administered allogeneic red cells labelled with ^{51}Cr; for a variable period of time following such injections, the cells survive as well as autologous cells, but in the majority of cases normal survival eventually gives way to accelerated destruction, presumably as a result of immunization. In the following experiments observations have been made on the survival of A-positive red cells injected into A-negative rabbits in an attempt to define the normal response to different doses of cells and to compare responses following a first and subsequent injections. In this way, it has been possible to distinguish between primary and secondary responses to intravenously administered red cells. In addition, a small number of A-negative rabbits that showed little or no response to intravenously administered A-positive red cells has been studied.

Procedure

Red cell survival. Donor A-positive red cells (AA or AF) were labelled with ^{51}Cr using the citrate-wash method (see p.80); for each injection approximately 20 μCi ^{51}Cr was used and the amount of sodium chromate added contained less than 2 μg Cr per ml red cells. The washed, labelled cells were suspended in isotonic saline to give a suitable concentration for injection, depending upon the volume of

cells being given. Cells were injected into recipient A-negative rabbits into a marginal vein of one ear, and the first blood sample was taken 3-5 minutes later from the other ear. Further blood samples were taken at 24 hours, on two more occasions during the first week and once weekly thereafter. Sampling was discontinued when 10% or less of the injected radioactivity remained. Samples were stored at 4°C and those from individual rabbits were counted together at the end of each survival study. The blood was lysed with saponin and 0.5 ml volumes were pipetted into counting vials. ^{51}Cr activity was measured in a standard gamma counter (see p.78). For the earlier samples, sufficient counts were obtained to keep the counting error at less than 1%; towards the end of each survival study, when the remaining radioactivity was much lower, the counting error was about 3%. The haemoglobin concentration of each sample was measured by the cyanmethaemoglobin method and radioactivity was expressed as ct/min per g Hb. These results were converted to a percentage of the activity found in the first sample. No corrections were applied for Cr elution nor for sampling as all comparisons were made with data for autologous red cell survival uncorrected for these factors.

Injection schedules

Five groups of A-negative rabbits were given i.v. injections of A-positive red cells according to the schedule shown in Table 32. The size of the first dose varied from 0.01 to 1 ml per kg body weight. Subsequent doses were either 0.01 or 0.02 ml/kg (except in the two rabbits shown, one of which was given 0.005 ml/kg and the other a total of 0.5 ml), and were given at various times after the preceding dose of

red cells had been cleared from the circulation. Some rabbits were given additional i.v. injections of red cells, accompanied in certain cases by the simultaneous i.m. administration of cells with Freund's complete adjuvant (FCA); details of these injections have been omitted from the table for the sake of simplicity but are given for the appropriate animals together with the results.

Description of red cell survival curves. Following an initial period during which essentially normal survival of the injected red cells was observed, many of the survival curves indicated a variable degree of accelerated destruction. For the present purposes, these curves could not be adequately described in terms of any single quantity such as the T_{50} or the time taken for all the cells to be cleared. However, it seemed that a much more appropriate measurement in these circumstances was that of the initial period of normal survival. It was possible to determine quite accurately the time at which accelerated disappearance from the circulation began by comparing each individual curve with data for autologous red cell survival in normal rabbits (see p.103). Survival was defined as subnormal (sn) when the percentage of allogeneic cells remaining in the circulation fell to 10% or more below the normal lower limit for autologous cells (set at 2 s.d. below the mean). The time taken for survival thus to become subnormal, T_{sn}, was used to compare the responses observed in different groups of animals.

Serological tests for antibody. The methods used to screen for serum antibodies have been described on p.77 . Samples were usually taken on more than one occasion, the first within a few days following clearance of the injected allogeneic red cells and the others within the next 30 days or so. Serum samples were also obtained before any injection had been given, for use as controls in antibody screening tests.

Estimation of serum IgG concentration. A single phase, radial diffusion method was used in which anti-rabbit IgG was incorporated in agar (see p.87).

Sites of sequestration of red cells undergoing accelerated clearance. Two A-negative rabbits (S85, R17) in which progressive impairment of survival of successive i.v. injections of A-positive red cells had been observed and in which anti-A had been detected serologically, were given a further injection of ^{51}Cr-labelled A-positive cells (0.02 ml per kg body weight). When less than 1% of the injected radioactivity remained in the circulation, namely 5 and 3 days respectively after injection, the rabbits were killed. In both cases, the spleen, liver, kidneys and the long bones from both hind limbs were removed, each tissue was digested separately in concentrated nitric acid and the radioactivity in aliquots of the digests was estimated. At the same time, dilutions of the injection suspension were counted. From these measurements, the proportion of the injected red cells cleared by each of the tissues examined could be estimated.

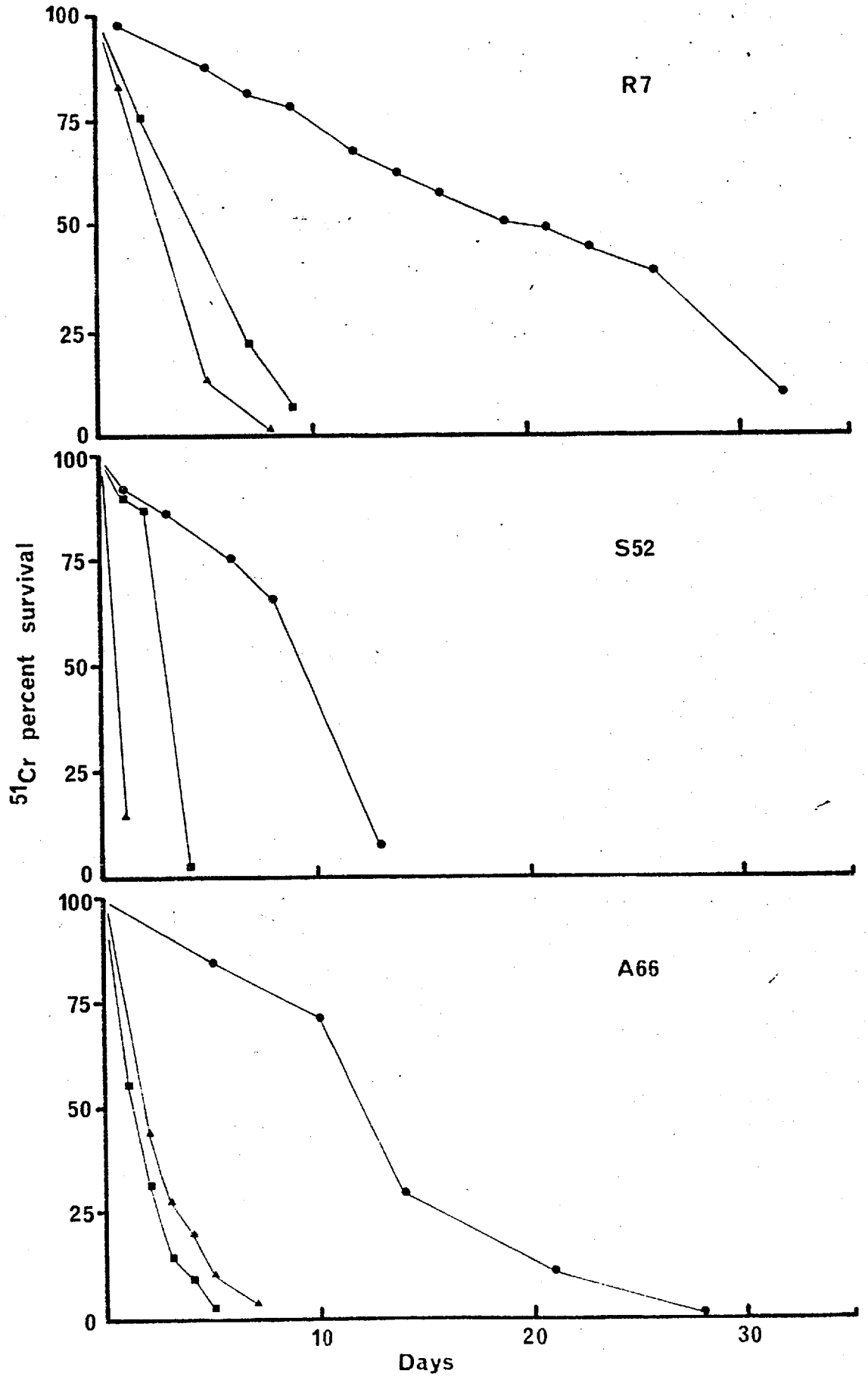
It was assumed that the amount of red cells cleared by the mononuclear phagocyte system in the long bones was typical of that cleared by the system in the bone marrow as a whole; the fraction of total bone marrow present in the long bones was obtained from the data of Deitz (1944).

Results

Survival of allogeneic red cells. Two main patterns of survival could be distinguished. In the first (normal responders) the survival of successive injections of red cells tended to become progressively shorter, whereas in the second (non-responders or poor responders), survival was either normal throughout or else the survival of initial injections was subnormal and that of later injections normal.

(i) Normal responders. Forty-four out of 51 rabbits showed progressive shortening of survival; in 41 of the 44, following the first injection of red cells, a "collapse" curve was observed, that is, survival was initially normal but after a period of between 3 and 33 days (mean about 17 days), there was an abrupt increase in the rate of disappearance of the injected cells; in the remaining 3 cases the survival of the first injection was normal but following the second injection a collapse curve was observed. Fig.18 shows three examples of collapse curves, with collapse occurring at about days 28, 10 and 12 respectively.

Figure 18. Survival of three successive i.v. injections, (●) 1, (■) 2 and (▲) 3, of A-positive red cells in three different "normally-responding" A-negative rabbits. In all three animals the first survival curve "collapses" between 10 and 28 days after injection. In R7 and S52, the remaining cells are cleared rapidly but in A66 the remaining cells are cleared slowly. All three rabbits show far more rapid clearance following a second injection; in R7 and S52 there is even more rapid clearance following a third injection, but in A66 clearance is slightly less rapid than following a second injection. No antibody was detectable serologically in R7 and A66 even after the third injection of cells had been cleared; anti-A was found in S52 following the second injection of cells.


R7
S52
A66
100
75
50
25
0
^{51}Cr percent survival
10
20
30
Days

Of the 41 rabbits which showed a collapse curve after their first injection, 39 received a second injection and in all 39 survival was again impaired; in 37 cases, T_{sn} was distinctly shorter after the second injection than after the first and in the remaining 2 cases (S31, S51) it was essentially the same (3 or 4 days) on both occasions. Of the 39 rabbits, 35 received a third injection; clearance was rapid in all cases, the longest T_{sn} being 5 days. The T_{sn} values observed on these three occasions are shown in Table 33.

Following collapse of the survival curve, clearance of the remaining cells was relatively slow in about 25% of cases and could be described by a simple exponential (e.g. Fig.18, rabbit A66); in the others (e.g. Fig.18, rabbit S52) clearance generally occurred more rapidly and the slope appeared to be approximately linear (but see later).

The data relating to antibody detection following successive injections of A-positive red cells is incomplete, in that not all of the recipients were tested during the month following clearance of the injected cells, and in those tested, the same serological methods were not used regularly throughout the period of study. However, in those cases in which more than one method was used for detection and titration of antibody, no significant difference in sensitivity was found (see Discussion). The results of antibody testing are summarized in Table 34 and give some idea of the incidence of serologically detectable antibody following impaired survival of injected red cells. Anti-A was found in only 3 out of 21 rabbits tested 2-3 times during the month

after they had shown accelerated clearance of a first injection. Following the accelerated clearance of a second injection, anti-A was identified in a further 7 cases. In 8 other rabbits, weak antibody reacting with the donor's red cells was detected but the specificity was not determined; in 3 of these cases, the antibody was shown to react with A-negative as well as with A-positive red cells. Following a third injection, antibody was detected in a further 6 out of 16 rabbits that had not previously had detectable antibody and in 2 it was shown to be anti-A. Thus, the cumulative incidence of serologically detectable antibody after three injections in the group of rabbits which showed progressive shortening of red cell survival was 24 out of 34 animals tested; in at least 11 cases, the antibody was anti-A. The maximum titre of anti-A, as judged by the agglutination of untreated red cells suspended in magnesium sulphate solution, did not exceed 8 and as already mentioned, the results obtained with enzyme-treated cells and with the indirect antiglobulin test did not differ significantly.

Of the 10 (out of 34) rabbits in which no antibody could be detected after three injections of red cells, despite progressive shortening of red cell survival, 3 produced antibody after further injections of cells but the specificity of the antibody was not determined.

(ii) Non-responders or very poor responders. As mentioned above, in 7 rabbits there was no progressive shortening of red cell survival following successive i.v. injections of red cells. Details of survival curves and a summary of injections given to each of these

rabbits is shown in Figs. 19 to 25. The patterns of response observed varied from strictly normal survival of the injected cells on the one hand, to apparent immunization by the first injection, followed by gradual improvement in survival of subsequent injections on the other hand. These extremes are illustrated by the responses of R9 (see Fig.19) and S94 (see Fig.25) respectively. However, it was possible to classify the responses of the 7 rabbits in this group into 3 main types.

(a) Three rabbits (R9, A77 and S41; Figs. 19, 20 and 21) showed essentially normal survival of three successive injections of red cells. Two of these animals (R9 and S41) were given further i.v. injections of labelled cells, together with i.m. injections of unlabelled cells with FCA. Both animals ultimately produced anti-A. In addition, R9 produced an antibody which reacted weakly in vitro with one A-negative (FF) sample of red cells (from R20); when these cells were injected i.v., more than 90% of a dose of 0.02 ml/kg was cleared within 4 days. In this particular rabbit, antibody was detectable serologically only after red cells had been injected six times i.v. and twice i.m. with FCA.

(b) In 3 rabbits (S67, S44 and S82; Figs. 22, 23 and 24) survival following the first injection was definitely subnormal by day 10 but the rate of destruction thereafter returned to normal and in all 3 animals the survival of a second and third injection of cells was essentially normal. All 3 animals received a fourth dose of cells i.v., together with an i.m. dose of cells with FCA; in 2 animals (S67 and S44),

Figure 19. Survival of six successive i.v. injections (●, ■, ▲, 0, ◆, □) of A-positive red cells in a poorly-responding A-negative rabbit, R9. Additional injections of red cells mixed with FCA were given i.m. at the same time as the fourth injection (0) and 11 days after the sixth injection (□). The interrupted line indicates the lower limit of normal survival for autologous red cells (also in subsequent Figures). The first three injections survived normally; the fourth injection showed slightly impaired survival from about day 20, and the fifth showed impaired survival from the outset; the sixth injection was normal up to day 25 but then "collapsed". Anti-A was detectable serologically only after the sixth injection of cells had been cleared; in addition, an antibody reacting weakly with one example of A-negative red cells was detected.

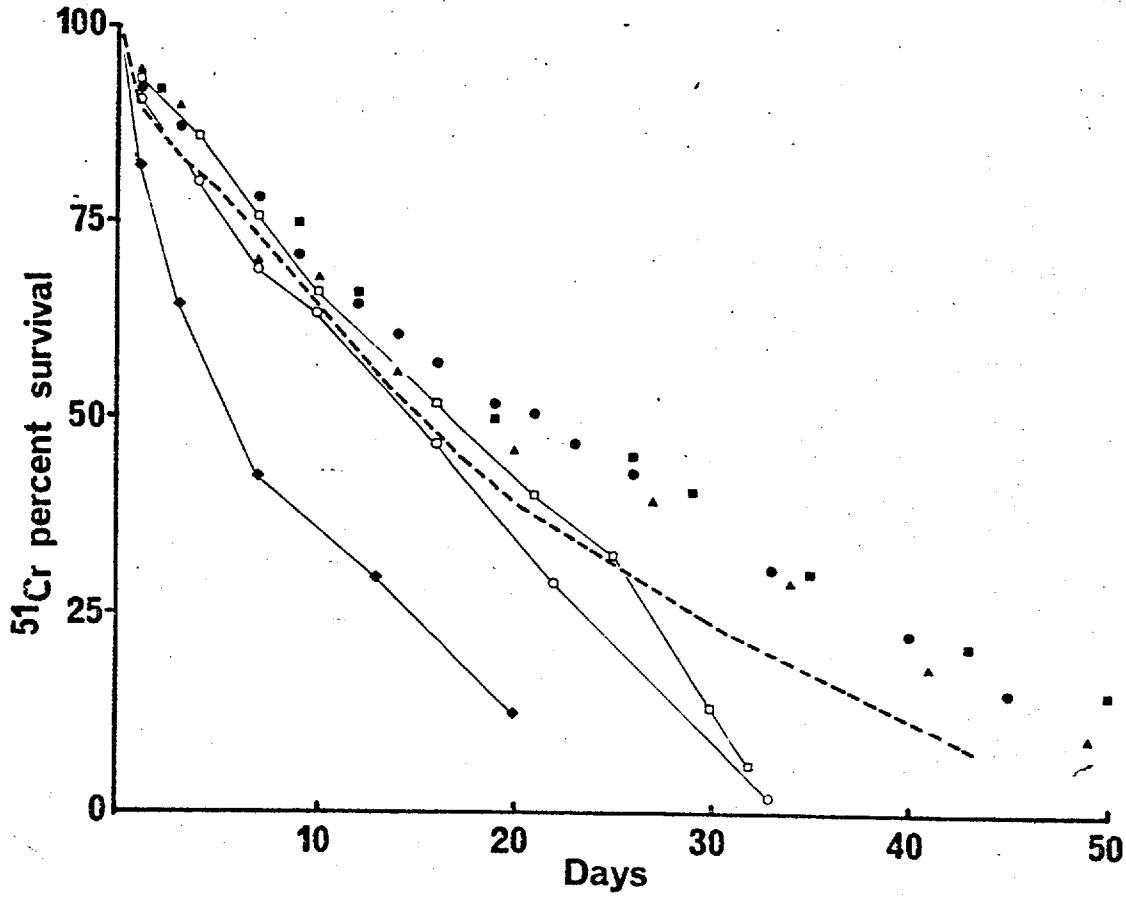

100
75
50
25
0
^{51}Cr percent survival
10
20
30
40
50
Days

Figure 20. Survival of three successive i.v. injections (●, ■ , ▲) of A-positive red cells in a poorly-responding A-negative rabbit, A77. All three injections show essentially normal survival. No antibody could be detected serologically in this rabbit.

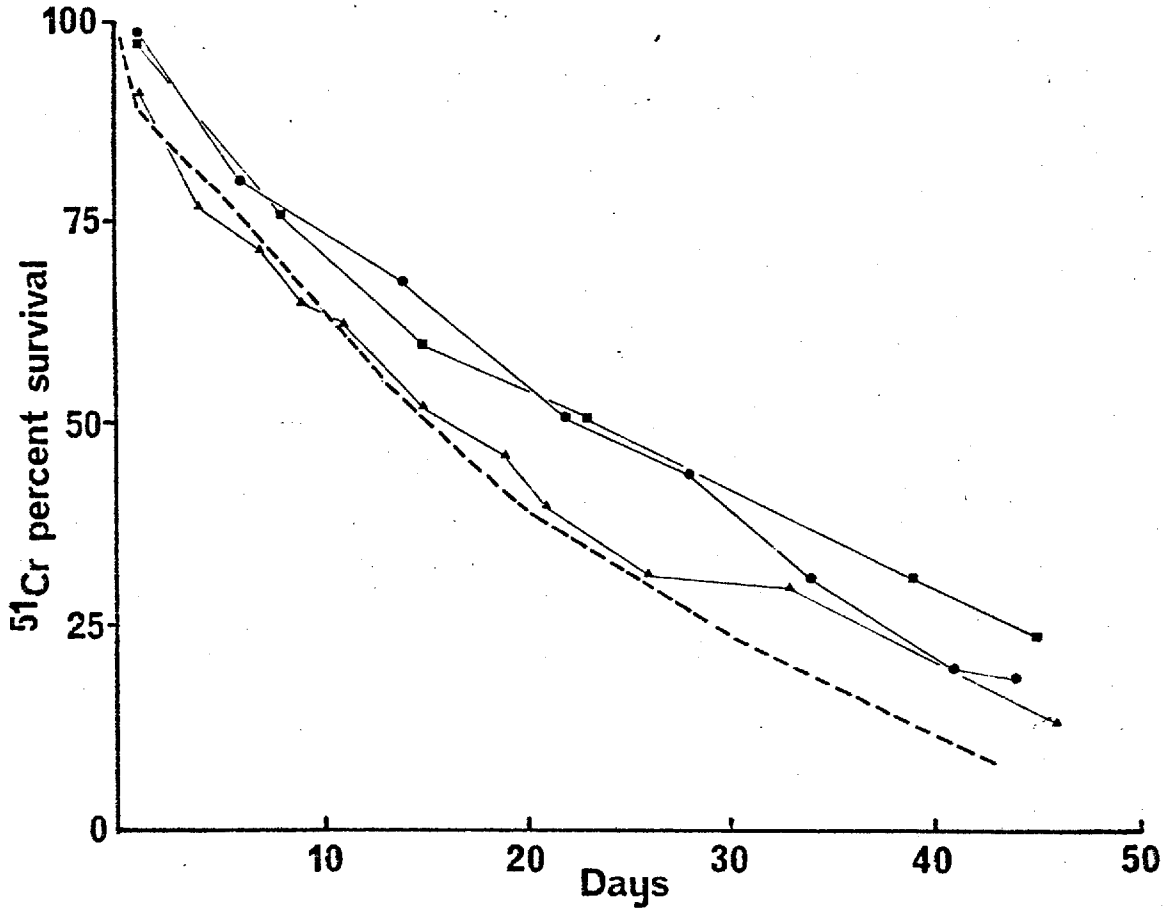

100
75
50
25
0
^{51}Cr percent survival
10
20
30
40
50
Days

Figure 21. Survival of five successive i.v. injections (●, ■, ▲, 0, ◆) of A-positive cells in a poorly-responding A-negative rabbit, S41. An additional injection of red cells mixed with FCA was given with the fourth injection (0). Survival of the first injection was slightly subnormal but the second and third injections survived normally; the fourth injection "collapsed" on about day 20 and was then cleared rapidly; the fifth injection survived normally for about 3 days and was then cleared rapidly. Anti-A was detectable serologically after the fifth injection of cells had been cleared.

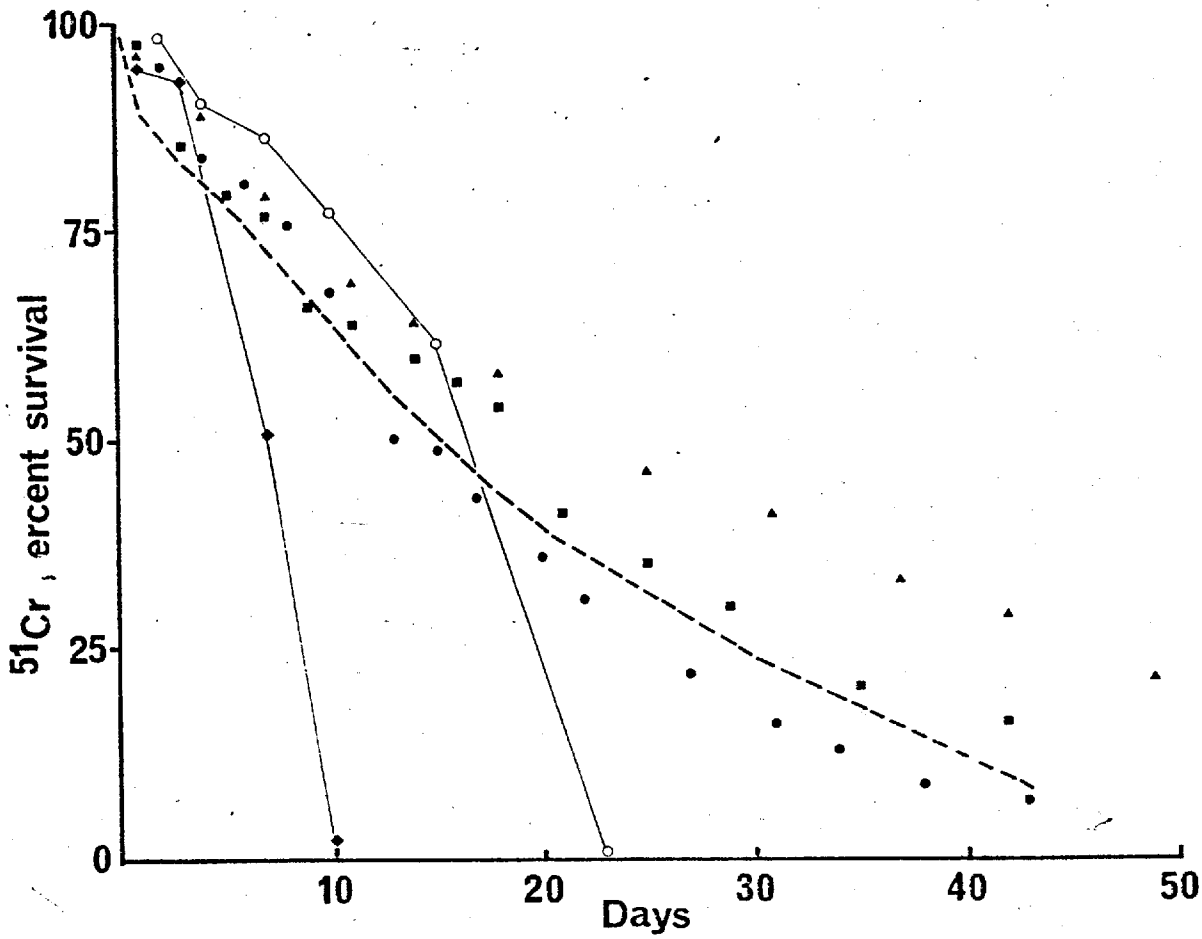

100
75
50
25
0
51Cr , ercent survival
10
20
30
40
50
Days

Figure 22. Survival of four successive i.v. injections (●, ■, ▲, 0) of A-positive red cells in a poorly-responding A-negative rabbit, S67. An additional injection of red cells mixed with FCA was given 11 days after the fourth injection (0). Survival of the first injection was markedly subnormal initially, but gradually improved over the first 15 days, after which the remaining cells survived normally; the second and third injections showed essentially normal survival; the fourth injection survived normally up to day 21 but had been cleared completely by day 25. Anti-A was detectable serologically only after the fourth injection of cells had been cleared.

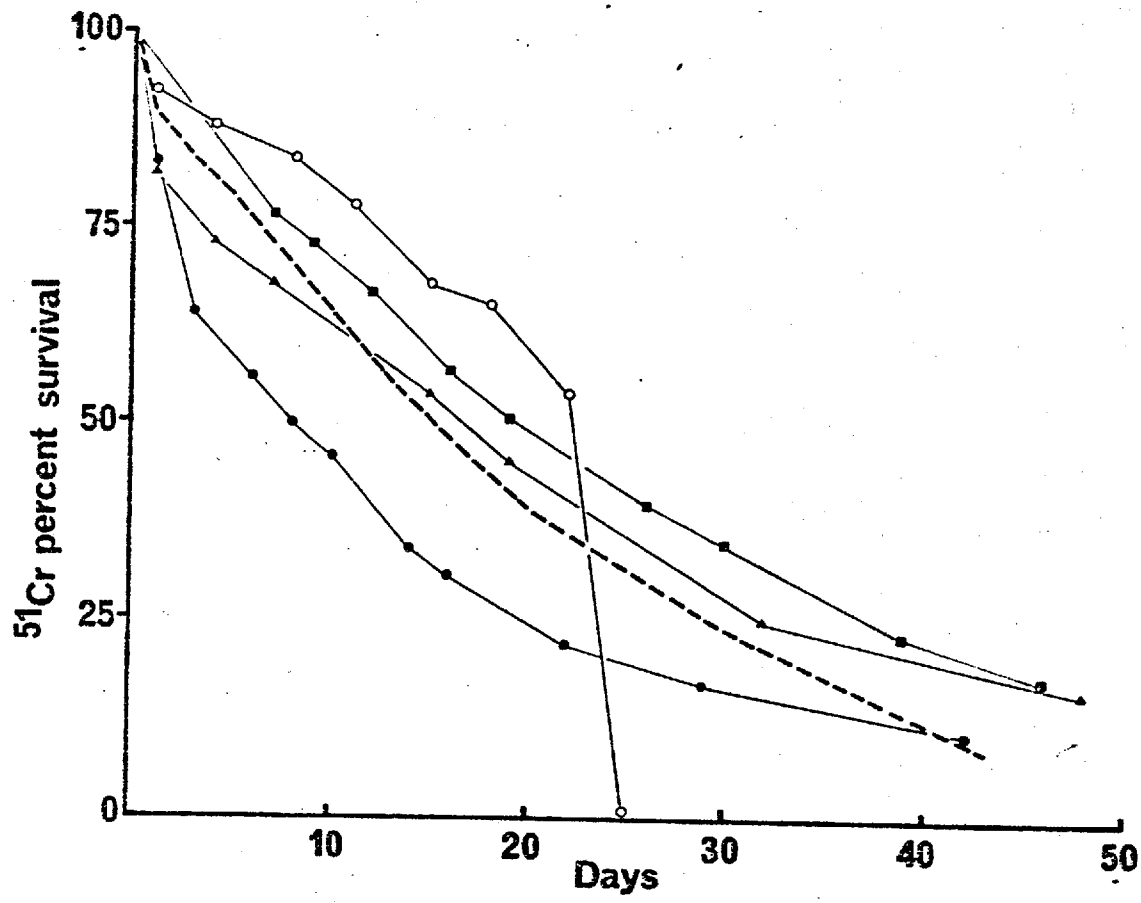

100
75
50
25
0
^{51}Cr percent survival
10
20
30
40
50
Days

Figure 23. Survival of four successive i.v. injections (●, ■, ▲, 0) of A-positive red cells in a poorly-responsive A-negative rabbit, S44. An additional injection of red cells mixed with FCA was given i.m. at the same time as the fourth injection (0). The first injection showed accelerated destruction by day 7, but after a few days the rate of destruction gradually decreased until about day 20 when survival of the remaining 15% of cells was again normal; the second and third injections survived normally; survival of the fourth injection was normal initially but the curve "collapsed" on about day 20. Anti-A was detectable serologically only after the fourth injection of cells had been cleared.

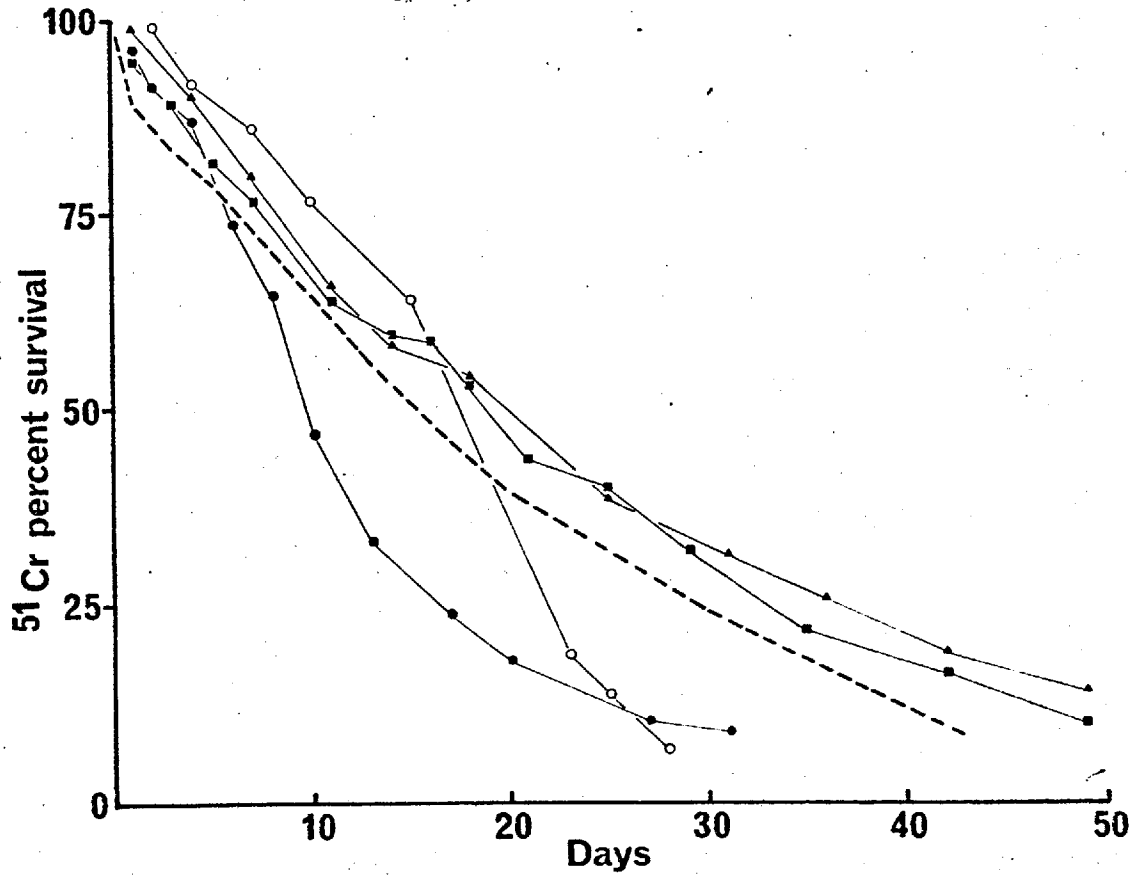
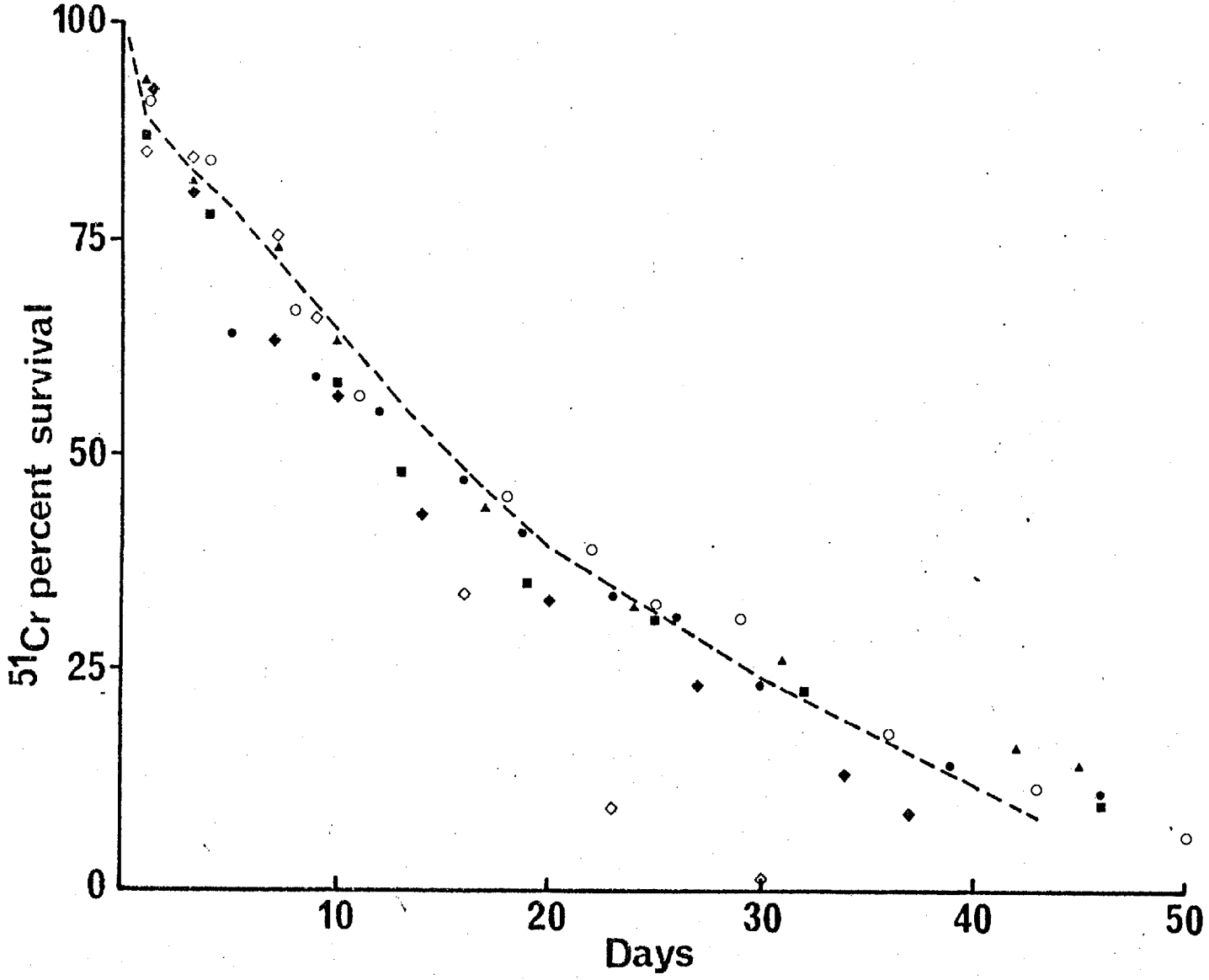

100
75
50
25
0
51Cr percent survival
10
20
30
40
50
Days

Figure 24. Survival of six successive i.v. injections (●, ■, ▲, 0, ◇, ◆) of A-positive red cells in a poorly-responding A-negative rabbit, S82. Additional injections of red cells mixed with FCA were given 11 days after the fourth injection (0) and at the same time as the fifth injection (◇). Survival of the first injection was definitely subnormal by day 5 but thereafter the remaining cells survived normally; the second, third and fourth injections survived normally or were only slightly subnormal; the fifth injection showed slightly accelerated clearance from about day 10 onwards; survival of the sixth injection was only slightly subnormal throughout. At no time was anti-A demonstrated serologically in this rabbit.


100
75
50
25
0
^{51}Cr percent survival
10
20
30
40
50
Days

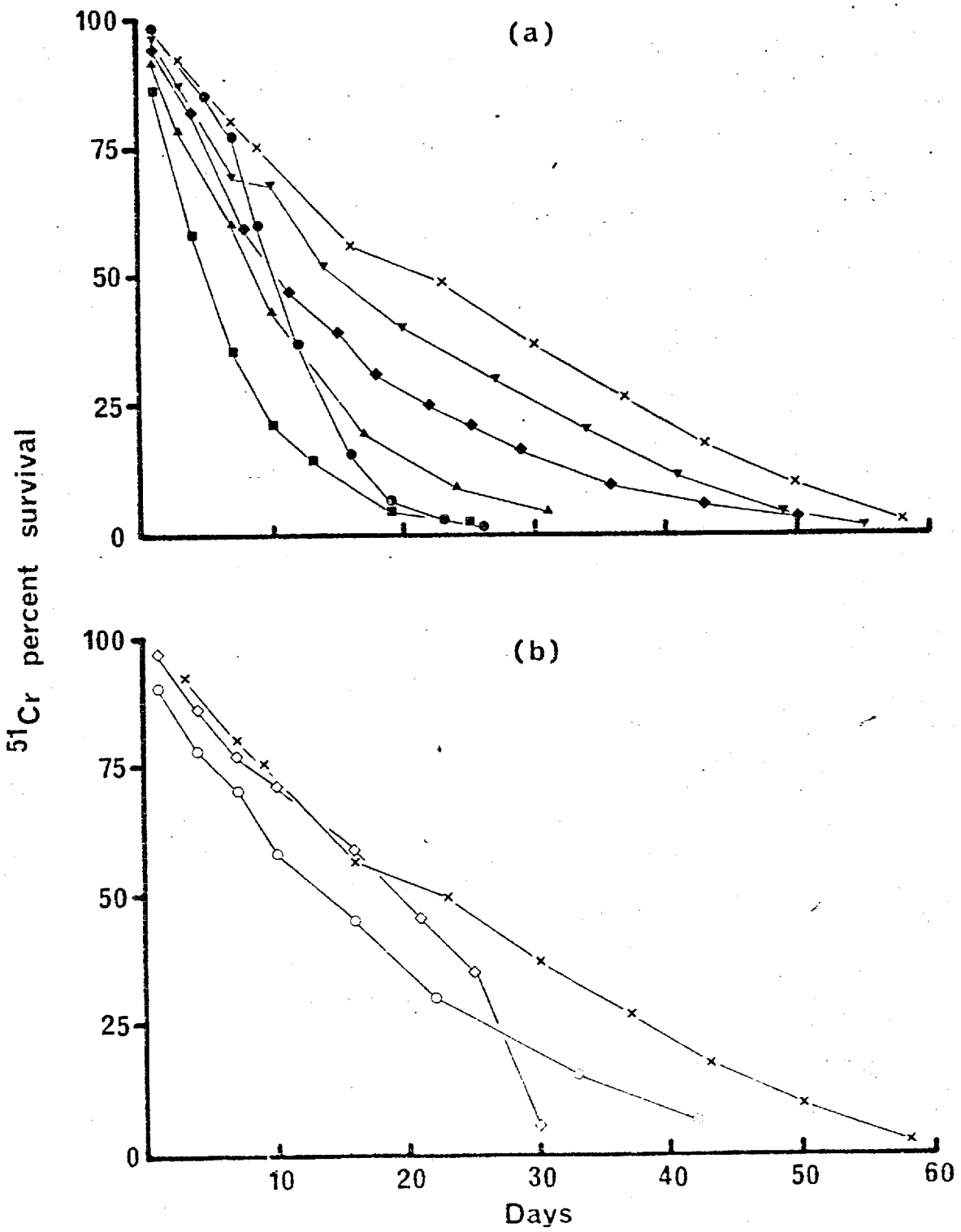
the survival curve showed collapse and, after clearance of the cells, serologically detectable anti-A was demonstrable. In the other rabbit (S82), the survival of the fourth injection of cells was normal, but after a further i.v. injection of labelled cells, together with a second i.m. injection of cells with FCA, survival was definitely impaired by day 15 and clearance was complete by day 30. No anti-A was detectable serologically. A sixth injection of cells given i.v. 2 months later showed almost normal survival.

(c) One rabbit (S94; Fig.25) at first appeared as if it were a "normal" responder: thus, following the first injection, the survival curve collapsed at about day 10, and following the second injection, there was impaired survival from the outset and clearance was approximately exponential with a $T_{\frac{1}{2}}$ of about 4 days. On the other hand, following injections 3 and 4 survival became progressively longer and following a fifth injection survival was only just below the limits of normal. A sixth i.v. injection was accompanied by an i.m. injection of cells with FCA, but survival was only slightly subnormal throughout. No antibody could be detected in the serum at any time. After a further five months a seventh i.v. injection of cells was given. At day 10, when survival was strictly normal, an i.m. injection of cells with FCA was given; there was collapse of the survival curve at about day 26 and only 5% of cells remained in the circulation on day 30. Unfortunately, no serum was available for further antibody testing at this time.

Figure 25. Survival of seven successive i.v. injections (●, ■, ▲, ◆, ▼, 0, ◇) of A-positive red cells in a poorly-responding A-negative rabbit, S94.

a) The first survival curve "collapsed" on day 10; survival of all of the next four injections was impaired but there was a progressive improvement in survival with each injection.

b) An additional injection of red cells mixed with FCA was given at the same time as the sixth injection and 11 days after the seventh injection. The sixth injection survived normally up to day 20 when the curve "collapsed"; the seventh injection showed slightly impaired survival throughout. Anti-A could not be demonstrated following the sixth injection of cells; serum was not available for testing following the seventh injection of cells. Survival of autologous cells (X) was also measured during the course of these experiments.


(a)
100
75
50
25
0
(b)
100
75
50
25
0
10
20
30
40
50
60
Days
^{51}Cr percent survival

Comparison of responses to different doses of red cells. In order to ascertain whether the injection of different doses of red cells on the first occasion substantially altered the observed responses, red cell survival curves were analysed as follows: T_{sn} values from the 41 rabbits which exhibited the collapse phenomenon with the first injection of cells were used. Simple inspection of the data in the larger groups indicated a skew distribution, with an excess of T_{sn} values below the arithmetic mean; the numbers are admittedly small, but the same observation was also made while analysing results from other experiments (see p.245). A rather better distribution was found using logarithms of the T_{sn} values, and consequently a lognormal distribution has been assumed in analysing these data. The mean T_{sn} values so obtained in the different groups of rabbits with the first, second and third injections of cells are shown in Table 33. It can be seen that with the first injections, mean T_{sn} ranges from 7.3 (s.d. 1.7) days in group 3, to 19.3 (s.d. 1.4) days in group 2; an analysis of variance of the results of the first injections confirms that these are indeed heterogeneous population samples ($p < 0.001$). It should be noted that the two groups with the lowest mean values had originally contained rabbits showing normal survival of a first injection of red cells (2 examples in group 1; 1 example in group 3). It was thus impossible to accommodate these animals in the terms of the present analysis, but their removal consequently decreases the means observed in their respective groups. When the second injections were analysed there was no significant difference in T_{sn} between the groups, as might be expected from the calculated means and s.d. In all groups, there was a very significant difference between the T_{sn} values observed with a first and second injection of cells, as shown in the Table.

In the 7 rabbits which failed to respond, or responded only poorly, to allogeneic red cells, there was no evidence of any relation to the amount of cells injected ($p > 0.5$; chi-square).

Comparison of responses to AA and AF red cells. These experiments were not specifically designed to compare the effects of homozygous and heterozygous red cells in inducing immunization and only in the group given 0.02 ml per kg were the two types of cell given to different animals. T_{sn} values in 9 recipients of AA and 3 recipients of AF cells in this group did not differ significantly ($p = 0.21$, t-test).

Relationship between accelerated clearance and antibody detection. In those cases in which the serum was screened for antibody within a few days of the cells being cleared at an accelerated rate, the increased rate of clearance following "collapse" was expressed in one of the two ways already described (see p.214). Antibody was detected in only 1 of the 20 tests in animals showing slow ("exponential") clearance, but in 36 out of 71 tests following rapid ("linear") clearance. Once rapid collapse had been established, the time taken for removal of the remaining cells varied from 1-8 days (mean 3.4; s.d. 1.5) in those cases in which antibody was detectable serologically within 1 month of the cells being cleared, and from 2-12 days (mean 4.6; s.d. 1.5) in those cases without detectable antibody (see Table 35). There was thus considerable overlap between the two groups, but the populations were significantly different at the 1% level (t-test). In cases showing relatively rapid "collapse", the apparently linear slope was possibly due to a paucity of experimental points; in some cases the rate of clearance may well have been accelerating progressively.

Comparison of serum IgG concentrations in responders and non-responders. Serum IgG concentrations were determined in 8 of the A-negative rabbits showing a "normal" response to successive injections of A-positive red cells, and in 4 of the rabbits classified as non-responders or very poor responders. (These two groups correspond to groups 1 and 2 respectively in Table 26 on p.175). Determinations were carried out on a single serum sample from each rabbit at varying times after the type of response to allogeneic red cells had been established. No significant difference in IgG concentrations between the two groups was demonstrated ($p > 0.8$, t-test), the mean values being respectively 12.2 mg per ml (s.d. 1.3) and 12.4 mg per ml (s.d. 1.7) for "responders" and "non-responders".

Sequestration sites of red cells undergoing accelerated clearance. The percentage of injected radioactivity found in the different tissues examined following clearance of ^{51}Cr-labelled A-positive red cells injected into two immunized rabbits is shown in Table 36. The relatively efficiency of the spleen and liver in removing A-positive red cells under these circumstances differed considerably in the two rabbits investigated. In the case of S85, the spleen contained the biggest proportion of radioactivity, amounting to 36% of the injected dose, whereas in R17, the liver predominated with 54% of the injected activity; the ratio of the activity recovered from the spleen to that from the liver (per g tissue) in each animal was 148 for S85 and 4 for R17. The amount of activity found in the bones varied between 3.1% and 7.3% of the injected dose; negligible amounts were found in the kidneys.

It should be pointed out that the total recovery in these experiments was only 51% and 66%; a large proportion of the deficit was probably lost by elution of ^{51}Cr from the organs of sequestration during the 3-5 days between injection and clearance of the cells (see Hughes-Jones, 1961).

Discussion

Early reports of attempts at immunization to red cell alloantigens in rabbits are difficult to interpret because of the confusion surrounding the identification and nomenclature of rabbit blood groups prevailing at that time (see p.39). Although it has been generally agreed that production of high titre alloantibodies following i.v. injection of appropriate red cells is uncommon (Cohen, 1954; Anderson, 1955) it would appear that when A-positive red cells are injected into A-negative rabbits, a substantial proportion of recipients will form detectable anti-A. Thus, Anderson (1955) was able to detect antibody by serological means in just over 40% of A-negative rabbits given a course of 20 or 40 intravenous injections of saline-suspended red cells obtained from 1-3 ml blood of A-positive donors; one half to three quarters of the responding rabbits had anti-A titres of less than 4 and only 1 animal had a titre in excess of 128. Similar results have been reported following a single intravenous injection of a small volume of red cells. Thus, Pollack et al (1968) injected 10^9 A-positive red cells (about 0.1 ml packed cells) into 21 New Zealand White rabbits and detected antibody in 11 cases (52%); no details of antibody titres or timing of the response were given. Similarly, Elson et al (1971) injected 2×10^9 A-positive cells into 32 Californian rabbits and found antibody in 18 cases (56%), titres varying between 1 and 32 in all but one

case, in which the titre was 256; all responses were found to occur within 30 days of injection. In the present series of experiments, the dose of A-positive red cells injected on the first occasion varied between 0.01 and 1 ml per kg body weight; subsequent doses were either of 0.01 or 0.02 ml per kg. Although antibody was detected in only 3 out of 21 rabbits (14%) tested after the first injection, the incidence had risen to 24 out of 34 (70%) by the time of the third injection and the antibody was identified as anti-A in 11 of these cases (32%). In fact, it should be recalled that these rabbits had been selected on the basis of impaired survival of the injected red cells; if the 7 "non-responders" are included in this assessment, the incidence of serologically detectable antibody after each of the 3 injections becomes 3 out of 28 (11%) for the first, 18 out of 46 (39%) for the second, and 24 out of 41 (58%) for the third injection. Antibodies produced in these experiments were of low titre and consequently determination of their specificity was sometimes difficult or impossible.

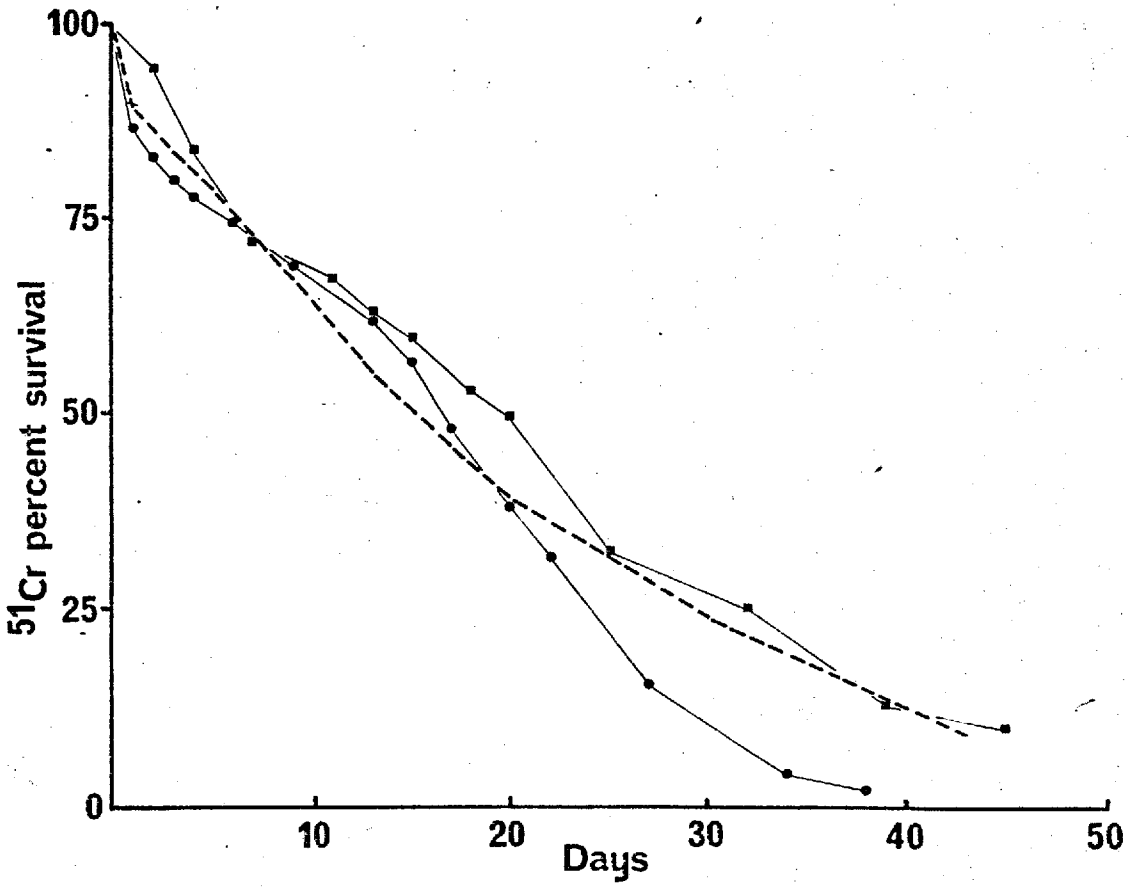
In order to be certain that low titre antibodies were not being missed as a result of local technical factors in performing the screening tests, it was decided to seek an independent assessment of a number of the sera obtained during these experiments. Dr. J.C. Woodrow in Liverpool kindly agreed to an exchange of relevant rabbit sera. Results of tests performed on 12 sera from each laboratory, without prior knowledge of the results obtained in the laboratory of origin, are shown in Table 37. The tests were performed using papain-treated detector red cells. With one exception (serum L), for which no explanation was apparent, substantial agreement in antibody detection was obtained, suggesting that technical failure to identify antibody in the present experiments was unlikely to account for the lower incidence of antibody detected.

The relatively high incidence of failure to respond to a first or second injection of red cells in the present experiments, as judged by the production of serologically detectable antibody, may perhaps be a feature of the particular strain of rabbits used; certainly, the response of rabbits to allogeneic red cells, assessed by this criterion has been shown to be genetically determined (Cohen, 1960(a); Woodrow et al., 1973). On the other hand, as judged by "collapse" of the first survival curve, 42 out of 51 rabbits (82%) presumably formed antibody following their first injection; almost all of these animals did in fact show progressively shorter survival following successive injections of A-positive red cells and just over half did eventually have serologically detectable antibody. "Collapse" curves in the absence of serologically detectable antibody following the injection of allogeneic red cells have been interpreted as a manifestation of primary immunization. In a number of reports, "collapse" curves have been observed following transfusion of red cells compatible by conventional cross-matching tests (Loutit, Mollison and Young, 1943; Stohlman and Sneiderman, 1956; Goudsmit and Krijnen, 1957; Adner and Sjölin, 1957; Brown and Smith, 1958; Kaplan and Hsu, 1961); it was assumed that an unrecognised antigenic difference existed between donor and recipient, and in some cases specific antibody was detected serologically following further injections of the donor cells. Similarly, following spaced injections of small amounts of Rh-positive cells to Rh-negative subjects, accelerated clearance of the red cells in the absence of serologically detectable antibody has often been seen (Woodrow, Finn and Krevans, 1969; Mollison et al., 1969).

In experiments in which known small amounts of IgG anti-Rh were injected into Rh-negative subjects followed by injections of small volumes of ^{51}Cr-labelled Rh-positive red cells, it was shown that the lowest concentration of anti-Rh which would bring about accelerated clearance of red cells was about 10 times lower than the lowest concentration that could be detected serologically (Mollison and Hughes-Jones, 1967). Similarly, it has been shown in rabbits that passively administered IgM anti-A will bring about the clearance of small volumes of A-positive red cells with concentrations of antibody which are about 10 times below the minimum which can be detected serologically (Burton and Mollison, 1968). Direct proof that the accelerated clearance of red cells seen during the "collapse" phenomenon is mediated by a plasma factor, presumably antibody, has not yet been obtained. Two plasma transfer experiments were undertaken during the course of this work. In one experiment, plasma was taken from an A-negative rabbit after the survival curve of a first injection of A-positive red cells had collapsed; plasmapheresis was undertaken as soon as the injected cells had been cleared from the circulation. Anti-A was not detectable serologically and the transfer of 100 ml of this plasma to another A-negative rabbit immediately after the injection of a small dose of labelled A-positive red cells (0.005 ml/kg body weight) produced no impairment of red cell survival (see Fig.26; R5). In the second experiment antibody was readily detectable in neat plasma from the donor rabbit; transfusion of 100 ml of this plasma into an A-negative recipient immediately after a first injection of ^{51}Cr-labelled A-positive red cells, was followed only by a small initial impairment of red cell survival, (see Fig.26; R3).

Figure 26. ●: survival of A-positive red cells (0.02 ml/kg) in an A-negative rabbit, R3. Shortly after injection the recipient was transfused rapidly with 100 ml plasma from another A-negative rabbit, R8, in which the survival curve of A-positive red cells from the same donor had "collapsed".

■ : survival curve from a similar experiment in which A-positive donor cells (0.005 ml/kg) were injected into another A-negative rabbits, R5; the donor of the "collapse" plasma in this case was R12.


100
75
50
25
0
^{51}Cr percent survival
10
20
30
40
50
Days

Although "collapse" curves are generally considered to result from primary immunization, it is puzzling that further injections from the same donor to the same recipient have frequently not been followed by the formation of detectable antibody. Moreover, in some cases, a progressive improvement in survival has been noted with succeeding injections (Adner and Sjolin, 1957; Adner et al., 1963; Holburn et al., 1971). In the present series of experiments there were several examples of this phenomenon (see, for example, S94, Fig.25).

Failure to respond (or to maintain a response) to allogeneic red cells, as judged by normal or almost normal survival curves, was demonstrated in 7 out of the 51 rabbits described here. However, when 6 of these animals were given i.m. injections of A-positive cells with FCA they subsequently showed impaired survival of intravenously administered cells. In 2 rabbits (S82, R9) it appeared that there was only a transient increase in the rate of red cell destruction and neither of these formed detectable antibody; in the remaining 4 rabbits anti-A was demonstrated serologically after accelerated clearance of A-positive cells. In these cases, then, it seems that unresponsiveness is not due to complete absence of the appropriate immunologically competent cells required to mount a response to the A antigen, but possibly results from inadequate or inappropriate stimulation of the immune system by red cells introduced by the intravenous route. By analogy, those Rh-negative subjects who are classified as non-responders because Rh-positive red cells injected intravenously survive normally in their circulation may, similarly, be only poor responders who would form anti-Rh if injected by another route or if stimulated by the use of adjuvants.

Results of serum IgG concentrations and sequestration sites of cells cleared rapidly as a result of active immunization will be discussed later.

Table 32. Red Cell Injection Schedule

Group of rabbits	First Injection		Second Injection		Third Injection	
	Number of recipients	Dose of red cells (ml/kg)	Number of recipients	Dose of red cells (ml/kg)	Number of recipients	Dose of red cells (ml/kg)
1	7	0.01	7	0.01	5	0.01
2	15	0.02	12	0.02	9	0.02
			1	0.005	1	0.02
			1	0.5*	1	0.02
3	15	0.04	15	0.02	13	0.02
4	5	1.0	4	0.01	4	0.01
5	9	0.5*	9	0.02	9	0.02
Total no. injected	51		49		42	

* In these cases animals received a total dose of 0.5 ml red cells

Table 33(a). Individual T_{sn} values observed following successive injections of Hg^A-positive red cells into Hg^A-negative rabbits. The dosage schedule for the different groups is shown in Table . Means and standard deviations (s.d.) have been calculated for the different groups using data from the rabbits showing "collapse" of a first injection of cells. The "p" value refers to comparisons between T_{sn} values following a first and second injection of cells to each group of rabbits (t-test). A lognormal distribution has been assumed (see text).

Group 1				Group 2			
	T_{sn} (days)				T_{sn} (days)		
Rabbit number	Injection 1	2	3	Rabbit number	Injection 1	2	3
A29	5	1	1	A73	10	9	4
A53	6	4	2	R 8	15	1	1
A60	8	4	1	R12	15	1	2
A61	12	1	1	R 1	18	1	-
A62	14	1	1	A72	18	6	3
A33	NS	11	-	A70	20	9	5
A30	NS	14	-	A97	20	5	-
				R 6	20	1	-
				R11	21	1	2
				A98	22	-	-
				R 7	30	2	2
				R 4	33	4	1
Total	7	7	5	Total	12	11	8
n	5	5	5	n	12	11	8
mean	8.3	1.7	1.1	mean	19.3	2.45	2.2
s.d.	1.5	2.1	1.4	s.d.	1.4	2.6	1.8
"p"	<0.01			"p"	<0.001		

Table 33b (contd.)

Group 3				Group 4			
	T_{sn} (days)				T_{sn} (days)		
Rabbit number	Injection 1	2	3	Rabbit number	Injection 1	2	3
S31	3	4	2	A67	12	-	-
S51	3	3	1	A66	13	1	1
S54	5	2	1	A74	18	1	1
S16	7	2	1	A63	20	1	1
S56	7	4	2	A64	24	2	3
S36	8	2	-				
S 5	8	3	1				
S52	9	3	1				
S38	10	8	4				
S 2	10	3	1				
S 1	11	2	-				
S55	17	8	1				
S57	NS	8	1				
Total	13	13	11	Total	5	4	4
n	12	12	11	n	5	4	4
mean	7.3	3.2	1.3	mean	16.8	1.2	1.3
s.d.	1.7	1.6	1.6	s.d.	1.3	1.4	1.7
'p'	<0.001			'p'	<0.001		

Table 33c(cont'd)

Group 5			
	T_{sn} (days)		
Rabbit number	Injection 1	2	3
S87	7	1	1
S76	13	2	4
S88	17	3	4
S79	21	8	4
S73	21	2	2
S85	23	1	2
S91	30	1	2
Total	7	7	7
n	7	7	7
mean	17.3	1.9	2.4
s.d.	1.6	2.1	1.7
'p'	< 0.001		

Table 34. Summary of results of serological tests obtained in Hg^A-negative rabbits during the month following the elimination of an i.v. injection of Hg^A-positive red cells. The pattern of accelerated red cell clearance, and the clearance time are also indicated.

+ The time taken for clearance of the residual cells from the circulation at the accelerated rate following "collapse" of the survival curves; the pattern of clearance was either
  a) "rapid" and apparently linear-expressed in days, or
  b) "slow" and approximately exponential-expressed as $T\frac{1}{2}$ (days).

N.S. Normal survival.

* Antibody identified as anti-Hg^A.

Table 34 (a) Group 1

Rabbit number			A29	A30	A33	A53	A60	A61	A62
First injection	Clearance time +	(a)	8	NS	NS	3	9	7	7
		(b)	-	-	-	-	-	-	-
	Antibody titre	$MgSO_4$	0	-	-	-	0	0	0
		Papain	-	-	-	-	-	-	-
		IAGT	0	-	-	-	0	0	0
Second injection	Clearance time +	(a)	3	3	-	3	3	5	-
		(b)	-	-	3.2	-	-	-	3
	Antibody titre	$MgSO_4$	0	0	0	0	1	0	0
		Papain	-	-	-	-	-	-	-
		IAGT	0	-	-	0	1	0	0
Third injection	Clearance time +	(a)	1	-	-	2	2	4	-
		(b)	-	-	-	-	-	-	1.3
	Antibody titre	$MgSO_4$	4	-	-	2	2	2	2
		Papain	-	-	-	-	-	-	-
		IAGT	4	-	-	0	-	8	0

Table 34 (b) Group 2

			Rabbit number	A70	A72	A73	A97	R1	R4	R6	R7	R8	R11	R12
First injection	Clearance time +	(a)		-	4	6	5	4	4	3	4	4	6	-
		(b)		3.4	-	-	-	-	-	-	-	-	-	2
	Antibody titre	$MgSO_4$		-	-	-	0	-	-	-	-	-	-	-
		Papain		-	-	-	-	0	0	0	0	1*	-	0
		IAGT		-	-	-	0	-	-	-	-	-	-	-
Second injection	Clearance time +	(a)		4	4	4	4	6	-	-	7	3	-	-
		(b)		-	-	-	-	-	3.5	1.7	-	-	2	2.4
	Antibody titre	$MgSO_4$		-	0	-	1	-	-	-	-	-	-	-
		Papain		-	-	-	-	0	0	0	0	1	0	-
		IAGT		0	0	0	0	-	-	-	-	-	-	-
Third injection	Clearance time +	(a)		3	5	3	-	-	-	-	5	4	6	-
		(b)		-	-	-	-	-	4.4	-	-	-	-	3.5
	Antibody titre	$MgSO_4$		0	0	0	-	-	-	-	-	1	-	-
		Papain		-	-	-	-	-	0	-	0	-	0	0
		IAGT		0	0	0	-	-	-	-	-	-	-	-

Table 34(c) Group 3

			Rabbit number	S1	S2	S5	S16	S31	S36	S51	S52	S54	S55	S56	S57
First injection	Clearance time +	(a)		5	5	14	4	4	6	6	4	4	-	6	NS
		(b)		-	-	-	-	-	-	-	-	-	5.5	-	-
	Antibody titre	$MgSO_4$		0	0	-	0	-	-	-	-	-	-	-	-
		Papain		-	-	-	-	-	-	-	-	-	-	-	-
		IAGT		0	0	-	0	-	-	-	-	-	-	-	-
Second injection	Clearance time +	(a)		3	5	5	3	2	4	4	2	3	4	2	6
		(b)		-	-	-	-	-	-	-	-	-	-	-	-
	Antibody titre	$MgSO_4$		1*	0	0	wk	-	0	1*	1*	1*	0	0	0
		Papain		-	-	-	-	-	-	1	1	-	1	1*	1
		IAGT		0	0	0	0	0	0	2	1	0	0	0	0
Third injection	Clearance time +	(a)		-	-	-	-	4	-	2	2	3	-	5	-
		(b)		-	-	-	-	-	-	-	-	-	1	-	2
	Antibody titre	$MgSO_4$		-	-	-	-	8	-	8	8	1	0	1	0
		Papain		-	-	-	-	-	-	-	-	-	-	-	-
		IAGT		-	-	-	-	4	-	16	8	-	-	-	-

Table 34 (d) Groups 4 and 5

			Group 4					Group 5						
Rabbit number			A63	A64	A66	A67	A74	S73	S76	S79	S85	S87	S88	S91
First injection	Clearance time +	(a)	12	-	-	-	12	3	5	5	4	5	9	5
		(b)	-	6	4	2.8	-	-	-	-	-	-	-	-
	Antibody titre	$MgSO_4$	0	0	0	0	0	0	-	-	-	1*	-	-
		Papain	-	-	-	-	-	1*	-	-	-	-	-	-
		IAGT	0	0	0	0	0	-	-	-	-	-	-	-
Second injection	Clearance time +	(a)	-	-	-	-	-	6	3	8	3	3	5	3
		(b)	1.6	6	1.2	-	0.7	-	-	-	-	-	-	-
	Antibody titre	$MgSO_4$	-	0	0	-	0	0	1	0	0	2	0	0
		Papain	-	-	-	-	-	1	-	1*	wk	2	1*	1
		IAGT	-	0	0	-	0	-	-	-	-	-	-	-
Third injection	Clearance time +	(a)	-	-	-	-	4	5	4	4	5	3	6	3
		(b)	2.5	7.5	1.6	-	-	-	-	-	-	-	-	-
	Antibody titre	$MgSO_4$	0	0	0	-	2	0	0	0	0	2	0	0
		Papain	-	-	-	-	-	1	1	1	2*	4	1	0
		IAGT	0	0	0	-	2	-	-	-	-	0	-	-

Table 31. Relationship between the results of antibody tests in samples taken during the month following clearance of Hg^A-positive red cells injected into Hg^A-negative rabbits, and the pattern of clearance of red cells following "collapse" of the survival curves (see text). Analyses were performed assuming a lognormal distribution of clearance times.

	Serology	Positive antibody test		Negative antibody test	
	Clearance	"Rapid"	"Slow"	"Rapid"	"Slow"
	Numbers of tests	36	1	35	19
"Rapid" Clearance Time (days)	Range	1-8		2-12	
	Mean ± s.d.	3.4 ± 1.5		4.6 ± 1.5	

Table 36. Recovery of radioactivity from various organs following clearance of ^{51}Cr-labelled A-positive red cells from the circulation of previously immunized A-negative rabbits.

Rabbit number		S85	R17
Weight (kg)		4.25	4.55
Volume of cells injected (ml/kg)		0.02	0.02
Percent circulating at death		< 1	< 1
Time of death (days after injection)		5	3
Percentage recovery	Spleen	36.5	4.6
	Liver	10.5	54.0
	Kidneys	0.6	0.3
	Bones	3.1	7.3
	Total	50.7	66.2
Spleen:liver ratio ct/min per g		148	4

Table 37. Comparison of results obtained in two laboratories of serological tests on 24 rabbit sera (see text). Tests were performed using papain-treated Hg^A-positive red cells.

	Liverpool sera			London sera	
	Liverpool results	London results		Liverpool results	London results
A	-	-	M	-	-
B	-	wk	N	-	-
C	16	32	O	wk	wk
D	4	1	P	2	1
E	8	8	Q	1	wk
F	-	-	R	2	1
G	-	-	S	1	-
H	4	1	T	-	1
I	-	-	U	-	1
J	2	2	V	2	2
K	8	8	W	-	-
L	16	1	X	2	1

IMMUNIZATION BY RED CELL ALLOANTIGENS IN SANDY LOP RABBITS

THE EFFECT OF PASSIVELY-ADMINISTERED IgG ANTIBODY ON PRIMARY IMMUNIZATION

Methods and procedure

- Anti-A immunoglobulin
- Injection schedule
- Analysis of results of red cell survival studies
- Calculation of the amount of passive antibody bound to injected A-positive red cells

Results

- Relationship between antibody concentration in plasma and indirect antiglobulin titre
- Relationship between initial antibody concentration in plasma and red cell clearance
- Sites of sequestration of red cells
- Binding of passive antibody to A-positive red cells
- Responses to injections of red cells alone
- Non-responders to A
- Production of serologically-detectable antibody

Discussion

Methods and procedure

Most of the methods used in the present series of experiments have already been described. However, certain details concerning procedure and analysis of the results will be given.

Anti-A immunoglobulin. Preparation of the IgG antibody used in the present experiments and the determination of its characteristics have been described elsewhere (Frame and Mollison, 1972). In summary, the antibody was raised in a single A-negative rabbit by the i.m. injection of A-positive red cells mixed with Freund's complete adjuvant; subsequently i.v. injections of A-positive cells in isotonic saline were given. Serum from the immunized rabbit (A25) was obtained by bleeding on several occasions; the indirect antiglobulin titre (IAT) varied between 4,000 and 6,000. IgG was separated by diethylaminoethyl (DEAE) cellulose chromatography. The fraction eluted with 0.0175 M phosphate buffer, pH 6.8, was shown to contain only IgG by immuno-electrophoresis against anti-whole rabbit serum. The final preparation contained 9.75 mg IgG per ml, of which 10% was specific anti-A. In addition to the very potent anti-A (titre 1 in 50,000 by indirect antiglobulin test), the preparation contained a weak antibody of undetermined specificity directed against 6 out of 20 A-negative samples of red cells. The equilibrium constant, K, for the anti-A estimated on two occasions was found to be 1×10^8 and 1.56×10^8 l/mole respectively.

Injection schedule

Injections of cells and antibody given to the different groups of rabbits are summarised in Table 38. The intervals between injections were planned to be sufficient for the level of passively administered antibody to decline to less than 0.001 µg/ml plasma or to allow the complete elimination of any red cells injected previously. In fact, in the rabbits in group 5, given the largest dose of antibody i.e. 300 µg per kg body weight, the amount remaining in the plasma at the time of injection with cells alone was calculated to be approximately 0.001 µg per ml; this calculation assumes that no antibody is consumed during the destruction of simultaneously injected red cells and that the h.d.t. of the antibody in plasma is 9 days (see p.160). In groups 1-4, all injections were of AA cells, whereas in groups 5-7, AF cells were used throughout. A dose of 0.02 ml red cells per kg was used for all "challenge" injections of cells alone.

Analysis of results of red cell survival studies. A total of 40 rabbits received injections of A-positive cells and passive antibody followed by an injection of 0.02 ml red cells per kg alone. Five other animals, similarly treated, were excluded from analysis because the cells injected alone survived normally; they will be described separately in Results. Red cell survival curves were described in terms of T_{sn}, i.e. the time required for survival to become definitely subnormal as compared with the survival of autologous red cells (but see later). This method of expressing survival times has been described fully in a previous section (see p.210). As before, results were better fitted by

a log normal distribution. The values for T_{sn} in any one group of rabbits were compared (Student's 't'-test) with values obtained in control animals that had not been given passive antibody with the first injection (or first two injections in groups 5, 6 and 7) of A-positive red cells. Thus, in group 1, results were compared with those obtained in a group of A-negative rabbits that had received an i.v. injection of 0.02 ml A-positive red cells per kg 83 days following a first i.v. injection of 0.5 ml cells. In addition, the results for each experimental group were compared with those in a group of A-negative rabbits receiving 0.02 ml A-positive cells per kg for the first time.

Calculation of the amount of passive antibody bound to injected A-positive red cells. The amount of antibody bound to red cells, x mole, can be obtained from the following equation (Eisen and Siskind, 1964):

$$\frac{x}{Ab_T - x} = K(Ag_T - x)^a$$

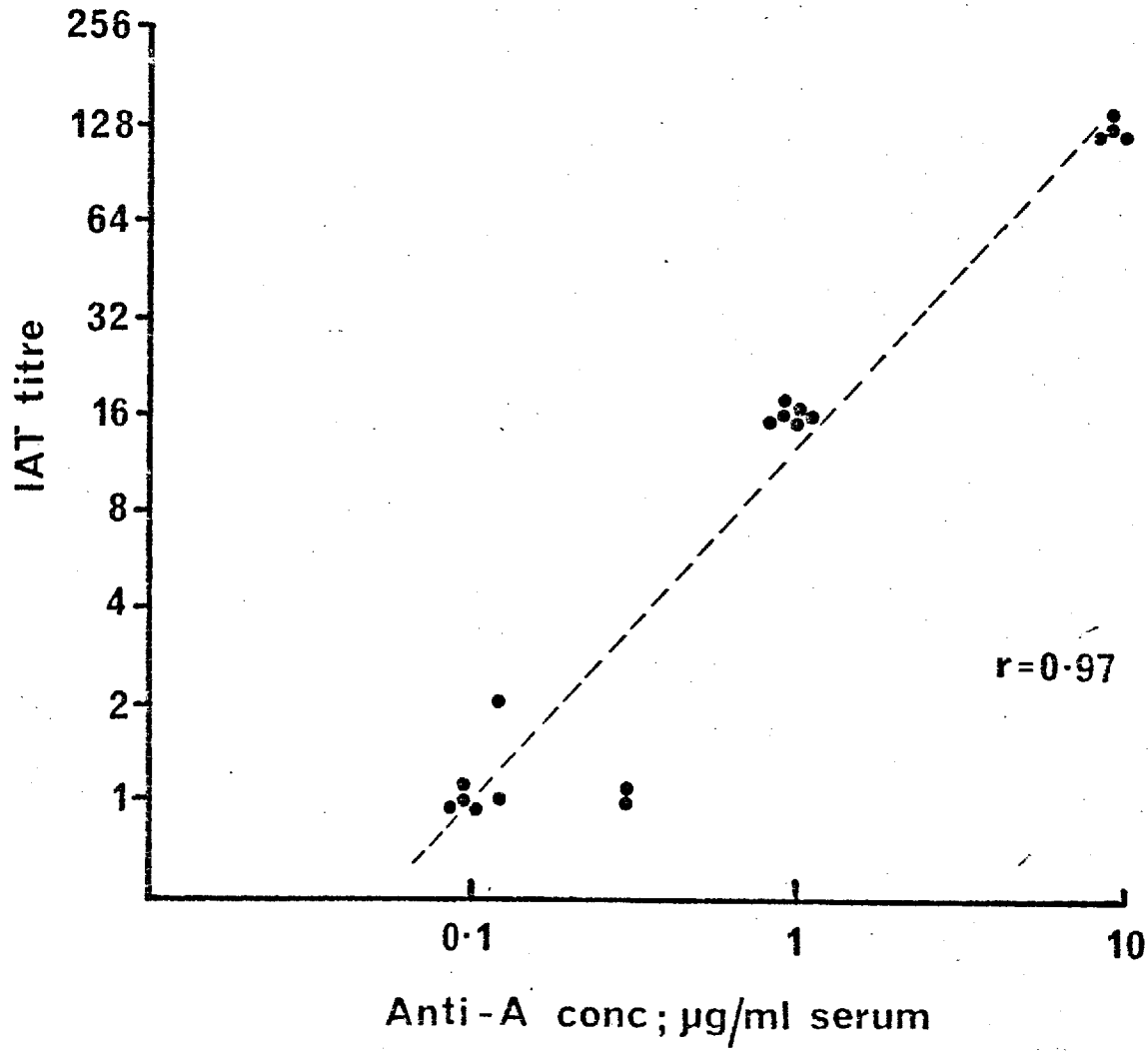
where Ab_T and Ag_T are the initial molar concentrations of injected antibody and antigen respectively, K is the equilibrium constant (1/mole) and a is the index of heterogeneity of the antibody. Relevant characteristics of the anti-A preparation used in the present experiments have already been given (see p.243); the values used for the number of A antigen sites per red cell were 1.7×10^5 for AF cells and 3.4×10^5 for AA cells (Frame and Mollison, 1972).

In applying the formula, it was assumed that antigen and antibody had come into equilibrium before the red cells were removed from the circulation. Furthermore, when the rate of red cell destruction was rapid (h.d.t. less than a few hours), it could be assumed that no appreciable amount of antibody had left the plasma and that the reaction had taken place entirely within the plasma, whereas, when red cell destruction was relatively slow (h.d.t. 1 day or more), a substantial amount of injected IgG must have left the plasma before red cell destruction was complete. In practice, calculations were made (a) assuming that the antibody was confined to the plasma and (b) assuming that it was distributed throughout the IgG space, to indicate the likely limits of the amount of antibody bound to the cells at equilibrium.

Results

Relationship between antibody concentration in plasma and indirect antiglobulin titre. During the course of these experiments samples of serum were obtained from a number of rabbits within a few hours of injection of known amounts of passive antibody and the titre of anti-A was determined by an indirect antiglobulin test (IAT). It was thus possible to compare the concentration of anti-A present in the serum (calculated by assuming a plasma volume of 34 ml per kg body weight; see p.119) and the observed titre. The results are plotted in Fig.27. It can be seen that there is a good correlation between IAT titre and log concentration of anti-A ($r = 0.97$) and furthermore that the IAT did not detect antibody at a concentration much below 0.1 µg/ml serum.

Figure 27. The relationship between the indirect antiglobulin test titre and the calculated concentration of passively-administered anti-Hg^A present in the serum (see text). Note logarithmic scales.


256
128
64
32
16
8
4
2
1
IAT titre
r=0·97
0·1
1
10
Anti-A conc; µg/ml serum

These results are similar to those obtained *in vitro* by Frame and Mollison (1972); the apparent lower sensitivity of the IAT in the present experiments may be explained in part by the fact that in estimating antibody concentrations no allowances were made for antibody bound to injected A-positive red cells, nor for antibody lost to the extravascular space.

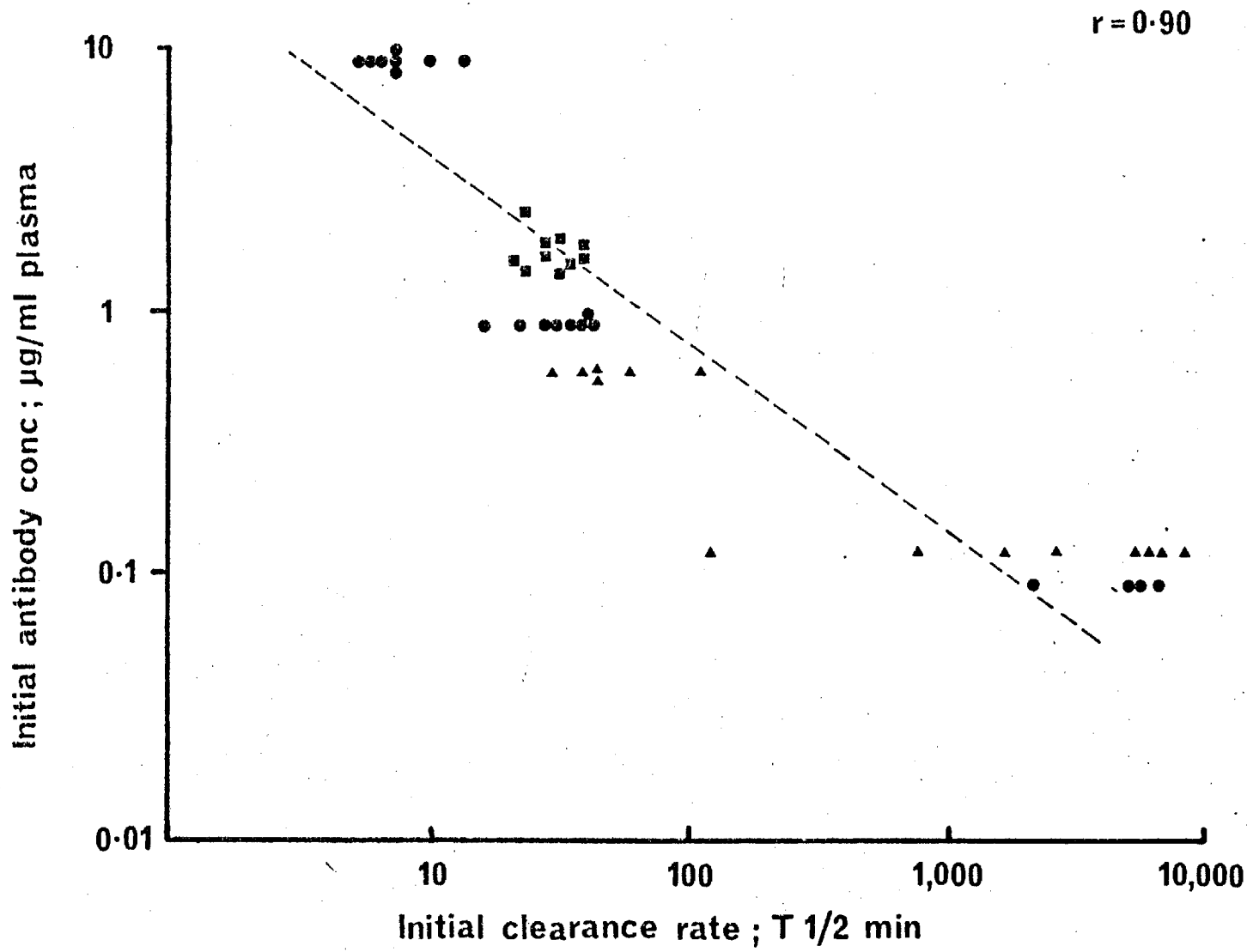
Relationship between initial antibody concentration in plasma and the rate of red cell clearance. In those A-negative rabbits given i.v. injections of IgG anti-A in amounts sufficient to bring about the complete clearance of a small dose of A-positive red cells within a few hours, the pattern of red cell removal could be described by a simple exponential function and the clearance rate expressed as $T\frac{1}{2}$. On the other hand, in rabbits given smaller amounts of passive antibody, the rate of clearance of A-positive red cells was much slower and although accelerated clearance was seen initially, the rate gradually declined towards normal over the first few days. Presumably in these cases, the changing rate of red cell clearance was a reflection, at least in part, of decreasing plasma levels of anti-A as a result of equilibration of the antibody in the total IgG space. Because of a paucity of early samples in these cases, only approximate estimates of the initial rate of clearance could be made. In all cases, the initial anti-A concentration in plasma following injection of the antibody was calculated assuming a plasma volume of 34 ml per kg body weight (see p.119). These results are plotted in Fig.28.

Figure 28. The relationship between the calculated concentration of passively-administered anti-Hg^A present in the plasma immediately after injection, and the initial clearance rate of a small volume of red cells injected at about the same time. The doses of red cells were as follows:

● 0.02 ml/kg; ▲ 0.04 ml/kg

■ 0.5 ml total dose.

Note log scales


r = 0·90
10
1
0·1
0·01
Initial antibody conc ; µg/ml plasma
10
100
1,000
10,000
Initial clearance rate ; T 1/2 min

Sites of sequestration of red cells. In 5 A-negative rabbits given ^{51}Cr-labelled A-positive red cells followed by a dose of IgG anti-A sufficient to destroy the cells with a $T_{\frac{1}{2}}$ of 1.2-2.7 hours in 4 cases and with a T_{50} of 12 hours in the remaining case, 52-86% of the injected radioactivity (mean 64%) was found in the liver and spleen combined and 4-27% (mean 14%) in the bones. Expressed in terms of activity per g tissue, the spleen contained between 13 and 175 times more than the liver. In these experiments the mean total recovery of radioactivity from the tissues sampled was 82% (range 68-104%). These results are shown in Table 39.

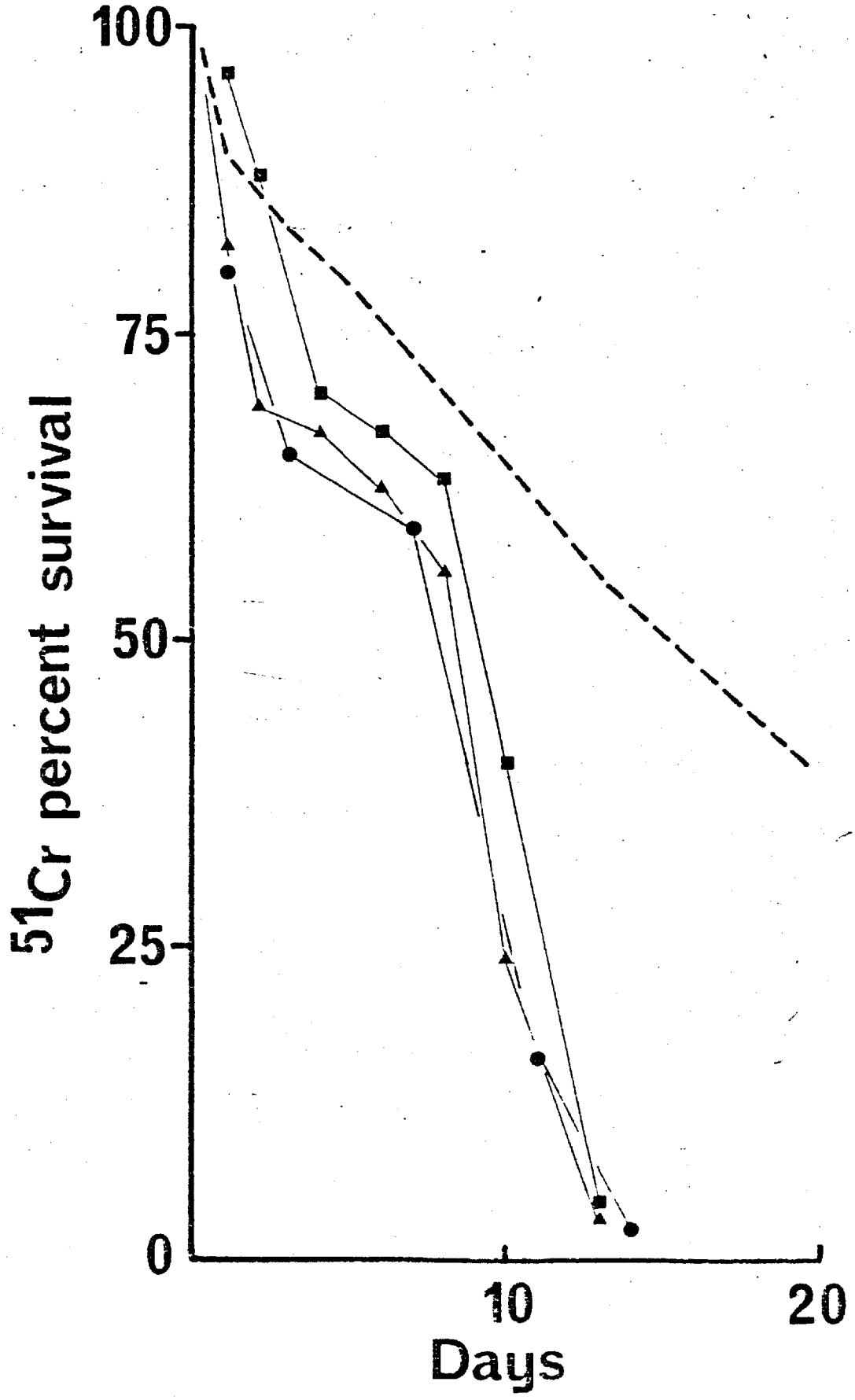
Binding of passive antibody to A-positive red cells. The proportion of A sites bound by anti-A at equilibrium is given by the molar ratio of bound antigen to total antigen. In the present experiments, it was calculated that the proportion of A sites on the injected cells bound by anti-A varied between 2% when 6 µg antibody and 0.12 ml AA red cells were injected, and 96% when 900 µg antibody and 0.06 ml AF red cells were injected. In Table 40 the amount of antibody bound to A-positive red cells at equilibrium in the different groups of rabbits is expressed as µg/ml red cells.

As mentioned previously, the maximum concentration of antibody remaining in the plasma at the time of the first "challenge" injection of red cells alone, even if it is assumed that no antibody was consumed during antibody-mediated destruction of the red cells given with the antibody, can be shown to be about 0.001 µg per ml (group 5). This concentration of antibody would bind less than 0.075% of the A sites

present on 0.06 ml AF red cells injected into a rabbit with a plasma volume of 100 ml. In group 5, with these conditions prevailing, there was no evidence of accelerated clearance immediately following injection of the first "challenge" injection of red cells.

Responses to injections of red cells alone. In attempting to express the results of red cell survival following the first injection of red cells given without antibody, it became apparent that the term T_{sn}, as originally defined (see p.210), was inappropriate in certain cases. In some rabbits, following injection of red cells alone, there was an initial phase of rapid clearance starting presumably at the time of injection and persisting for 1-3 days; this phase was followed by a period of several days, during which the rate of clearance was essentially normal, and was then superseded by a second phase of rapid elimination, leading to complete clearance of the remaining red cells within a few days. In most of these cases, the value of T_{sn} determined as described, would have been dependent upon the second phase of accelerated clearance, but in 3 cases (S18, S48, S53; Fig.29) the initial fall in the survival curve was sufficiently marked for the T_{sn} value to be a reflection of the first phase of rapid clearance. It was thus apparent that the T_{sn} was measuring different responses in these two circumstances. For the purposes of the present analysis, the second phase of accelerated clearance was considered to give a better indication of the immunological response to the second injection of red cells so that in the 3 cases mentioned above, "T_{sn}" is really the time at which this second phase of accelerated clearance had become definitely established. The significance of this type of clearance curve is discussed later (see p.265).

Figure 29. Triphasic red cell survival curves seen in three Hg^A-negative rabbits, S18 (●), S48 (■) and S53 (▲) following a first challenge injection of Hg^A-positive cells (0.02 ml/kg) given alone. All three rabbits had received a first injection of Hg^A-positive cells (0.04 ml/kg) together with passive IgG anti-Hg^A (20 µg/kg) three months previously.


100
75
50
25
0
^{51}Cr percent survival
10
20
Days

Consequently, it was found that for any groups of A-negative rabbits injected with a particular dose of A-positive red cells and varying amounts of passive antibody, the mean T_{sn} of A-positive cells (0.02 ml/kg) subsequently injected alone appeared to be positively related to the amount of antibody given with the first injection(s) of cells. In all the groups, the mean T_{sn} value was significantly lower than that found in a group of A-negative rabbits given 0.02 ml A-positive cells per kg for the first time ($p<0.001$). On the other hand, when the mean T_{sn} results for each group were compared with those found in control rabbits which had previously received equivalent injections of cells without passive antibody, significantly higher T_{sn} values were found in groups 1 ($p<0.025$), 2 ($p<0.0005$), 5 ($p<0.0005$) and 6 ($p<0.05$). These results are shown graphically in Fig.30.

A further injection of A-positive red cells was given to 31 rabbits and in 28 cases was cleared more quickly than the first "challenge" injection. In the 3 remaining cases, the first "challenge" injection had been cleared very rapidly with T_{sn} values of 1 day; following the second "challenge" injection, T_{sn} values were 1, 2 and 3 days.

Non-responders to A

Following the injection of A-positive red cells and passive antibody into 5 A-negative rabbits (omitted from Table 40), survival of the first "challenge" injection of A-positive cells alone was normal or almost normal. In one of these rabbits (S45, see Fig.31), further injections of A-positive red cells survived normally until, following an injection

Figure 30. Individual T_{sn} values observed following the first challenge injection of A-positive red cells given alone (0.02 ml/kg) to rabbits in groups 1-7, all of which had received a previous injection (two previous injections in groups 5-7) of A-positive red cells with anti-A (●). Details of preceding injections of cells and antibody given to these groups of rabbits are shown in Table 38. The T_{sn} values of groups 1-7 may be compared with those found in control A-negative rabbits following a first injection of A-positive red cells without antibody (■) or with those found following a series of injections of A-positive red cells equivalent to those given to the corresponding experimental groups but without passive antibody (0). The horizontal lines in each column indicate 95% confidence limits and mean values for each group. Note the logarithmic scale.

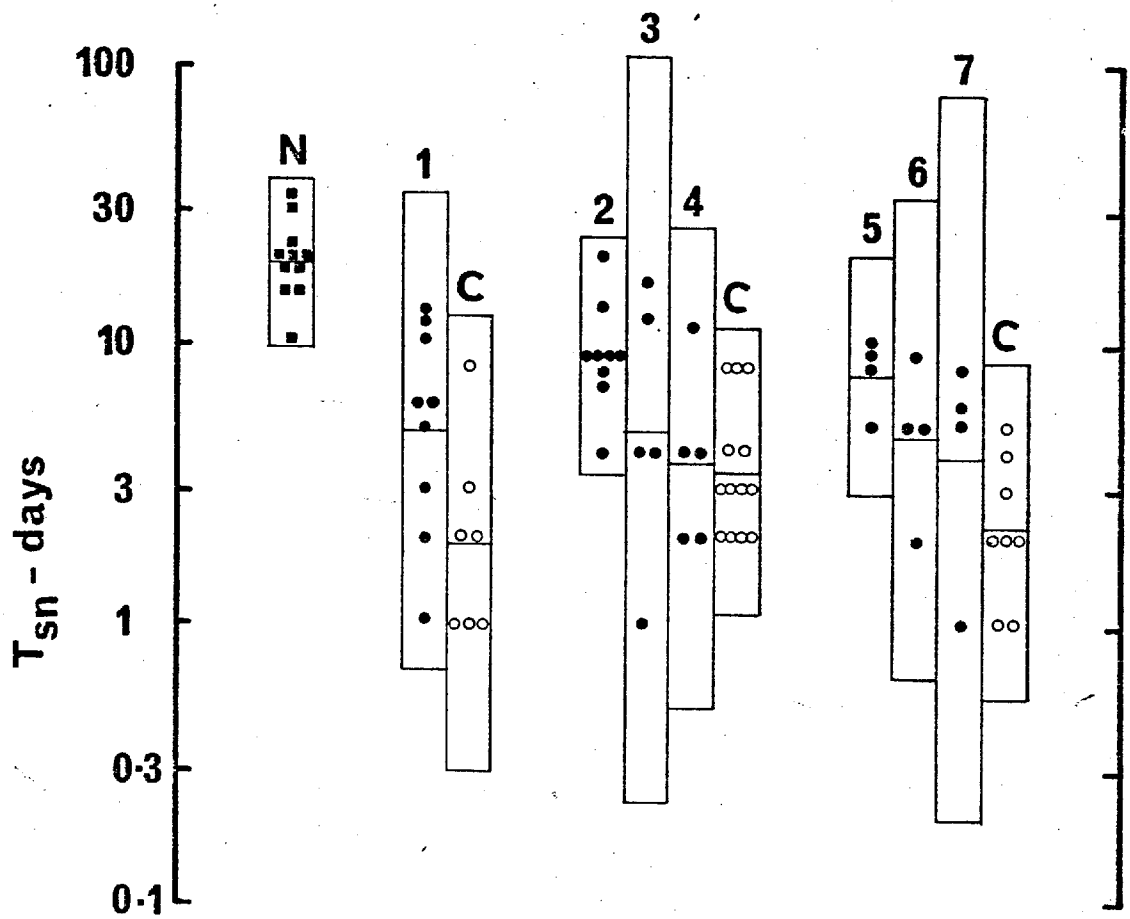
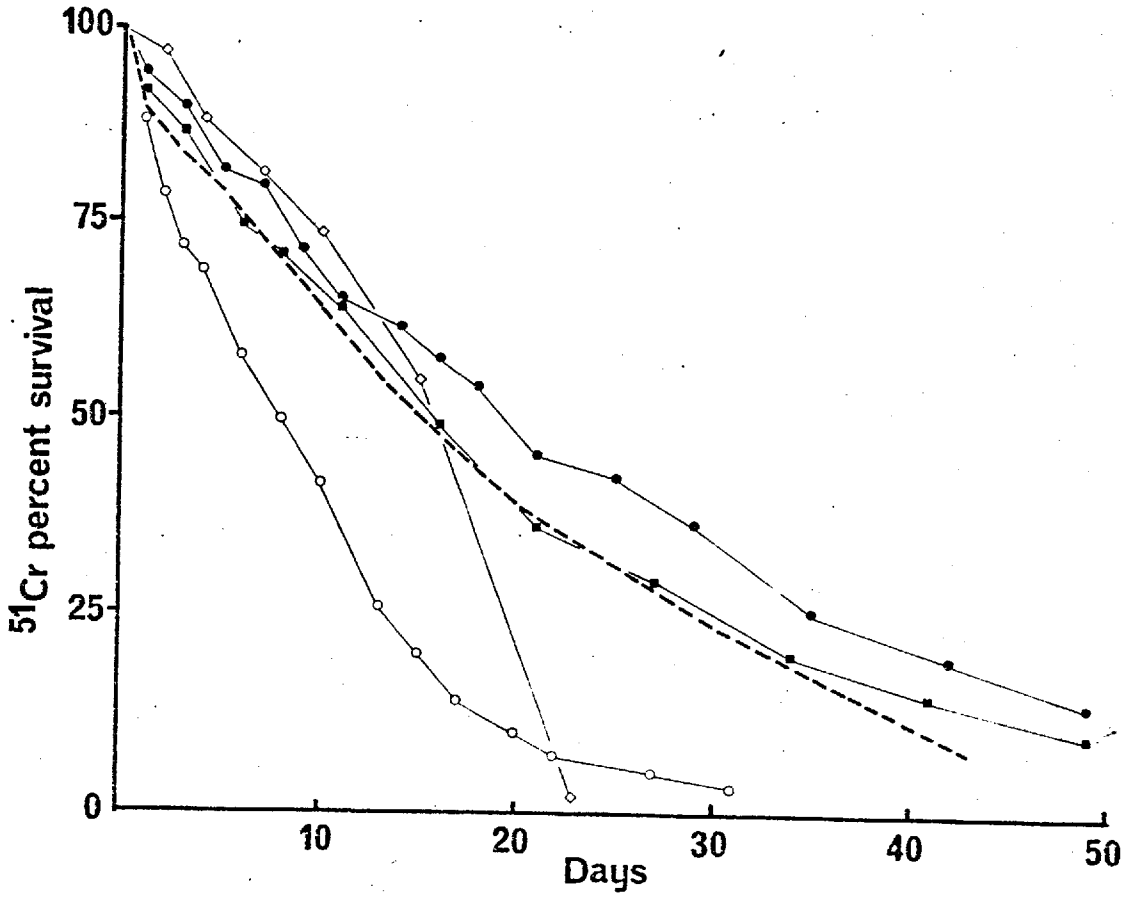

100
30
10
3
1
0·3
0·1
T_{sn} - days
N
1
C
2
3
4
C
5
6
7
C

Figure 31. Survival of four successive i.v. injections (○, ●, ■, ◇) of A-positive red cells into a poorly-responding A-negative rabbit, S45. IgG anti-A (4 µg/kg) was given with the first injection (○) and an additional injection of cells mixed with FCA was given i.m. at the same time as the fourth injection (◇). The second and third injections survived normally; the fourth injection survived normally to about day 19 when the survival curve "collapsed".


100
75
50
25
0
^{51}Cr percent survival
10
20
30
40
50
Days

of donor cells mixed with FCA given i.m. at the same time as the third i.v. dose of cells alone, the survival curve "collapsed" on about day 19. The remaining 4 rabbits (S14, S15, S20, S39) were also considered to be examples of non-responders, although no further red cell survival studies were performed in these animals; the relevant survival curves are shown in Fig.32.

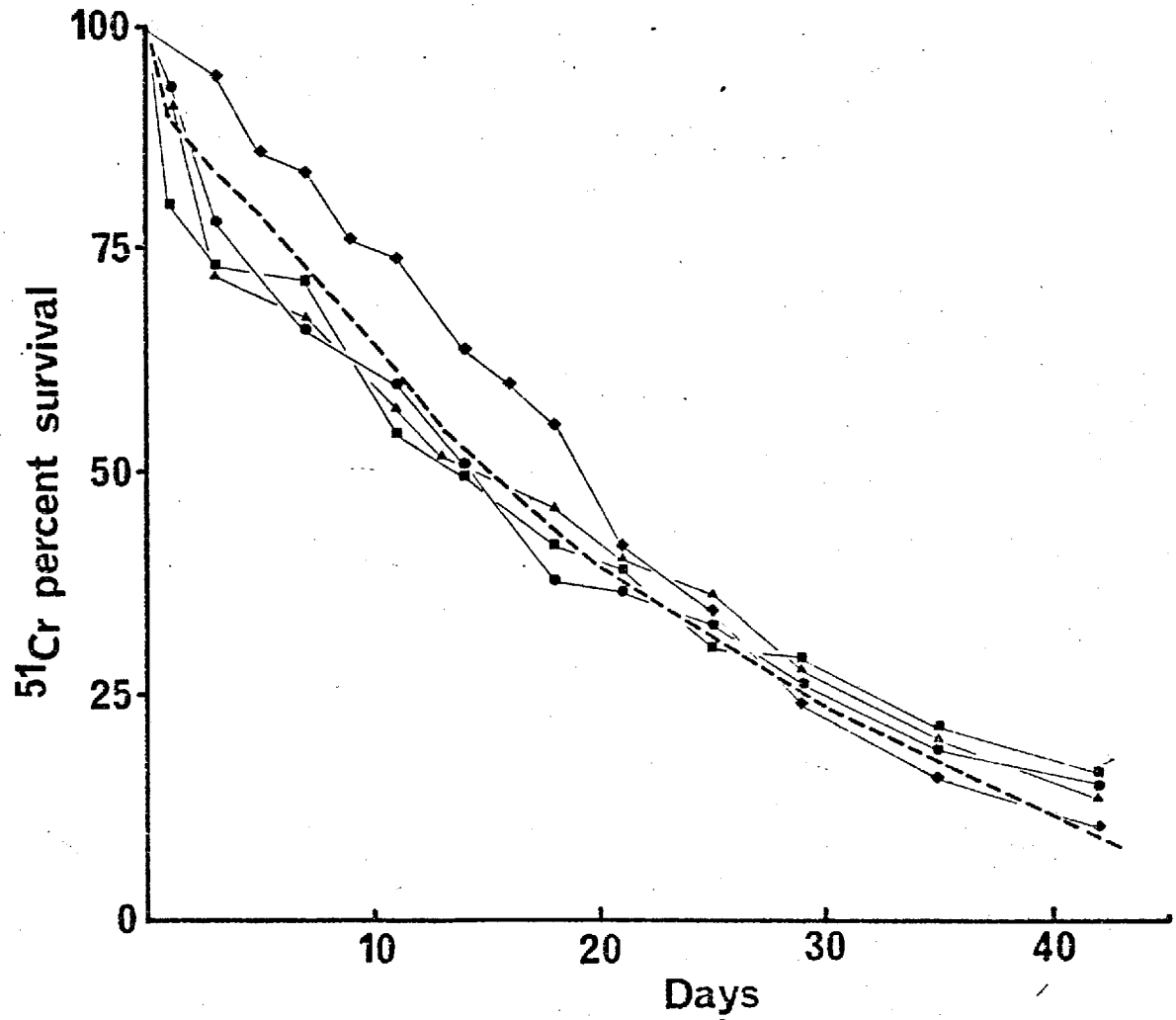
The incidence of non-responders in the present experiments was therefore about 11% (5/45), which is similar to the figure of 14% (7/51) found in rabbits not given passive antibody with the first injection of red cells (see p.233).

Production of serologically-detectable antibody. Following the first injection(s) of red cells given with passive antibody, sequential testing in several rabbits showed a decline in antibody titre until no antibody was detectable. In no case out of 19 animals tested was any evidence obtained by serological tests of active immunization following injections of cells given with passive antibody.

Sera from 27 rabbits were obtained on several occasions during the month following clearance of a first i.v. injection of red cells given alone. Anti-A was detected in 6 of these rabbits; in one other animal, an antibody reacting with donor cells was found but the specificity was not determined.

Following a second i.v. injection of cells alone, sera from 20 rabbits that had not had antibody present following the previous injection were tested. Eight out of 20 rabbits developed antibody reacting with donor

Figure 32. Survival of the first challenge injection of A-positive red cells (0.02 ml/kg) in four poorly-responding A-negative rabbits, S14 (●), S15 (■), S20 (▲) and S39 (◆). All four rabbits had received a first injection of cells (0.04 ml/kg) together with IgG anti-A (20 µg/kg in the case of S20, 4 µg/kg in the remaining cases) three months previously.

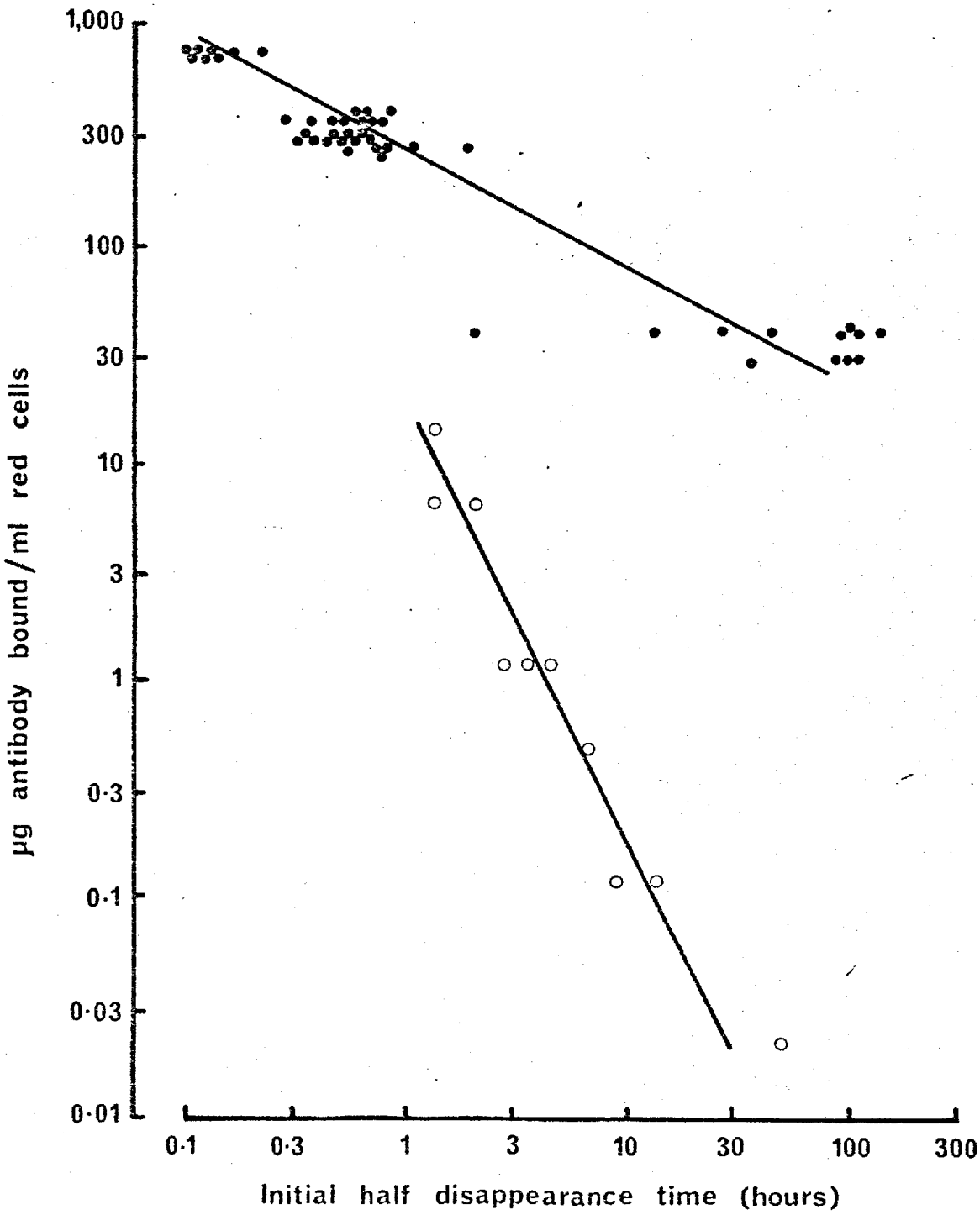

100
75
50
25
0
^{51}Cr percent survival
10
20
30
40
Days

red cells, and in 7 cases this was shown to be anti-A. Thus, the cumulative incidence of serologically-detectable antibody in 27 rabbits following two "challenge" injections of A-positive cells in the present experiments was 15 (56%). Although 4 out of the 5 non-responders were not given a second "challenge" injection of red cells alone, it can reasonably be assumed that they would have failed to respond again, so that a more realistic estimate of the proportion of rabbits producing detectable antibody would be 15 out of 32 (47%).

Discussion

From the present observations, the relation between the rate of clearance of a small dose of A-positive red cells and the amount of IgG anti-A bound to the cells can be deduced. In Fig.33 these data are plotted together with those of Mollison and Hughes-Jones (1967) for Rh-positive cells and anti-Rh in man. It is apparent that for any given clearance rate, far more antibody has to be bound to the rabbit red cells than to the human red cells. This finding presumably reflects differences in the interactions between antibody-coated red cells and the mononuclear phagocytic cell systems in the rabbit and man. One obvious way in which this difference manifests itself is in the tissues sequestering the antibody-coated red cells; in man sequestration takes place predominantly in the spleen until the amount of bound anti-Rh exceeds about 20 µg per ml Rh-positive red cells (Mollison et al., 1965) whereas, in the rabbit, amount of anti-A inducing comparable rates of red cell clearance are associated with sequestration predominantly in the liver. The rabbit spleen is about 5-10 times smaller

Figure 33. Relationship between the calculated amount of passive IgG antibody bound to the red cells and the initial red cell clearance rate. ●: data for anti-Hg^A (present work). O: data for anti-Rh(D) (Mollison and Hughes-Jones, 1967). The lines were fitted by a "least squares" method. Note the logarithmic scales.


1,000
300
100
30
10
3
1
0·3
0·1
0·03
0·01
µg antibody bound/ml red cells
0·1
0·3
1
3
10
30
100
300
Initial half disappearance time (hours)

in relation to total body weight than the spleen in man and although this may account in part for the slower red cell clearance rates with low doses of passive antibody, the spleen remains relatively inefficient at sequestering red cells on a weight for weight basis compared with the liver, even with higher doses of antibody.

Another point of interest is the relationship between the amount of passive antibody injected, the indirect antiglobulin titre found in the plasma immediately following injection, and the rate of clearance of the A-positive red cells. It is perhaps surprising that when sufficient IgG anti-A was injected to give a positive IAT in these rabbits, the initial rate of red cell clearance was frequently not very different from that observed in other rabbits during the phase of accelerated clearance of red cells injected alone, yet in the latter, actively immunized rabbits, antibody was rarely detected following accelerated clearance of a first injection of allogeneic red cells. One probable explanation is that during the early phase of the primary response, specific IgM antibody predominated, that is, antibody which, on a molar basis, is some 50 times more effective than specific IgG in bringing about the clearance of A-positive red cells (Burton and Mollison, 1968).

The rates of clearance observed in the present work in relation to the amount of antibody injected are rather different from those reported by Pollack et al. (1968) and Elson et al. (1971). The first group of authors injected i.v. 40 µg anti-A 5 hours before giving 10^9 A-positive red cells (i.e. approximately 0.03 ml cells/kg in a rabbit

weighing 3.3 kg) and found that the cells were cleared with a $T_{50}Cr$ of 7.4 days. Similarly, Elson et al. (1971) gave 80 µg anti-A 5 hours before 2×10^9 cells and found that the $T_{50}Cr$ was 120 hours (5 days). Assuming that the equilibrium constant of different examples of anti-A is much the same, and also assuming that in these cases the injected antibody was distributed throughout the IgG space at the time when equilibrium was attained, the amount of anti-A bound to the cells at equilibrium in these two series can be calculated to be approximately 120 and 190 µg per ml respectively. The dose of red cells injected in groups 5-7 of the present series and in these other two series was within the range of approximately 0.02-0.06 ml cells/kg, and it would therefore be expected that the main factor determining the rate of clearance would be the amount of antibody bound to the cells rather than any overloading of the mononuclear phagocyte system. It is therefore not clear why, in the present series, cells coated with approximately 30 µg antibody per ml were cleared as rapidly as cells which in the other two series appear to have been coated with 120-190 µg per ml. The discrepancy raises the possibility that the estimates of antibody concentration were incorrect, either in the present series or in the two previous series, although it is of course possible that the antibody used in the present work was relatively more effective in bringing about red cell clearance. An important objection to this latter explanation is that a similar relation to that found in the present work between the anti-A titre in plasma and the rate of clearance of A-positive red cells was found by Burton and Mollison (1968) using a different example of IgG anti-A.

In the observations published by Pollack et al. (1968) and Elson et al. (1971), the criterion of suppression of primary immunization was failure to detect antibody by serological tests; following a single injection of red cells and passive antibody, it is possible that antibody formation was only partially suppressed and that this might have been revealed by estimating the survival of a subsequent injection of Hg^A-positive red cells. In the present observations, the criterion of complete suppression of primary immunization was a survival curve for a subsequent injection of A-positive red cells which was indistinguishable from a first survival curve in a previously uninjected rabbit. By this criterion, no evidence was obtained that the doses of passive antibody used were able to induce complete suppression. However, as already indicated, it was not always possible to test serologically the specificity of the response detected by means of curtailed red cell survival. Thus it is conceivable that in certain cases, impaired survival of A-positive red cells was in fact due to the response of the recipient to a different red cell antigen present on the injected cells. That this may occur has already been demonstrated in a previous section (p.216), where examples were given of the impaired survival of A-negative red cells in A-negative rabbits previously transfused with A-positive red cells. In such circumstances, therefore, impaired survival of A-positive red cells might be observed, even if the response to the A antigen were completely suppressed.

On the other hand, if there had been no suppression of the response to the A antigen when passive antibody was given with the first injection of A-positive cells, it would be expected that responses to subsequent injections of cells would be indistinguishable from those in rabbits not

given passive antibody with the first injection. In fact, such comparisons between the treated and control groups have shown a graded improvement of T_{sn} values directly related to the amount of passive anti-A given with the initial injection(s) of cells (see Fig.30). These findings are taken to indicate that immunization to the A antigen has been at least partially suppressed. For the reasons already given, it is not possible from these data to ascertain whether suppression was complete in any of the groups of rabbits given the higher doses of passive antibody.

Apart from the use of different criteria for detecting immunization in the present work, another possible explanation for the apparent different capacity of passive antibody to suppress the immune response compared with that reported in other published work could be the timing of the injections of antibody in relation to antigen. Whereas the other authors gave the antibody several hours before the red cells, in the present work, red cells were always given first, up to an hour before antibody was injected. If, as seems possible, passive antibody acts directly on immunocompetent cells to suppress immunization to specific antigen (see p.64), it could well be that time is needed for concentration of antibody in strategic sites in order to enable complete suppression of the immune response to specific antigen. However, this is clearly not so in the case of Rh immunization and its suppression by passive antibody.

The relatively poor response of the rabbits used in these experiments to allogeneic red cells as judged by antibody detection <u>in vitro</u> has been dealt with in a previous section (see p.235) and is one reason why

so much reliance has had to be placed upon red cell survival curves in interpreting the data presented. Nevertheless, for the sake of completeness, the incidence of antibody detection following sequential injections of A-positive red cells is shown in Table 41 both for the present groups of rabbits given passive antibody with the first injection of red cells and for those control rabbits given red cells alone on each occasion. It is appreciated that a comparison is being made between groups that are heterogeneous within themselves with respect to amount of red cells injected, and in the case of the treated group, with respect to the amount of passive antibody given initially, but the numbers of animals tested in each well-defined group was too small to permit any useful comparisons to be made. It can be seen from Table 41 that following each of the red cell injections, antibody was detected less frequently in the treated groups of rabbits, although the difference is not statistically significant.

As already mentioned in Results, several examples of triphasic survival curves were observed following the first injection of red cells alone into some of the rabbits given passive antibody with the initial injection of red cells. This phenomenon was seen in 8 out of 9 rabbits in group 2, in 4 out of 9 rabbits in group 3, in 1 out of 5 rabbits in group 4 and in 1 out of 9 rabbits in group 1. Apart from the solitary rabbit in group 1 showing this characteristic, all of the other rabbits listed had been given 0.04 ml red cells per kg from donor H4. It is of interest that following the initial accelerated phase of destruction in two rabbits (S14, S15), survival of the remaining red cells was entirely normal. Of 15 control rabbits given a first injection of 0.04 ml red cells/kg

from this same donor, none showed any significant degree of early impairment of the survival curve. Although it is possible that the less pronounced examples based upon few experimental points may reflect artefacts of the method, this explanation will not do for the majority of cases. A different explanation, which is mere speculation, is that the initial phase of accelerated clearance was mediated by IgM antibody (see, for comparison, Burton and Mollison, 1968) produced in response to the first injection of red cells. This unusually protracted albeit weak response might perhaps reflect the effect of the passive antibody given with the first injection of cells. The fact that cells from the donor H4 had been used in most cases could also be relevant in that the response may have been to an antigen other than A present on H4 cells. In the two cases subsequently showing normal survival of H4 cells, the initial response, whether to the A antigen or to some other determinant(s) was presumably followed by a state of tolerance.

Table 38. Schedule of injections of A-positive* red cells given to A-negative rabbits

Group	Injection of red cells with antibody			Injections of red cells alone			
				First injection of cells alone		Second injection of cells alone	
	Number of recipients	Dose of red cells (ml/kg)	Dose of antibody (µg/kg)	Number of recipients	Interval (d) since previous injection	Number of recipients	Interval (d) since previous injection
1	9	0.5 total	200 total	9	83	9	67
2	5	0.4	20	5	106	5	30
	4			4	91	4	34
3	1	0.04	4	1	91		
	4			4	55	2	100
4	5	0.04	2	5	91	-	-
5	4	0.02 (repeated after 126 d)	300	4	113	3	32
6	4	0.02 (repeated after 124 d)	30	4	107	4	32
7	4	0.02 (repeated after 125 d)	3	4	100	4	32
Total	40			40		31	

* AA cells in groups 1-4, AF cells in groups 5-7

Table 39. Recovery of radioactivity from various organs following clearance of A-positive red cells injected into A-negative rabbits at the same time as passive IgG anti-A.

Rabbit number		A75	A78	A84	A93	R27
Volume of cells injected (ml)		0.06	0.07	0.06	0.06	0.10
Amount of anti-A injected (μg)		30	51	52	21	60
Clearance rate $T_{\frac{1}{2}}$ Cr (hours)		2.6	1.2	1.5	12*	2.7
Percent circulating at death		< 1	< 1	2	10	5
Time of death (hours after injection)		24	24	24	48	18
Percent recovery	Spleen	22	10	31	41	18
	Liver	42	51	55	11	38
	Kidneys	-	-	-	-	3
	Gut	-	-	-	-	0
	Bones	27	11	16	14	4
	Total	91	72	104	76	68
Spleen:liver ratio (ct/min per g tissue)		47	13	28	175	30

* In this case, clearance is expressed as T_{50} Cr (hours).

Table 40. Calculated amounts of anti-A bound to A-positive* red cells at equilibrium and rates of red cell clearance in relation to amounts of A-positive red cells and anti-A given to A-negative rabbits.

Group	Total amounts injected		Antibody bound (μg/ml cells)		Antigen sites bound (%)		Clearance rate ($T_{1/2}$)
	Antibody (μg)	Red cells (ml)	(a)	(b)	(a)	(b)	
1	200	0.5	296	245	21.0	17.0	≯ 1 hr
2	60	0.12	260	187	18.0	13.0	≯2.5 hr
3	12	0.12	55	39	4.0	3.0	2-130 hr
4	6	0.12	28	20	2.0	1.5	9-11 days†
5	900	0.06	750	660	96.0	91.0	≯ 13 min
6	90	0.06	345	238	48.0	33.0	≯ 40 min
7	9	0.06	46	29	6.0	4.0	2-8 days †

* AA cells in groups 1-4, AF cells in groups 5-7

† Clearance described by T_{50} in these cases

(a) assuming antibody confined to plasma

(b) assuming antibody distributed throughout IgG space

Table 41. Cumulative incidence of antibody detection in A-negative rabbits after sequential injections of A-positive red cells. In the control groups, red cells alone were given on each occasion, whereas in the treated groups, specific IgG antibody was given with the first injection of red cells.

Number of red cell injections	Groups of rabbits	Antibody present	Antibody absent	Number tested	Percentage with antibody
1	Treated	0	19	19	0
	Control	3	25	28	11
2	Treated	7	25	32	22
	Control	18	28	46	39
3	Treated	15	17	32	47
	Control	24	17	41	58

IMMUNIZATION TO RED CELL ALLOANTIGENS IN SANDY LOP RABBITS

THE RESPONSE TO RED CELLS TREATED WITH N-ETHYLMALEMIDE (NEM)

Methods and procedure

- Treatment of red cells with NEM
- Osmotic fragility of NEM-treated red cells
- Effect of NEM treatment on the serological reactions of the red cells
- Injections of NEM-treated A-positive red cells into rabbits already immunized to the A antigen
- Injections of NEM-treated and untreated A-positive red cells given in the main experiment
- Description of red cell survival curves
- Sites of sequestration of NEM-treated red cells

Results

- Osmotic fragility of NEM-treated red cells
- Absorption of antibody by NEM-treated red cells
- Rate of clearance and sites of sequestration of NEM-treated red cells
- Effect of NEM-treated red cells in "primed" rabbits
  - (i) Effect on antibody production
  - (ii) Effect on the survival of untreated cells
- Survival of untreated red cells in rabbits previously injected with NEM-treated cells
  - (i) Progressive shortening of red cell survival
  - (ii) Accelerated clearance followed by normal or almost normal survival
  - (iii) Normal or almost normal survival
- Serum IgG concentrations in responders and non-responders

Discussion

In experiments in which passive IgG anti-A was given to A-negative rabbits at the same time as A-positive red cells in an attempt to suppress primary immunization to the A antigen (see p.243), two control groups of animals were used; in one group the usual procedure of giving equivalent doses of donor cells without passive antibody was followed, whereas in the second group, an attempt was made to mimic more closely the experimental group by inducing accelerated clearance of donor cells from the recipient's circulation at a rate similar to that produced by giving passive antibody. To this end, the donor cells given to the second control group were treated with N-ethylmaleimide (NEM); this sulphydryl blocking agent has been shown to induce a variety of metabolic changes in red cells, leading to their altered osmotic fragility and impaired survival when reinjected into the circulation In fact, it was found that many of the recipients in this group given NEM-treated cells exhibited a marked degree of immunological unresponsiveness to subsequent injections of untreated A-positive red cells. This observation was considered worthy of further investigation and the results of relevant experiments are reported in this section.

Methods

In addition to the methods already described, the following procedures were used in the experiments reported in this section.

Treatment of red cells with NEM. One volume of 50% saline-suspended A-positive red cells (previously labelled with ^{51}Cr) was incubated with one half-volume of normal saline solution containing 10 μmoles NEM per ml.

The mixture was kept at 37°C for 1 hour with continuous gentle shaking. The cells were then washed twice and resuspended in normal saline for injection. In some of the experiments different amounts of NEM were used and this is indicated in the appropriate sections below.

Osmotic fragility of NEM-treated red cells. Heparinized venous blood from a normal rabbit (S62) was centrifuged and the serum discarded. The cells were washed twice with dextrose-isotonic saline (200 mg dextrose per 100 ml) and a 50% suspension in this medium was prepared. To 1 ml amounts of this suspension was added 0.5 ml isotonic saline solution containing respectively 5, 8, 12 and 20 μmoles NEM. As a control, 0.5 ml dextrose-saline was added to a further 1 ml cell suspension. These mixtures were incubated at 37°C for 2 hours with constant gentle shaking. Osmotic fragility was then determined for each preparation of NEM-treated red cells, as described previously (see p.101).

Effect of NEM treatment on the serological reactions of the red cells. The ability of AA and AF red cells to absorb anti-A from a potent antiserum was tested using untreated and NEM-treated red cells. FF red cells were used as a control. One volume of anti-A serum was incubated at 37°C for 30 min with an equal volume of a 50% suspension of AA red cells or with two volumes of a 50% suspension of AF red cells. After absorption, the antiserum was tested for residual anti-A using the indirect antiglobulin test.

Injections of NEM-treated A-positive red cells into rabbits already immunised to the A antigen. Two experiments were performed in vivo in order to establish that treatment of red cells with NEM in the doses used to induce rapid clearance from the circulation did not substantially affect the potential antigenicity of the red cells.

In one experiment, two A-negative rabbits which had received injections of A-positive red cells and had formed anti-A were kept for several months, during which time the titre fell and ultimately the antibody could no longer be detected. At this time, each animal was given an i.v. injection of 0.2 ml A-positive red cells treated with 10 µmoles NEM per ml cells. Samples of blood were taken on days 8 and 12 and tested for anti-A.

In another experiment, 3 A-negative rabbits that had responded to i.v. injections of untreated A-positive red cells by showing a progressive increase in the rate of clearance of three successive injections and had also produced weak, serologically-detectable anti-A, were each given an i.v. injection of 0.5 ml A-positive red cells treated with 10 µmoles NEM per ml cells. After 5 weeks, the survival of 0.02 ml/kg of ^{51}Cr-labelled A-positive untreated cells was studied.

Injections of NEM-treated and untreated A-positive red cells given in the main experiment. Seventeen previously uninjected A-negative rabbits were each given an i.v. injection of 0.5 ml of ^{51}Cr-labelled, NEM-treated A-positive red cells. Nine rabbits received cells from an

AA donor and 8 (injected on a later occasion) received cells from an AF donor. Between 1 and 3 months after the injection of NEM-treated cells, all animals were given an injection of 0.02 ml untreated ^{51}Cr-labelled A-positive red cells per kg body weight. Further injections of untreated red cells were given at varying times after the complete clearance of the preceding dose of red cells.

Description of red cell survival curves. The term "T_{sn}", i.e. the time taken for survival of injected cells to become significantly subnormal, has been described fully in the previous section (see p.210); it has been used to describe results in the present experiments in certain cases in which survival was initially normal, but was followed, after a period of some days, by a phase of accelerated destruction. In other cases, for example, when survival was throughout normal or only slightly subnormal, results were expressed as the $T_{50}Cr$, i.e. the time taken for the Cr counts to fall to 50% of the immediate post-injection value.

Sites of sequestration of NEM-treated red cells. A number of rabbits were injected with ^{51}Cr-labelled, NEM-treated red cells; within 24 hours of injection, at a time when 96% or more of the injected radioactivity had been cleared from the circulation, the animals were killed. The spleen, liver and in some cases kidneys, small gut and long bones from the hind limbs were removed, dissolved in concentrated nitric acid and the radioactivity in aliquots of the digested tissue was estimated. At the same time, dilutions of the injection suspension were counted. From these measurements, the amount of red cells cleared by each of the tissues examined could be estimated, as described in the previous section (see p.211).

Results

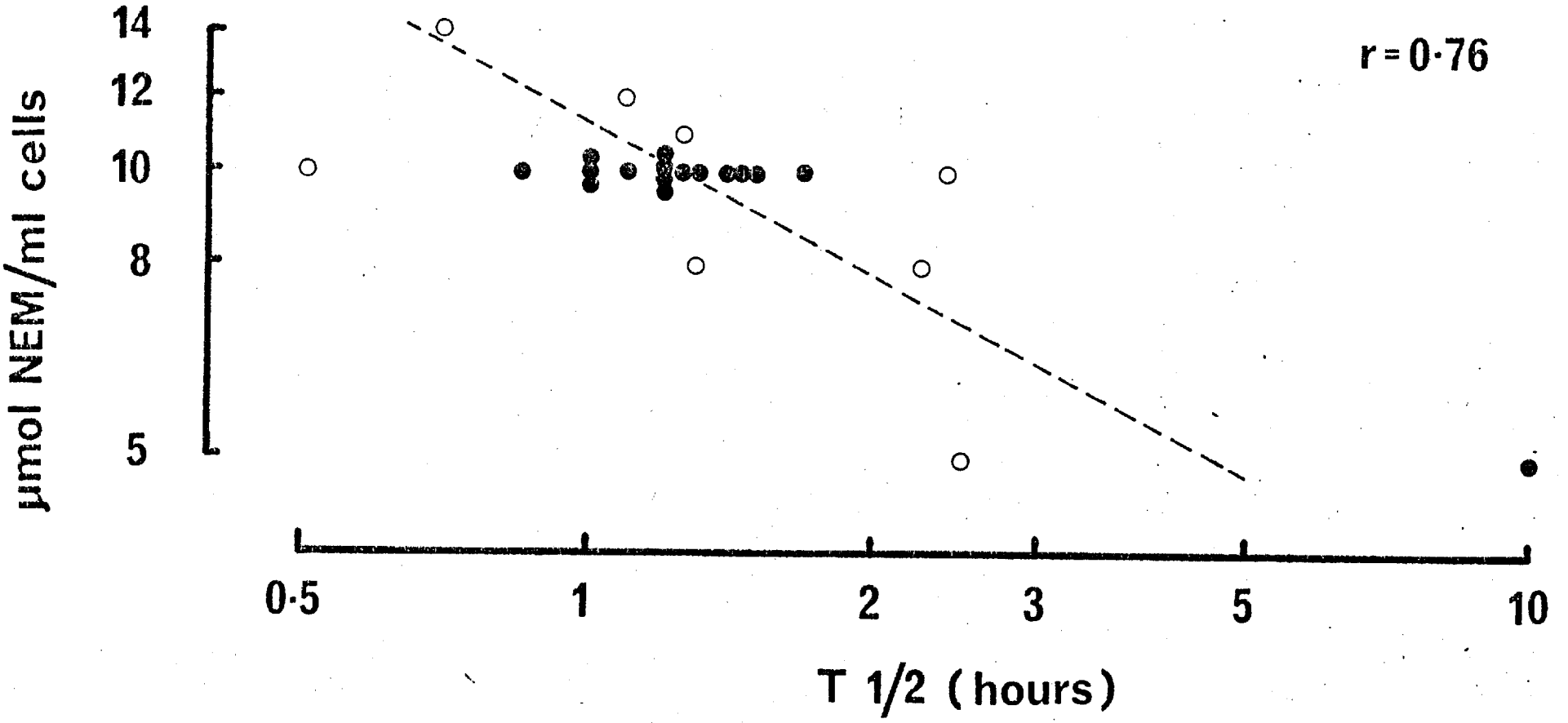
Osmotic fragility of NEM-treated red cells. Table 42 shows the per cent haemolysis at different concentrations of NaCl found with the red cell suspensions exposed to different amounts of NEM. Compared with the control suspension, the effect of NEM in these experiments was to increase the resistance of the cells to osmotic lysis at concentrations of 5, 8 and 12 μmoles per ml cells, and to increase fragility at 20 μmoles per ml cells.

Absorption of antibody by NEM-treated red cells. No reduction in the capacity to adsorb anti-A was observed. Thus, the titre of anti-A was reduced from 2048 to 128 by untreated AA cells and to 64 by NEM-treated AA cells. Similarly, using AF cells, the titre after absorption was 64 both for treated and untreated cells.

Rate of clearance and sites of sequestration of NEM treated red cells. The rate of clearance of NEM-treated red cells was determined in 24 rabbits. In 8 cases, autologous cells were used whereas in the remaining cases, A-positive donor cells were injected into 4 A-positive and 12 A-negative recipients; 11 of these 12 A-negative rabbits given allogeneic red cells were participants in the main experiment to be described below. In each case, between 0.19 and 0.5 ml red cells, previously incubated with 5-14 μmoles NEM per ml cells was injected. There appeared to be an inverse correlation between the amount of NEM used in treatment and the rate of clearance of the cells, as shown in Fig.34. All but two of the 17 observations made using cells treated with 10 μmoles NEM per ml gave half-times ranging from 0.8-1.7 hours.

Figure 34. Relationship between the amounts of NEM to which red cells are exposed in vitro and the rates at which they are cleared from the circulation of normal, unimmunized rabbits. Observations made using autologous (O) and allogeneic (●) cells. The line was fitted by a "least squares" method.

Note the logarithmic scales.


14
12
10
8
5
µmol NEM/ml cells
r = 0·76
0·5
1
2
3
5
10
T 1/2 (hours)

Sequestration studies were performed in 11 rabbits in this experiment and the results are summarized in Table 43. In 4 rabbits given between 0.19 and 0.34 ml red cells treated with 5, 8, 11 and 14 µmoles NEM per ml cells respectively, 28% or less of the injected radioactivity was recovered in spleen and liver combined at 24 hours, when less than 1% of the activity was present in the circulation. These results prompted experiments with more extensive tissue sampling. Thus, in 5 further rabbits given 0.5 ml NEM-treated red cells and killed within 24 hours, at a time when 96% or more of the injected radioactivity had been cleared from the circulation, bones, kidneys and small gut in addition to spleen and liver were tested for radioactivity. In 1 case, 5 µmoles NEM, and in the other 4 cases, 10 µmoles NEM per ml red cells had been used. The total activity recovered from the tissues examined in these animals ranged from 74-113% (mean 87%). The largest proportion of the injected radioactivity was present in the bones and ranged from 46-67%. The proportion in spleen and liver combined was 11, 24, 25, 26 and 57% respectively. Expressed in terms of radioactivity per gram of tissue, the amount in the spleen was greater by 51-285 times that in the liver. Insignificant amounts were found in the kidneys and small gut.

In order to ascertain whether the observed distribution of sequestered NEM-treated cells was a function of the volume of cells injected, two further experiments were performed in which the dose of cells (treated with 10 µmoles NEM per ml cells) was only 0.05 ml. The fraction of cells recovered from the bone marrow was 25% and 61% respectively, indicating that even with small doses of cells there was the same tendency for a substantial proportion to be sequestered in the bone marrow.

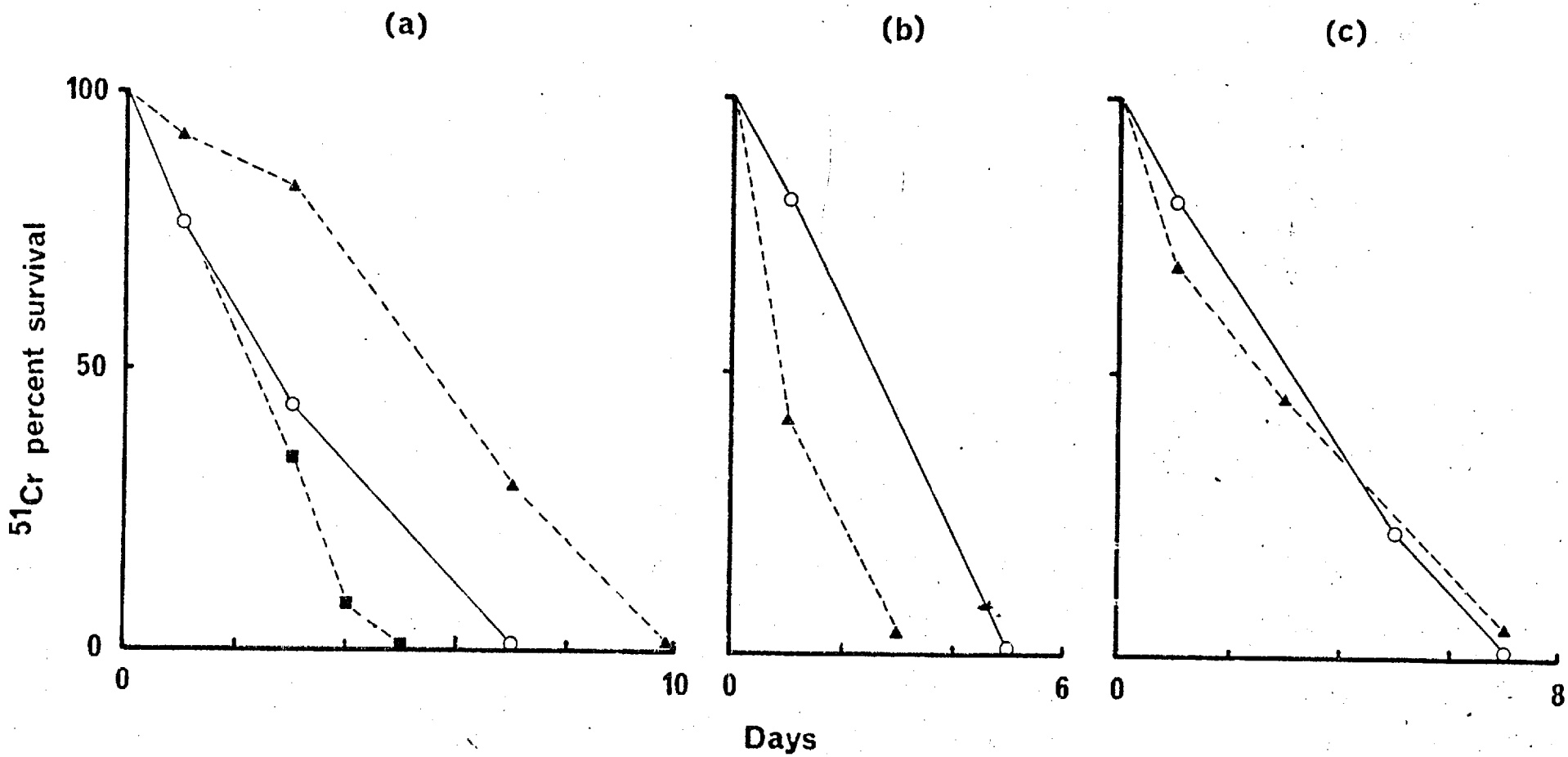
Effect of NEM-treated cells in "primed" rabbits

(i) Effect on antibody production. In 2 rabbits (S41, S57) which had previously had a trace of anti-A in their serum but had no detectable anti-A in a sample taken immediately before the present experiment, anti-A was readily detected (titre 4-8) in the serum 8 days after the injection of NEM-treated A-positive cells.

(ii) Effect on the survival of untreated red cells. Three A-negative rabbits (S85, S90, S97), in which survival of A-positive red cells was impaired and in which anti-A had also been demonstrated in the serum, were each given an injection of NEM-treated A-positive cells, followed 5 weeks later by an injection of untreated ^{51}Cr-labelled A-positive cells. The untreated cells were cleared more rapidly in S90 ($T_{50}Cr$ 2.5 days before NEM-treated cells and 0.8 days 5 weeks later) and at essentially the same rate in S97 ($T_{50}Cr$ 3.1 and 2.7 days respectively); in the remaining case, S85, although clearance of the untreated cells occurred at a slightly slower rate following the NEM-treated cells ($T_{50}Cr$ 5.5 days compared with the preceding observation of 2.5 days), a further injection of untreated A-positive cells given 6 months after the NEM treated cells again showed rapid clearance ($T_{50}Cr$ 2.3 days). The relevant survival curves in these rabbits are illustrated in Fig.35.

Survival of untreated red cells in rabbits previously injected with NEM-treated cells. Following a single injection of 0.5 ml A-positive red cells, treated with 10 μmoles NEM per ml cells into previously uninjected rabbits, three patterns of survival could be distinguished.

Figure 35. Survival of i.v. injections of A-positive red cells given before (O) and 5 weeks after (▲) the injection of NEM-treated A-positive red cells into three rabbits, S85 (a), S90 (b) and S97 (c) already immunized to the A antigen. In the case of S85, a further injection of cells was given (■). There was no evidence of a sustained improvement in survival of the untreated A-positive cells given after the NEM-treated cells.


(a)
(b)
(c)
100
50
0
^{51}Cr percent survival
0
10
0
6
0
8
Days

(i) Progressive shortening of red cell survival. Five rabbits exhibited accelerated clearance of the first injection of untreated allogeneic cells, with T_{sn} ranging from 2-30 days (mean 12.4; s.d. 11.3), and with one exception, showed progressive shortening of T_{sn} with each successive injection of red cells. The exception (S97) had T_{sn} values of 5, 17 and 2 days and clearance of the first two of these injections was approximately exponential.

All 5 rabbits produced antibody against the donor red cells and in 4 the specificity was shown to be anti-A. Although serum from the fifth rabbit agglutinated only red cells from the donor used for injection, another sample of A-positive red cells (not agglutinated in vitro) was very rapidly destroyed in vivo.

(ii) Accelerated clearance followed by normal or almost normal survival. In 4 rabbits, following the first i.v. injection of untreated red cells, the survival curves "collapsed" on days 17, 17, 21 and 34 respectively. In 3 out of 4 cases, a second injection of untreated cells was cleared more rapidly than the first, with T_{sn} values of 3, 4 and 7 days; in the remaining rabbit, the second injection survived normally. In all 4 animals, normal or almost normal survival of a third injection of cells was observed ($T_{50}Cr$ 17 days in 3 cases and 15 days in the remaining case). A fourth i.v. injection of red cells from a different A-positive donor was given, and on day 11, when survival was still within normal limits, A-positive donor red cells and FCA were injected i.m. Between 1 and 3 weeks following the i.m. injections, the survival curves "collapsed" in all 4 rabbits and specific

anti-A could be demonstrated serologically for the first time; a fifth i.v. injection (of cells from the second donor) was cleared very rapidly in all 4 cases. In addition to anti-A, the sera from these 4 rabbits gave a weak reaction with one example of A-negative cells (R20) used in the typing panel. Furthermore, an i.v. injection of these A-negative red cells was cleared from the circulation within 20 days in 3 out of the 4 rabbits (T_{50}Cr 2, 3 and 10 days); in the fourth animal, very slight impairment of survival (T_{50}Cr 14 days) was observed. As an example of this type of response, the survival curves observed in R16 are given in Fig.36.

(iii) Normal or almost normal survival. In 8 rabbits, the survival of A-positive cells following i.v. injections was repeatedly normal or only slightly subnormal. Thus, the range of T_{50}Cr observed following the first and second injections was 14-23 days and 15-21 days respectively, compared with the range of 19-24 days found for autologous cells (see p.191). Survival curves following the third injections are shown in Fig.37; T_{50}Cr was 17-20 days in 6 of the 8 rabbits and was slightly subnormal in the remaining 2 (10 and 13 days). One of these rabbits, R2, was given an i.m. injection of donor cells with FCA on day 11 of the survival study illustrated, and the curve collapsed about two weeks later as shown; two subsequent i.v. injections of untreated cells were cleared rapidly but antibody to the donor cells was not detected serologically.

In 5 rabbits, an i.m. injection of donor cells with FCA was given for the first time with the fourth i.v. injection of untreated red cells. The survival curves collapsed in 3 of these rabbits and in 1 of the 3 (R19; see Fig.38) anti-A plus another antibody reacting <u>in vitro</u> with

Figure 36. Survival of five successive injections (●, ▲, ■, 0, ▼) of untreated A-positive red cells (0.02 ml/kg) in an A-negative rabbit, R16, which had been injected previously with 0.5 ml NEM-treated A-positive cells. An additional injection of red cells mixed with FCA was given i.m. at the same time as the fourth injection (0). The first survival curve "collapsed" at about day 15; survival of the second injection was impaired initially but gradually improved, and survival of the third injection was normal; the fourth survival curve was normal to day 21 and then "collapsed"; the fifth injection was cleared very rapidly. Anti-A was demonstrable serologically after the fourth injection of cells had been cleared and antibody reacting weakly with A-negative cells was also detected at this time. A sixth injection, this time of A-negative cells (+), was given and showed markedly impaired survival.

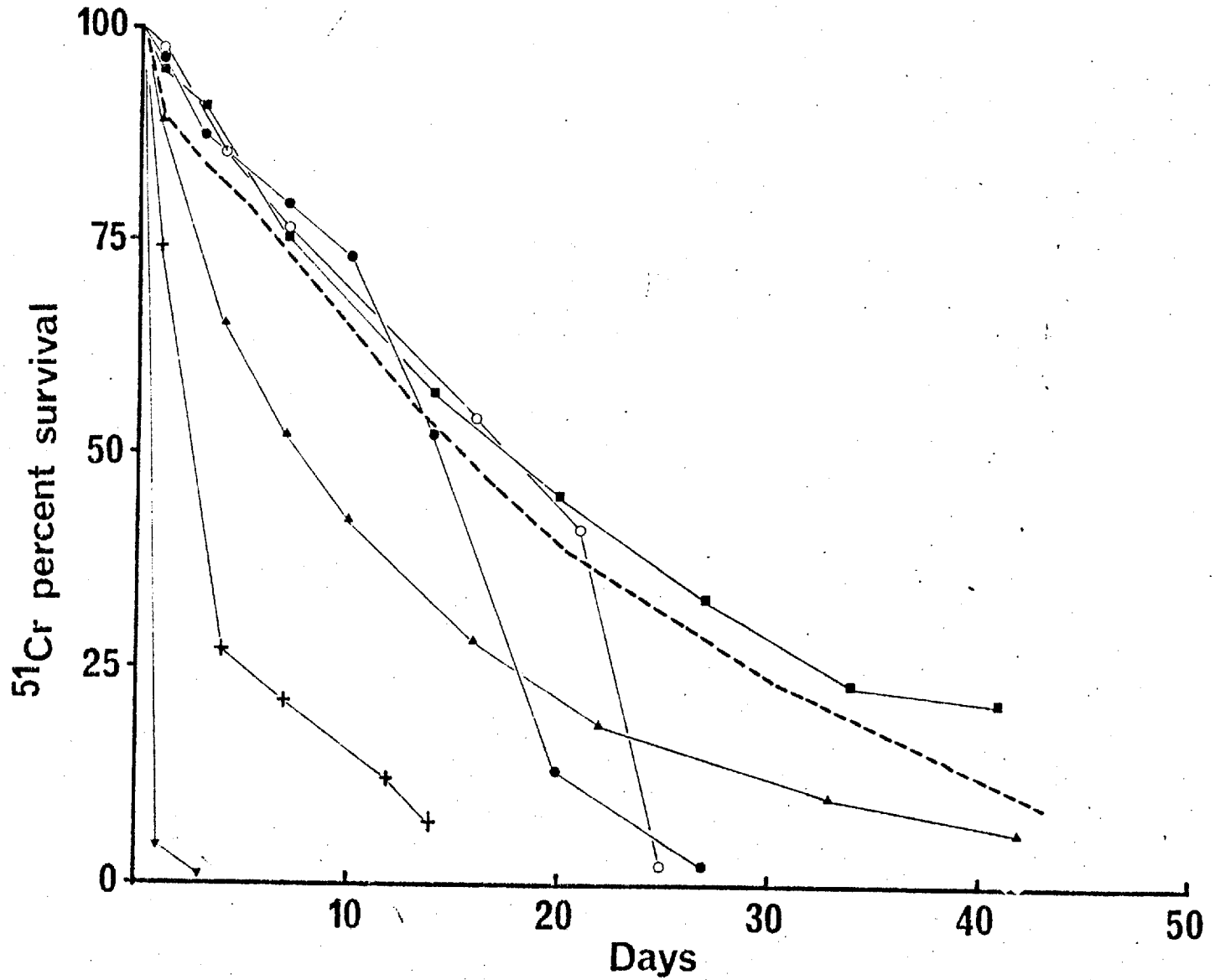

100
75
50
25
0
^{51}Cr percent survival
10
20
30
40
50
Days

Figure 37. Survival of a third injection of untreated A-positive red cells (0.02 ml/kg) in rabbits which had been injected previously with 0.5 ml NEM-treated, A-positive cells. In five rabbits (○, △, □, ◇ and ▲) survival was normal throughout. In one other rabbit (+) survival was normal at day 11 when an i.m. injection of donor cells mixed with FCA was given; the survival curve subsequently "collapsed" at about 25 days. Two rabbits (● and ◆) showed slightly subnormal survival of donor cells throughout.

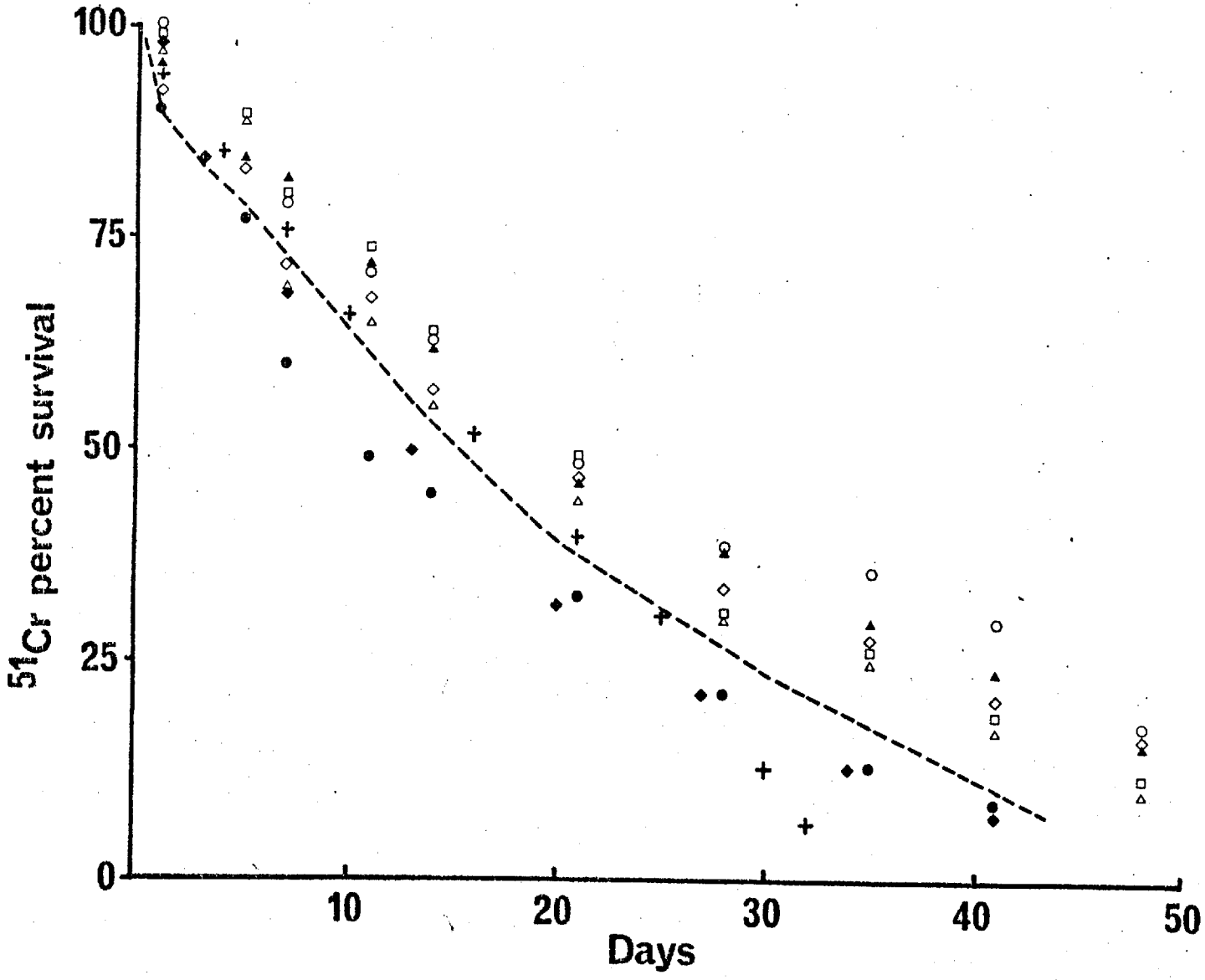
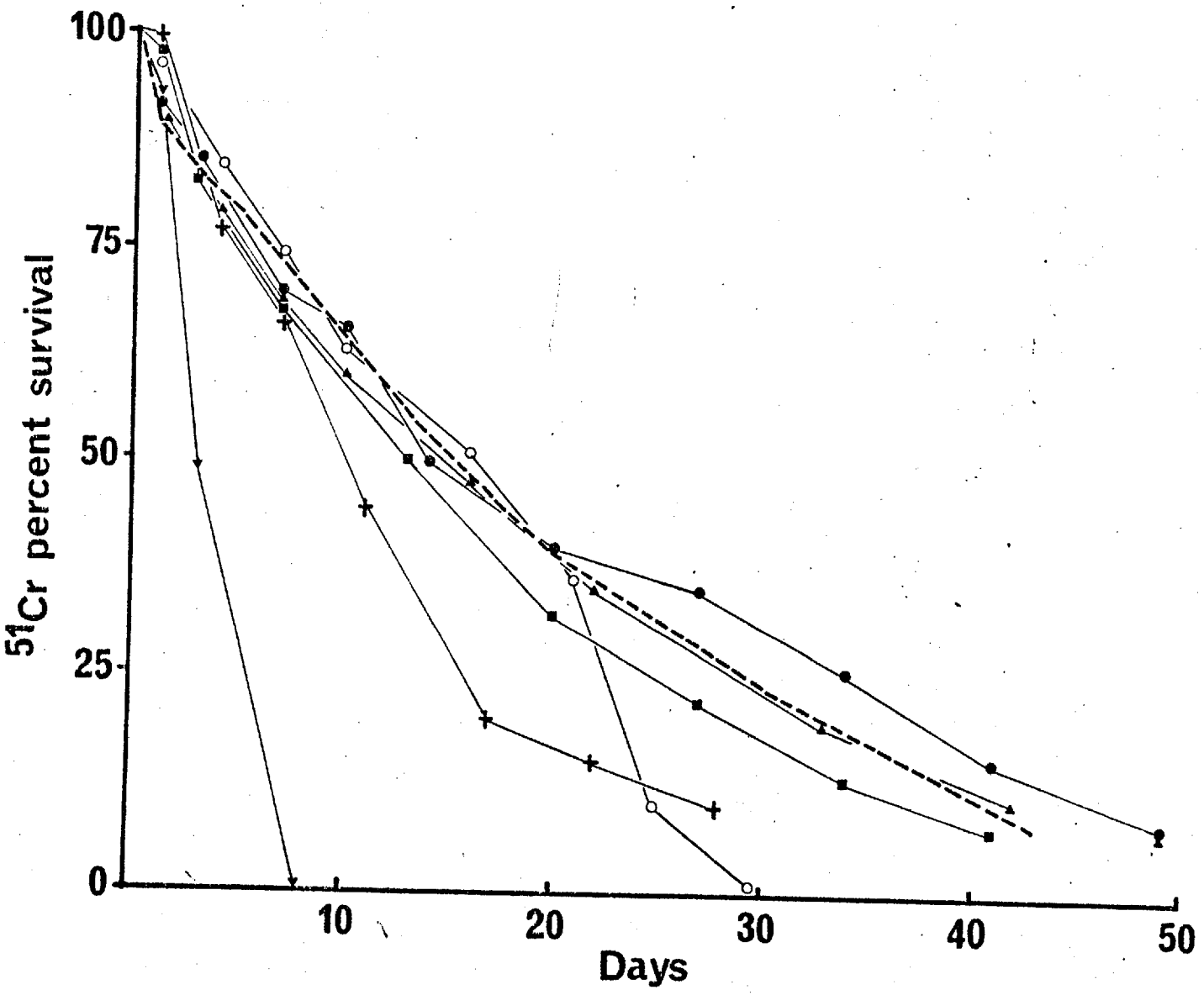

100
75
50
25
0
^{51}Cr percent survival
10
20
30
40
50
Days

Figure 38. Survival of 5 successive i.v. injections (●, ▲, ■, 0, ▼) of A-positive red cells (0.02 ml/kg) in an A-negative rabbit, R19, which had previously been injected with 0.5 ml NEM-treated A-positive cells. An additional injection of donor cells mixed with FCA was given i.m. 11 days after the fourth injection (0). The first three injections showed normal or almost normal survival; the fourth injection survived normally to day 21 when the curve "collapsed"; the survival of the fifth injection was markedly impaired. Anti-A was demonstrable serologically after the fourth injection of cells had been cleared, and an antibody reacting weakly with A-negative cells (from R20) was also detected. A sixth injection, this time of A-negative cells (+) was given and survival was definitely impaired (see text).


100
75
50
25
0
^{51}Cr percent survival
10
20
30
40
50
Days

1 example of A-negative cells (R20) was demonstrated; in 1 of the 2 rabbits without demonstrable antibody (S78; see Fig.39) two further i.v. injections of A-positive cells showed only slightly impaired survival until a second i.m. injection of donor cells with FCA was given (i.e. with the sixth i.v. injection of untreated cells), following which anti-A was demonstrated serologically. In the 2 remaining rabbits survival curves were normal or only slightly impaired following the fourth i.v. and first i.m. injection (with FCA). Likewise, survival of a fifth i.v. injection was normal at 11 days when a second i.m. injection of donor cells with FCA was given; 2-3 weeks later, the survival curves collapsed and anti-A was demonstrated serologically in one case (S81; see Fig.40).

Serum IgG concentrations in responders and non-responders.

The concentration of IgG in serum from 6 of the 8 A-negative rabbits that had shown repeatedly normal, or almost normal, survival of A-positive red cells (pattern (c) above) was determined and compared with values for 8 normal "responders" that had not received NEM-treated red cells (see Table 26, p.175). Determinations were made on a single sample of serum obtained after the pattern of response to allogeneic red cells had been established. There was no significant difference between the mean values for the two groups ($p > 0.4$, t-test), the values being 12.2 mg per ml (s.d. 1.3) and 11.8 mg per ml (s.d. 0.8) for responders and non-responders respectively.

Figure 39. Survival of 7 successive i.v. injections (●, ▲, ■, ○, ▼, ◆, +) of A-positive red cells (0.02 ml/kg) in an A-negative rabbit, S78, which had previously been injected with 0.5 ml NEM-treated A-positive cells. Additional injections of donor cells mixed with FCA were given i.m. 11 days after the fourth injection (○) and at the same time as the sixth injection (◆). The first three injections survived normally; survival of the fourth injection was slightly subnormal between days 4 and 10, and by day 15 was definitely subnormal; the fifth injection showed accelerated destruction from the outset but the rate of destruction gradually diminished; survival of the sixth injection was slightly subnormal up to day 10 and markedly subnormal thereafter; the seventh injection was destroyed rapidly. Anti-A was demonstrable serologically only after the sixth injection of cells had been cleared.

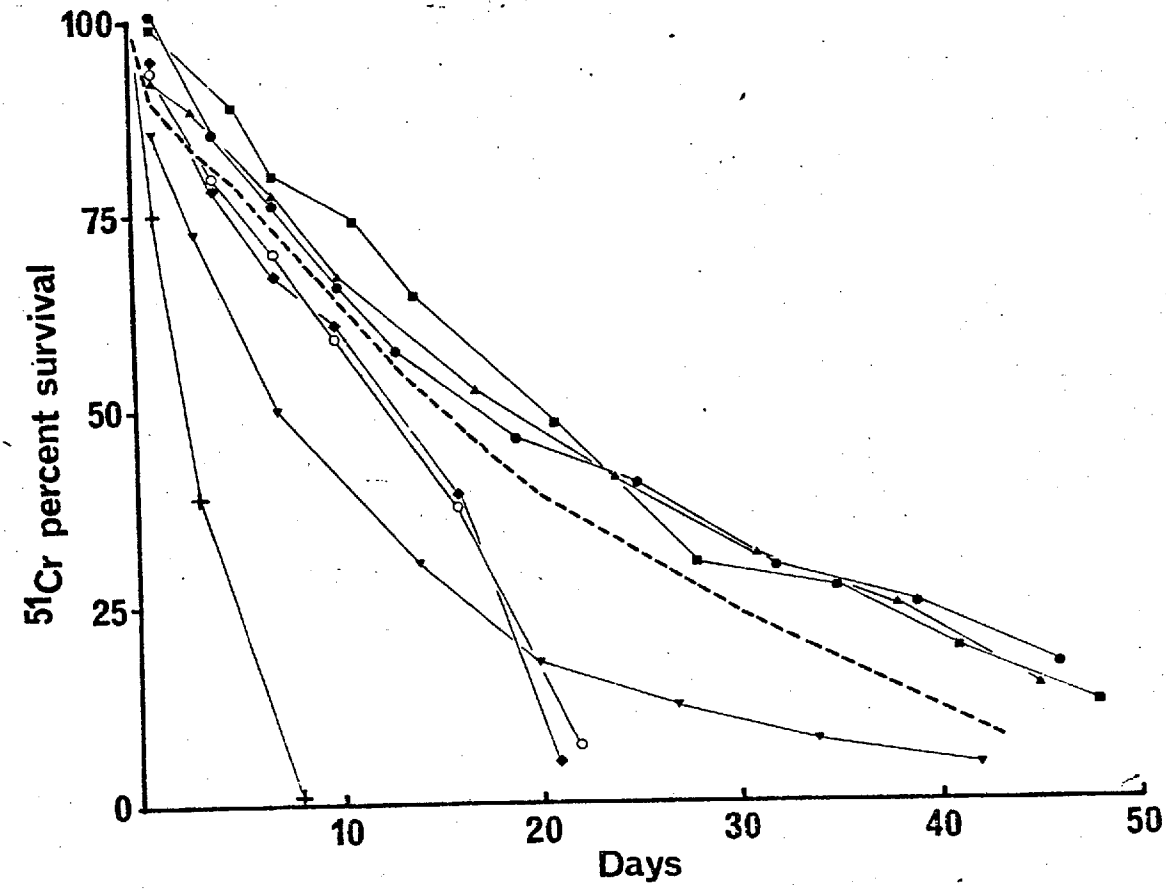
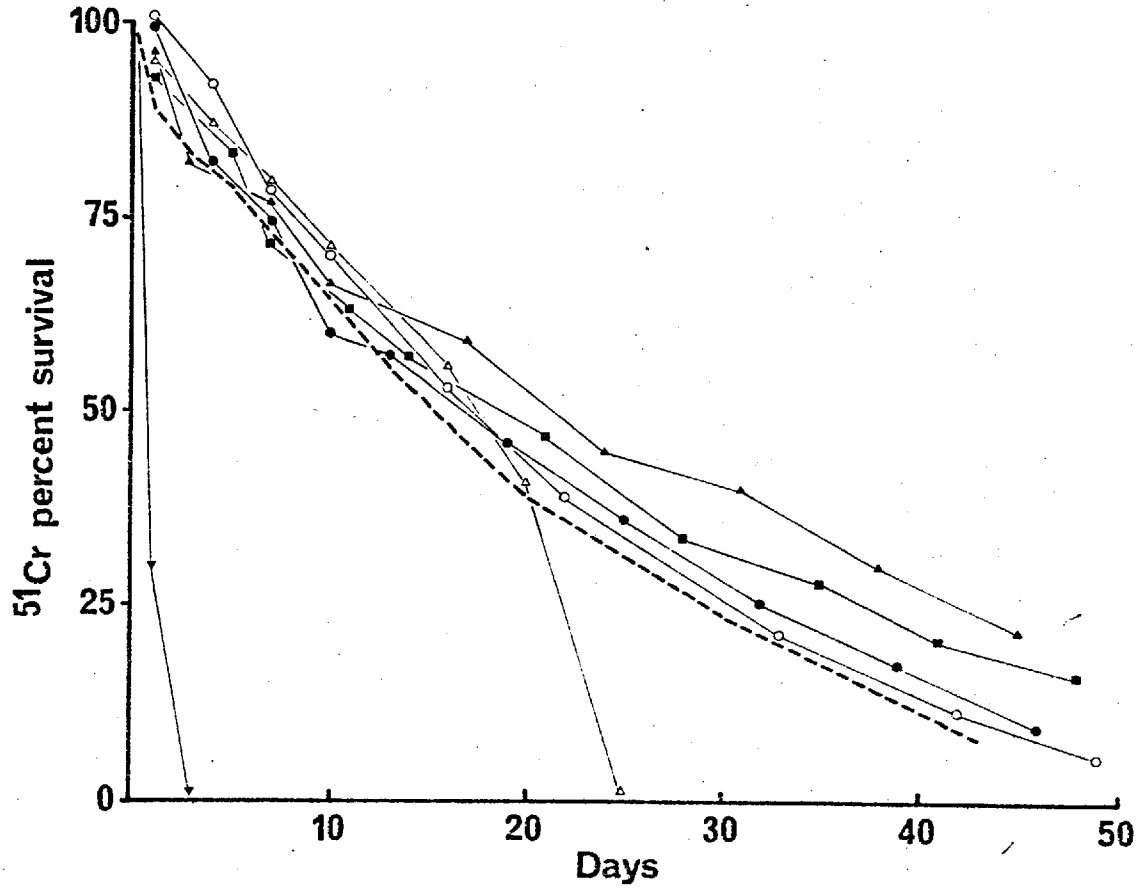

100
75
50
25
0
51Cr percent survival
10
20
30
40
50
Days

Figure 40. Survival of 6 successive i.v. injections (●, ▲, ■, 0, △, ▼) of A-positive red cells in an A-negative rabbit, S81, which had previously been injected with 0.5 ml NEM-treated A-positive cells. Additional injections of donor cells mixed with FCA were given i.m. at the same time as the fourth injection (0) and 11 days after the fifth injection (△). The first four injections survived normally; survival of the fifth injection was normal up to day 20 but the remaining cells were cleared rapidly thereafter; the sixth injection was cleared very rapidly. Anti-A was demonstrable serologically after the fifth i.v. injection had been cleared.


100
75
50
25
0
^{51}Cr percent survival
10
20
30
40
50
Days

Discussion

As described in the previous section, when a series of i.v. injections of untreated $A(Hg^A)$-positive red cells was given to A-negative rabbits, the commonest finding (in 44 out of 51 animals) was progressive shortening of red cell survival followed, usually after 2 or 3 injections, by the appearance of serologically-detectable antibody. By contrast, in the present series, in which the first injection given was of NEM-treated A-positive red cells, followed by injections of untreated A-positive cells, only 5 out of 17 rabbits showed progressive shortening of red cell survival. This difference is highly significant ($p < 0.0005$, t-test). It must be concluded that the injection of NEM-treated A-positive red cells induced in the majority of rabbits a considerable degree of tolerance towards the A antigen lasting for many months at least.

In 10 of the 12 rabbits in which survival of untreated red cells (after an initial injection of NEM-treated red cells) was repeatedly normal or almost normal, further i.v. injections of A-positive cells were given accompanied by i.m. injections of donor cells with FCA. All of these rabbits eventually responded by showing impaired survival of the i.v. injections and in addition some produced serologically detectable antibody. Nevertheless, in several cases there was considerable resistance to immunization even by this method; some rabbits produced only transient impairment of red cell survival following a first injection of cells with FCA (see for example S78, Fig.39) and others responded only poorly to a second such injection (see for

example S81, Fig.40). These patterns of response to cells given i.m. with FCA in this group of rabbits are very similar to those seen in the group of A-negative rabbits that failed to respond to repeated i.v. injection of untreated A-positive red cells when no initial injection of NEM-treated cells had been given (compare S94, Fig.25 and S82, Fig.24 with the two examples indicated above). Moreover, in both groups of non-responders a proportion of cases showed initial transient impairment of survival of untreated cells given alone before normal or almost normal survival was observed. These similarities suggest that in the group given untreated cells alone, non-responders may have been rendered tolerant by the first injection (of untreated cells), rather than being examples of a primary inability to respond. It is interesting to speculate whether some of the cases described in man in which allogeneic red cells survive normally or have only transiently impaired survival (Adner and Sjolin, 1957; Adner et al., 1963; Krevans et al., 1964; Mollison et al., 1970; Holburn et al., 1971) might not also be examples of induced unresponsiveness.

Agents such as NEM and p-chloromercuribenzoate (PCMB), which are capable of binding sulphydryl groups, have been shown to affect the properties of certain red cell antigens. Using human red cells, Baird and Bove (1966) and Bove and Baird (1967) found that PCMB impaired the ability of intact D-positive and Kell-positive cells to absorb specific antibody, whereas the antigens A, B, H, I, Fy^a, M, $\bar{c}$ and $\bar{e}$ were unaffected. Likewise, Green (1967) observed a gradual loss of Rh activity over 24 hours in lyophilised red cell membranes treated with NEM. Presumably, combination of the sulphydryl binders with groups forming

parts of specific antigenic determinants accounts for these observations. No evidence was obtained in the present experiments that incubation of rabbit red cells with NEM in a dose of 10 μmoles per ml cells at 37°C for 1 hour altered the A antigen: A-positive red cells treated in this way absorbed anti-A just as well as untreated red cells and they also elicited normal secondary antibody response, in "primed" A-negative rabbits.

It seems unlikely that NEM-treated red cells caused generalised non-specific paralysis of immunocompetent cells since, in the present experiments, rabbits in which impaired responses to A had been induced remained in good health for periods of more than a year. Serum IgG levels also remained normal after induction of the unresponsive state. Furthermore, in rabbits that had already shown impaired survival of A-positive cells, injection of NEM-treated cells did not result in any substantial improvement in survival of subsequent injections of untreated cells.

The induction of tolerance by NEM-treated A-positive cells given before untreated cells is unlikely to be related merely to the rapid destruction of the first injection of antigen-carrying cells. Elson et al. (1971) gave injections of 2×10^9 heated A-positive red cells (approximately 0.125 ml cells) to A-negative rabbits, following which the cells were removed from the circulation with a mean T_{50} of 15 min. Such injections were no less effective in inducing immune responses to the A antigen than were injections of untreated red cells. On the other hand, there have been several reports of experiments suggesting that the

site of antigen sequestration may affect humoral antibody response and even play a role in the induction of tolerance. Souhami (1972) showed that when 5 mg colloidal carbon was injected i.v. into mice, the subsequent phagocytosis in the liver of sheep red cells, also given by the i.v. route, was depressed, whereas a relatively large proportion of the cells was sequestered in the spleen. This change in the distribution of phagocytosed red cells was associated with an increase in antibody production and in the number of spleen plaque-forming cells when low doses of sheep cells were given, and with a slight depression of antibody responses when higher doses were used. By contrast, depression of antibody responses by "blockade" of the mononuclear phagocytic system(MPS) (and presumed alteration of the distribution of injected antigen) has been reported in rabbits. Cruchaud (1968) induced "blockade" of the MPS in rabbits by the i.v. injection of either colloidal carbon or saccharated iron oxide, and found that if bovine serum albumin (BSA) was then injected in a dose of 10 mg/kg body weight, primary humoral antibody production was depressed in most animals. Further i.v. injections of 5 mg BSA/kg 40 days later were associated with decreased secondary responses in many animals even though the "blockading" treatment had been withdrawn some 3 weeks previously. Furthermore, in 2 out of 11 rabbits that had been treated with colloidal carbon and 4 out of 8 that had received saccharated iron oxide, specific tolerance to BSA was induced; these rabbits retained the ability to respond normally to human gamma globulin. In the present experiments it has not been possible to demonstrate the specificity of the tolerant state because donor rabbits of appropriate blood groups were not available.

In the present experiments it is possibly relevant that the NEM-treated red cells were rapidly removed from the circulation predominantly in the bone marrow. This distribution was unexpected in view of the findings reported for humans and rats. Thus in man Jacob and Jandl (1962) treated ^{51}Cr-labelled autologous red cells from 25 ml whole blood with 5 or 20 µmole NEM per ml cells; by surface counting over spleen, liver and praecordium, it was found that the cells were trapped preferentially in the spleen. The special role of the spleen in trapping the treated cells was further emphasised by the demonstration that survival time of the injected cells was prolonged by a factor of more than 30 in 2 splenectomised subjects. These findings have been confirmed by Kimber, Lander and Robson (1965). Similarly in rats, the larger part of a dose of 0.23 or 0.5 ml ^{51}Cr-labelled red cells treated respectively with 25 or 20 µmole NEM per ml red cells was recovered from the spleen and negligible amounts of radioactivity were found in the bone marrow (Jacob and Jandl, 1962; Ultmann and Gordon, 1965). The tendency in rabbits for NEM-treated red cells to be sequestered in bone marrow was observed over the range of cell doses studied in the present experiments, namely 0.05-0.5 ml packed cells per rabbit. Removal of A-positive red cells at comparable rates from the circulation of A-negative rabbits by passively injected IgG anti-A has been found to result in sequestration of cells predominantly in the spleen and liver; in some rabbits treated similarly with cells and antibody, there has been some delay in the response to subsequent injections of cells given alone (see p.252), but no increase in the incidence of long-lasting unresponsiveness comparable to that described in the present experiments has been observed.

Table 42. Percentage haemolysis of rabbit red cells which had been treated with different amounts of NEM before being exposed to different concentrations of sodium chloride solution.

NEM µmol/ml red cells	NaCl concentration (g/dl)						Mean Corpuscular Fragility
	1	0.6	0.5	0.4	0.3	0	
0	0	2.7	46	97	100	100	0.495
5	0	2.5	26	87	98.5	100	0.460
8	0	1.6	18	69	93	100	0.430
12	0	3.4	18	68	94	100	0.430
20	0	4.3	80	93.5	96.5	100	0.585

Table 43. Recovery of radioactivity from various organs following clearance of NEM-treated ^{51}Cr-labelled red cells from the circulation.

Rabbit number		S32	S43	S50	S52	M67	S41	S43	S44	R21	R18	R23
Volume of cells injected (ml)		0.19	0.26	0.34	0.29	0.5	0.5	0.5	0.5	0.5	0.05	0.05
NEM treatment (μmole/ml cells)		5	8	11	14	5	10	10	10	10	10	10
Clearance rate (h.d.t. - hours)		2.5	2.25	1.25	0.7	10	1.7	1.2	1.0	1.25	0.8	0.7
Time of death (hours after injection)		24	24	24	24	24	5	24	24	6	5	5
Per cent circulating at death		<1	<1	<1	<1	4	3	1	2	2	1	1
Percentage recovery	Spleen	24	12	9	9	17	23	18	3	23	44	12
	Liver	4	3	3	8	7	34	7	8	3	6	1
	Kidneys	-	-	-	-	-	1	0	0	-	-	-
	Gut	-	-	-	-	-	-	0	0	-	-	-
	Bone	-	-	-	-	53	52	61	67	46	25	61
	Total	28	15	12	17	81	113	87	80	74	76	75
Spleen:liver ratio (ct/min per g tissue)		263	219	99	68	70	51	264	73	285	180	348

SOME ADDITIONAL OBSERVATIONS ON THE EFFECTS OF ALLOANTIBODIES ON RED CELL SURVIVAL

EFFECTS OF NATURALLY-OCCURRING ANTIBODIES

Observations in vitro

Antibodies

Antigens

Observations in vivo

Plan of experiment

Results

(i) Red cell survival

(ii) Antibody titre

Conclusion

Several examples of naturally-occurring red cell antibodies have been found in newly-acquired rabbits that had not been involved in any experiments. Some of the *in vitro* and *in vivo* characteristics of these antibodies are briefly summarized in this section.

Observations *in vitro*

Antibodies

Sera from 12 FF rabbits were tested against a panel of red cells from 25 rabbits (FF and AF). Eight were found to give positive results with 4 of the panel cells when tested using a saline agglutination method at room temperature; none of the samples showed autoagglutination under these conditions. The strongest of these reactions was observed with serum from rabbit S58 and was investigated further.

S58 serum gave a titre of 1 in 16 in a saline medium at room temperature; the titre was inversely proportional to the temperature and at 39°C (rabbit body temperature) there was no reaction. The titre was also reduced by using an isotonic $MgSO_4$ solution medium instead of saline, and there was no enhancement of the reaction when tested by the indirect antiglobulin method (anti-rabbit IgG). Antibody activity was abolished by treatment with 2-mercapto-ethanol.

Antigens

In the initial tests, 4 out of 25 red cell samples (3 FF, 1 AF) were agglutinated by the antibodies. Tests with samples of red cells taken from these 4 rabbits at different times showed that the strength of the reaction with a given antiserum decreased over a period of about 4 months, by which time agglutination was barely detectable; on the contrary, there was no decrease in titre using the same antiserum with the early red cell samples which had been stored in liquid nitrogen.

Observations in vivo

Plan of experiment

As described above, serum from S58 gave the strongest in vitro reaction with test red cells from 4 rabbits. A sample of cells from one of these donors (S50) was labelled with ^{51}Cr and was injected i.v. into S58 in a dose of 0.02 ml red cells per kg body weight. Survival of the injected cells was studied. Samples of serum were taken from S58 before and on 2 occasions during the red cell survival study. These sera were stored at -20°C and titrated in parallel against S50 cells at the end of the experiment.

Results

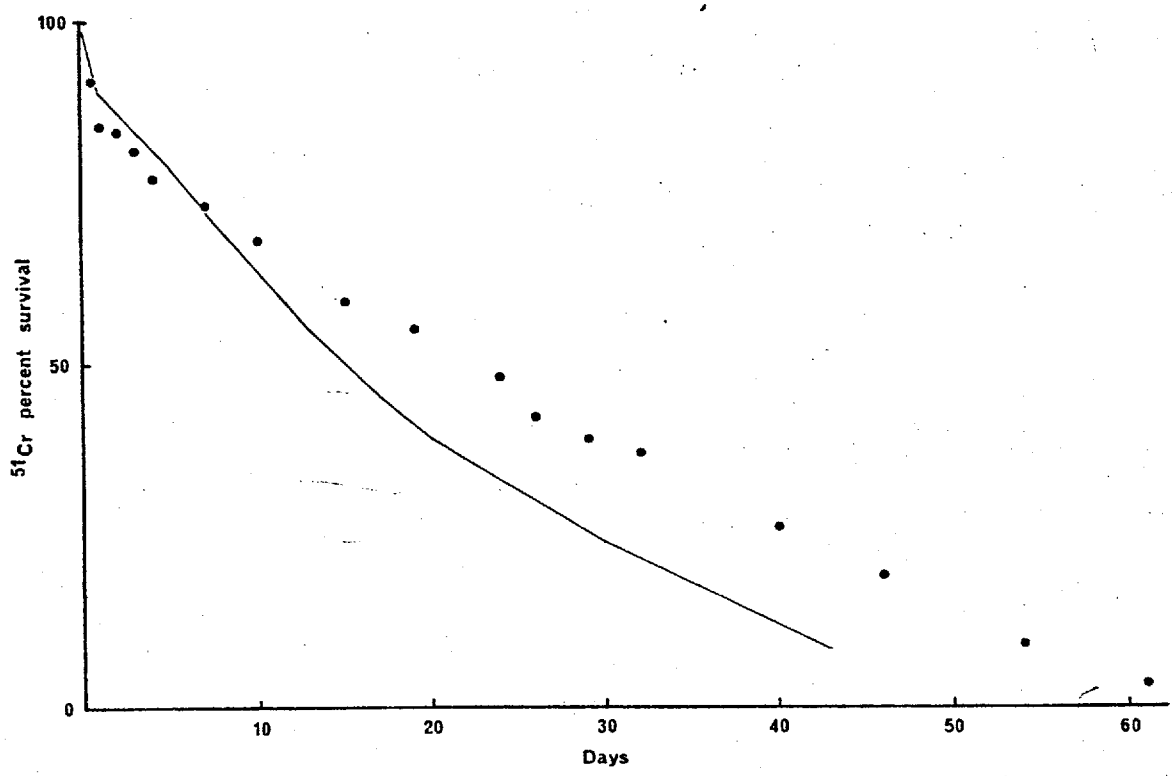
(i) Red cell survival. Following the injection of labelled cells there was a rapid loss of about 10% of the initial ^{51}Cr activity over the first 6 hours but thereafter, survival appeared normal. Consequently, the survival curve lay just below the normal range for autologous red cells during the first 6 days of the study but was within normal limits thereafter (see fig. 41).

(ii) Antibody titre. There was no significant difference between antibody titres in sera obtained 3 days before and 31 and 35 days after the injection of S50 cells (see table 44).

Conclusion

The findings reported in this section suggest that in a substantial proportion of rabbits, naturally-occurring IgM antibodies of low thermal amplitude may be found. The specificity of these antibodies was not investigated extensively but the corresponding red cell antigen was shown to weaken gradually over several months and eventually to become undetectable serologically in all 4 out of 25 rabbits in which it was found. There are several precedents for this loss of red cell antigen, e.g. the Ii system in man. There were no obvious features, such as immaturity, which were typical of the rabbits with red cells positive for the antigen.

Figure 41. The survival of ^{51}Cr-labelled red cells from S50 following injection into S58. The continuous line indicates the normal lower limit for autologous red cell survival.


100
50
0
^{51}Cr percent survival
10
20
30
40
50
60
Days

There are several possible explanations for the initial rapid fall of ^{51}Cr activity, leading to transient subnormal values for red cell survival in this experiment.

(i) It may represent an extreme example of the normal pattern of ^{51}Cr survival curves, most of which show a variable initial rapid loss of ^{51}Cr activity.

(ii) The fall may represent antibody-mediated red cell destruction with exhaustion of antibody after the first few hours following the injection of red cells. This explanation is most unlikely; the volume of red cells injected was very small, and furthermore, the antibody titre remained unchanged during the course of the experiment at a time when red cell survival was normal.

(iii) Antibody may have been bound to red cells immediately following their injection at room temperature, only to be eluted subsequently from the majority of the injected cells at 39°C. In fact, the very short time during which the cells would be circulating at a temperature of less than 39°C makes this explanation most unlikely.

(iv) It is possible that a small proportion of the population of injected red cells was subject to antibody-mediated destruction under these circumstances. Red cell heterogeneity in relation to IgM mediated destruction has been described by Burton and Mollison, 1968 (see p.35).

Table 44. Antibody titre in serum taken from S58 at different times in relation to the injection of S50 red cells. Titrations were performed by the methods indicated using S50 test cells.

Time (days) in relation to cell injection	Agglutination in saline medium	Agglutination in $MgSO_4$ medium	Indirect antiglobulin (-IgG) titre
-3	16	2	2
+31	32	4	8
+35	16	4	4

SOME ADDITIONAL OBSERVATIONS ON THE EFFECTS OF ALLOANTIBODIES ON RED CELL SURVIVAL

EXPERIMENTS WITH IgG ANTIBODIES LYTIC IN VITRO

Procedure

- Immunization schedules
- In vitro characteristics of antisera
- Donor red cells
- Injection protocol
- Calculations

Results

- Red cell survival
- Plasma radioactivity

Discussion

During the course of this work, approximately 50 rabbits responded to injections of allogeneic red cells by producing serologically-detectable antibody to the donor cells. Of these antibodies, only 3 were found which caused lysis of appropriate red cells *in vitro*. Two of the rabbits producing lytic antibodies were given i.v. injections of the corresponding allogeneic red cells in order to investigate the characteristics of red cell destruction *in vivo*.

Procedure

Immunization schedules. Neither the donor cells used for immunization nor the way in which they were given appeared to be in any way peculiar to the three rabbits producing lytic antibody. Thus, S37 was given three standard i.v. injections of red cells (0.04, 0.02 and 0.02 ml/kg) in connection with red cell survival studies, followed by 3 i.m. injections of 0.25 ml cells with FCA at about 2-monthly intervals, with small i.v. injections in between; all injections were of cells from the same donor, H4. S51 received 3 similar i.v. injections initially, then 1 i.m. injection with FCA and another 3 i.v. injections; again, the donor was H4 on each occasion and the injections were spread over 4 months. S54 was given 3 i.v. injections of red cells from H4; about a year later an injection of 0.25 ml red cells from S62 mixed with FCA was given i.m., followed within a few weeks by a small i.v. injection of cells from the same donor. These injection protocols were followed in immunizing several other rabbits and did not result in the production of lytic antibody.

In vitro characteristics of antisera. Details of the properties of the 3 lytic antisera have been reported by Frame and Mollison (1972). Briefly, all three antibodies were shown to be haemolytic in the presence of rabbit complement and, using an anti-rabbit-C3 anti-serum, the presence of C3 on sensitized HgA-positive red cells was demonstrated. In addition, the rate of haemolysis of HgA-positive red cells induced by S37 serum in vitro was determined using the ^{51}Cr release method (Polley, Adinolfi and Mollison, 1963); by comparison with the rate of haemolysis of human A_1 red cells by a potent haemolytic anti-A, the rabbit antibody system was much less efficient. These findings are summarized in Tables 45 and 46.

In the present in vivo experiments rabbits S37 and S51 were used. At the time of injection, antibody titres against HgA-positive red cells suspended in magnesium sulphate solution and by IAT were respectively 64 and 128 in S37, and 64 and 1024 in S51.

Donor red cells. A-positive red cells which had been shown by in vitro tests to undergo lysis in the presence of serum from the two recipient animals were used. The cells were labelled with ^{51}Cr by the citrate-wash method (see p.80) and were re-suspended in isotonic saline for injection.

Injection protocol. (a) S37 was given 3 i.v. injections of A-positive red cells during the present experiments. The first injection was of 0.03 ml cells from donor H4. Two days later 0.03 ml cells from another donor, S59, was given followed half an hour later by 10.4 ml cells from S59.

(b) S51 was given a single injection of 0.03 ml A-positive red cells from donor S59. In addition, red cell volume was measured in this rabbit using autologous red cells labelled with ^{32}P (see p.80).

In both cases the volumes of the red cell suspensions injected were noted.

Blood samples. For the red cell survival studies 1 ml samples of venous blood were taken at the times indicated in the graphs (see figs. 42 and 43); EDTA was used as anticoagulant. For each sample, 0.5 ml was pipetted into 5 ml phosphate-buffered isotonic saline. After mixing, the suspension was centrifuged and the supernatant was saved for radioactive counting. The red cells were washed twice with phosphate-buffered saline and then lysed with 0.04% ammoniated water, the final volume being adjusted to 5 ml. 2 ml aliquots of the diluted plasmas and red cell lysates prepared in this way were pipetted for radioactive counting. Known dilutions of the injection suspensions were prepared and 2 ml portions were counted.

Calculations. Because the injected red cells were cleared rapidly from the circulation, the initial radioactivity (100% survival) had to be calculated from the measured amount of activity injected and the total blood volume of the recipient. Similarly, the amount of free haemoglobin in the plasma was calculated as a percentage of

Figure 42. Survival of ^{51}Cr-labelled A-positive red cells (0.03 ml) in two A-negative rabbits, S37, (a), and S51, (b), whose sera contained anti-A lytic *in vitro*. Total plasma ^{51}Cr activity, expressed as a percentage of the total radioactivity injected, is shown by the interrupted line.

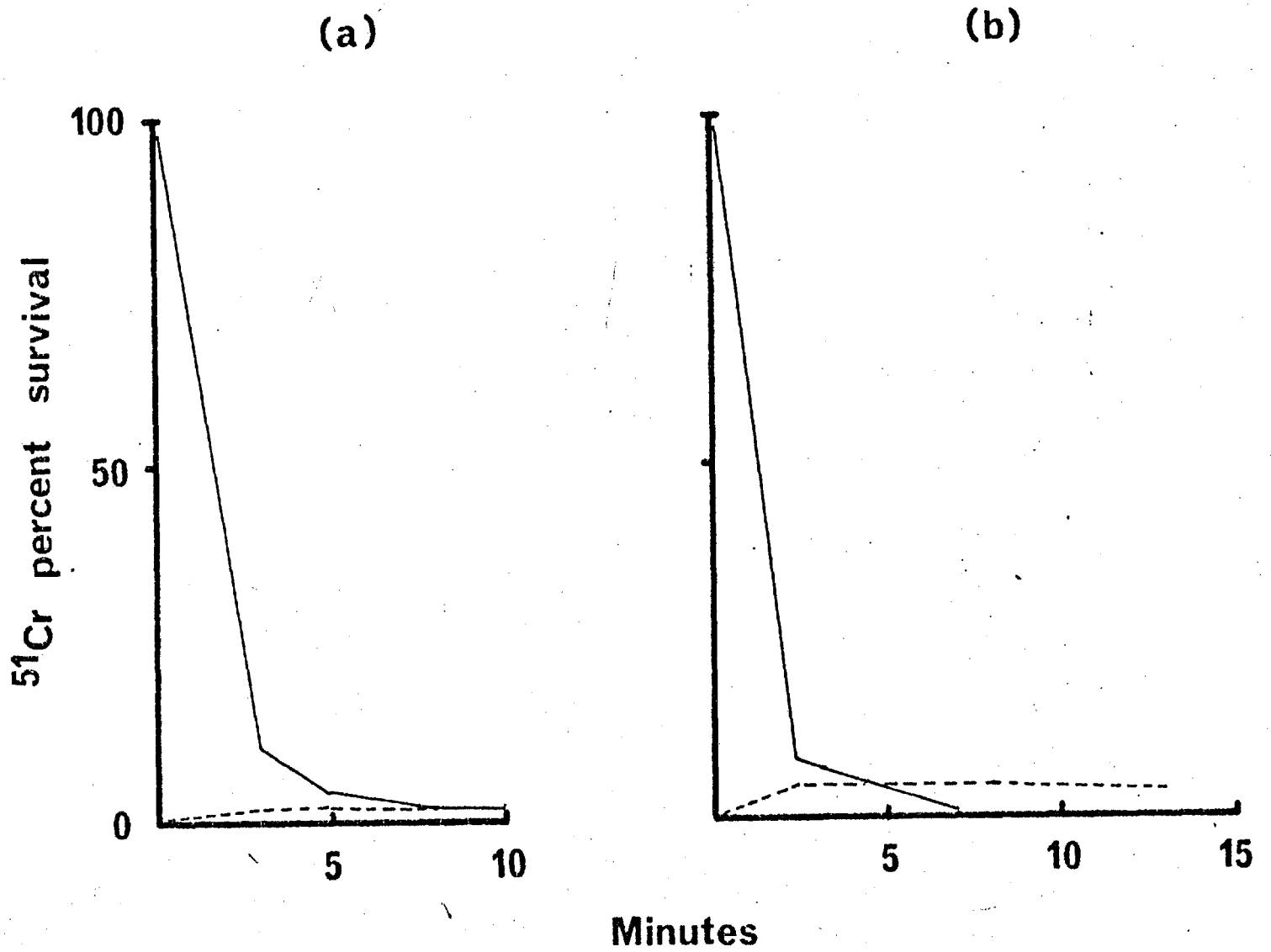
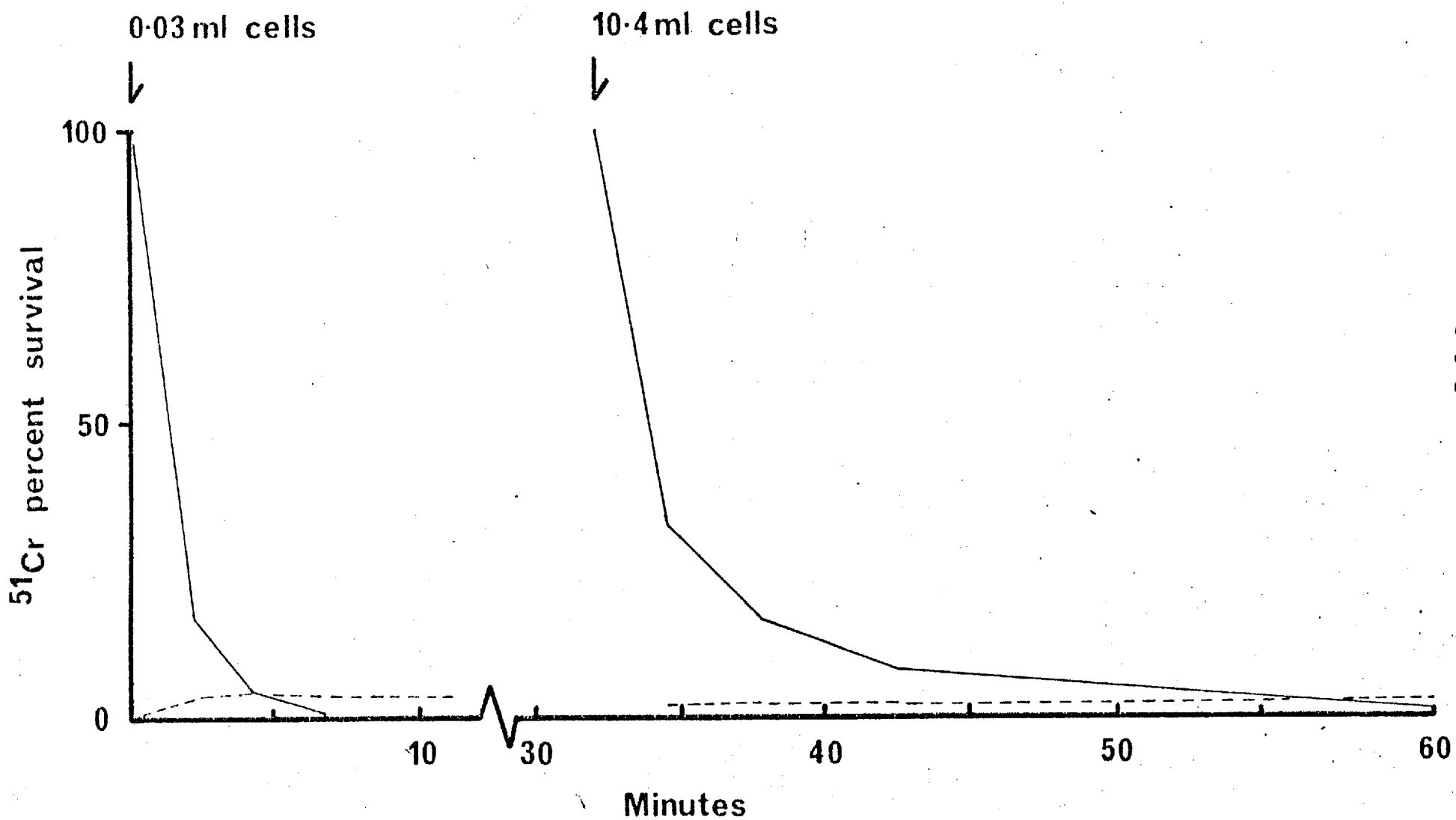

(a)
(b)
100
50
0
^{51}Cr percent survival
5
10
5
10
15
Minutes

Figure 43. Survival of a small (0.03 ml) and a large (10.4 ml) dose of A-positive red cells in S37, whose serum contained anti-A lytic *in vitro*. Total plasma ^{51}Cr activity, expressed as a percentage of the total amount of radioactivity injected on each occasion, is shown by the interrupted line.


0·03 ml cells
10·4 ml cells
100
50
0
^{51}Cr percent survival
10
30
40
50
60
Minutes

the activity that would have been observed if all the injected radioactivity were to have appeared instantaneously in the plasma. In the case of S37, a total blood volume of 150 ml and plasma volume of 100 ml were assumed; for S51 the respective volumes were deduced from the measured red cell volume and were found to be 151.9 ml and 103.7 ml. In both cases it was further assumed that the total blood volume, plasma volumes and hence the venous haematocrit did not change during the period of observation.

Results:

Red cell survival. 90% of a small dose (0.03 ml) of A-positive red cells was cleared from the circulation of each recipient in about 3 minutes. Consequently, the number of observations made during the removal of the majority of the injected cells was small and the pattern of removal could not be accurately determined. However, the data up to 5 minutes after injection could be fitted approximately by a single exponential, the half disappearance time varying between about 0.7 and 0.9 minutes (see Fig 42).

When a dose of 10.4 ml A-positive red cells was injected into S37, the rate of clearance gradually decreased over the period of observation so that by 30 minutes less than 2% of the injected red cells remained in the circulation. From the plot illustrated in Fig. 43 it is inferred that immediately following injection, the rate of red cell clearance was similar to that of the small dose of red cells ($t_{\frac{1}{2}}$ about 0.8 min); by about $8\frac{1}{2}$ minutes after injection, when 90% of the injected cells had been cleared, the clearance rate was markedly reduced ($t_{\frac{1}{2}}$ about $4\frac{1}{4}$ min).

Plasma radioactivity. The appearance of radioactivity in the plasma following each injection of red cells is shown in Figs. 42 and 43, together with the corresponding red cell survival plot (see, also, Table 47). It can be seen that, at a time when 90% or more of the injected red cells had been cleared from the circulation, in no instance did plasma activity amount to more than 4.5%. The highest value for plasma activity (5.8%) was found in S37, 20 hours following the injection of 10.4 ml A-positive red cells.

Discussion

These experiments indicate that in the two recipient rabbits, S37 and S51, haemolysis of the injected red cells was predominantly extravascular, even when clearance of a proportion of the cells from the circulation was relatively slow following the larger injection of cells to S37. Haemoglobinaemia and haemoglobinuria were seen after the large dose of red cells, and a comparable low level of ^{51}Cr activity was found in the plasma following large and small injections. These findings probably represent haemoglobin release after the labelled red cells had been removed from the circulation, an explanation suggested by Jandl et al. (1957) for the release of similar proportions of radioactivity into the plasma following the extravascular destruction of ^{51}Cr-labelled red cells by a non-lytic antibody in man. No attempt has been made to quantitate the amount of haemoglobin liberated into the plasma in terms of the proportion of injected cells this represents, but it is clear that intravascular lysis of the injected cells played little part in their initial rapid disappearance from the circulation.

The sites in which the red cells were sequestered were not determined in these experiments. By analogy with other C-binding IgG antibodies the sequestration of IgG-coated cells seems to be largely dependent upon complement fixation and, to a small extent, directly upon IgG fixation to the cell membrane (Schreiber and Frank, 1972a). Using rabbit anti-guinea-pig erythrocyte IgG to coat guinea-pig red cells these authors showed that in normal guinea-pigs as few as 1.4 C-fixing sites would bring about accelerated clearance of cells, predominantly in the spleen, whereas when the number of sites was increased to 117, the liver also played a significant part in the removal of cells. On the other hand, cells with 90 IgG C-fixing sites injected into C-4 deficient animals showed only slightly impaired survival. It has been demonstrated in vitro that macrophages have receptors for IgG (Lo Buglio, Cotran and Jandl, 1967; Huber and Fudenberg, 1968) but the work of Schreiber and Frank clearly indicates that clearance of red cells is quantitatively much less dependent on the IgG receptor than on the C3 receptor.

Table 45. Results of *in vitro* tests with antisera from 3 Hg^A-negative rabbits which had produced lytic anti-Hg^A antibodies in response to immunization with Hg^A-positive red cells. From Frame & Mollison, 1972.

	Haemolysis		Indirect antiglobulin titre	
Rabbit number	Macroscopic	Cr release(%)	Anti-C3	Anti-C4
S37	+	32	16	1024
S51	+	43	16	512
S54	+	69	16	128

Table 46. Comparison of the rates of antibody-mediated haemolysis *in vitro* in two blood group systems. In the rabbit system, Hg^A-positive red cells and anti-Hg^A from S 37 were used; in the human system, group A red cells and a lytic anti-A were used. From Frame & Mollison, 1972.

	Percentage haemolysis	
Time (minutes)	Anti-Hg^A	Anti-A
3	3	81
10	11	92
60	44	93
180	60	93

Table 47. Survival of Hg^A-positive red cells in the circulation of Hg^A-negative rabbits (S37, S51), whose serum contained antibody which caused lysis of the donor cells in vitro. Calculation of the percentage survival and plasma radio-activity are described in the text.

(a) S37

Sample number	Time	Red cell survival (%)	Plasma activity (%)
	Zero - 0.03 ml red cells (H4) injected i.v.		
1	3 m	10.8	1.9
2	5	3.9	1.9
3	8	1.9	1.8
4	10	1.9	1.5
5	17	1.2	1.3
6	30	<1	1.2
7	45	<1	1.1
8	1 h	<1	<1
9	3	<1	1.2
10	24	<1	<1

Table 47 (cont'd).

(b) S37

Sample number	Time	Red cell survival (%)	Plasma activity (%)
	Zero - 0.03 ml red cells (S59) injected i.v.		
1	2 m	16.8	3.2
2	4	3.9	3.3
3	6	<1	3.1
4	8	<1	3.1
5	11	<1	2.9
	32 - 10.4 ml red cells (S59) injected i.v.		
6	35	32.5	1.8
7	38	17.4	1.8
8	42	7.7	1.9
9	1 h	1.6	2.4
10	$1\frac{1}{4}$	<1	2.7
11	$1\frac{1}{2}$	<1	2.8
12	2	<1	3.5
13	$2\frac{1}{2}$	<1	3.6
14	$3\frac{3}{4}$	<1	5.0
15	5	<1	5.6
16	20	<1	5.8
17	44	<1	3.1
18	92	<1	1.3

Table 47 (cont'd).

(c) S51

Sample number	Time	Red cell survival (%)	Plasma activity (%)
	Zero - 0.03 ml red cells (S59) injected i.v.		
1	2 m	8.0	4.5
2	3	6.6	4.5
3	7	1	4.1
4	13	1	3.4

SOME ADDITIONAL OBSERVATIONS ON THE EFFECTS OF ALLOANTIBODIES ON RED CELL SURVIVAL

EXPERIMENTS WITH NON-COMPLEMENT-BINDING IgM ANTIBODY

Methods and procedure

IgM antibody preparation

Characteristics of the antibody preparation

Complement depletion of the recipient rabbit

Plan of experiments

Handling of blood samples

Results

Blood volumes

Red cell clearance patterns

Peripheral blood counts

Sites of sequestration of the injected red cells

Hepatic and splenic histology

Discussion

One high-titre antiserum produced by the repeated injection of A-positive red cells into an A-negative rabbit was fractionated to give a preparation containing predominantly IgM antibody. In vitro tests indicated that this was a non-complement-binding antibody (see Burton and Mollison, 1968). In the following experiments the ability of this antibody preparation to bring about the clearance of a small dose of A-positive red cells injected into a normal and a complement(C)-depleted A-negative recipient was examined.

Methods and procedure

IgM antibody preparation. The antiserum used for these experiments was raised in a single FF rabbit (A25) by injection with A-positive red cells; the first injection was given i.m. with FCA and subsequent injections were of cells alone given i.v. (see p.72). Serum was harvested over a period of several weeks. The pooled serum was fractionated using DEAE-cellulose (DE52) and phosphate buffer, pH 6.5, of different concentrations for elution of different molecular species; 0.0175 M buffer was used to elute IgG, following which, elution with 0.3 M buffer gave a fraction containing IgM relatively free of IgG. After concentration by pressure dialysis, this IgM-containing fraction was put through a second fractionation procedure on a DE52 column. Examination of the 0.3 M eluate by immunoelectrophoresis indicated that some IgG was still present (see Fig.44); the effect of 2-mercaptoethanol (2-me) treatment on the anti-A activity of the preparation was therefore tested, as described below.

Figure 44. Immuno-electrophoresis of normal rabbit serum (on the left of each channel) and the "IgM" preparation from A25 serum (on the right of each channel). 1: goat anti-rabbit whole serum. 2: sheep anti-rabbit IgG (SK 16-1-P1). 3: sheep anti-rabbit IgM (Sk 15-2-P1).

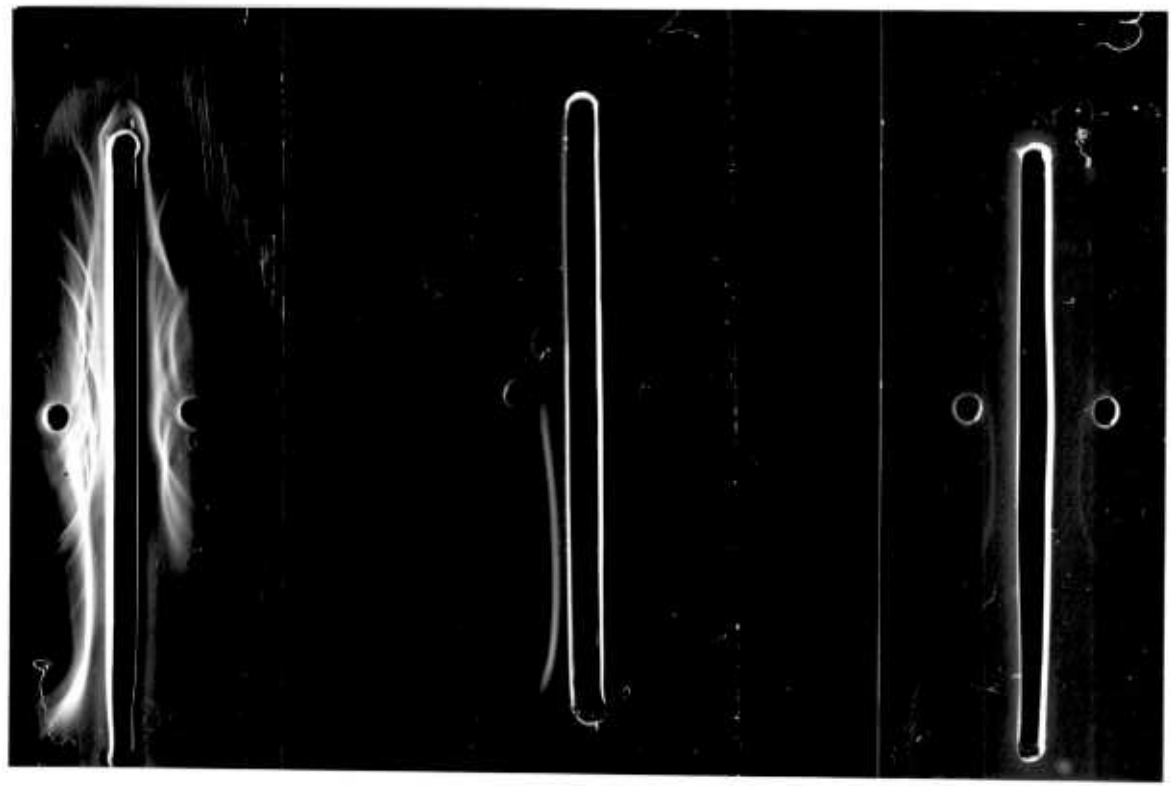


Figure 44

The fraction was dispensed into several containers and stored at -50°C. Before use, the material was thawed, spun in a bench centrifuge at maximum speed for 15 minutes and was then passed through a millipore filter. The titre of antibody against A-positive red cells was unchanged by storage.

Characteristics of the antibody preparation. The concentrated IgM fraction agglutinated AA and AF red cells suspended in magnesium sulphate solution at 37°C to a titre of 1 in 320; in addition, agglutination of some samples of FF cells was observed to a dilution of 1 in 40. In the presence of fresh rabbit serum, no macroscopic lysis was observed and no significant lysis could be demonstrated using the sensitive ^{51}Cr-release method (Polley, Adinolfi and Mollison, 1963).

The effect of 2-me on the anti-A activity was tested on a sample which had been diluted approximately 1 in 2 during the course of the experiments. Allowing for this dilution, the titre against AA cells suspended in magnesium sulphate solution was 1 in 256, and this was reduced to 1 in 16 following 2-me treatment. In addition, using an indirect antiglobulin test (anti-rabbit IgG), the titre of the 2-me treated serum was enhanced to 1 in 16 compared with 1 in 8 for the saline control. It is clear therefore that the preparation contained some anti-A activity due to IgG antibody, but it can be shown that the amount present would be insufficient to bring about accelerated clearance

of A-positive cells in the present experiment. A titre of 1 in 16 by IAGT represents an IgG antibody concentration of about 1.6 µg/ml (see Fig. 27, p.); 5 ml of the preparation containing 8 µg IgG anti-A was injected, together with 1.7 ml A-positive red cells, so that even if all the IgG antibody were to combine with the red cells, the maximum amount bound would be less than 5 µg/ml red cells; as shown in Fig 33, this amount of IgG antibody is unlikely to have any significant effect on the clearance of the coated cells.

Complement depletion of the recipient rabbit. One of the recipient rabbits was made deficient in complement components (C3-9) by the use of cobra venom factor (Brown et al., 1970). The cobra venom preparation was kindly provided by Dr. D.L. Brown, Department of Immunology, Royal Postgraduate Medical School, London W.12. Four i.v. injections of 0.4 ml cobra venom preparation were given at 0, 8, 24 and 32 hours. C3 levels in samples of EDTA plasma taken before each of these injections and also at 48 hours (at the start of the present experiment) were determined by Dr. Brown, using a single-phase radial diffusion agar-gel technique in which specific anti-rabbit-C3 was incorporated in the gel (see Brown, 1970). It was shown that immediately before the injection of A-positive red cells and IgM anti-A, the C3 level was less than 6% of the value found before treatment with cobra venom factor; the residual C3 determined by the immunological technique is thought to represent breakdown products of C3 which have no biological activity in standard haemolytic assay systems (D. L. Brown, personal communication).

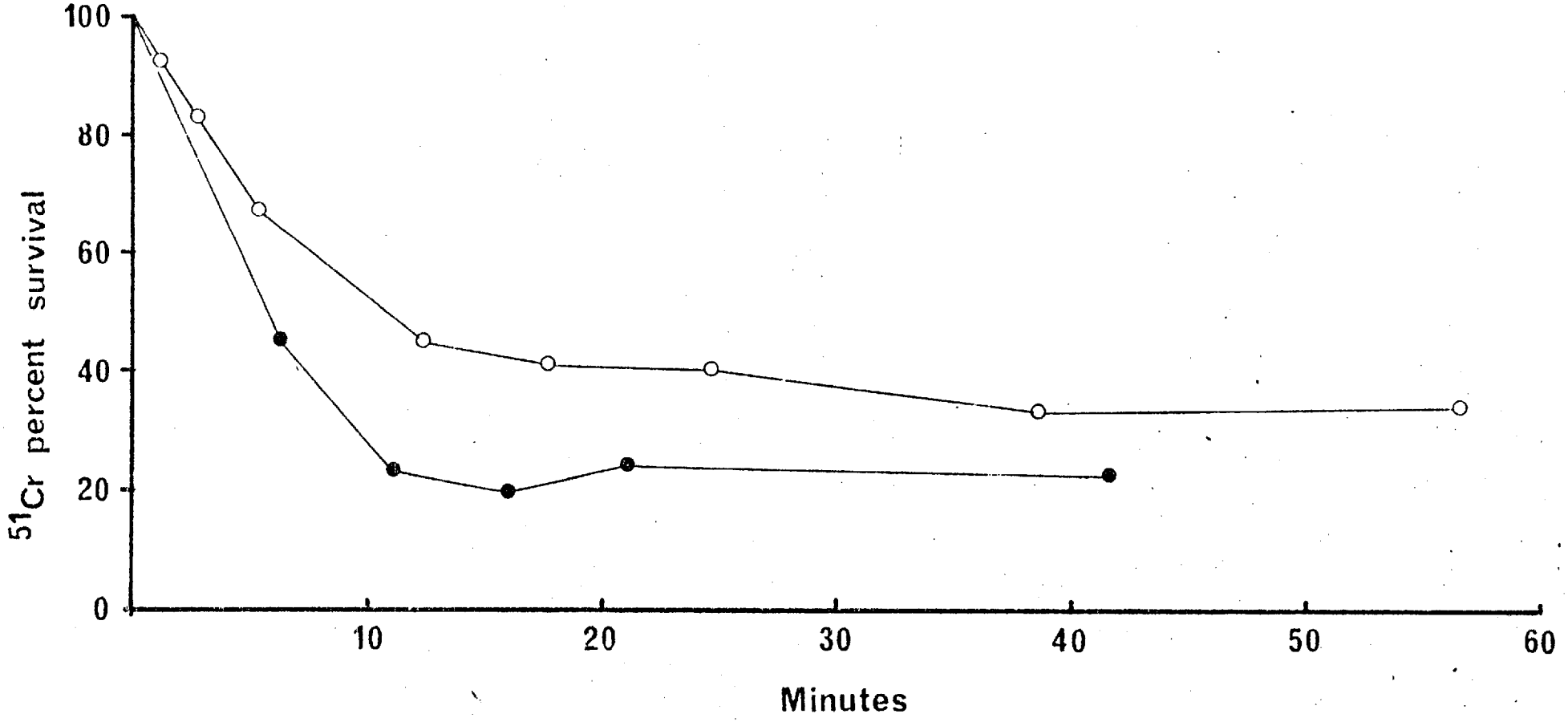
Plan of experiments. Two young A-negative (FF) Sandy Lop rabbits, each weighing about 3 kg, were used. One (R50) was rendered C-deficient as described above and the other (R39) was untreated. A single AA rabbit (R28) was used as red cell donor for both experiments.

For each experiment, donor cells were labelled with ^{51}Cr by the citrate-wash method and a known volume of saline suspension containing approximately 1.7 ml of the Cr-labelled cells was injected i.v. A blood sample was taken into EDTA 5 minutes later for determination of red cell volume, total blood volume and venous haematocrit.

5 ml of the IgM preparation was then injected i.v. Blood samples were taken at various times thereafter (see Fig 45 and Table 49). In the case of the normal rabbit, approximately 1.3 ml blood was taken on each occasion into a syringe containing about 0.1 ml liquid heparin; all blood samples from the C-depleted rabbit were anticoagulated with solid EDTA.

After it had been established that ^{51}Cr activity in the blood of the recipients had stabilized following the IgM injections, the animals were killed by a sharp blow to the base of the skull. The spleen and a small portion of liver from the normal rabbit were fixed in formol-saline for preparation of paraffin sections, and the remainder of the liver was digested in conc. HNO_3. In the case of the C-depleted rabbit, the liver was perfused *in situ* with formol-saline before removal from the carcass; after sampling of both liver and spleen for histology they were digested separately in conc. HNO_3.

Figure 45. Survival of ^{51}Cr-labelled A-positive red cells (1.7 ml) injected i.v. into two A-negative rabbits at the same time as 5 ml of an IgM anti-A preparation which did not bind complement *in vitro*. Rabbit R50 (O) had been rendered complement-deficient by prior treatment with cobra venom factor (see text); the other rabbit, R39 (●), was an untreated control.


100
80
60
40
20
0
^{51}Cr percent survival
10
20
30
40
50
60
Minutes

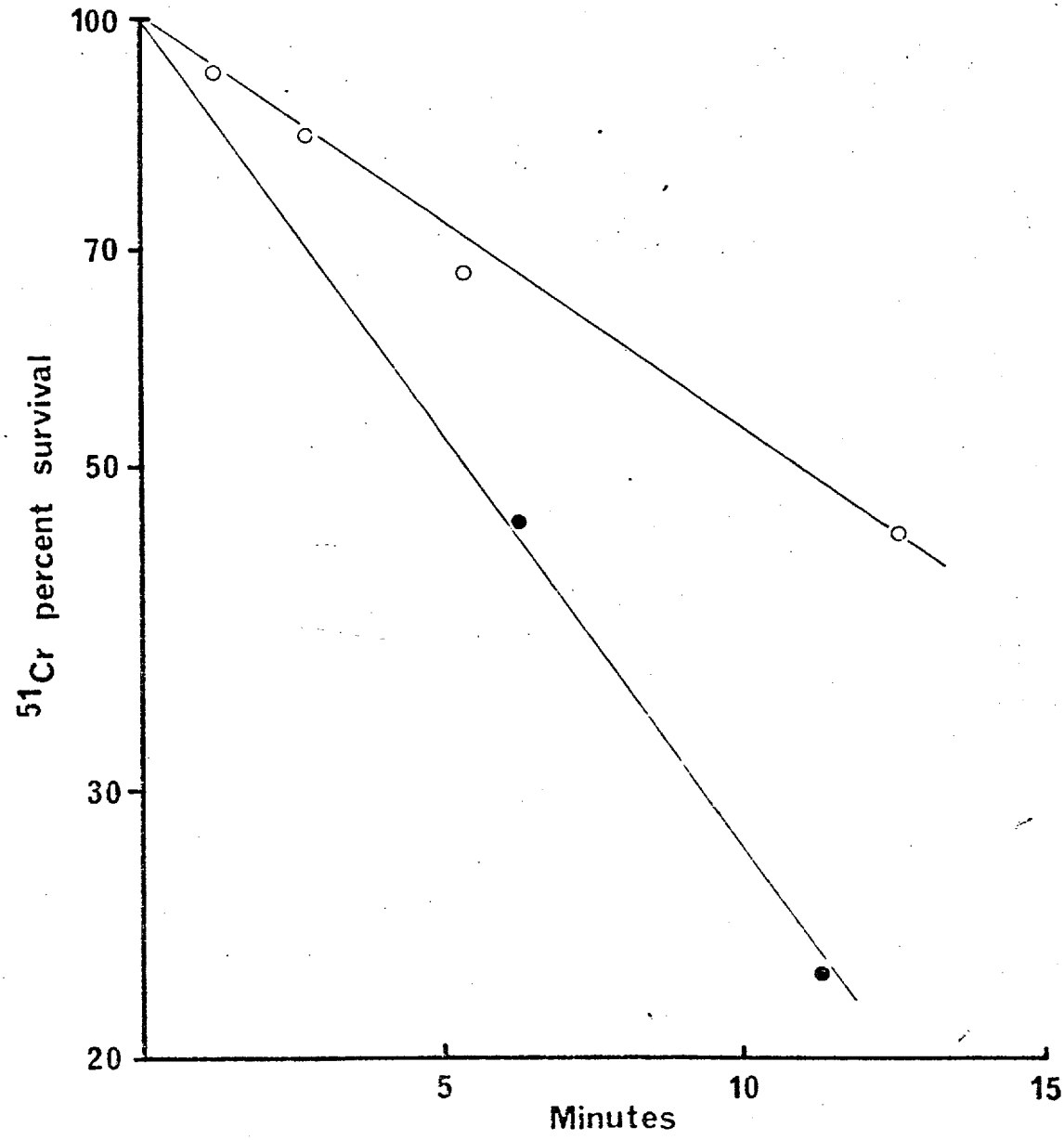
Handling of blood samples. Manual dilutions of whole blood samples in Isoton were made for estimation of blood counts using a Coulter Electronic Counter, Model S. The following measurements were made: WBC, RBC, Hb, PCV, MCV, MCH, MCHC. A part of each sample was removed and the plasma was separated for radioactive counting. The whole blood samples were then lysed with saponin and 0.5 ml amounts were pipetted into counting vials for estimation of ^{51}Cr activity. Haemoglobin concentration of the lysed samples was determined using the cyanmet-haemoglobin method. Results were expressed as ct/min per gm Hb. Known dilutions of the injection suspensions of labelled cells were also counted. The volumes of the spleen and liver digests were measured and aliquots of each were pipetted for counting.

Results

Blood volumes. Measured red cell volumes and venous haematocrits are shown in Table 48 together with deduced values for total blood volumes and plasma volumes. It can be seen that for plasma volume and total blood volume similar values were obtained in the two rabbits.

Red cell clearance patterns. The percentage of A-positive red cells remaining in the circulation of each rabbit at various times after injection of IgM anti-A is shown in Table 49 and Fig. 45. In the normal control rabbit, clearance appeared to be approximately exponential during the first 10-15 minutes during which time 75-80% of the injected red cells were removed with a $T_{\frac{1}{2}}$ of 5.5 minutes (see Fig. 46); the remaining A-positive red cells underwent no significant removal during

Figure 46. Initial data from the survival curves shown in Fig.45. plotted on a semi-logarthmic plot to determine the rates of red cell destruction. The data for the complement-depleted (O) and the normal (●) rabbits were fitted by straight lines using a "least squares" method ($r = 0.99$ in each case).


100
70
50
30
20
^{51}Cr percent survival
5
10
15
Minutes

the subsequent 30 minutes. The overall pattern of clearance was similar in the C-depleted rabbit, although the red cells were removed rather more slowly and a slightly larger proportion was left in the circulation. Thus about 60% of the injected cells were removed with a $T\frac{1}{2}$ of 9.8 minutes; by 15-20 minutes the rate of clearance had decreased markedly and there was no substantial removal during the ensuing 50 minutes of observation.

Counts in plasma samples from both rabbits indicated that no significant amount of haemoglobin was liberated from the injected red cells during the period of observation.

Peripheral blood counts. Following the injection of IgM anti-A, leucocyte counts fell from 12.0×10^9 to 5.8×10^9 per litre in the control rabbit and from 14.3×10^9 to 6.2×10^9 per litre in the C-depleted rabbit; this reduction was apparent in the first samples tested, i.e. at about 11 and 5 minutes respectively in the normal and C-depleted rabbits. There was no substantial change in any of the other measurements and the leucocyte count remained fairly steady at the reduced level for the duration of the observations.

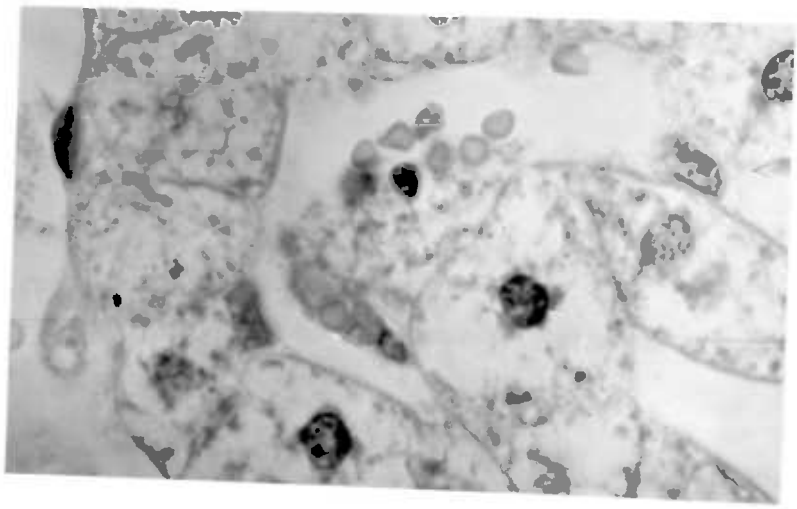
Sites of sequestration of the injected red cells. The livers of the normal and C-depleted rabbits contained 47% and 34% respectively of the injected radioactivity. The spleen of the C-depleted rabbit was also examined and 5% of the injected radioactivity was detected. In both animals a substantial proportion of the cleared activity was not accounted for in the organs tested.

Hepatic and splenic histology. Microscopic examination of liver from both the control and the C-depleted rabbits revealed, in a few areas, collections of red cells in close proximity to, or sometimes apparently within, Küpfer cells lining the hepatic sinusoids (see Fig. 47). However, similar appearances were also found in sections of liver taken from a rabbit that had not received any injections of antibody or red cells in the preceding weeks. Splenic sections were also found to be difficult to interpret. No specific histological appearances could therefore be assigned to the tissues examined in these rabbits.

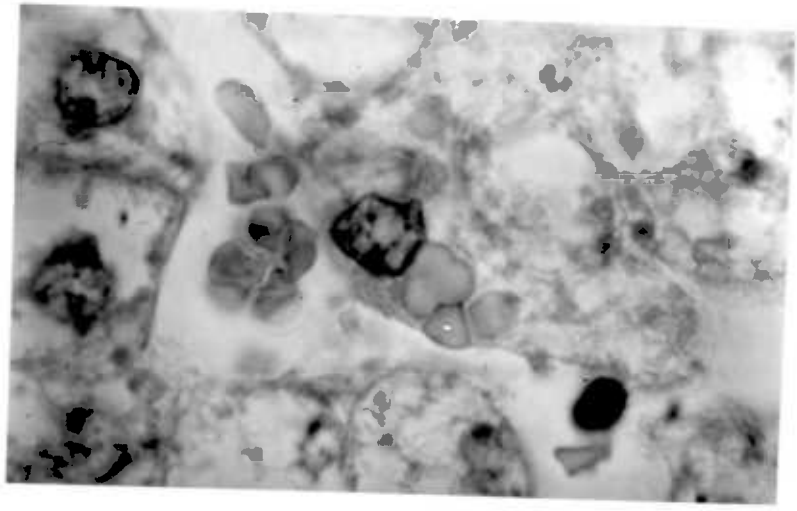
Discussion

These experiments provide little evidence to indicate that complement played any significant part in the clearance of A-positive red cells by this particular example of IgM anti-A. The small quantitative differences in the rates of red cell clearance seen in the normal and the C-depleted rabbit were possibly the result of normal variation in the recipients. Certainly, the weights of the livers differed substantially (120 g and 101 g respectively in the normal and C-depleted rabbits) which in part accounts for the apparent difference in hepatic sequestration of red cells; the volume of cells sequestered per g of liver tissue was not very different in the two animals, namely 0.0066 ml and 0.0057 ml.

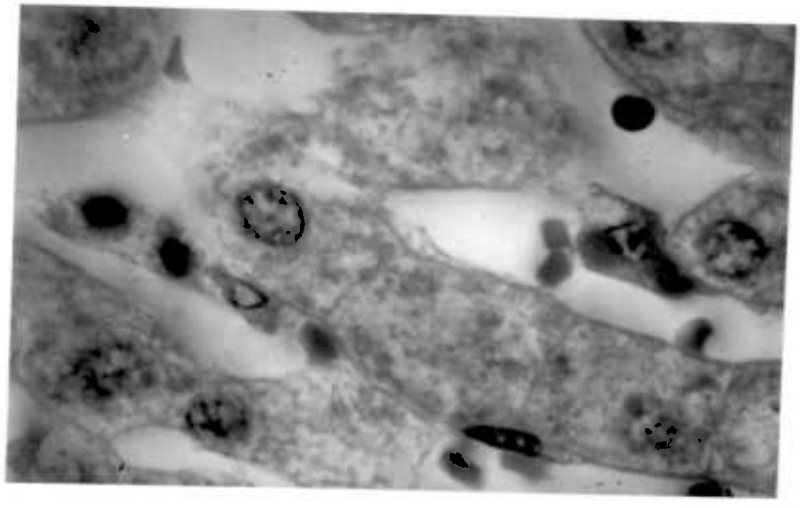
Figure 47. Histological sections of liver taken from (a) R50, the complement-depleted rabbit, after 80% of the injected A-positive cells had been cleared; (b) R39, the control rabbit, after 65% of the injected cells had been cleared; (c) another rabbit which had not been injected with allogeneic red cells for many weeks. Small aggregates of red cells adjacent to or apparently within the Küpfer cells are seen in each case. (H & E; x 1000)



(a)



(b)



(c)

Figure 47

The behaviour of the antibody *in vivo* might have been predicted from its *in vitro* characteristics. It is a high-affinity IgM antibody capable of causing agglutination but not lysis of appropriate red cells, and it appears not to bind C; as discussed on p.318 , the survival of a small volume of allogeneic red cells in the presence of this type of antibody would be expected to follow a 2- or multi-component clearance pattern. Using this antibody preparation, Burton and Mollison (1968) did in fact observe 2-component clearance curves, and these findings were confirmed in the present experiments.

The factors involved in producing two-component clearance curves are not understood. Burton and Mollison (1968) concluded that both antibody exhaustion and heterogeneity of antigen distribution on the red cells were in part responsible for the phenomenon. These two-component curves undoubtedly depend upon factors different from those operating when C-binding IgM antibodies are involved; in the latter circumstances the sequestration of red cells in the liver and their subsequent release back into the circulation is associated with inactivation of the bound complement (Brown *et al.*, 1970; Schreiber and Frank, 1972b). It has been postulated that intravascular agglutination as a mechanism for the destruction of small volumes of allogeneic red cells might result in two-component survival curves (see p.35). The present experiments suggest that, in addition to the liver and spleen, other sites were involved in the removal of the antibody-coated cells from the circulation, a finding which would be expected if agglutinates were trapped in the microcirculation. Furthermore, histological examination of the liver and spleen provided no convincing evidence for an antibody-mediated interaction between red cells and the cells of the mononuclear phagocyte system, although it must be admitted that the histological sections were very difficult to interpret.

Table 48. Blood volumes in the normal control and C-depleted rabbit.

	Control (R39)	C-depleted (R50)
Venous haematocrit	0.338	0.324
Red cell volume (ml)	60.5	54.4
Total blood volume (ml)	207.0	199.3
Plasma volume (ml)	146.5	144.9

Table 49. Survival of Hg^A-positive red cells in the circulation of normal control and C-depleted Hg^A-negative rabbits following the i.v. injection of IgM anti-Hg^A.

	Sample number	Time after antibody injection (min),	Percent survival
Control-R39	1	0	100.0
	2	6.3	45.8
	3	11.3	22.6
	4	16.3	19.8
	5	21.3	24.1
	6	41.8	23.0
C-depleted-R50	1	0	100.0
	2	1.2	92.8
	3	2.8	83.7
	4	5.4	67.6
	5	12.6	45.2
	6	17.7	41.8
	7	24.8	41.0
	8	38.7	34.0
	9	56.8	34.5
	10	71.1	36.1

GENERAL CONCLUSIONS

The Rh and Hg blood group systems

"Collapse" curves and immunization

The effect of NEM-treated red cells and the non-responder

Further work

The Rh and Hg blood group systems

The choice of the rabbit as an experimental model for the study of alloimmunization and its suppression relates to certain similarities which the Hg blood group system bears to the Rh system in man, at least in so far as alloimmunization to the respective major antigens, A and D, are concerned. These similarities have been stressed by Pollack et al. (1968).

(i) Naturally occurring alloantibodies are found very rarely, if at all, in these two blood group systems.

(ii) Intravenous injection of red cells bearing the alloantigen into individuals lacking it, leads in the majority of cases to the production of serologically detectable antibody; there is considerable variation in the time taken for different individuals to produce antibody.

(iii) The antibody produced is predominantly IgG and, in the majority of cases does not bind complement.

(iv) It is possible to suppress primary immunization, at least partially, by the administration of specific IgG antibody at the time of injection with red cells.

(v) A proportion of individuals do not produce serologically-demonstrable antibody in response to i.v. injections of red cells bearing the alloantigens, so-called non-responders.

Two important differences between these blood group systems are also well-recognised.

(i) The number of antigen sites per red cell is about 10 times greater for A than D.

(ii) The pattern of tissue sequestration of red cells following specific IgG antibody-mediated destruction is different; the spleen is the major site of removal in man whereas in the rabbit, the liver plays a much more important role.

With few exceptions, it has not been possible in the present work to characterize extensively the alloantibodies produced in response to i.v. injections of red cells because the antibody titres have been very low. In most cases, no significant difference in titres of antibody have been found using three serological techniques - cells suspended in magnesium sulphate solution, enzyme-treated cells and IAGT using anti-rabbit IgG (see Table 34). Nor have the patterns of the red cell survival curves been helpful in determining the class of antibody involved in these circumstances.

The concept of the non-responder has been more clearly defined. It has been shown that failure to detect antibody serologically is very frequently associated with the impaired survival of allogeneic red cells and that in many of these cases, antibody may be detected following subsequent injections of cells, suggesting that immunization had occurred long before antibody could be detected. By contrast, in other cases

in which antibody could not be detected serologically even after repeated i.v. injections of allogeneic red cells, the survival of the injected cells had been normal on all occasions. These individuals have been called non-responders although most of them will respond to cells injected i.m. with adjuvant.

"Collapse" curves and immunization.

Reasons why red cell survival curves have been used in this work as indicators of an immune response have already been given and an attempt has been made to justify the assumption that in the circumstances under which the experiments were carried out, impaired red cell survival could be equated with an immunological response to the A antigen (see p.42). Nevertheless, the possibility remained that in the absence of serologically detectable antibody, a response to antigens other than A might be responsible for some of the "collapse" curves observed. Indeed, in one case, the appearance of a second antibody in addition to anti-A was demonstrated serologically and was found to cause the accelerated clearance of a particular example of A-negative red cells (see p.216). Since this work was completed, Mollison (personal communication) has carried out a small number of experiments in which red cells from a single FF donor rabbit were injected i.v. into several FF recipients; in 4 out of 10 cases, the survival curves collapsed and no antibody could be detected serologically.

The lack of specificity of this method for detecting immunization has meant therefore that some of the results obtained were not so clear-cut as they might at first sight appear. This has been taken into account in the discussion and interpretation of the experimental findings. However, that it was possible to delay collapse of a survival curve when specific anti-A had been given with a preceding dose of red cells suggests that immunization to the A antigen was being monitored in a substantial proportion of cases. Much more certain was the interpretation that no significant humoral antibody response to the injected red cells had occurred when survival curves were repeatedly normal.

The effect of NEM-treated red cells and the non-responder

The response in the rabbit to certain red cell antigens had been shown to involve an early interaction between antigens and antigen-reactive cells located in the bone marrow (see p.49). In the present studies, a large part of an injection of NEM-treated red cells was found to be sequestered rapidly in the bone marrow and it was argued that an excess of antigen in this site might have been responsible for the state of tolerance observed under these circumstances.

Some of the features of this unresponsive state following NEM-treated cells were similar to those observed when rabbits which had been given only untreated allogeneic red cells by i.v. injection failed to respond. In both cases, some animals appeared to respond initially by showing increased rates of red cell destruction which subsequently reverted to normal. It has now been established at a cellular level that the

induction of tolerance may be preceded by an early immune response (Chiller et al., 1973) and this provides a possible explanation for the transient impairment of red cell survival seen in some of these rabbits. Both types of non-responders resisted immunization by repeated i.v. injections of red cells given over many months but when cells were given i.m. with FCA, the survival curves collapsed and antibody could be detected serologically in many cases; in a few cases amongst both types of non-responder rabbits showing a transient response (as described above) were found.

These similarities suggest that, at least in some of the rabbits studied, failure to respond to i.v. injection of untreated A-positive red cells might represent a form of tolerance induced by the injected cells. While it is unlikely that "innate" unresponsiveness is brought about by the same mechanism as that responsible for NEM-associated unresponsiveness, it is conceivable that the clearance of untreated cells may occur in such a way that tolerance rather than immunization occurs.

Further work

Much of the speculation in the preceding paragraphs is amenable to experimental testing, and some areas which might be profitably explored are outlined briefly below.

1). The lack of specificity of the collapse phenomenon might be overcome by studying the response of "immunized" rabbits at the cellular level. Spleen cells or even peripheral blood leucocytes could be used in a system such as the haemolytic plaque assay with a panel of red cells

in an attempt to determine specificity of a response. In addition, studies of this kind might provide information about the cells involved in the unresponsive states and also in the suppression of immunization by passive antibody.

2). The mechanism of "collapse" of red cell survival curves has hitherto been assumed to involve specific antibody-mediated destruction of the injected cells. No evidence implicating a serologically undetectable humoral factor was obtained in the two experiments described on p.231. Alternatively, a cell-bound antibody could be responsible. Further plasma exchange experiments of the type performed in this work, and in vitro cell adherence experiments might resolve this question. The possibility of a non-specific mechanism of allogeneic cell clearance has been raised by the observations of Mollison cited above, but it seems more likely that the accelerated clearance of "compatible" red cells was due to immunization to unrecognised alloantigens.

3). It would be of considerable interest to investigate in greater detail, the nature of the "innate" and "NEM-associated" unresponsive states using cell transfer experiments of the kind carried out by Richter and his colleagues (see p.49). This might enable the unresponsive cell(s) to be identified.

4). Whether the NEM effect is due to the concentration of antigen at a particular site rather than to an effect of NEM on antigen or antigen-reactive cell could possibly be established by quantitating the response to different volumes of cells treated with a standard dose of NEM in terms of the ability of such injections to induce an unresponsive state.

5). Breeding experiments to establish the influence of heredity on the "innate" unresponsive state and on the NEM-associated unresponsive state would be of interest.

6). The way in which NEM-treated cells bring about prolonged immunological unresponsiveness in the rabbit is not known from the present work. If the site of sequestration of the removed cells is important, there is little reason to believe that in man NEM-treated allogeneic cells would result in tolerance for it is known that in man such cells are removed predominantly by the spleen. However, it has been shown in vitro that sulphydryl-blocking agents, including NEM, can interfere with immunoglobulin receptor sites on macrophages (Lo Buglio et al., 1967) and it is therefore possible that this type of mechanism may be involved in inducing tolerance under these conditions. The injection of NEM-treated Rh(D)-positive red cells into Rh(D)-negative volunteers would be a simple and a safe procedure; if it were to result in longstanding tolerance to the D antigen, the observation might form the basis of a prophylactic procedure.

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Normal Red Cell Survival in the Rabbit

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Red cell survival in the rabbit was estimated using both ^{51}Cr to label cells in vitro and $DF^{32}P$ to label cells in vivo. The results suggest that, after about the first 7 days, there was no elution of $DF^{32}P$ from the red cells and that, accordingly, the rate of ^{51}Cr elution could be obtained by plotting the $^{51}Cr/DF^{32}P$ ratio from day 7 onwards. If the assumption is made that the rate of Cr elution was the same between days 1 and 7 as it was after day 7, then observed Cr values from day 1 onwards can be corrected for elution and an estimate of true red cell survival obtained. Analysis of the resulting curves indicates that the initial slope is linear on an arithmetical scale for about the first 17 days, becoming curvilinear thereafter. It is concluded that the curvilinear slope of red cell survival in rabbits, hitherto ascribed to random red cell destruction, is more likely to be due to a rather wide scatter of red cell life-span combined with superimposed elution of the red cell label. In the present series, mean red cell life-span in the rabbit was estimated to be 50.4 days; as in previous series, the survival of the longest-lived cells was found to be about 65–70 days.

During the course of experiments in rabbits on alloimmunization to the red cell antigen Hg^A (to be reported elsewhere), it was necessary to define normal red cell survival as estimated with autologous ^{51}Cr-labelled red cells. Preliminary observations indicated that in our hands T_{50} Cr was longer than had been reported by previous observers, presumably because with the citrate-wash mehod of ^{51}Cr labelling Cr elution was relatively slow. It was decided to try to estimate the rate of elution directly by labelling red cells simultaneously with ^{51}Cr and $DF^{32}P$ in the same animal. In a small subsidiary study, autologous ^{51}Cr red cell survivals after labelling with the citrate-wash method and with an ACD method were compared.

MATERIALS AND METHODS

Animals

Adult Sandy-Lop rabbits of both sexes, supplied from an inbred colony kept at the Radiobiological Research Unit, Harwell, were used. The animals had not been bled for a period of at least 6 months preceding the experiments recorded here.

Labelling of red cells with ^{51}Cr

In 7 rabbits, the citrate-wash method (Mollison 1961, Garby & Mollison 1971) was used. In two cases, the results were simply compared with those obtained in two cases in which the red cells were labelled with ^{51}Cr by an ACD method. In the remaining five cases, $DF^{32}P$ was used simultaneously to label red cells in vivo (see below).

In the citrate-wash method, approximately 2 ml of blood was drawn from a marginal ear vein into a syringe containing a drop of liquid heparin. The blood was mixed with 10–15 ml of citrate-phos-

phate-dextrose, pH 6.9 (Mollison et al 1958) and centrifuged. After discarding the supernatant, approximately 50 μCi ^{51}Cr as sodium chromate was added and mixed gently; the amount of chromate added was less than the equivalent of 1 μg Cr/ml red cells. After incubating at 37° C for 10 min, the cells were washed twice with isotonic saline. The washed red cells were resuspended to a volume of about 6 ml of which 5 ml was injected into the donor animal. Samples of about 1 ml of blood were taken from a different vein at 5 to 10 min after injection and thereafter approximately twice a week for 4 weeks. In the ACD method, blood was mixed with one-quarter its volume of ACD (2.5 g disodium citrate, 3.0 g dextrose, distilled water 120 ml). The blood was centrifuged and ^{51}Cr was added to the packed cells. From this point, the method was exactly the same as in the citrate-wash method.

Estimation of red cell volume

Red cell volume was deduced from measurements of ^{51}Cr activity in blood samples taken 5 min after the injection of a measured amount of ^{51}Cr-labelled red cells. In practice, the haemoglobin concentration of the 5 min blood sample was estimated and PCV was deduced, assuming the MCHC of normal rabbit red cells to be 34.5 g/100 ml (based on unpublished observations).

Labelling with DF^{32}P

In five cases, about 10 min after a sample of the red cells labelled with ^{51}Cr by the citrate-wash method had been injected, a solution of DF^{32}P, freshly diluted in isotonic saline was infused at a constant rate over about 10 min. The total amount of DFP injected into each animal was 77 μg (28.6 μCi).

Blood samples

1 ml samples were taken 1 h after the DFP infusion and subsequently at increasing intervals for about 6 weeks, as indicated in the graphs. Samples were taken into dry syringes and transferred into tubes containing 2.5 mg dry EDTA. When only ^{51}Cr was to be counted, whole blood samples were lysed with saponin. When DF^{32}P was to be counted as well, 0.5 ml of the unlysed sample was washed into a tube containing approximately 20 volumes of 1 % phosphate-buffered saline. After mixing, the samples were centrifuged. The supernatant was removed as completely as possible, without disturbing the cell button. Two further washes were carried out in this way. The packed cells were then lysed with 0.04 % ammoniated water containing 0.01 % sodium azide, the final volume being adjusted to 10.5 ml. The lysed samples were stored at 4° C.

Radioactivity counting

Samples containing both DF^{32}P and ^{51}Cr. Each complete 10.5 ml lysed sample prepared as described above was transferred to a liquid sample Geiger-Müller tube for counting. Initially, sufficient counts were obtained to ensure a counting error of less than 2 % but in some of the later samples an error of up to 3 % had to be accepted because of the low counting rates. Corrections were applied for counts due to ^{51}Cr.

After the ^{32}P activity had been measured, 2 ml of the lysed samples was counted in a gamma counter as described below. A correction was applied for counts due to ^{32}P.

Samples containing ^{51}Cr alone. Whole blood samples were lysed with saponin and 0.5 ml amounts counted in a Packard Autogamma Spectrometer, sufficient counts being recorded to keep the counting error at less than 1 %.

Expression of results

The Hb concentration of each sample was determined by the cyanmethaemoglobin method and results were expressed as ct/min per g Hb.

Observed results were corrected for cumulative blood loss due to sampling as suggested by Mollison (1972, pp 31–32), either using derived values for whole blood volume based on estimates of red cell volume or assuming blood volume to be 150 ml.

Corrected values were expressed as percentages of the value of the first sample taken after injection.

RESULTS

Red cell volume

In the five rabbits injected both with DF^{32}P and ^{51}Cr, red cell volumes varied from 43.4

to 57.5 ml, or 15.3 to 16.9 ml/kg (mean 16.2 ml/kg). Deduced PCVs ranged from 32.2 to 35.6 % (mean 33.7 %).

^{51}Cr survival curves

Figure 1 shows all the results. With the citrate-wash method, values for T_{50} Cr were as follows: 24.2, 19.5, 23.0, 18.8 and 20.1 d for the five cases in which $DF^{32}P$ was also injected and 21.5 and 22.2 d for the two cases in which only ^{51}Cr was injected; in the two cases in which an ACD method was used, T_{50} Cr was 17.9 and 18.9 d. Although the mean T_{50} Cr for the seven cases in which the citrate-wash method was used is 21.3 d and that for the two cases in which an ACD method was used is 18.4 d, the difference is not statistically significant (p = approximately 0.09).

$DF^{32}P$ survival curves

As Figure 2 shows, about 20 % of the injected $DF^{32}P$ was lost from the red cells in the first 24 h and the rate of loss became negligible only after about the first 7 d.

From the amount of $DF^{32}P$ injected and the red cell volume estimates, it was calculated that uptake of $DF^{32}P$ into the red cell mass at 1 h ranged from 6.8–9.0 % (mean 8.0 %) of the injected dose, corresponding

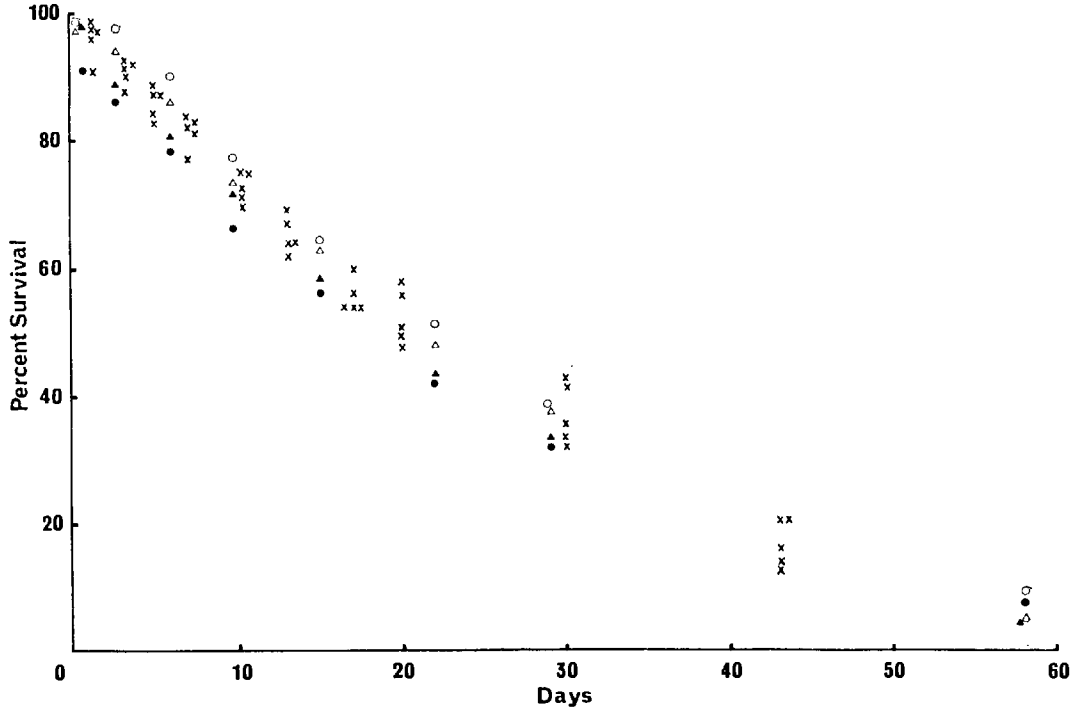


Figure 1. ^{51}Cr red cell survival curves.
×: 5 cases in which the citrate-wash method was used and in which red cell survival was also estimated with $DF^{32}P$ (see Figure 2). △, ○: 2 further cases in which the citrate-wash method was used for comparison with 2 cases (▲, ●) in which an ACD method was used.

to 0.119–0.132 μg DFP/ml red cells (average 0.124 μg).

Comparison of ^{51}Cr and $DF^{32}P$ survival curves

In the five cases in which both labels were used, the ratio Cr/DFP rose slightly over the first few days. However, from day 7 onwards the ratio fell progressively in all but one case, in which a decrease was not seen until day 10. These results indicate that, during the first 7 d or so, elution of DFP from the red cells occurs more rapidly than elution of ^{51}Cr and that, thereafter, the converse applies. From a consideration of our survival data, the most likely explanation of this observation is that there is rapid elution of DFP initially but negligible elution after about day 7 and that, by contrast, the rate of Cr elution remains constant throughout (except for the slightly increased loss noted in the first 24 h). Accordingly, Cr/DFP ratios from day 7 (10) onwards were used to determine the rate of Cr elution and it was then assumed that the rate of Cr elution was in fact constant from day 1 onwards. Values for Cr elution, based on measurements from day 7 (10) onwards, ranged from 0.49–1.40 %/day (mean 1.00 %/d).

Calculation of true red cell survival

Using the values for Cr elution described above, true red cell survival was calculated from the Cr results in the five cases in

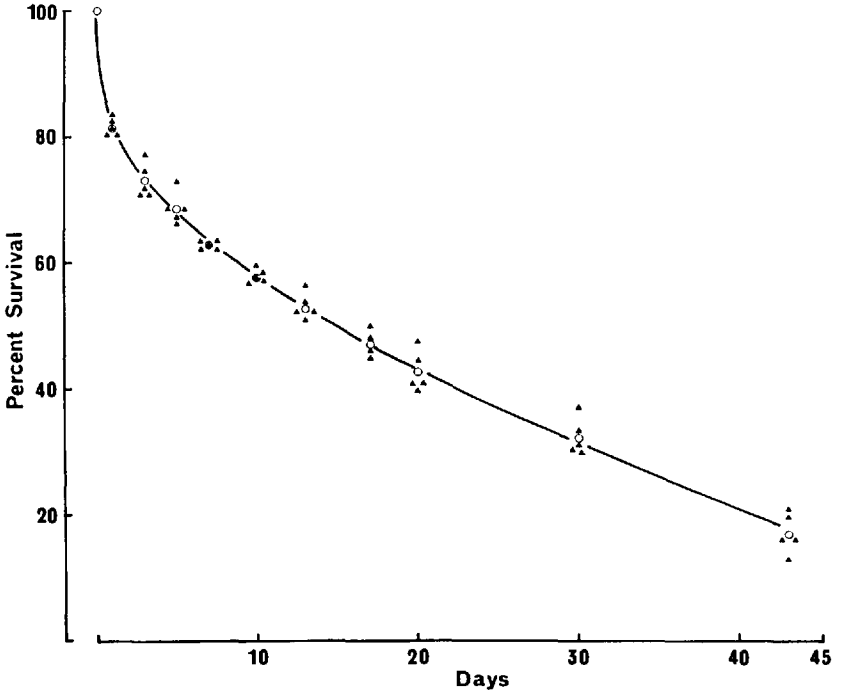


Figure 2. $DF^{32}P$ red cell survival curve.
○: mean values; ▲: individual values in 5 rabbits. The line has been fitted by eye.

which both Cr and DFP were injected; the results are shown in Figure 3. As the figure shows, the data can be fitted by a straight line over about the first 17 d. Mean life-span deduced by extrapolating this line to the time axis varied from 41.6 to 59.7 d, with a mean value of 50.4 d.

DISCUSSION

The T_{50} Cr observed in the present series following labelling by the citrate-wash method averaged 21.3 d for 7 cases (range 18.8–24.2 d). This is slightly longer than the figures of 19 d (6 cases) reported by Marvin & Lucy (1957), 17 d (5 cases) reported by Heimpel et al (1964), and of 16–17 d (31 cases) reported by Miescher (1956), and considerably longer than the figure of 12 d (17 cases) reported by Gardener et al (1961). Presumably, as in humans, the method of treating the red cells before incubation with ^{51}Cr affects the subsequent rate of Cr elution (Mollison 1961). In the present series, there was in fact a suggestion that T_{50} Cr was shorter when ACD blood was labelled than when the citrate-wash method was used although the difference was not significant (p = approximately 0.09).

Simple inspection of the Cr survival data (Figure 1) shows that over about the first 20 d, decrease in Cr activity is only very slightly curvilinear; thereafter, the curvilinearity increases. This is in marked contrast to the $DF^{32}P$ disappearance curve (Figure 2) which shows maximal curvilinearity initially, is substantially linear between days 7 and 20, and thereafter shows slight curvilinearity to the final observations on day 43.

Analysis of these data indicates that after injection of the labels, the rate of loss of $DF^{32}P$ is greater than that of ^{51}Cr for about the first 7 d and that from this time onwards, the rate of loss of ^{51}Cr exceeds that of $DF^{32}P$ by approximately 0.8 % per d.

The initial uptake of DFP observed in the present series, namely 0.124 μg/ml red cells, is well within the range previously reported for the maximum amount which

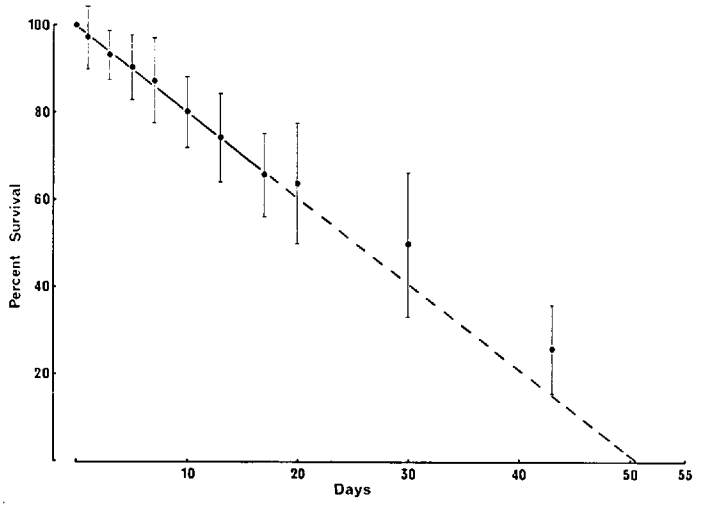


Figure 3. True red cell survival curve obtained by correcting observed Cr survival for Cr elution (see text). Each point shows the mean (± 2 SD) of observations in 5 rabbits. The continuous line was fitted by a 'least squares' method to the mean values over the first 17 d (r = 0.99); extrapolation of this line to the time axis gives an estimate of mean red cell life-span.

can be bound irreversibly to human red cells namely 0.1–0.15 μg/ml (Bratteby & Wadman 1968, Matsuda 1969).

The extent of loss of $DF^{32}P$ from the circulating red cell mass in the first 24 h, namely 20 %, in the present series is similar to that of 25 % found in rabbits by Heimpel et al (1964). It is distinctly greater than the rate of 8 % found in man by Wadman (1972) but this latter author used doses of DFP in the range of 0.2–3.0 μg/kg, which is far less than that of about 25 μg/kg used in the present experiments.

Heimpel et al (1964) in their experiments in rabbits assumed that there was no further elution of $DF^{32}P$ after 24 h and, taking the 24-h values as 100 %, found T_{50} to be 19–21 d in five rabbits. If the same assumptions are applied to the present data, T_{50} is approximately 22 d. However, as already described, our data indicate that elution of DFP continues for about 7 d, so that T_{50} DFP calculated in this way does not correctly indicate the T_{50} of true red cell survival. Incidentally, in man it has been found that elution continues for a period of several days (Bove & Ebaugh 1955) and even under optimum labelling conditions still amounts to about 0.5 % on day 4 (Wadman 1972).

In the present series, the rate of Cr elution calculated from the $^{51}Cr/DF^{32}P$ ratios from day 7 onwards was 0.49–1.40 (average 1.00) %/day. This rate is slightly less than that observed in humans, namely 0.6–2.0 (average 1.19) %/day, also using the citrate-wash method of ^{51}Cr labelling (Garby & Mollison 1971).

In calculating true red cell survival from the present results, it has been assumed that the rate of elution of Cr was constant from day 1 onwards. As shown in Figure 3, the mean corrected Cr data between days 1 and 17 are well fitted by a straight line (correlation coefficient 0.99); the three subsequent observations exceed by increasing amounts the values predicted by extrapolation of the earlier data. Survival curves in which the slope is initially linear on an arithmetical plot, thereafter becoming curvilinear, are expected when red cell destruction is due predominantly to senescence but with a large variation in red cell life-span (Dornhorst 1951). In the past it has been generally assumed that the curvilinear slopes of red cell survival observed in rabbits either with random labelling or with cohort labelling (Neuberger & Niven 1951, Brown & Eadie 1953, Card & Valberg 1967) are due to random red cell destruction superimposed on red cell death from senescence (see Brown & Eadie 1953), but this interpretation now seems less likely. So far as the cohort-labelling experiments are concerned, analysis of the data is in any case rendered difficult by the fact that labelling continues for a period of many days.

In principle, it should be possible to confirm that the initial slope of red cell survival is linear on an arithmetical plot by determining survival by differential agglutination using compatible red cells. Cross-transfusion experiments were performed by Wright et al (1955) but unfortunately their published data are not sufficiently extensive to permit precise analysis.

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Responses in Rabbits to the Red Cell Alloantigen Hg^A

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Summary. Successive injections of Hg^A-positive red cells were given at intervals of 2–3 months to Hg^A-negative rabbits. In forty-four out of fifty-one animals, red cell survival became progressively shorter and, in the majority of instances, anti-Hg^A was found in the serum. Characteristically, the first survival curve was initially normal but showed 'collapse' after a mean interval of 17 days; following subsequent injections, cells were cleared at an accelerated rate. In the remaining seven Hg^A-negative rabbits the survival of Hg^A-positive red cells was either repeatedly normal or was subnormal after the first one or two injections and became almost normal following further injections. In four out of six of these animals, following additional injections of Hg^A-positive cells given i.m. with FCA, anti-Hg^A became detectable in the serum. Thus, some animals which appeared to be non-responders to Hg^A as judged by normal survival of Hg^A-positive red cells after i.v. injection alone showed some response when antigen was also given i.m. with adjuvant.

INTRODUCTION

Suppression of the primary response to Rh(D) in man by passively-administered antibody has become a routine procedure. However, many details concerning the process, such as the minimum effective dose of antibody and the maximum interval which can elapse between the injection of antigen and the successful injection of antibody, remain unknown. Since experiments in man are subject to many restrictions, it would be most helpful if a suitable animal model existed. The present study represents part of a programme to explore in rabbits the suitability of using responsiveness to the red cell alloantigen Hg^A as a model for responsiveness to the human antigen Rh(D).

MATERIALS AND METHODS

Animals

Adult Sandy lop rabbits of both sexes were used. They were obtained from an inbred colony kept at the MRC Radiobiological Unit, Harwell, and from Hylyne Commercial Rabbits, Cheshire. A few black Havana rabbits were used as red cell donors in some of the experiments.

Blood groups

Antisera provided by Mrs Valerie Joysey and Dr Carl Cohen, detecting the red cell antigens Hg^A, Hg^D and Hg^F (terminology of Cohen, 1958) were used. The majority of

the rabbits tested had red cells of the phenotype Hg(A-D-F+) and were of the presumed genotype Hg^FHg^F. For convenience, red cells of this group are designated FF and anti-Hg^A is referred to as anti-A. A few rabbits belonged to group AA or AF.

Sera from test animals were screened for antibody by one or more of the three methods described below. In all tests, red cells were suspended in 3·3 per cent magnesium sulphate to avoid non-specific agglutination (Heard, Hinde and Mynors, 1949).

1. For detection of antibodies capable of agglutinating untreated red cells suspended in magnesium sulphate solution, the ratio of serum to cells used was 100:1 and tests were read microscopically after incubation at 37° for 1 hour.

2. For incomplete antibodies, an indirect antiglobulin test was used with a specific anti-IgG reagent. The ratio of serum to cells was 20:1 and the mixture was incubated at 37° for 1½ hours. After washing, the results were read by a standard tile technique.

3. Serum was incubated at 37° for 5 minutes and papain-treated cells, also pre-warmed to 37°, were then added; the ratio of serum to cells was 20:1. Incubation was continued for 1 hour and results were read microscopically on heated slides.

The specificity of any antibody detected in the screening procedure was investigated by testing with a panel of red cells containing at least four A-positive and four A-negative samples.

Red cell survival

Donor red cells were labelled with ^{51}Cr using the citrate-wash method (Garby and Mollison, 1971). The amount of sodium chromate (^{51}Cr) added was such that each rabbit received about 20 μCi of ^{51}Cr; the amount of Cr per ml red cells was less than 2 μg. The washed, labelled cells were re-suspended in normal saline to give a suitable concentration for injection, depending upon the volume of cells being given. Cells were injected into a marginal vein of one ear and the first blood sample was taken into heparin 3–5 minutes later from the other ear. Further samples, each of about 1 ml, were taken at 24 hours, on two more occasions during the 1st week, twice during the 2nd week and once weekly thereafter. Sampling was discontinued when 10 per cent or less of the injected radioactivity remained. Samples were stored at 4° and those from individual rabbits were counted together at the end of each survival study. The blood was lysed with saponin and 0·5 ml volumes were pipetted into counting tubes. ^{51}Cr activity was measured in a standard gamma counter. For the earlier samples, sufficient counts were obtained to keep the counting error at <1 per cent; towards the end of each survival study, when remaining radioactivity was much lower, the counting error was about 3 per cent. The haemoglobin concentration of each sample was measured by the cyanmethaemoglobin method (Dacie and Lewis, 1968) and radioactivity was expressed as counts per minute per gram of haemoglobin. These results were converted to a percentage of the activity found in the first sample. No corrections were applied for Cr elution nor for sampling as all comparisons were made with data for autologous red cell survival, uncorrected for these factors.

Immunization using Freund's complete adjuvant (FCA)

An attempt was made to induce humoral antibody formation and a shortening of cell survival by injecting certain rabbits with allogeneic red cells and FCA. 0·5 ml of a 50 per cent suspension of washed red cells was mixed with 0·5 ml FCA and the mixture was injected intramuscularly (i.m.) into both hind quarters, and subcutaneously into the skin overlying the back of the neck.

Injection protocols

Five groups of A-negative (FF) rabbits were given A-positive (AA or AF) cells i.v. according to the protocol shown in Table 1. The size of the first dose varied from 0·01 to 1 ml per kg body weight. Subsequent doses were either 0·01 or 0·02 ml/kg (except in the two rabbits given 0·005 ml/kg or a total dose of 0·5 ml respectively), and were given at various times after the preceding dose of red cells had been cleared from the circulation. Some rabbits were given additional intravenous (i.v.) injections of red cells (accompanied in certain cases by the simultaneous administration of cells with adjuvant i.m. as described above). Details of these injections have been omitted from the table for the sake of simplicity but are given below (see the Results section).

TABLE 1

RED CELL INJECTION SCHEDULE

Group of rabbits	First injection		Second injection		Third injection	
	Number of recipients	Dose of red cells (ml/kg)	Number of recipients	Dose of red cells (ml/kg)	Number of recipients	Dose of red cells (ml/kg)
1	7	0·01	7	0·01	5	0·01
2	15	0·02	12	0·02	9	0·02
			1	0·005	1	0·02
			1	0·5*	1	0·02
3	15	0·04	15	0·02	13	0·02
4	5	1·0	4	0·01	4	0·01
5	9	0·5*	9	0·02	9	0·02
Total number injected	51		49		42	

* In these cases animals received a total dose of 0·5 ml red cells.

Samples for antibody screening were usually taken on more than one occasion, the first within a few days following clearance of the injected allogeneic red cells and the others within the next 30 days or so. Serum samples were also obtained before any injections had been given, for use as controls in antibody screening tests.

Description of red cell survival curves

Many of the survival curves showed a variable rate of removal of injected red cells from the circulation and thus could not be satisfactorily described by a simple parameter such as the T_{50} or the time taken for all the cells to be cleared. It was, however, possible to determine quite accurately the time at which accelerated disappearance from the circulation began by comparing each individual curve with data for autologous red cell survival in normal rabbits (Smith and Mollison, 1973). Survival was defined as subnormal (sn) when the percentage of allogeneic cells remaining in the circulation fell to 10 per cent or more below the normal lower limit for autologous cells (set at 2 s.d. below the mean). The time taken for survival thus to become subnormal, T_{sn}, was used to compare the responses observed in different groups of animals.

Estimation of serum IgG concentration

A single-phase radial diffusion method was used in which anti-rabbit IgG was included in the agar. A standard curve was prepared by estimating dilutions of a purified preparation of rabbit IgG, the protein concentration of which had been determined by measuring the optical density at 280 nm.

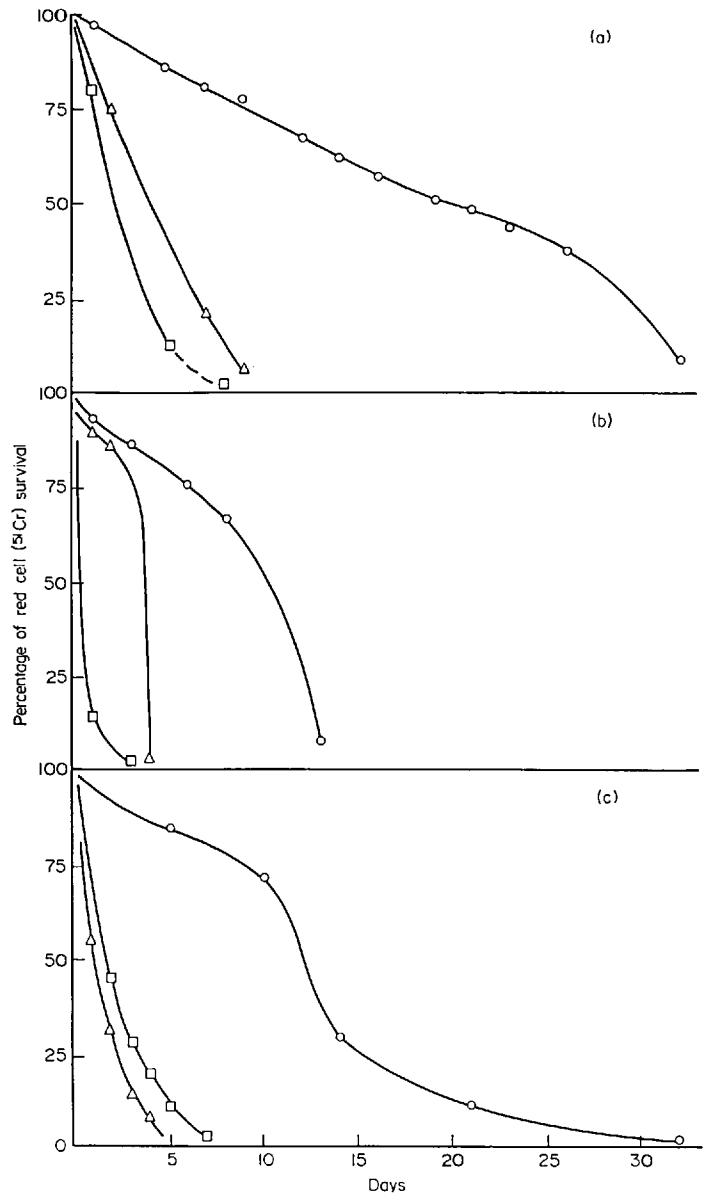


FIG. 1. Survival of three successive i.v. injections, (○)1, (△)2 and (□)3, of Hg^A-positive red cells in three different 'normally-responding' Hg^A-negative rabbits. In all three animals the first survival curve 'collapses' between 10 and 28 days after transfusion. In rabbits (a) R7 and (b) S52 the remaining cells are cleared rapidly but in rabbit (c) A66 the remaining cells are cleared slowly. All three rabbits show far more rapid clearance following a second injection; in R7 and S52 following a third injection there is even more rapid clearance but in A66 clearance is slightly less rapid than following a second injection.

RESULTS

Two main patterns of survival could be distinguished: in the first ('normal' responders), the survival of successive injections of red cells tended to become progressively shorter whereas in the second (non-responders or poor responders), survival was either normal throughout or else the survival of initial injections was subnormal and that of later injections normal.

'NORMAL' RESPONDERS

Forty-four out of fifty-one rabbits showed progressive shortening of survival; in forty-one of the forty-four, following the first injection of red cells, a 'collapse' curve was observed, that is, survival was initially normal, but after a period of between 3 and 33 days (mean about 17 days), there was an abrupt increase in the rate of disappearance of the injected cells; in the remaining three cases, the survival of the first injection was normal but following the second injection a collapse curve was observed. Fig. 1 shows three examples of collapse curves, with collapse occurring at about days 28, 10 and 12 respectively.

Of the forty-one rabbits which showed a collapse curve after their first injection, thirty-nine received a second injection; in all thirty-nine survival was again diminished and in thirty-seven cases T_{sn} was distinctly shorter after the second injection than after the first. Of the thirty-nine rabbits, thirty-five received a third injection; clearance was rapid in all cases, the longest T_{sn} being 5 days.

Following collapse of the survival curve, clearance of the remaining cells was relatively slow and could be described by a simple exponential in about 25 per cent of cases (Fig. 1c); in the others (Fig. 1b) clearance generally occurred more rapidly and the slope appeared to be approximately linear (but see below).

Anti-A was detectable serologically in only two out of twenty-one rabbits tested two to three times during the month after they had shown accelerated clearance of a first injection. Following the accelerated clearance of a second injection, anti-A was identified in a further seven cases. In eight other rabbits, antibody reacting with the donor's red cells was detected but the specificity was not determined; in three of these cases the antibody reacted with A-negative as well as with A-positive red cells. Following the third injection, anti-A was detected in a further seven out of seventeen rabbits tested. Thus, the cumulative incidence of serologically detectable antibody after three injections in the group of rabbits which showed progressive shortening of red cell survival was twenty-four out of thirty-four animals tested; in at least sixteen cases, the antibody was anti-A.

The maximum titre of anti-A, as judged by the agglutination of saline-suspended red cells, did not exceed 8. Results obtained with enzyme-treated red cells or with the indirect antiglobulin test did not differ significantly.

Of the ten (out of thirty-four) rabbits in which no antibody could be detected after three injections of red cells despite progressive shortening of red cell survival, three produced antibody after further injections of cells but the specificity of the antibody was not determined.

NON-RESPONDERS OR VERY POOR RESPONDERS

As mentioned above, in seven rabbits there was no progressive shortening of red cell survival following successive injections of red cells. Amongst these seven, three slightly

different patterns could be recognized. (a) Three rabbits (A77, S41 and R9) showed normal survival of three successive injections of red cells. Two of these animals (S41 and R9) were given further i.v. injections of labelled cells together with i.m. injections of unlabelled cells with FCA. Both animals ultimately produced anti-A. In addition, R9 produced an antibody which reacted weakly *in vitro* with one FF sample of red cells (from R20); when these cells were injected i.v. more than 90 per cent of a dose of 0·02 ml/kg was cleared within 4 days. Details of the injections of A-positive cells given to R9 are shown in Fig. 2; in this rabbit, antibody was detectable serologically only after red

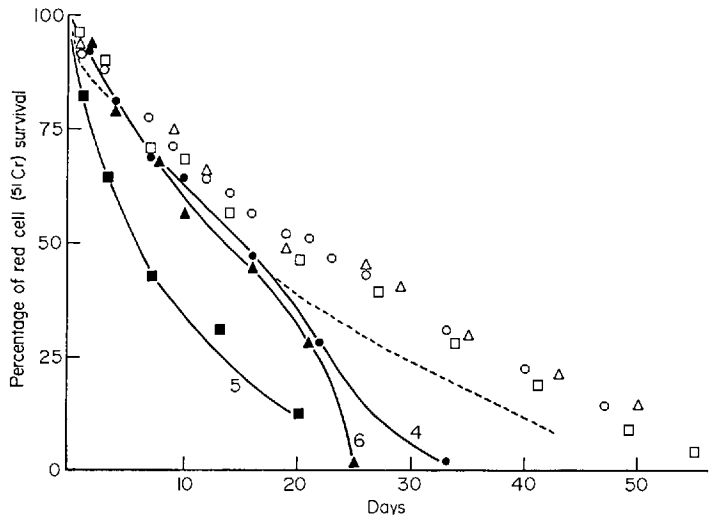


FIG. 2. Survival of six successive i.v. injections (○, △, □, ●, ■, ▲) of Hg^A-positive red cells in a poorly responding Hg^A-negative rabbit (R9). Additional injections of red cells, mixed with FCA, were given i.m. on the same day as the fourth injection (●) and 11 days after the sixth injection (▲). The interrupted line indicates the lower limit of normal survival for autologous red cells (see description of red cell survival curves).

cells had been injected six times i.v. and twice i.m. with FCA. (b) In three rabbits (S67, S44 and S82), survival following the first injection was definitely subnormal by day 10 but the rate of destruction thereafter returned to normal and in all three animals the survival of a second and third injection of cells was essentially normal. All three animals received a fourth dose of cells i.v. together with an i.m. dose of cells with FCA; in two animals (S44 and S67) the survival curves showed collapse and, after the clearance of the cells, serologically detectable anti-A was demonstrable. In the other rabbit (S82) the survival of the fourth injection of cells was normal but after a further i.v. injection of labelled cells together with a second i.m. injection of cells with FCA, survival was definitely impaired by day 15 and clearance was complete by day 30. No anti-A was detectable serologically and a sixth injection of cells given i.v. 2 months later showed almost normal survival. Results in S82 are shown in Fig. 3. (c) One rabbit (S94) at first appeared as if it were going to have an antibody response to the A antigen; following the first injection, the survival curve collapsed at about day 10 and following the second injection there was impaired survival from the outset and clearance was approximately exponential with a $T_{\frac{1}{2}}$ of about 4 days. On the other hand, following injections three and four, survival became progressively longer and following a fifth injection survival was only just below the limits of normal; see Fig. 4a. A sixth i.v. injection was accompanied

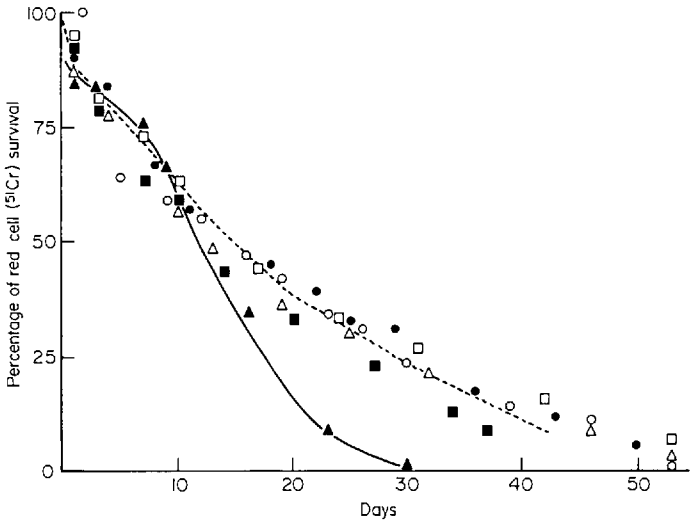


FIG. 3. Survival of six successive i.v. injections (○, △, □, ●, ▲, ■) of A-positive red cells in a poorly responding A-negative rabbit (S82). Additional injections of red cells, mixed with FCA, were given i.m. 11 days after the fourth injection (●) and on the same day as the fifth injection (▲). Anti-A was never demonstrated and a sixth injection (■) of A-positive red cells showed only slightly subnormal survival (T_{50}Cr 12 days). The interrupted line indicates the lower limit of normal survival for autologous red cells (see description of red cell survival curves).

by an i.m. injection of cells with FCA but survival was only slightly subnormal throughout. No antibody could be detected in the serum at any time. After a further 5 months, a seventh i.v. injection of cells was given. At day 10, when survival was strictly normal, an i.m. injection of cells with FCA was given; there was collapse of the survival curve at about day 26 and only 5 per cent of cells remained in the circulation on day 30 (Fig. 4b). Unfortunately, no serum was available for further antibody testing at this time.

COMPARISON OF RESPONSES TO DIFFERENT DOSES OF RED CELLS

In order to ascertain whether the use of different volumes of red cells for injection on the first occasion substantially altered the observed responses, red cell survival curves were analysed as follows. T_{sn} values from the forty-one rabbits exhibiting the collapse phenomenon were used. An analysis of variance was performed on all the first T_{sn} values for the different dose groups, and also on the second T_{sn} results. This indicated that the different groups given a first injection of cells were heterogeneous with respect to the first T_{sn}, but there was no obvious relationship between the dose of red cells and T_{sn}. There was no evidence of any statistical difference between the groups when second T_{sn} values were analysed.

With regard to the seven animals that failed to respond or responded only very poorly, their distribution between the different dosage groups studied showed no evidence of a dose effect ($P = >0{\cdot}5$, chi-square).

COMPARISON OF RESPONSES TO AA AND AF RED CELLS

These experiments were not specifically designed to compare the ability of homozy-

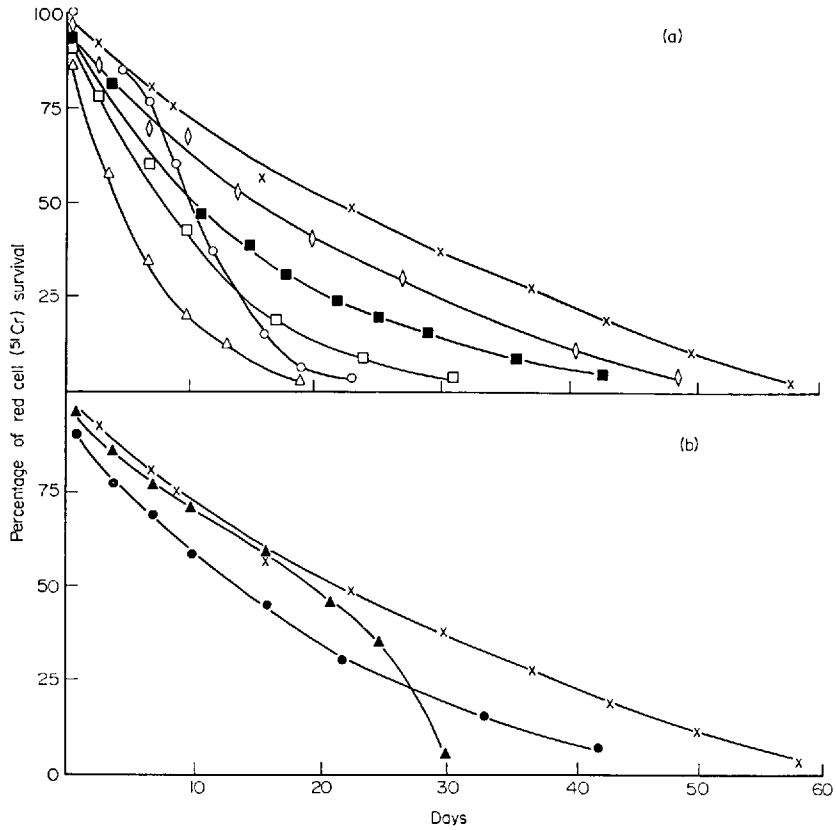


FIG. 4. Survival of seven successive i.v. injections (○, △, □, ■, ◊, ●, ▲) of A-positive red cells in a poorly responding A-negative rabbit (S94). The first injection was of 0·5 ml cells but all subsequent injections were of 0·02 ml cells/kg. (a) shows the survival of five successive i.v. injections of A-positive red cells. (b) shows the survival of two further i.v. injections of A-positive red cells; additional injections of cells, mixed with FCA, were given i.m. on the same day as the sixth injection (●) and 11 days after the seventh injection (▲). Survival of autologous red cells (×) was also measured during the course of these experiments.

gous and heterozygous red cells to induce immunization and only in a group given 0·02 ml per kg were both types of cell used. T_{sn} values in recipients of AA and AF cells in this group did not differ significantly ($P = 0{\cdot}21$, Student's t-test).

RELATIONSHIP BETWEEN ACCELERATED CLEARANCE AND ANTIBODY DETECTION

In those cases in which the serum was screened for antibody within a few days of the cells being cleared at an accelerated rate, the increased rate of clearance following 'collapse' was expressed in one of the two ways already described, depending upon whether rapid or slow clearance was observed. Antibody was detected in only two of the twenty-one animals showing slow clearance, but in thirty-one out of seventy-one with rapid clearance. Once rapid collapse had been established, the time taken for removal of the remaining cells varied from 1–8 days (mean 3·6±s.d. 1·4 days) in those cases with serologically detectable antibody within 1 month, and from 2–12 days (mean 4·9±s.d. 2·3 days) in those cases without detectable antibody within 1 month. There was thus

considerable overlap between the two groups but the populations were significantly different at the 5 per cent level (Student's *t*-test). In cases showing relatively rapid 'collapse', the apparently linear slope was thought to be due to a paucity of experimental points; in some cases the rate of clearance may have been accelerating progressively.

COMPARISON OF SERUM IGG CONCENTRATIONS IN 'RESPONDERS' AND 'NON-RESPONDERS'

Serum IgG concentrations were determined in eight of the A-negative rabbits showing a 'normal' response to successive injections of A-positive red cells, and in four of the rabbits classified as 'non-responders' or very poor 'responders'. Determinations were carried out on a single serum sample from each rabbit at varying times after the type of response to allogeneic red cells had been established. No significant difference in IgG concentrations between the two groups was demonstrated ($P>0{\cdot}8$, Student's *t*-test), the mean values being respectively 12·2 mg per ml (s.d. 1·3 mg) and 12·4 mg per ml (s.d. 1·7 mg) for 'responders' and 'non-responders', respectively.

DISCUSSION

Intravenous injections of A-positive (Hg^A-positive) red cells have been found to induce rather poor antibody responses in A-negative rabbits (Anderson, 1955; Cohen and Allton, 1962) although Pollack, Gorman, Hager, Freda and Tripodi (1968) found anti-Hg^A in eleven out of twenty-one rabbits after a single dose of 10^9 red cells (approximately 0·1 ml packed red cells). In the present work, only about 10 per cent of rabbits developed serologically detectable antibody after a single intravenous injection, usually of 0·02–0·04 ml cells/kg, and even after three injections most animals developed only weak antibodies (titres 1–4). On the other hand, as judged by 'collapse' of the first survival curve, forty-two out of fifty-one rabbits presumably formed antibody following their first injection and almost all these animals did in fact show progressively shorter survival following successive injections of A-positive red cells. Accelerated clearance of red cells in the absence of serologically detectable antibody is often seen in man when spaced injections of small amounts of Rh-positive cells are given to Rh-negative subjects (Woodrow, Finn and Krevans, 1969; Mollison, Hughes-Jones, Lindsay and Wessely, 1969). From experiments in which known small smounts of IgG anti-Rh were injected into Rh-negative subjects followed by injections of small volumes of ^{51}Cr-labelled Rh-positive red cells, it is known that the lowest concentration of anti-Rh which will bring about accelerated clearance of red cells is about ten times lower than the lowest concentration which can be detected serologically (Mollison and Hughes-Jones, 1967).

Similarly, it has been shown that in rabbits, passively administered IgM anti-Hg^A will bring about the clearance of small volumes of Hg^A-positive red cells with concentrations which are about ten times below the minimum which can be detected serologically (Burton and Mollison, 1968).

In the present series of experiments, following a first injection of A-positive red cells to an A-negative recipient, survival was initially normal (with a single exception among fifty-one cases) but after a period ranging from 3 to 33 days there was an abrupt acceleration of the rate of destruction (in forty-one out of fifty-one cases). Survival curves of this type were first described in dogs (Stohlman and Schneiderman, 1956) and are well

known in man (Adner, Foconi and Sjölin, 1963; Mollison, 1972). Although curves of this kind appear to indicate primary immunization, it is puzzling that, in man at least, further injections from the same donor to the same recipient are frequently not followed by the formation of detectable antibody. Moreover, in some recipients a progressive improvement in survival is noted with succeeding injections (Adner, Foconi and Sjölin, 1963; Holburn, Frame, Hughes-Jones and Mollison, 1971). In the present series of experiments there were several examples of this phenomenon (e.g. S94, Fig. 4).

In the present series, there were seven (out of fifty-one) A-negative rabbits which showed no progressive shortening of red cell survival following successive i.v. injections of A-positive red cells. As judged by a strictly normal survival of the second injection of A-positive red cells, six of the animals might be classified as non-responders to the A antigen. On the other hand, all of these animals showed impaired survival after i.m. injections of A-positive red cells with FCA. In two animals (e.g. S82, Fig. 3), it appeared that there was only a transient increase in the rate of red cell destruction and neither of these formed anti-A. In the remaining four animals, anti-A was demonstrated serologically after accelerated clearance of A-positive red cells. Strictly speaking, then, these animals should be classified as poor responders and this observation suggests the possibility that Rh-negative human subjects who are classified as non-responders because Rh-positive red cells injected i.v. survive normally in their circulation (Woodrow *et al.*, 1969; Mollison, Frame and Ross, 1970) may, similarly, really be poor responders who might form anti-Rh if given i.m. injections of Rh-positive red cells with FCA.

In a paper published after this work was completed, Woodrow, Elson and Donohoe (1973) demonstrated that the ability of A(Hg^A)-negative rabbits to form detectable anti-A after a single dose of red cells was genetically determined.

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No 3

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The Immune Response of Hg^A-negative Rabbits to Hg^A-positive Red Cells Treated with *N*-Ethylmaleimide

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Summary. Hg^A-positive red cells were treated *in vitro* with approximately 10 μmoles *N*-ethylmaleimide (NEM) per ml cells for 1 hour at 37° and then injected into the circulation of Hg^A-negative rabbits; all the cells were cleared within 24 hours. After 1–3 months the animals were given a series of injections of untreated Hg^A-positive red cells at 3-monthly intervals. Five out of seventeen rabbits showed progressively diminishing survival of Hg^A-positive red cells with the development of anti-Hg^A. On the other hand, in eight rabbits red cell survival following three consecutive injections of untreated red cells was normal or almost normal and in four further rabbits survival was initially subnormal but became progressively longer and was almost normal following a third injection of cells. Thus, in as many as twelve out of seventeen animals (70 per cent) the survival of Hg^A-positive red cells following a third injection of untreated cells was normal or almost normal whereas this pattern was previously observed in only seven out of fifty-one animals (14 per cent) not given an initial injection of NEM-treated cells. It is concluded that the injection of NEM-treated Hg^A-positive red cells to Hg^A-negative rabbits induces a degree of immunological tolerance to the Hg^A antigen.

INTRODUCTION

In the course of studying responses in rabbits to the red cell alloantigen Hg^A (referred to hereafter as A), the effect of injecting red cells treated with *N*-ethylmaleimide (NEM) was investigated. The main purpose was to see whether the rapid clearance of treated red cells from the circulation would be associated with a diminished immune response.

MATERIALS AND METHODS

The methods were the same as those used in the preceding paper (Smith and Mollison, 1974a), with the following additional procedures:

Treatment of red cells with N-*ethylmaleimide* (*NEM*)

One volume of a 50 per cent suspension of saline-suspended A-positive red cells (previously labelled with ^{51}Cr) was incubated with one half-volume of normal saline solution

containing 10 μmoles NEM per ml. The mixture was kept at 37° for 1 hour with continuous gentle shaking. The cells were then washed twice and resuspended in normal saline for injection. In some of the experiments different amounts of NEM were used and this is indicated in the appropriate sections below.

Effect of NEM treatment on the serological reactions of the red cells

The ability of AA and AF red cells to absorb anti-A from a potent antiserum was tested using untreated and NEM-treated red cells. FF red cells were used as a control. One volume of anti-A serum was incubated at 37° for 30 minutes with an equal volume of a 50 per cent suspension of AA red cells or with two volumes of a 50 per cent suspension of AF red cells. After absorption, the antiserum was tested for residual anti-A using the indirect antiglobulin test.

Injections of NEM-treated and untreated A-positive red cells

Seventeen previously uninjected A-negative rabbits were each given an i.v. injection of 0·5 ml of ^{51}Cr-labelled, NEM-treated A-positive red cells. Nine rabbits received cells from an AA donor and eight (injected on a later occasion) received cells from an AF donor. Between 1 and 3 months after the injection of NEM-treated cells, all animals were given an injection of 0·02 ml untreated ^{51}Cr-labelled A-positive red cells per kg body weight. Further injections of untreated red cells were given at varying times after the complete clearance of the preceding dose of red cells.

Description of red cell survival curves

The term 'T_{sn}', i.e. the time taken for survival of injected cells to become significantly subnormal, has been described fully in the previous paper (Smith and Mollison, 1974a); it has been used to describe results in the present experiments in those cases in which survival was initially normal, but was followed, after a period of some days, by a phase of accelerated destruction. When survival was throughout normal, or only slightly subnormal, it was expressed as the T_{50}Cr, i.e. the time taken for the Cr counts to fall to 50 per cent of the immediate post-injection value.

Sites of sequestration of NEM-treated red cells

Rabbits were injected with ^{51}Cr-labelled, NEM-treated red cells; within 24 hours of injection, at a time when 96 per cent or more of the injected radioactivity had been cleared from the circulation, the animals were killed. The spleen, liver and in some cases kidneys, small gut and long bones from the hind limbs were removed, dissolved in concentrated nitric acid and the radioactivity in aliquots of the digested tissue was estimated. At the same time, dilutions of the injection suspension were counted. From these measurements, the amount of red cells cleared by each of the tissues examined could be estimated. It was assumed that the amount of red cells cleared by the reticulo-endothelial tissue in the long bones was typical of that cleared by the reticulo-endothelial tissue in bone marrow as a whole; the fraction of total bone marrow present in the long bones was obtained from the data of Deitz (1944).

Injections of NEM-treated, A-positive red cells into rabbits already immunized to the A antigen

Two A-negative rabbits which had received injections of A-positive red cells and had formed anti-A were kept for several months, during which time the titre fell and ultimately

the antibody could no longer be detected. At this time, each animal was given an i.v. injection of 0·2 ml A-positive, NEM-treated cells. Samples of blood were taken on days 8 and 12 and tested for anti-A.

In another experiment, three A-negative rabbits that had responded to i.v. injections of untreated A-positive red cells by showing a progressive increase in the rate of clearance of three successive injections and had also produced weak, serologically detectable anti-A, were each given an i.v. injection of 0·5 ml A-positive NEM-treated cells. After 5 weeks, the survival of 0·02 ml/kg of ^{51}Cr-labelled A-positive untreated cells was studied.

RESULTS

EFFECT OF NEM ON CELLS *in vitro*

No reduction in the capacity to absorb anti-A was observed. Thus, the titre of anti-A was reduced from 2048 to 128 by untreated AA cells and to 64 by NEM-treated AA cells. Similarly, using AF cells, the titre after absorption was 64 both for treated and untreated cells.

Incidentally, under the conditions of NEM treatment chosen, the red cells beacme more resistant to lysis by hypotonic saline, as described by others (Jacob and Jandl, 1962; Ultmann and Gordon, 1965).

EFFECT OF NEM-TREATED CELLS IN 'PRIMED' RABBITS

In two rabbits which had previously had a trace of anti-A in their serum but had no detectable anti-A in a sample taken immediately before injection, anti-A was readily detected (titre 4–8) in the serum 8 days after the injection of NEM-treated, A-positive cells.

Three A-negative rabbits in which survival of A-positive red cells was impaired and in which anti-A had also been demonstrated in the serum, were each given an injection of NEM-treated, A-positive cells, followed 5 weeks later by an injection of untreated ^{51}Cr-labelled A-positive cells. The untreated cells were cleared more rapidly in one case (T_{50}Cr, 2·5 days before NEM-treated cells and 0·8 days 5 weeks later) and at essentially the same rate in another case (T_{50}Cr, 3·1 and 2·7 days respectively); in the remaining case, although clearance of the untreated cells occurred at a slightly lower rate following the NEM-treated cells (T_{50}Cr, 5·5 compared with the previous observation of 2·5 days), a further injection of A-positive cells given 6 months later again showed rapid clearance (T_{50}Cr, 2·3 days).

RATE OF CLEARANCE AND SITES OF SEQUESTRATION OF NEM-TREATED RED CELLS

Between 0·19 and 0·5 ml red cells, previously incubated with 5–14 μmoles NEM per ml cells, was injected into eight rabbits. There appeared to be an inverse correlation between the amount of NEM used in treatment and the rate of clearance of the cells. The longest $T_{\frac{1}{2}}$ observed (10 hours) was in a rabbit given 0·5 ml red cells treated with 5 μmoles NEM per ml cells; the shortest $T_{\frac{1}{2}}$ (0·7 hours) followed injection into another rabbit of 0·29 ml cells treated with 14 μmoles NEM per ml cells.

In four rabbits given between 0·19 and 0·34 red cells treated with 5, 8, 11 and 14 μmoles NEM per ml cells respectively, only about 20 per cent of the injected radioactivity was

recovered in spleen and liver combined at 24 hours, when less than 1 per cent of the activity was present in the circulation. These results prompted experiments with more extensive tissue sampling. Thus, in four further rabbits given 0·5 ml NEM-treated red cells and killed within 24 hours, at a time when 96 per cent or more of the injected radioactivity had been cleared from the circulation, bones, kidneys and small gut in addition to spleen and liver were tested for radioactivity. In one case 5 μmoles NEM and in the other three cases 10 μmoles NEM per ml red cells had been used. The total activity recovered from the tissues examined in these animals ranged from 80–112 per cent (mean 90 per cent). The largest proportion of the injected radioactivity was present in the bones and ranged from 52–67 per cent. The proportion in spleen and liver combined was 11, 24, 25 and 57 per cent respectively. Expressed in terms of radioactivity per gram of tissue, the amount in the spleen was greater by 51–264 times that in the liver. Insignificant amounts were found in the kidneys and small gut.

In order to ascertain whether the observed distribution of sequestered NEM-treated cells was a function of the volume of cells injected, two further experiments were performed in which the dose of cells (treated with 10 μmoles NEM per ml cells) was only 0·05 ml. The fraction of cells recovered from the bone marrow was 25 per cent and 61 per cent respectively, indicating that even with small doses of cells there was the same tendency for a substantial proportion to be sequestered in the bone marrow.

SURVIVAL OF UNTREATED RED CELLS IN RABBITS PREVIOUSLY INJECTED WITH NEM-TREATED CELLS

Three patterns of survival could be distinguished.

(1) *Progressive shortening of red cell survival.* Five rabbits exhibited accelerated clearance of the first injection of untreated allogeneic cells, with T_{sn} ranging from 2–30 days (mean 12·4, s.d. 11·3 days) and, with one exception, showed progressive shortening of T_{sn} with each successive injection of red cells. The exception (S97) had T_{sn} values of 5, 17 and 2 days and clearance of the first two of these injections was approximately exponential.

All five rabbits produced antibody against the donor red cells and in four the specificity was shown to be anti-A. Although serum from the fifth rabbit agglutinated only red cells from the donor used for injection, another sample of A-positive red cells (not agglutinated *in vitro*) was very rapidly destroyed *in vivo*.

(2) *Accelerated clearance followed by normal, or almost normal, survival.* In four rabbits, following the first injection of untreated red cells, the survival curves 'collapsed' on days 17, 17, 21 and 34 respectively. In three out of four cases, a second injection of untreated cells was cleared more rapidly than the first, with T_{sn} values of 3, 4 and 7 days; in the remaining rabbit, the second injection survived normally. In all four animals, normal, or almost normal, survival of a third injection of cells was observed (T_{50}Cr, 17 days in three cases and 15 days in the remaining case). A fourth i.v. injection of red cells from a different A-positive donor was given and, on day 11, when survival was still within normal limits, A-positive donor red cells and FCA were injected i.m. Between 1 and 3 weeks following the i.m. injections, the survival curves 'collapsed' in all four rabbits and specific anti-A could be demonstrated serologically for the first time; a fifth i.v. injection (of cells from the second donor) was cleared very rapidly in all 4 cases. In addition to anti-A, the sera from these four rabbits gave a weak reaction with one example of A-negative cells (R20) used in the typing panel. Furthermore, an i.v. injection of these A-negative

red cells was cleared from the circulation within 20 days in three out of the four rabbits (T_{50}Cr, 2, 3 and 10 days); in the fourth animal, very slight impairment of survival (T_{50}Cr, 14 days) was observed.

(3) *Normal, or almost normal, survival.* In eight rabbits, the survival of A-positive cells following i.v. injections was repeatedly normal or only slightly subnormal. Thus, the range of T_{50}Cr observed following the first and second injections was 14–23 days and 15–21 days respectively, compared with the range of 19–24 days previously found for autologous cells (Smith and Mollison, 1973). Survival curves following the third injection are shown in Fig. 1; T_{50}Cr was 17–20 days in six of the eight rabbits and was slightly subnormal in the remaining two (10 and 13 days).

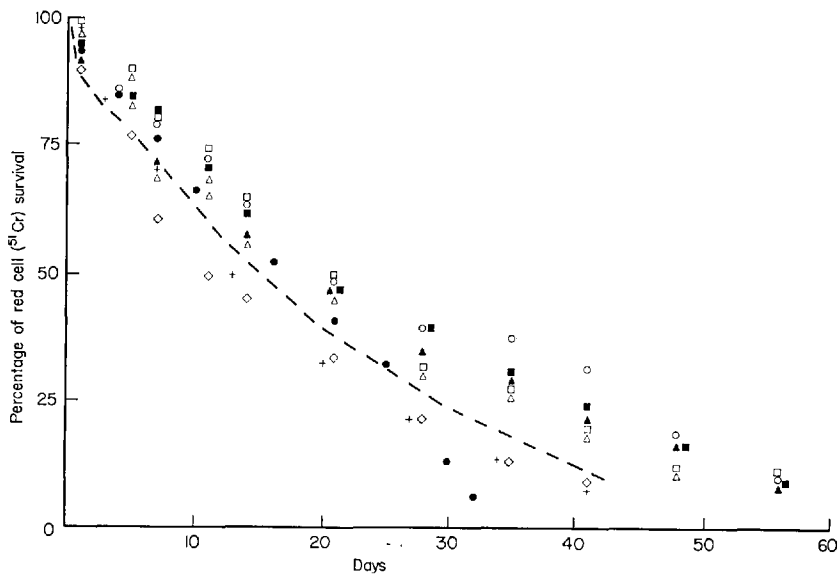


FIG. 1. Survival of a third injection of untreated, A-positive red cells (0·02 ml/kg) in rabbits given an initial injection of 0·5 ml A-positive red cells treated with 10 μmoles NEM/ml cells. In five rabbits (○, △, □, ▲, ■) survival was normal throughout. In one other rabbit (●) survival was normal at day 11 when an i.m. injection of donor cells mixed with FCA was given; the survival curve subsequently 'collapsed' at about 25 days. Two rabbits (◇, +) showed slightly subnormal survival of donor cells throughout. The interrupted line indicates the lower limit of normal survival of autologous cells (mean 25 days) observed in other rabbits (Smith and Mollison, 1973).

Following one i.m. injection of cells with FCA into six of these rabbits, serologically detectable anti-A was found in only one animal. Although antibody could not be detected *in vitro* in sera from the remaining five rabbits, the survival of intravenously-administered A-positive cells was impaired in all but one of these animals. Moreover, the rabbit showing normal survival of A-positive cells on this occasion, and one other given a second i.m. injection of cells with FCA, subsequently produced anti-A.

SERUM IgG CONCENTRATIONS IN 'RESPONDERS' AND 'NON-RESPONDERS'

The concentration of IgG in serum from six of the eight A-negative rabbits that had shown repeatedly normal, or almost normal, survival of A-positive red cells (pattern (3) in the preceding section) was determined and compared with values reported in the

previous paper for eight normal 'responders' that had not received NEM-treated red cells (Smith and Mollison, 1974a). Determinations were made on a single sample of serum obtained after the pattern of response to allogeneic red cells had been established. There was no significant difference between the mean values for the two groups ($P>0{\cdot}4$, Student's t-test), the values being 12·2 mg per ml (s.d. 1·3 mg) and 11·8 mg per ml (s.d. 0·8 mg) for 'responders' and 'non-responders' respectively.

DISCUSSION

As described in the preceding paper (Smith and Mollison, 1974a), when a series of i.v. injections of untreated A(Hg^A)-positive red cells was given to A-negative rabbits the commonest finding (in forty-four out of fifty-one animals) was progressive shortening of red cell survival followed, usually after two or three injections, by the appearance of serologically detectable anti-A. By contrast, in the present series in which the first injection given was of NEM-treated A-positive red cells, followed by injections of untreated A-positive cells, only five out of seventeen rabbits showed progressive shortening of red cell survival. This difference is highly significant ($P<0{\cdot}0005$, Student's t-test). It must be concluded that the injection of NEM-treated, A-positive red cells induced in the majority of rabbits a considerable degree of tolerance towards the A antigen lasting for many months at least.

In ten of the twelve rabbits in which survival of a third injection of untreated red cells (after an initial injection of NEM-treated red cells) was normal, or almost normal, a fourth injection of untreated red cells was given followed by an injection of FCA. Nine of the ten rabbits produced serologically detectable anti-A or showed an impaired survival of A-positive red cells and the tenth rabbit produced anti-A after a second injection of cells and FCA. In responding to the A antigen only after receiving FCA with A-positive cells, the rabbits in which tolerance was induced by NEM-treated red cells behaved like 'non-responder' rabbits given only untreated A-positive red cells (Smith and Mollison, 1947a).

No evidence was obtained that incubation of red cells with NEM in a dose of 10 μmoles per ml cells at 37° for 1 hour altered the A antigen. A-positive red cells treated in this way absorbed anti-A just as well as untreated red cells and they also elicited normal secondary antibody responses in 'primed' A-negative rabbits.

It seems unlikely that NEM-treated red cells caused generalized non-specific paralysis of immunocompetent cells since, in the present experiments, rabbits in which impaired responses to A had been induced remained in good health for periods of more than a year. Serum IgG levels also remained normal after induction of the unresponsive state. Furthermore, injection of NEM-treated, A-positive cells into rabbits already immunized to A did not significantly impair the responses to subsequent injections of untreated A-positive cells.

The induction of tolerance by NEM-treated, A-positive cells given before untreated cells is unlikely to be related merely to the rapid destruction of the first injection of antigen-carrying cells. Elson, Woodrow and Donohoe (1971) gave injections of 2×10^9 heated A-positive red cells (approximately 0·125 ml cells) to A-negative rabbits, following which the cells were removed from the circulation with a mean T_{50} of 15 minutes. Such injections were no less effective in inducing immune responses to the A antigen than were injections of untreated red cells. On the other hand, there have been several reports of

experiments suggesting that the site of antigen sequestration may affect humoral antibody response and even play a role in the induction of tolerance. Souhami (1972) showed that when an appropriate dose of colloidal carbon was given to mice, the subsequent phagocytosis of sheep red cells in the liver was depressed and a relatively large proportion of the cells was sequestered in the spleen. This change in the distribution of phagocytosed red cells was associated with an increase in antibody production when low doses of cells were given and a slight depression of antibody response when higher doses were used.

Depression of antibody responses by 'blockade' of the RES (and presumed alteration of the distribution of injected antigen) has also been reported in rabbits. Cruchaud (1968) induced 'blockade' of the RES in rabbits by injecting colloidal iron and found that, if bovine serum albumin (BSA) was then injected, humoral antibody production was depressed in all animals and tolerance to further injections of BSA, persisting for varying periods of time, was induced in four out of six rabbits. Moreover, this tolerance was specific for the antigen injected at the time of 'blockade'.

In the present experiments, it is possibly relevant that the NEM-treated red cells were rapidly removed from the circulation predominantly in the bone marrow. Removal of A-positive cells at comparable rates from the circulation of A-negative rabbits by passively injected IgG anti-A has been found to result in sequestration of cells predominantly in the spleen and liver; an increased incidence of unresponsiveness to subsequent injections of cells given without specific antibody has not been observed (Smith and Mollison, 1974c).

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No 4

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Suppression of Primary Immunization to the Rabbit Red Cell Alloantigen Hg^A by Passively Administered Anti-Hg^A

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Summary. Hg^A-negative rabbits were given an intravenous injection of Hg^A-positive red cells followed within 30 minutes by an intravenous injection of IgG anti-Hg^A. Several weeks later a second injection of Hg^A-positive red cells, without antibody, was given; the survival of these red cells was compared with the survival of red cells given to animals which had received no passive antibody. As judged by a relatively good survival of the second injection of red cells, primary immunization to Hg^A was at least partially suppressed when the dose of anti-Hg^A given with the first injection of red cells was such that 260–750 μg antibody was bound per ml red cells. In three groups of animals given Hg^A-positive cells and anti-Hg^A in doses such that 20–40 μg antibody was bound per ml red cells there was apparently no suppression of the antibody response.

It is concluded that the amount of specific IgG antibody required for the suppression of primary immunization is at least ten times greater, in terms of μg antibody per ml red cells, for the rabbit antigen Hg^A than for the human antigen Rh; the difference may be due partly to the greater number of Hg^A antigen sites on the rabbit red cell compared with the number of Rh(D) sites on the human red cell.

INTRODUCTION

In investigating the suppression of primary immunization to red cell antigens by passively administered antibody, two methods can be used to determine antibody formation, namely serological testing and estimation of the survival of red cells carrying the relevant antigen. When only low concentrations of antibody are formed in the primary response, estimation of the survival of incompatible red cells may be the only method of demonstrating that antibody has been formed.

Since, in the particular strain of rabbits used in the present work, only a minority of animals developed serologically detectable anti-A (Hg^A) after a single small injection of A(Hg^A)-positive red cells, the effect of passively administered anti-A on primary immunization by A-positive red cells was assessed by determining the survival of a subsequent injection of A-positive cells.

MATERIALS AND METHODS

Methods were the same as those used in the preceding papers (Smith and Mollison, 1974a, b), with the following additional procedures.

Anti-A(Hg^A) immunoglobulin

A preparation of purified IgG containing 975 μg anti-A per ml and having an indirect antiglobulin titre of 50,000 was used. The anti-A was found to have an equilibrium constant (K) of $1{\cdot}28 \times 10^8$ l/mole and an index of heterogeneity (a) of 0·7 (Frame and Mollison, 1972).

Experimental groups of animals

Injections of cells and antibody given to several different groups of rabbits are summarized in Table 1. The intervals between injections were planned to be sufficient for

TABLE 1

SCHEDULE OF INJECTIONS OF A-POSITIVE* RED CELLS GIVEN TO A-NEGATIVE RABBITS

	Injections of red cells with antibody			Injections of red cells alone			
				First injection of cells alone		Second injection of cells alone	
Group	Number of recipients	Dose of red cells (ml/kg)	Dose of antibody μg/kg)	Number of recipients	Interval since previous injection (days)	Number of recipients	Interval since previous injection (days)
1	9	0·5 total	200 total	9	83	9	67
2	5	0·04	20	5	106	5	30
	4			4	91	4	34
3	1	0·04	4	1	91		
	4			4	55	2	100
4	5	0·04	2	5	91	—	—
5	4	0·02 (repeated after 126 days)	300	4	113	3	32
6	4	0·02 (repeated after 124 days)	30	4	107	4	32
7	4	0·02 (repeated after 125 days)	3	4	100	4	32
Total	40			40		31	

* AA cells in groups 1–4, AF cells in groups 5–7.

the level of passively administered antibody to decline to $<0{\cdot}001$ μg/ml plasma and to allow the complete elimination of any red cells injected previously. In groups 1–4, all injections were of AA cells, whereas in groups 5–7, AF cells were used throughout. A dose of 0·02 ml red cells per kg was used for all 'challenge' injections of cells alone.

Analysis of results of red cell survival studies

A total of forty rabbits received injections of A-positive cells and passive antibody followed by an injection of 0·02 ml red cells per kg alone (see Table 1). Five other animals, similarly treated, were excluded from analysis because the cells injected alone survived normally (see Results). Red cell survival curves were described in terms of the time, 'T_{sn}', required for survival to become definitely subnormal (Smith and Mollison, 1974a). The values for T_{sn} in any one group of rabbits were compared (Student's t-test) with values

obtained in control animals that had not been given passive antibody with the first injection (or first two injections in groups 5, 6, 7) of A-positive red cells. Thus, in group 1, results were compared with those obtained in a group of A-negative rabbits that had received an i.v. injection of 0·02 ml A-positive red cells per kg 83 days following a first i.v. injection of 0·5 ml cells. In addition, the results for each experimental group were compared with those in a group of A-negative rabbits receiving 0·02 ml A-positive cells per kg for the first time. Log transforms were used in the analysis of T_{sn} data.

Calculation of the amount of passive antibody bound to injected A-positive red cells

The amount of antibody bound to red cells, x mole, can be obtained from the following equation (Eisen and Siskind, 1964):

$$\frac{x}{Ab_T - x} = \left[K\,(Ag_T - x\right]^a$$

where Ab_T and Ag_T are the initial molar concentrations of injected antibody and antigen respectively, K is the equilibrium constant (l/mole) and a the index of heterogeneity of the antibody.

In applying the formula, it was assumed that antigen and antibody had come into equilibrium before the red cells were removed from the circulation. When the rate of red cell destruction was rapid ($T_{\frac{1}{2}}$ less than a few hours), it could be assumed that no appreciable amount of antibody had left the plasma and that the reaction took place entirely within the plasma. On the other hand, when red cell destruction was relatively slow ($T_{\frac{1}{2}}$ 1 day or more), a substantial amount of injected IgG must have left the plasma before red cell destruction was complete. In practice, calculations were made (a) assuming that the antibody was confined to the plasma and (b) assuming that it was distributed throughout the IgG space, to indicate the likely limits of the amounts of antibody bound to the cells at equilibrium.

RESULTS

RATE OF RED CELL CLEARANCE RELATED TO TITRE OF ANTI-A

In Fig. 1 the rate of clearance, in A-negative rabbits, of a dose of 0·01–0·02 ml A-positive red cells per kg is plotted against the titre of passively administered IgG anti-A in the plasma. Two sets of results are shown: first, those obtained in the present work following the injection of 3, 30 and 300 μg anti-A per kg and, second, those reported previously by Burton and Mollison (1968). The present results can be given as antibody concentrations (μg/ml) rather than as titres, if it is assumed that within 30 minutes of the i.v. injection of IgG anti-A there was no loss of antibody from the plasma and if the plasma volume is assumed to have been 34 ml/kg in all cases (the mean value for Sandy lop rabbits found by Smith (1973)). The predicted concentrations of anti-A after doses of 3, 30 and 300 μg anti-A per kg respectively would then be approximately 0·09, 0·9 and 9 μg anti-A per ml. The titres of anti-A actually found were 1, 16 and 128 respectively. These findings suggest that an indirect antiglobulin titre of 1 should correspond approximately to a concentration of 0·08 μg antibody per ml. This value is in fact similar to the mean value of 0·04 μg/ml reported by Frame and Mollison (1972).

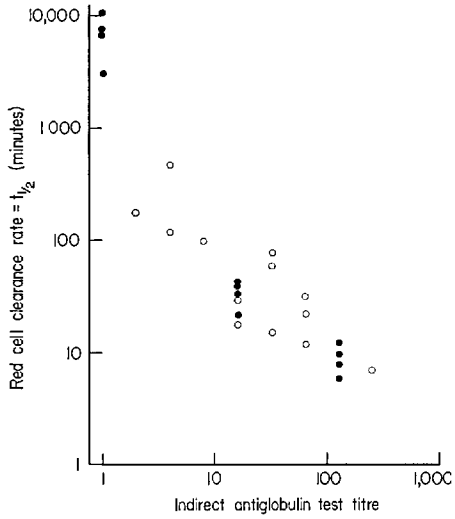


FIG. 1. Relationship between the rate of clearance of A-positive red cells (0·01–0·02 ml/kg) injected into A-negative rabbits and the prevailing titre of passively injected anti-A. (●) Values observed in rabbits in groups 5, 6 and 7 of the present experiments (given respectively 300, 30 and 3 μg/kg anti-A). (○) Results obtained using a different example of anti-A and published previously by Burton and Mollison (1968).

SITES OF SEQUESTRATION OF RED CELLS

In five A-negative rabbits given ^{51}Cr-labelled, A-positive red cells followed by a dose of IgG anti-A sufficient to destroy the cells with a $T_{\frac{1}{2}}$ of 1·2–2·7 hours in four cases (T_{50}, 12 hours in the remaining case), 52–86 per cent of the injected radioactivity (mean 64 per cent) was found in the liver and spleen combined and 4–28 per cent (mean 15 per cent) in the bones. Expressed in terms of activity per gram of tissue, the spleen contained between 13 and 175 times more than the liver. In these experiments, the mean total recovery of radioactivity from the tissues sampled was 82 per cent (range 65–104 per cent) of the injected dose. In two further cases in which A-positive cells were injected into animals actively immunized against the A antigen, the cells were cleared completely within 3 and 5 days, and 47 per cent and 59 per cent respectively of the radioactivity was found in the liver and spleen combined, with 3 per cent and 7 per cent respectively in the bones.

BINDING OF PASSIVE ANTIBODY TO A-POSITIVE RED CELLS

The proportion of A sites bound by anti-A at equilibrium is given by the molar ratio of bound antigen to total antigen. In the present experiments, it was calculated that the proportion of A sites on the injected cells bound by anti-A varied between 2 per cent when 6 μg antibody and 0·12 ml AA red cells were injected, and 96 per cent when injections were of 900 μg antibody and 0·06 ml AF red cells. In Table 2, the amount of antibody bound to A-positive red cells at equilibrium in the different groups of rabbits is expressed as μg/ml red cells.

The maximum concentration of antibody remaining in the plasma at the time of the first 'challenge' injection of red cells alone, even if it is assumed that no antibody was consumed during antibody-mediated destruction of the red cells given with the antibody,

TABLE 2

AMOUNTS OF ANTI-A AND A-POSITIVE* RED CELLS GIVEN TO A-NEGATIVE RABBITS, CALCULATED AMOUNT OF ANTIBODY BOUND AT EQUILIBRIUM AND RATES OF CLEARANCE OF RED CELLS

Group	Total amounts injected		Antibody bound (μg/ml cells)		Antigen sites bound (percentage)	Clearance rate ($T_{\frac{1}{2}}$)
	Antibody (μg)	Red cells (ml)	(a)†	(b)‡		
1	200	0·5	296	245	21·0 (a)	≯ 1 hour
2	60	0·12	260	187	18·0 (a)	≯ 2·5 hours
3	12	0·12	55	39	3·0 (b)	2–8 days
4	6	0·12	28	20	1·5 (b)	9–11 days
5	900	0·06	750	660	96·0 (a)	≯ 13 minutes
6	90	0·06	345	238	48·0 (a)	≯ 40 minutes
7	9	0·06	46	29	4·0 (b)	2–8 days

* AA cells in groups 1–4, AF cells in groups 5–7.
† (a) Assuming antibody confined to plasma.
‡ (b) Assuming antibody distributed throughout IgG space.

can be shown to be about 0·001 μg per ml (group 5). This concentration of antibody would bind less than 0·075 per cent of the A sites present on 0·06 ml AF red cells injected into a rabbit with a plasma volume of 100 ml. In group 5, with these conditions prevailing, there was no evidence of accelerated clearance immediately following injection of the first 'challenge' injection of red cells.

RESPONSES TO INJECTIONS OF RED CELLS ALONE

For any groups of A-negative rabbits injected with a specific dose of A-positive red cells and varying amounts of passive antibody, the mean T_{sn} of A-positive cells (0·02 ml/kg) subsequently injected alone appeared to be positively related to the amount of antibody given with the first injection(s) of cells. In all the groups, the mean T_{sn} value was significantly lower than that found in a group of A-negative rabbits given 0·02 ml A-positive cells per kg for the first time ($P<0·001$). On the other hand, when the mean T_{sn} results for each group were compared with those found in control rabbits which had previously received equivalent injections of cells without passive antibody, significantly higher T_{sn} values were found in groups 1 ($P<0·025$), 2 ($P<0·0005$), 5 ($P<0·0005$) and 6 ($P<0·05$). These results are shown graphically in Fig. 2.

A further injection of A-positive red cells was cleared more quickly than the first 'challenge' injection in twenty-eight out of thirty-one cases tested. In the three remaining cases, clearance of the first 'challenge' injection was very rapid with T_{sn} values of 1 day; following the second 'challenge' injection, T_{sn} values were 1, 2 and 3 days.

'NON-RESPONDERS' TO Hg^{A}

Following the injection of A-positive red cells and passive antibody into forty-five A-negative rabbits, survival of the first 'challenge' injection of A-positive cells alone was normal, or almost normal, in five animals. In one of these rabbits, further injections of A-positive red cells survived normally until, following an injection of donor cells mixed with FCA given i.m. at the same time as the third i.v. dose of cells alone, the survival curve 'collapsed' on about day 19. The remaining four rabbits were also considered to be

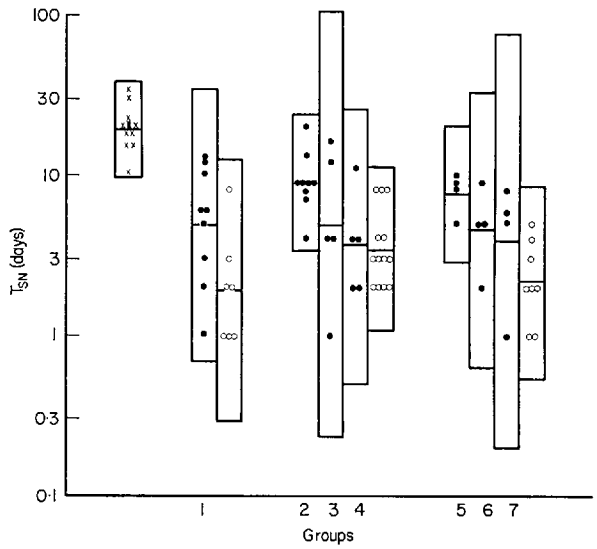


FIG. 2. Individual T_{sn} values observed following the first injection of A-positive red cells alone (0·02 ml/kg) to rabbits in groups 1–7, all of which had received a previous injection (two previous injections in groups 5–7) of A-positive red cells with anti-A (●). Details of preceding injections of cells and antibody given to these groups of rabbits are shown in Table 1. The T_{sn} values of groups 1–7 may be compared with those found in control A-negative animals following a first injection of A-positive red cells without antibody (×) or with those found following a series of injections of A-positive red cells (○) equivalent to those given to the corresponding experimental groups but without passive antibody. The horizontal lines in each column indicate 95 per cent confidence limits and mean values for each group.

examples of 'non-responders' although no further red cell survival studies were performed in them.

The incidence of 'non-responders' in the present experiments was therefore about 11 per cent (5/45) which is comparable with the figure of 14 per cent (7/51) reported previously for rabbits not given passive antibody with the first injection of red cells (Smith and Mollison, 1974a).

PRODUCTION OF SEROLOGICALLY DETECTABLE ANTIBODY

Following the first injection(s) of red cells given with passive antibody, sequential testing in several rabbits showed a decline in antibody titre until no antibody was detectable. In no case out of nineteen animals tested was any evidence obtained by serological tests of active immunization following injections of cells given with passive antibody.

Sera from twenty-seven rabbits were obtained on several occasions during the month following clearance of a first i.v. injection of red cells given alone. Anti-A was detected in six of these rabbits; in one other animal, an antibody reacting with donor cells was found but the specificity was not determined.

Following a second i.v. injection of cells alone, sera from twenty rabbits that had not had antibody present following the previous injection were tested. Eight out of twenty rabbits developed antibody reacting with donor red cells and in seven cases this was shown to be anti-A. Thus, the cumulative incidence of serologically detectable antibody in twenty-seven rabbits following two 'challenge' injections of A-positive cells in the present experiments was 15 (56 per cent).

DISCUSSION

The relation between the serum concentration of anti-A and the rate of clearance of a small dose of A-positive red cells can be deduced from the present data. As Fig. 1 shows, with plasma concentrations of approximately 9·0, 0·9 and 0·09 μg antibody per ml, the $T_{\frac{1}{2}}$ of clearance of a dose of 0·02 ml A-positive cells/kg was approximately 10 minutes, 40 minutes and 8000 minutes (5 days) respectively. Assuming that in the first two cases all the injected antibody was within the plasma space but that in the third case it had been distributed throughout the IgG space, the amount of antibody bound to the cells at equilibrium in the three cases was approximately 750, 350 and 30 μg/ml respectively.

Evidently, IgG anti-A(Hg^{A}) is relatively far less efficient in bringing about red cell clearance (in the rabbit) than is IgG anti-D in man, as discussed previously (Mollison, 1970).

The rates of clearance observed in the present work in relation to the amount of antibody injected do not agree very well with those reported by Pollack, Gorman, Hager, Freda and Tripodi (1968) and Elson, Woodrow and Donohoe (1971). The first of these authors injected 40 μg anti-A with 10^{9} A-positive red cells (i.e. approximately 0·03 ml cells/kg in a rabbit weighing 3·3 kg) and found that the cells were cleared with a $T_{50}Cr$ of 7·4 days. Similarly, Elson *et al.* (1971) gave 80 μg anti-A with 2×10^{9} cells and found that the $T_{50}Cr$ was 120 hours (5 days). The amount of anti-A bound to the cells at equilibrium in these two series, assuming that the equilibrium constant of different examples of anti-A is much the same and that in these cases the injected antibody was distributed throughout the IgG space at the time when equilibrium was attained, can be calculated to be approximately 120 and 190 μg per ml respectively. The dose of red cells injected in groups 5–7 of the present series and in these other two series was within the range of approximately 0·02 ml–0·06 ml cells/kg, and it would therefore be expected that the main factor determining the rate of clearance would be the amount of antibody bound to the cells rather than any overloading of the RE system. It is therefore not clear why in the present series cells coated with approximately 30 μg antibody per ml were cleared as rapidly as cells which in the other two series appear to have been coated with 120–190 μg per ml. The discrepancy raises the possibility that the estimates of antibody concentration were incorrect either in the present series or in the two previous series, although it is of course possible that the antibody used in the present work was relatively more effective in bringing about red cell clearance. The objection to this latter explanation is that, as shown in Fig. 1, the relation between the antibody titre in the plasma and the rate of clearance was similar for two different examples of anti-A.

At first sight, there appears to be another discrepancy between the present and previous observations, namely in the efficiency with which primary immunization was suppressed by a given dose of antibody, but it must be emphasized that different criteria were used to assess immunosuppression. In the observations published by Pollack *et al.* (1968) and Elson *et al.* (1971), the criterion of suppression of primary immunization was failure to detect antibody by serological tests; it is possible that antibody formation was only partially suppressed and that this might have been revealed by estimating the survival of Hg^{A}-positive red cells.

In the present observations, the criterion of complete suppression of primary immunization was a survival curve of a subsequent injection of A-positive red cells which was indistinguishable from a first survival curve in a previously uninjected rabbit. However, it

is possible that, following a second injection of A-positive cells, some shortening of red cell survival may indicate immunization to an antigen other than A. In fact, in the preceding paper (Smith and Mollison, 1974b), examples were given of the impaired survival of A-negative red cells in A-negative rabbits previously transfused with A-positive red cells. Thus, when A-positive red cells are injected together with anti-A, the impaired survival of a subsequent injection of A-positive red cells, when compared with that of a first injection of A-positive red cells in a previously uninjected animal, may be due to the formation of an antibody other than anti-A and is compatible with the complete suppression of the response to A. Conversely, when A-positive cells are injected with anti-A and the survival of a subsequent injection of A-positive red cells alone is compared with that of a second injection of A-positive red cells in an animal not given anti-A with the first injection, any improvement in survival indicates that the formation of anti-A has been at least partially suppressed and is consistent with the possibility that immunization to this antigen has been totally suppressed.

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