IN VITRO HISTOCOMPATIBILITY GROUPING

IN THE RABBIT

A Ph.D. Thesis to be submitted to the
University of London

Leonard Black, B.V.Sc., M.R.C.V.S.,
Surgical Unit, St. Mary's Hospital, London, W.2.

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ABSTRACT

A method for assessing histocompatibility has been described based on the matching of the leucocyte antigens demonstrable by cytotoxic antisera.

The dye exclusion test was used to manifest cytotoxicity and the factors affecting this technique were investigated. In heat-damaged suspensions stained counts were influenced by the cell type, cell concentration, staining time, incubation, dye concentration and total serum concentration. Undamaged suspensions were also affected by these factors but to a lesser extent by incubation and staining time. Counts were higher in damaged than undamaged suspensions except at the extreme dye concentrations, serum concentrations and staining times when the counts became similar.

With other known variables held constant, stained counts depended upon the concentrations of toxic antiserum, cells and complement. The toxicity of an antiserum depended upon the mode of preparation (i.e. the number, timing and type of stimuli).

A panel of iso-antisera, prepared by spleen cell inoculations, was used to screen the leucocytes of 52 rabbits. Most antisera were of narrow specificity and appeared to demonstrate antigens controlled at one genetic locus only and there were 3 or more alleles which could occupy this locus.

Every pair within batches of 7 to 12 rabbits was given an index number according to the similarity of the donor and host reactions with the antisera. Recipients were then grafted with skin from 2 to 6 donors in the same batch. Altogether 43 recipients received 137 grafts.

On 30 of 43 recipients (70%) the graft preselected as best survived as long or longer than the other on that animal and there was a significant correlation between the graft rejection orders and the serological assessments ($P < 0.02$).

This suggested that the locus which had been serologically demonstrated was strongly concerned in histocompatibility.

When each animal was retrospectively assigned a phenotype according to this locus, some recipients bore

grafts from compatible and incompatible donors. On 13 of the 14 recipients where the rejection times of the grafts on each differed by 2 days or more, a compatible graft lasted as long or longer than any other on the same individual.

It is suggested that panels of cytotoxic antisera may be of value in histocompatibility testing.

The implications and shortcomings of the method and its applicability to man have been discussed.

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PREFACE

The selection of the most suitable donor for homografting is important because the immune reaction which is responsible for the rejection of foreign tissues is most easily abrogated where histocompatibility differences are slight. Therapy aimed at the prolongation of the survival times of allografts has met with variable success and this variability has been attributed to the different degrees of histo-incompatibility between donor-host pairs.

The choice of the most compatible donor for any given recipient might result in maximal response to immunosuppressive therapy and might enable the dosage of the immunosuppressive agents to be held at relatively non-toxic levels.

In vivo techniques have successfully indicated histocompatibility relationships in laboratory animals but in man these techniques have been unreliable. *In vivo* methods are not ideal for use in man because

of the possibility of transferring pathogens and of sensitising the participants to tissue antigens. This might impair the survival of a homograft if such should be required subsequently.

In this thesis an *in vitro* method of matching rabbit tissues for histocompatibility is described. Blood leucocytes have been compared according to their susceptibilities to the cytotoxic action of a panel of isoantisera. Subsequently skin grafts were exchanged between the individuals matched in this way in order to test whether the antigens demonstrated were mainly the important histocompatibility antigens.

The first section describes investigations into the technical variables which affect the results of the dye exclusion test used in the measurement of cytotoxicity. The second section is devoted to investigations on the variables influencing cell damage and the third section describes the histocompatibility test and its application.

S E C T I O N I

The Dye Exclusion Test for Cell Viability

CHAPTER I

REVIEW

The ability to exclude certain dyes is generally regarded as an indication of cell viability. Damage to cells renders their surfaces more permeable and dyes which are normally excluded, e.g. trypan blue, eosin, nigrosin, lissamine green, are able to penetrate and stain them. A count of the percentage of stained cells forms a convenient method of estimating the degree of damage to a suspension.

The ability of trypan blue to differentiate between viable and non-viable cells was first investigated (Pappenheimer, 1917) after it was noticed during supravital staining experiments that the nuclei of damaged cells stained diffusely, while the nuclei of undamaged cells remained unstained

(Gross, 1911; MacCurdy and Evans, 1912; Evans and Schuleman, 1914; Rous and Jones, 1916). Richter and MacDowell (1933), and many others used the method to study the viability of both normal and neoplastic cells. In 1936 Schrek noted that eosin and methylene blue could also act as indicators of cell viability.

More recently the use of a number of dyes for this purpose has been investigated, for example, trypan blue (Hoskins, Meynell and Sanders, 1956; Eaton, Scala and Jewell, 1959; Craven, 1960; Tennant, 1964; Engelfriet, 1965), eosin (Hoskins et al., 1956; Hanks and Wallace, 1958; Eaton et al., 1959; Black and Berenbaum, 1964), nigrosin (Kaltenbach, Kaltenbach and Lyons, 1958), lissamine green (Holmberg, 1961), erythrosin B (Phillips and Terryberry, 1957) and acridine orange (Tennant, 1964).

There were four main considerations in these investigations, namely, the clarity with which stained cells were differentiated from unstained, the toxicity of the dyes, factors affecting the stained cell counts and finally the similarity of the viability assessments according to this and other methods.

A. The Clarity with which Stained Cells were differentiated from Unstained

Stained and unstained cells could be clearly distinguished from one another with most of the dyes investigated but some workers (Hoskins et al., 1956; Hanks and Wallace, 1958; Eaton et al., 1959; Holmberg, 1961; Tennant, 1964) reported that eosin was not fully satisfactory in this respect. Here the cells were sometimes stained with graded intensities or else the proportion which were stained increased gradually with the staining time.

These difficulties may have been due to unsatisfactory conditions of the tests. For example, Phillips and Terryberry (1957) suggested that the indistinct staining of Krebs 2 ascitic carcinoma cells noted by Hoskins et al. (1956) may have been due to the high concentration of eosin used (0.05%). When this was reduced to 0.02% the staining was clear-cut. Hanks and Wallace (1958) noted unclear staining of mouse fibrocytes only at high concentrations of eosin or in the presence of low concentrations

of serum. At very low concentrations of this dye (0.025% or less) Tennant (1964) noted indistinct staining of L-cells and human epithelial cells grown in culture. There was an increase in the numbers of stained cells with the staining time. Eaton et al. (1959) reported that eosin at final concentration 0.3% stained Ehrlich ascites tumour cells with varying intensities. Here the counts were performed after short staining intervals (2 to 5 minutes). An increase in the number of stained cells with the staining time was also noted when trypan blue was used (Pappenheimer, 1917), and this was especially the case where the proportion of stained cells was high.

When different dyes were compared, Schrek (1936) noted better differentiation of stained and unstained cells with eosin than with methylene blue, and Phillips and Terryberry (1957) found that erythrosin B, used in conjunction with a green light filter, gave a better contrast than eosin. Hewitt (1953) preferred trypan blue to eosin "because it gave more definite staining at non-toxic levels".

When lissamine green (unstated concentration) was added drop by drop to a suspension of L-cells "until the medium was coloured", the stained and unstained cells could be more clearly differentiated than when eosin was used in a similar manner (Holmberg, 1961). Kaltenbach et al. (1958) screened more than 50 dyes and concluded that nigrosin gave the greatest contrast, although the dye concentrations and other details of this work were not given.

B. Toxicity

Trypan blue was non-toxic at concentration 0.1% to 0.2% in the presence or absence of serum (Tennant, 1964). This was judged by the ability of L-cells and human epithelial cells to grow in culture after exposure to this dye for up to 20 minutes.

Eosin Y was more toxic. Exposure of L-cells and human epithelial cells to eosin at any of the concentrations tested (0.0125% to 0.25%) for more than 5 minutes in the absence of serum resulted

in the death of the cells as judged by their failure to grow subsequently in culture. In the presence of serum, eosin concentrations of 0.0125% to 0.025% were non-toxic and the cells recovered completely even after 10 minutes' exposure (Tennant, 1964).

As the term 'cytotoxicity' implies any form of cell damage, it was validly applied in Tennant's work to the impairment of cellular reproductive integrity. However, not all cell functions are necessarily impaired to the same extent by a deleterious agent and it may be possible for a cell to maintain the semipermeable nature of its membrane in spite of impaired ability to grow in vivo or in vitro (Cassel, 1957; Eaton et al., 1959; Craven, 1960).

Nigrosin. The stained counts were almost unchanged (about 55%) after 150 minutes when ascites sarcoma or carcinoma cells which had been modified by temporarily suspending them in hypotonic media were stained with 0.2% to 0.5% nigrosin in absence of serum (Kaltenbach et al., 1958). The authors took this to indicate that the dye was not toxic.

Lissamine green was stated to be "quite non-toxic" (Holmberg, 1961). The dye was less toxic than trypan blue *in vivo* (Goldacre and Sylven, 1959), although the experimental details upon which these opinions were founded were not given.

C. The Factors affecting the Stained Cell Counts

Certain factors may influence the stained counts irrespective of the percentage of cells originally damaged. These include:

(i) The concentration of serum in the suspending medium. (Pappenheimer, 1917; Phillips and Terryberry, 1957; Black and Berenbaum, 1964; Tennant, 1964.) Other factors being the same, the presence of high serum concentrations in the medium usually resulted in low stained counts and low serum concentrations in high counts. However, Hanks and Wallace (1958) noted little difference in the stained counts of L-cells stained with 0.15% eosin in the presence of 0.2% and 20% serum.

(ii) The concentration of the dye. Other factors being equal, more cells were stained where eosin concentrations

were high (Black and Berenbaum, 1964), but Engelfriet (1965) found that in presence of 55% serum the proportions of trypan blue stained human leucocytes increased as the dye concentrations increased to 0.18%, but decreased again as the concentration was raised further.

(iii) The type of cell. Rabbit leucocytes could not exclude 0.21% eosin dye (final concentration) in media devoid of protein and all the cells were stained (Black and Berenbaum, 1964) whereas Ehrlich ascites tumour cells in a similar medium were able to exclude an even higher concentration of the dye (0.3%). Here only 1% of the cells were stained (Eaton et al., 1959).

(iv) The staining time. When the cell suspensions were damaged the stained counts increased with time for the first 45 to 60 minutes when stained with erythrosin B (Phillips and Terryberry, 1957), eosin Y (Black and Berenbaum, 1964) and trypan blue (Pappenheimer, 1917). In the case of eosin and erythrosin B the counts remained relatively constant for at least 30 minutes after this. In undamaged

suspensions the staining time was less important and counts remained virtually unchanged for up to 90 minutes (Phillips and Terryberry, 1957; Black and Berenbaum, 1964). Engelfriet (1965) found that when trypan blue was incubated with cell-antiserum mixtures, the proportion stained increased during the first 30 minutes of staining, but then decreased. He ascribed this decrease to the lysis of damaged cells.

D. Comparisons between the Viability Assessments based on the Dye Exclusion Method and Other Tests

Fully viable cells may be defined as those capable of performing all their normal functions adequately. These functions may include the ability to grow and divide *in vivo* or *in vitro*, respiratory and metabolic functions, and the maintenance of the integrity of the cell membranes. Comparisons which were undertaken to check the accuracy of one or other of the tests usually consisted of carrying out the dye exclusion test in parallel with some other viability assessment, e.g. according to measurement of the growth

potential of malignant cells in vivo (Richter and MacDowell, 1933; Hewitt, 1953; Hoskins et al., 1956; Cassel, 1957; Eaton et al., 1959; Holmberg, 1961; Tennant, 1964) or in vitro (Craven, 1960; Tennant, 1964), the leak of radioactive K^+ from labelled cells (Klein and Perlman, 1963), the phagocytic ability of macrophages (Rous and Jones, 1916) or the various cellular metabolic activities such as respiration (Phillips and Terryberry, 1957; Eaton et al., 1959), ability to form lactic acid with glucose and substrate (Kaltenbach, 1956; Phillips and Terryberry, 1957) or dipeptidase, catheptic and glutathione activities (Holmberg, 1961).

The evaluation of the results is complicated because (a) technical variables not associated with the percentage of cells originally viable may affect the viability assessments. For example, the eosin concentration may influence the ability of cells to exclude dye (Black and Berenbaum, 1964), while the resistance of the host may influence assessments based on the growth of transplanted neoplasms (Hoskins et al., 1956). The results of any test

may have been as much a reflection of technical variables as of the proportion of damaged cells.

(b) Cell populations may vary in their amenability to study by different techniques. Hoskins et al. (1956) showed that with Krebs 2 ascites carcinoma suspensions, different methods gave assessments which varied between 17% and less than 0.1% viable. Sanders and Hoskins (1956) showed that monkey kidney cells which were able to multiply in vitro were all stained by eosin, and Stein and Gerarde (1950) showed that chick fibroblasts which grew vigorously in culture failed to reduce triphenyltetrazolium chloride.

(c) Each test demonstrated the integrity of certain special functions of the cells, for example, that of the cell membranes, the ability to divide, to respire, etc. In severely damaged suspensions all these functions may cease more or less simultaneously and here the results of different tests often correspond (Hoskins et al., 1956; Eaton et al., 1959). However, where the damage is less

severe some functions may fail while others remain at least temporarily intact (Kaltenbach, 1956; Eaton et al., 1959; Craven, 1960). The divergent results often observed between different viability assessments may reflect the relative sensitivity of the cell functions to the damaging regimen employed. For example, Eaton et al. (1959) showed that damage to ascites tumour cells by starvation, hypotonic suspension media, low O_2 tensions, cyanide, dinitrophenol, thyroxin, virus infections or unfavourable pH usually impaired first their ability to produce tumours in vivo, then their respiratory activity and after a variable interval their ability to exclude eosin. However, where the cells were damaged by Cl. welchii toxin they stained with eosin before their respiratory activity was impaired and it was suggested that this was due to early damage of the cell membranes by the lecithinase which this toxin contains.

These observations are supported by other workers (Hoskins et al., 1956; Cassel, 1957; Craven, 1960; Tennant, 1964), who found that cells which

seemed viable by dye exclusion tests were yet impaired in their capacity to produce tumours in susceptible hosts or to grow in culture after damage.

The work of Kaltenbach (1956) provides further evidence that the various cellular activities may function independently of one another. Ascites cells which were rendered permeable to nigrosin by temporarily suspending them in hypotonic media, were capable of glycolytic activity at a rate comparable to that of unmodified cells. On the other hand, L-cells became permeable to lissamine green only after their metabolic activities were severely impaired by freezing and thawing (Holmberg, 1961).

(d) Moderately damaged suspensions probably contain a heterologous population of severely damaged, partly damaged and undamaged cells, while severely damaged and intact suspensions contain relatively homogenous populations of functionless and fully functioning cells respectively. When tests which differentiate between the separate cell functions are applied to a range of suspensions damaged to varying degrees, they

might be expected to give divergent results in the moderately damaged suspensions and similar results in suspensions which are much or little damaged. This point is illustrated in the experiments of Hoskins et al. (1956) and Eaton et al. (1959). In Eaton's work, suspensions of Ehrlich ascites cells were damaged by exposing them to dinitrophenol or potassium cyanide for various periods up to 9 hours and then tested for viability according to their ability to exclude eosin and take up oxygen. The results of these two tests were divergent in the suspensions exposed to damage for the intermediate time ranges, but approached each other at the extremes.

Despite the limitations of the comparisons, some correlation was usually demonstrated between the different viability assessments. Where damaged suspensions were subjected to different tests those which were assessed as the more damaged by one technique were often so assessed by another (Richter and MacDowell, 1933; Phillips and Terryberry, 1957; Eaton et al., 1959). This correlation may have been

due to the fact that the severely damaged cells in the suspensions were detected with similar efficiency by the different techniques used.

CHAPTER II

MATERIALS AND METHODS

The animals were kept at St. Mary's Hospital Medical School, each species in a separate room.

Rabbits were outbred New Zealand Whites, Himalayan and crossbreds of various ages and both sexes, weighing 2 to 3 kg., from Ray Rabbitries, Lingfield, Surrey, and Glaxo Research Laboratories, Greenford, Middlesex. They were housed individually in standard galvanised iron cages 13" x 12" x 18" with wire mesh floors, and kept at temperature 65°F. They were fed diet SG.1, a small quantity of hay, and given water ad libitum.

Mice were one month old strain A2G of both sexes, obtained from Glaxo Research Farm, Harefield, Middlesex. They were housed in galvanised iron boxes 4½" x 6" x 13", six to a box and bedded on sawdust which was changed twice a week. Temperature was maintained at 70°F. They were fed diet 41.B and given water ad libitum.

Dogs were mongrels of both sexes, of various weights and ages. They were housed individually in metal cages 3' 8" square with wooden flooring. Temperature was maintained at 65°F. They were fed 1 to 1½ lbs. canned meat ("Pal" or "Lassie") and about 4 oz. biscuit meal ("Winalot") daily. Water was given ad libitum.

A. Mouse Lymphoid Cells

After killing mice with ether vapour the axillary and inguinal lymph nodes and thymuses were dissected out and placed in about 2 ml. Hank's Balanced Salt Solution. The tissues were teased apart with toothed dressing forceps and the suspensions were allowed to stand for a few minutes until the large tissue fragments either sank or floated to the surface. Those at the surface were removed and the suspensions were decanted to leave behind those at the bottom. The cells were counted and the suspensions were diluted as required with Hank's.

B. Rabbit Lymph Node Cells

Rabbits were anaesthetised with intravenous pentothal and the popliteal lymph nodes were excised.

After dissecting free of fat, the nodes were rinsed in Hank's and teased out in about 2 ml. Hank's. The cells were counted and suspensions diluted with Hank's as required.

C. Rabbit Leucocytes

Rabbit leucocytes were separated from whole blood by two alternative procedures:

(i) Albumin gradient method. This was a modification of the method of Spriggs and Alexander (1960). Twenty ml. of blood was taken from the marginal ear vein into 4 ml. 6% dextran in 0.9% saline (Dextraven, Benger Laboratories) and 1,000 units heparin (Heparin Injection BP.5,000 units per ml., Boots Pure Drug Co.). The mixture was allowed to settle in an upright 15 cm. by 1.5 cm. borosilicate glass conical centrifuge tube in a 37°C incubator for 1½ hours. The supernatant fluid was drawn off together with the topmost layer of cell deposit and centrifuged at 950 g. in a swing-out head for 20 minutes at 18°C to 20°C. The cell deposit was resuspended in 0.5 ml. of the supernatant. An albumin density gradient column was produced by layering 0.7 ml. of the supernatant

over 0.7 ml. concentrated bovine plasma albumin solution, prepared as described below, in a 9 mm. bore test tube and breaking down the interface by rapid perpendicular motions of a wire stirrer with a right angle bend at the end. The mixing usually took about 20 to 30 seconds and the correct degree of mixing was attained when there was a visible gradation of colour. The cell suspension was layered over the albumin gradient and the tube was centrifuged at 950 g. for 15 minutes at 18°C. The leucocytes, which lay in a band above the packed erythrocytes, were drawn off, washed twice in 0.15 M. saline and suspended in 2 ml. Hank's, or Hank's containing 10% serum from the cell donor. The suspension was counted and diluted as required. The preparation of the suspensions from time of bleeding until they were ready to be used usually took about 2½ hours.

The bovine plasma albumin solution was prepared by layering 25 G. Bovine Plasma Albumin (Fraction V, Armour Laboratories) over 33 ml. distilled water in a conical flask. This was kept for 2 days at 4°C and if any albumin had not dissolved a further

1 ml. distilled water was added at daily intervals until solution was completed. Two ml. 12.5% sodium bicarbonate solution was added and, if the pH was not neutral on the following day, small increments of the bicarbonate solution were added daily until neutrality was attained. A small volume of saline was then added to make the solution thin enough to draw into a pipette.

(ii) Sedimentation in dextran. This was a modification of the method of Jago (1956). Twenty ml. blood was taken from the marginal ear vein into 4 ml. 6% dextran in 0.9% saline (Dextraven, Benger Laboratories) and 750 units heparin (Heparin Injection BP. 5,000 units per ml., Boots Pure Drug Co.). The mixture was spun in a 15 cm. by 1.5 cm. borosilicate glass conical centrifuge tube at 405 g. at room temperature until the supernate appeared clear (usually 12 to 20 minutes). The buffy layer was drawn off and washed twice in 10 ml. 0.15 M. saline (spinning at 405 g. for 10 minutes), each time using only the cells drawn up from the buffy layer. These leucocytes tended to stick together and come off as a plaque. The leucocytes

were suspended in 2 ml. Hank's containing 10% fresh serum from the leucocyte donor. The suspension was counted and diluted as required with Hank's containing 10% fresh donor serum.

The albumin gradient method was used in the experiments of Section I. In Sections II and III the sedimentation method was preferred because this was simpler and the separation of leucocytes was at least as satisfactory.

D. Rabbit Peritoneal Macrophages

The peritoneal cavities of rabbits which had been injected intraperitoneally 4 days earlier with 40 ml. liquid paraffin (B.P.) were rinsed out under nembutal anaesthesia with about 60 ml. 0.2% ethyldiaminotetra-acetic acid (EDTA) in 0.15 M. saline. The cells in the harvested fluid were sedimented by spinning at 750 g. at 6°C for 10 minutes. The cells were washed twice in 0.2% ethyldiaminotetra-acetic acid in normal saline and resuspended in 10% autologous serum and 0.2% EDTA in normal saline. This fluid was also used to dilute the suspension to the required concentration of cells. Macrophages comprised

more than 90% of the leucocytes in these suspensions.

E. Rabbit Peritoneal Polymorphs

The peritoneal cavities of rabbits which had been injected intraperitoneally 4 hours earlier with 50 ml. 0.1% rabbit liver glycogen (British Drug Houses Ltd.) in 0.15 molar saline, were rinsed out under nembutal anaesthesia with about 60 ml. 0.2% ethyldiaminotetra-acetic acid in 0.15 M. saline. The cells were washed and suspended as described for peritoneal macrophages.

F. Dog Leucocytes

The leucocytes were separated using an albumin gradient as described for rabbit blood (paragraph C (i)).

G. Serum

Clotted blood was incubated at 37°C for 45 minutes and then spun at 1,200 g. at room temperature for 15 minutes. The supernatant serum was taken off and kept at 4°C until used.

H. Stain

Eosin Yellowish (John Gurr & Co.) was dissolved in Ringer's solution at the required concentration. Solutions were made up freshly on the day of a test.

J. Cell Counts

For estimating total numbers of leucocytes, the suspensions were diluted 20 times in white cell counting fluid. A well mixed aliquot was counted in a bright line (Lumiline) haemocytometer with improved Neubauer rulings.

For estimating the proportion of cells stained by eosin, an aliquot of undiluted suspension was taken up from the bottom of an undisturbed mixture of suspension and stain and run into the chamber of a haemocytometer. The cover slip was loosely attached and the chamber was overfilled so that the suspension overflowed into the bounding grooves. Counts were facilitated by using concentrated suspensions.

Each count was started at the edge and continued perpendicularly across both chambers of the haemocytometer. If half the required number

of cells was counted before reaching the central groove, the count was completed in the opposite chamber.

One hundred cells comprised each count in the experiments of Sections I and II, and 50 cells were counted in those of Section III. When there was any doubt about a count, e.g. if the leucocytes seemed excessively clumped or the proportion stained seemed to differ markedly in each half of the haemocytometer, the counts were repeated and the mean value calculated.

Three types of cell were observed (see Fig. 1):

(a) Erythrocytes were small, pale yellow and usually round, with refractile clearcut borders which were sometimes crenated.

(b) Unstained leucocytes were slightly larger, colourless and sometimes of irregular shape. Their borders were refractile. Their translucency, which was the most noticeable feature, was most marked when the lighting was maximal and the cells in focus.

(c) Stained leucocytes were larger than the others and

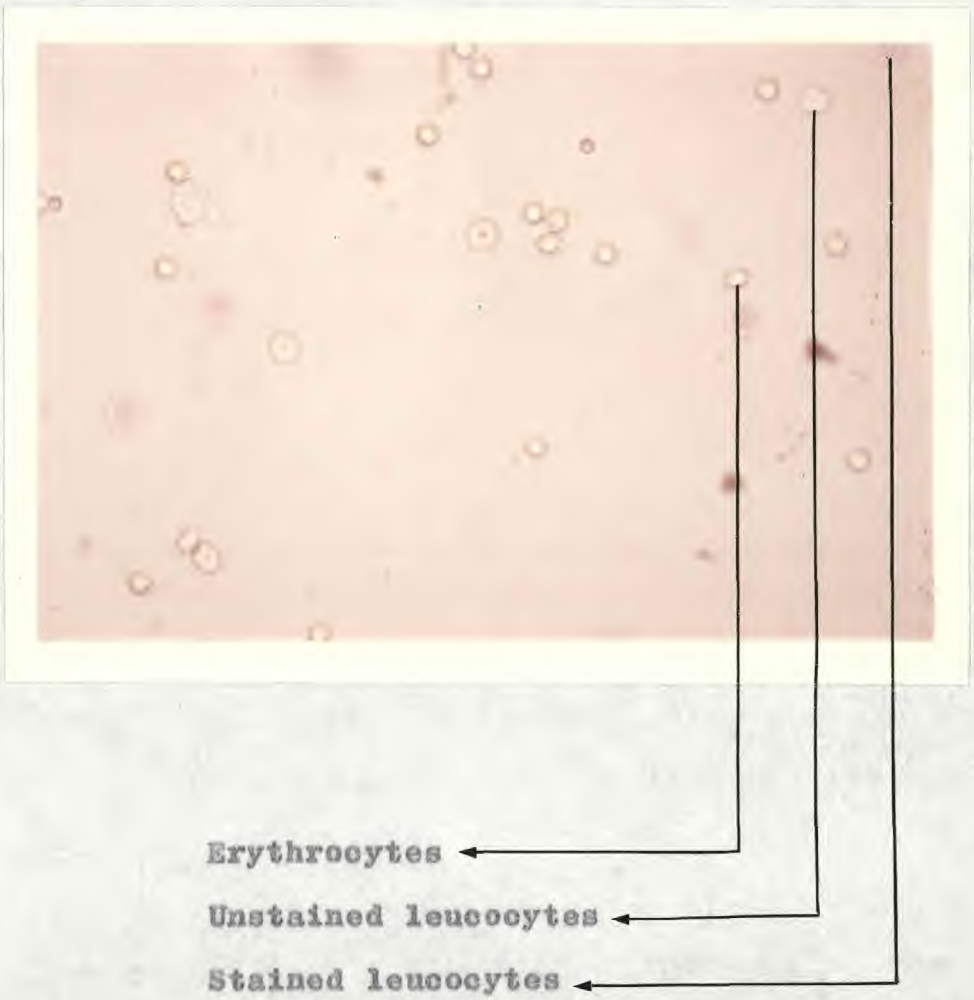


FIGURE 1

Rabbit leucocyte suspension 250 x magnified, stained with 0.21% eosin in presence of 8% autologous serum (final concentrations).
In reality the background staining was pale pink rather than mauve as it appears in this photograph.

their pink staining usually clearcut. The deep pink nucleus could usually be distinguished from a surrounding paler halo. Difficulty was seldom experienced in differentiating stained from unstained cells under optimal conditions, but intermediate staining was observed when the stain or serum concentrations were too high or too low. Slightly tinged cells were counted as stained.

Cell clumps were usually infrequent. Where they occurred the following rules were adopted.

1. If 100 cells were being counted,
   (i) the cells in clumps of up to 5 were counted as if unclumped.
   (ii) where all the cells in a clump of more than 5 were stained or unstained, only 5 were counted.
   (iii) where a clump of more than 5 consisted of stained and unstained cells, the clump was ignored.
2. Where 50 cells were being counted similar rules were adopted but here the maximum number counted in a clump was reduced to 3.

K. Damage

When damaged suspensions were compared with undamaged, the starting suspensions were divided into halves. A stoppered test tube containing one of these was placed in a waterbath at 45°C for 30 minutes while the 'undamaged' counterpart in a similar tube was placed in a 4°C refrigerator.

L. Mixing and Incubation

Mixing and incubation was carried out in the wells of a plastic World Health Organization agglutination tray. This was covered with a pad of moist tissue paper during incubation.

M. Washing the Apparatus

Glassware was unsiliconed and made from standard borosilicate pyrex. Glassware was soaked overnight in Haemosol (Heinicke) solution, 1 ounce to a gallon of water, brushed and rinsed copiously in warm tap water and then in 3 changes of distilled water and dried in a hot air oven.

The plastic agglutination trays were washed in a similar way but dried in a 37°C incubator.

CHAPTER III

SAMPLING

A preliminary investigation was carried out to establish whether the method of mixing and counting the cells in sample aliquots gave counts representative of the starting suspensions.

This was done by observing the repeatability of the counts in samples of various sizes drawn from

(i) the same suspensions, or

(ii) different suspensions all of which had been prepared similarly from the same stock of reagents.

It might be expected that in repeated counts on representative samples the variance would fall within established limits, while in non-representative samples the counts might vary widely.

REAGENTS

The following reagents were kept on ice during the experiment.

Mouse lymphoid cell suspension diluted to 5 x 10^6 cells/ml. in Hank's.

Rabbit serum diluted 1:4 in Hank's.

Dye : eosin Y 0.3% in Ringer's.

METHOD

Equal volumes (0.2 ml.) of each of the reagents were mixed together in a number of wells and the preparations were kept on ice for 1 hour. Samples were then drawn from the same or different mixtures and 200 cells counted each time. The proportion of stained cells was noted when the counts reached 50, 100 and 200.

RESULTS AND DISCUSSION

The results are shown in Table I.

(a) Samples from the same wells. When 50 cells were counted and 5 samples drawn from each well, the ranges were 5, 5, 13 and 8 for the samples from wells 1, 2, 3 and 4 respectively. When 100 were counted

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* on page 39:

S.D. = standard deviations. These were calculated using the mean percent of the counts of 200 cells as the ideal mean.

TABLE I

Final Concentrations: 1.67×10^6 cells
8% serum
0.1% eosin Y

Well No.	Number of Stained Cells in Counts of: 50 cells	100 cells	200 cells
1	15	35	45
	10	15	42
	12	24	52
	12	27	48
	15	24	56
Range	10 - 15	15 - 35	42 - 56
Mean	13	25	49
2	11	25	39
	11	29	47
	16	22	46
	12	29	38
	12	13	43
Range	11 - 16	13 - 29	38 - 47
Mean	12	24	43
3	8	29	37
	7	23	55
	20	29	49
	11	31	43
	8	22	51
Range	7 - 20	22 - 31	37 - 55
Mean	11	27	47
4	10	32	48
	16	22	51
	14	30	66
	10	23	51
	8	22	44
Range	8 - 16	22 - 32	44 - 66
Mean	12	26	52
$\bar{x}$	$\frac{\sum 2x}{n} = 23.8\%$	$\frac{\sum x}{n} = 25.3\%$	$\frac{\sum 0.5x}{n} = 23.8\%$
S.D.*	± 6.4%	± 5.5%	± 3.4%

the ranges were 20, 16, 9 and 10, and when 200 were counted the ranges were 14, 9, 18 and 22.

(b) Samples from different wells. The average proportions stained for all the cells counted in wells 1, 2, 3 and 4 were 24.3%, 21.3%, 23.5% and 26.0% respectively. These values fell within two standard deviations of the approximate ideal value (23.8 ± 1.35).

(c) Sample size. The means and standard deviations of all the 50 cell, 100 cell and 200 cell counts after each count had been brought to a percentage were 23.8% ± 6.58, 25.3% ± 5.46 and 23.8% ± 3.43 respectively.

Since ideal samples of K observations have variances $\propto \frac{1}{K}$, the variances of samples of 50, 100 and 200 cells should be in the proportions 4 : 2 : 1. The observed values were 43.3, 29.81 and 11.76, which approximates the expected ratio.

The moderate ranges in the counts on samples from the same wells (a), and the similarity of the mean counts in samples from different wells (b),

were not inconsistent with the hypothesis that the mixing and sampling technique resulted in counts which were representative of the starting suspensions.

CHAPTER IV

SERUM CONCENTRATION

The influence of serum concentration on the stained cell counts was investigated by counting the proportions stained in a series of mixtures which contained various concentrations of non-toxic serum. In each series the other known variables, e.g. stain concentration, cell concentration, species of origin of the cell and serum donors, staining time, were kept constant but these were sometimes varied from one series to another in order to establish whether the observations were generally applicable.

REAGENTS

Rabbit Cells

(i) 0.1 ml. damaged or undamaged leucocytes suspended in Hank's at 8.5×10^6 cells/ml.
0.96 ml. autologous serum or dilution in Hank's.
0.14 ml. 1.8% eosin Y in Ringer's.

(ii) 0.2 ml. undamaged leucocytes suspended in Hank's at 2.1 x 10^6 cells/ml.
0.2 ml. autologous serum or dilution in Hank's.
0.2 ml. 1.2% eosin Y in Ringer's.

(iii) 0.2 ml. cell suspension as above.
0.2 ml. serum as above.
0.2 ml. 0.3% eosin Y in Ringer's.

Mouse Cells

(iv) 0.2 ml. undamaged lymphoid cells suspended in Hank's at 2.1 x 10^6 cells /ml.
0.2 ml. rabbit serum or dilution in Hank's.
0.2 ml. 0.15% eosin Y in Ringer's.

(v) 0.2 ml. cell suspension as above.
0.2 ml. serum as above.
0.2 ml. 0.6% eosin Y in Ringer's.

METHOD

In each case the reagents were mixed together and left for 60 minutes on ice before counting. The experiments were repeated using cell-serum mixtures from different animals.

RESULTS AND DISCUSSION

The results are shown in Table II and Figures 2, 3 and 4.

TABLE II

Parallel columns show the results of repeated experiments and in Experiment 1 the columns below one another are the results of damaged and undamaged aliquots made from the same suspension.

Experiment 1 (see Figure 2)

Final Concentrations: 0.7×10^6 rabbit leucocytes/ml.
0 to 80% autologous serum
0.21% eosin Y

Serum Concentration	Damaged % Cells Stained					
0	96	98	90	98	94	100
2.5	-	-	-	27	50	64
5	62	-	-	36	56	46
10	46	62	42	28	50	46
20	42	70	26	34	40	44
40	22	40	0	34	-	-
60	0	6	0	-	-	-
80	-	0	0	-	-	-

Serum Concentration	Undamaged % Cells Stained					
0	96	100	100	98	100	100
2.5	-	-	-	6	6	6
5	20	-	-	0	8	6
10	2	34	26	2	14	12
20	2	12	20	0	14	6
40	0	0	0	0	0	0
60	0	0	0	-	-	-
80	-	0	0	-	-	-

Experiment 2 (see Figure 3)

Final Concentrations: 0.7×10^6 undamaged rabbit leucocytes/ml.

0 to 33% autologous serum

0.4% eosin Y

Serum Concentration %	% Cells Stained	
0	91	85
2.1	54	37
4.2	28	22
8.3	22	14
16.7	15	14
33.3	13	9

Experiment 3 (see Figure 3)

Final Concentrations: 0.7×10^6 undamaged rabbit leucocytes/ml.

0 to 33% autologous serum

0.1% eosin Y

Serum Concentration	% Cells Stained	
0	54	44
2.1	16	18
4.2	16	22
8.3	10	18
16.7	2	12
33.3	0	2

Experiment 4 (see Figure 4)

Final Concentrations: 0.7×10^6 mouse lymphoid cells/ml.

0 to 33% rabbit serum

0.05% eosin Y

Serum Concentration %	% Cells Stained	
0	31	58
2.1	20	37
4.2	25	29
8.3	25	12
16.7	7	0
33.3	1	0

Experiment 5 (see Figure 4)

Final Concentrations: 0.7×10^6 mouse lymphoid cells/ml.

0 to 33% rabbit serum

0.2% eosin Y

Serum Concentration	% Cells Stained		
0	100	70	84
2.1	72	63	25
4.2	77	69	33
8.3	76	80	68
16.7	90	82	76
33.3	89	70	40

When the serum concentrations were raised the proportion of stained cells decreased and vice versa.

This applied to damaged or undamaged rabbit leucocytes in autologous serum (Experiments 1, 2 and 3, Figures 2 and 3), and to mouse lymphoid cells in rabbit serum when stained with 0.05% eosin (Experiment 4, Figure 4).

At any given concentration of autologous serum more of the heat-damaged rabbit leucocytes were stained than were undamaged cells except at the extreme serum concentrations. Here the stained counts approached 100% and 0% at very low and very high serum concentrations respectively, whether or not the suspensions were damaged.

The proportions which stained at any given serum concentration were higher at the high than at the low eosin concentrations (Experiments 2 and 3, Figure 3).

In the case of mouse lymphoid cells mixed with rabbit serum, the counts behaved differently if the suspensions were stained with 0.05% or 0.2%

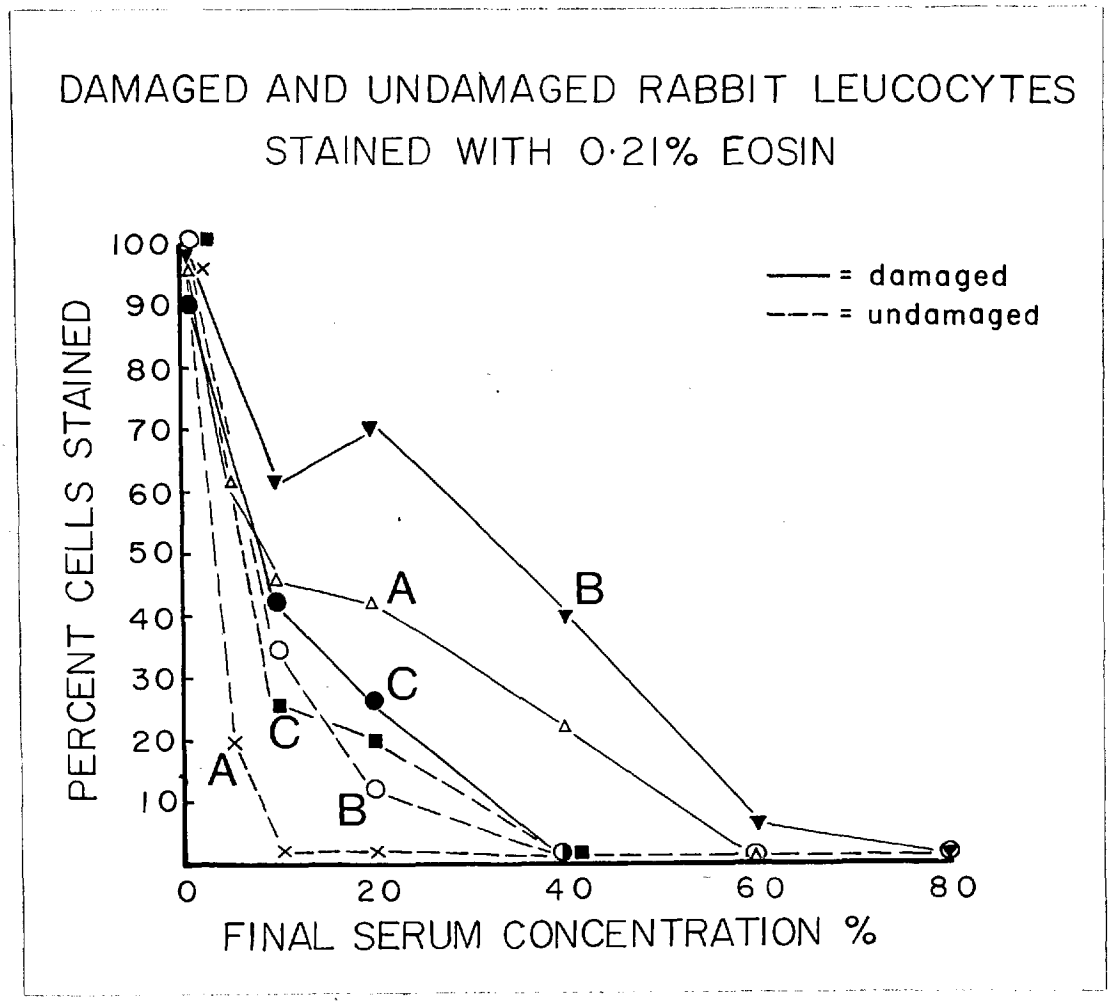


FIGURE 2

The effect of variation in the serum concentration. Lines marked with the same letter represent aliquots made from the same original suspension. Three of the six results are shown and these are the experiments where the serum concentrations were varied over the widest range. (See Table II, Experiment 1.)

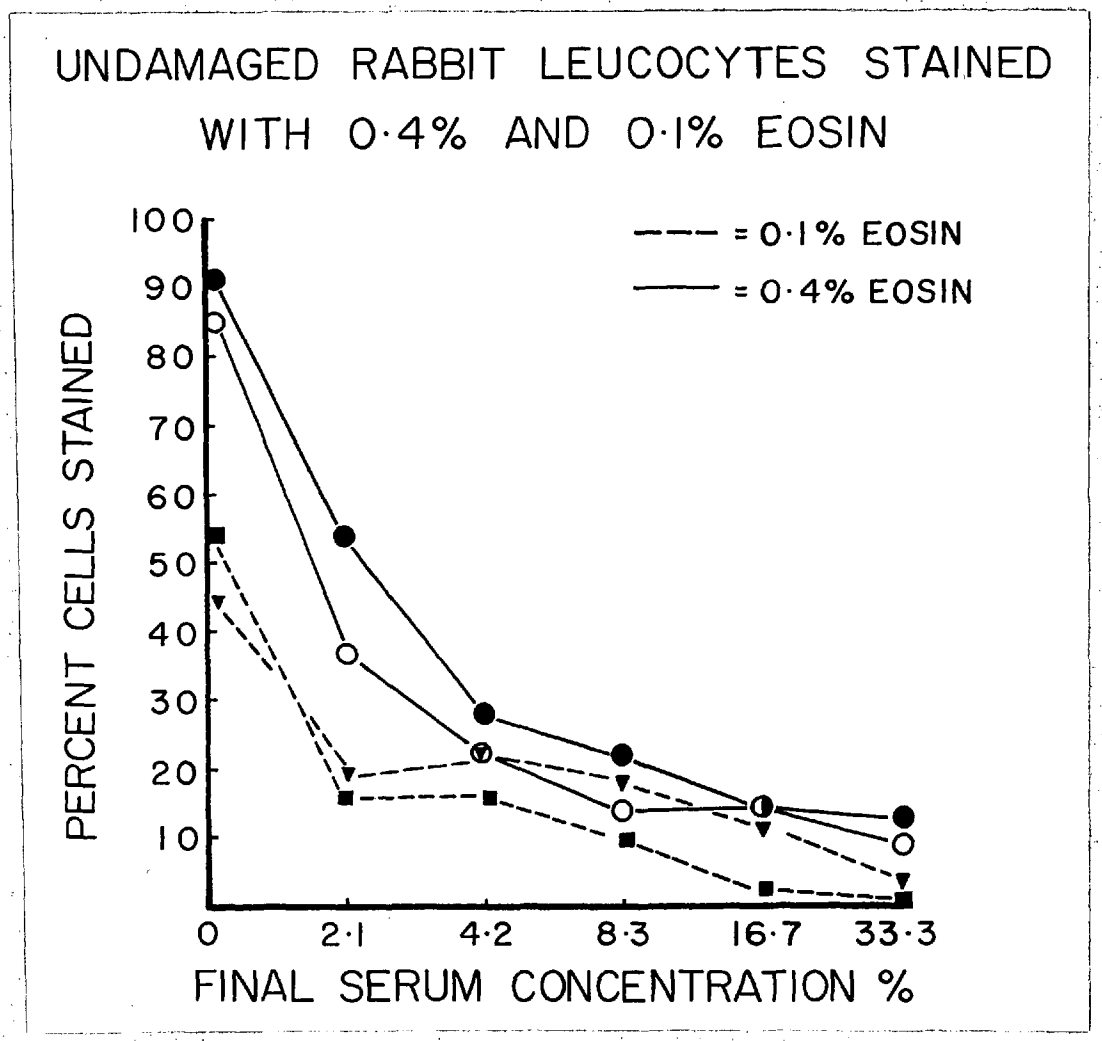


FIGURE 3

The effect of variation in the concentrations of autologous serum.
Solid lines : stained with 0.4% final concentration eosin.
Dotted lines: stained with 0.1% final concentration eosin.
(See Table II, Experiments 2 and 3.)

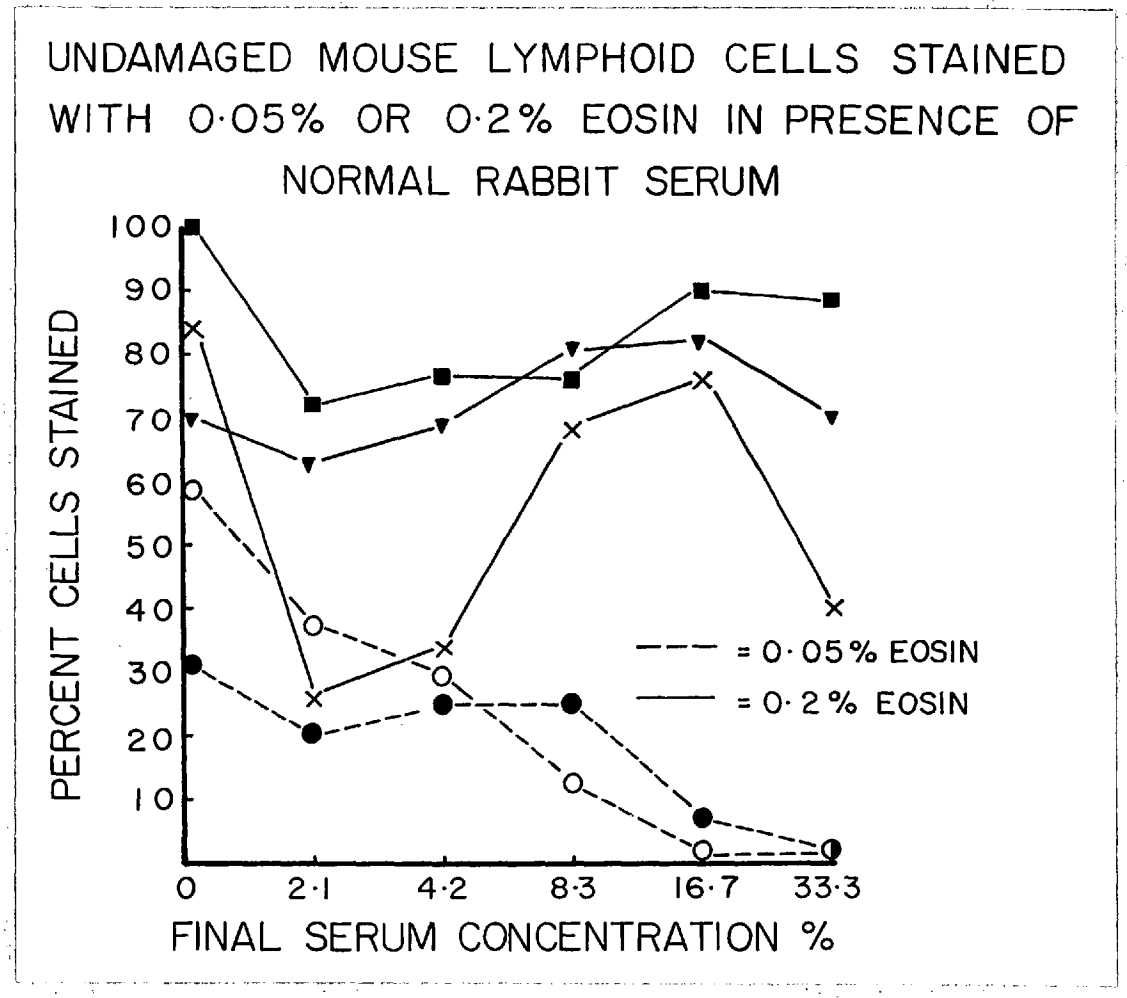


FIGURE 4

The effect of various concentrations of rabbit serum on mouse cells.
Solid lines : stained with 0.2% final concentration eosin.
Dotted lines : stained with 0.05% final concentration eosin.
(See Table II, Experiments 4 and 5.)

eosin (Figure 4). At 0.05% eosin the counts increased from 0% to 58% when the serum concentrations ranged between 33.3% and 0%.

When stained with 0.2% eosin there were 2 peaks in the stained counts, one at serum concentration 16.7% and another at 0% serum. Rabbit serum damages mouse cells (Terasaki, Esail, Cannon and Longmire, 1961b) and the peak at 16.7% may have been the result of toxic serum damage.

The interrelationships between the serum concentration, stain concentration and cell damage are considered further in Chapter VI.

CHAPTER V

STAIN CONCENTRATION

The influence of stain concentration was investigated by counting the proportion of stained cells in a series of preparations which contained a range of eosin concentrations, but were otherwise similar in respect of known variables.

Although these variables were held constant within a series, they were sometimes changed from one series to another to establish whether the observations were generally applicable.

The experiments were designed to assess the influence of eosin concentration on:

(i) damaged and undamaged rabbit leucocytes in presence of 8% autologous serum.

(ii) undamaged rabbit leucocytes at various serum concentrations.

(iii) damaged rabbit leucocytes at various serum concentrations.

(iv) rabbit leucocytes in absence of serum.

(v) damaged and undamaged dog leucocytes.

REAGENTS

(i) 0.1 ml. damaged or undamaged rabbit leucocytes suspended in Hank's at 8.5×10^6 cells/ml.
0.1 ml. autologous serum.
1 ml. eosin Y at various concentrations in Ringer's.

(ii) 0.1 ml. undamaged rabbit leucocytes in suspension as before.
0.48 ml. autologous serum or dilution in Hank's.
0.62 ml. eosin Y at various concentrations in Ringer's.

(iii) 0.1 ml. damaged rabbit leucocytes in suspension as before.
0.96 ml. autologous serum or dilution in Hank's.
0.14 ml. eosin Y at various concentrations in Ringer's.

(iv) 0.1 ml. damaged or undamaged rabbit leucocytes in suspension as before.
0.1 ml. Hank's.
1 ml. eosin Y at various concentrations in Ringer's.

(v) 0.1 ml. damaged or undamaged dog leucocytes suspended in Hank's at 8.5×10^6 cells/ml.

0.1 ml. autologous serum.

1 ml. eosin Y at various concentrations in Ringer's.

METHOD

The reagents were mixed together in each case and kept on ice for one hour before counting the proportions of stained cells. The experiments were repeated using cell-serum mixtures from different animals.

RESULTS AND DISCUSSION

(i) Damaged and undamaged rabbit leucocytes in presence of 8% serum

TABLE III (see Figure 5) The results of 4 repeated experiments

Final Concentrations: 0.7×10^6 cells/ml.
8% serum
2.5 to 0.01% eosin Y

Eosin Concentration %	% Cells Stained							
	D*	UD*	D	UD	D	UD	D	UD
0.01	0	0	50	0	0	0	0	0
0.2	86	0	92	6	94	18	76	20
0.5	72	20	94	14	92	10	78	18
1.0	86	54	100	62	96	38	82	64
1.7	86	84	100	74	100	18	88	88
2.5	92	98	100	92	100	100	96	94

* D = damaged
UD = undamaged

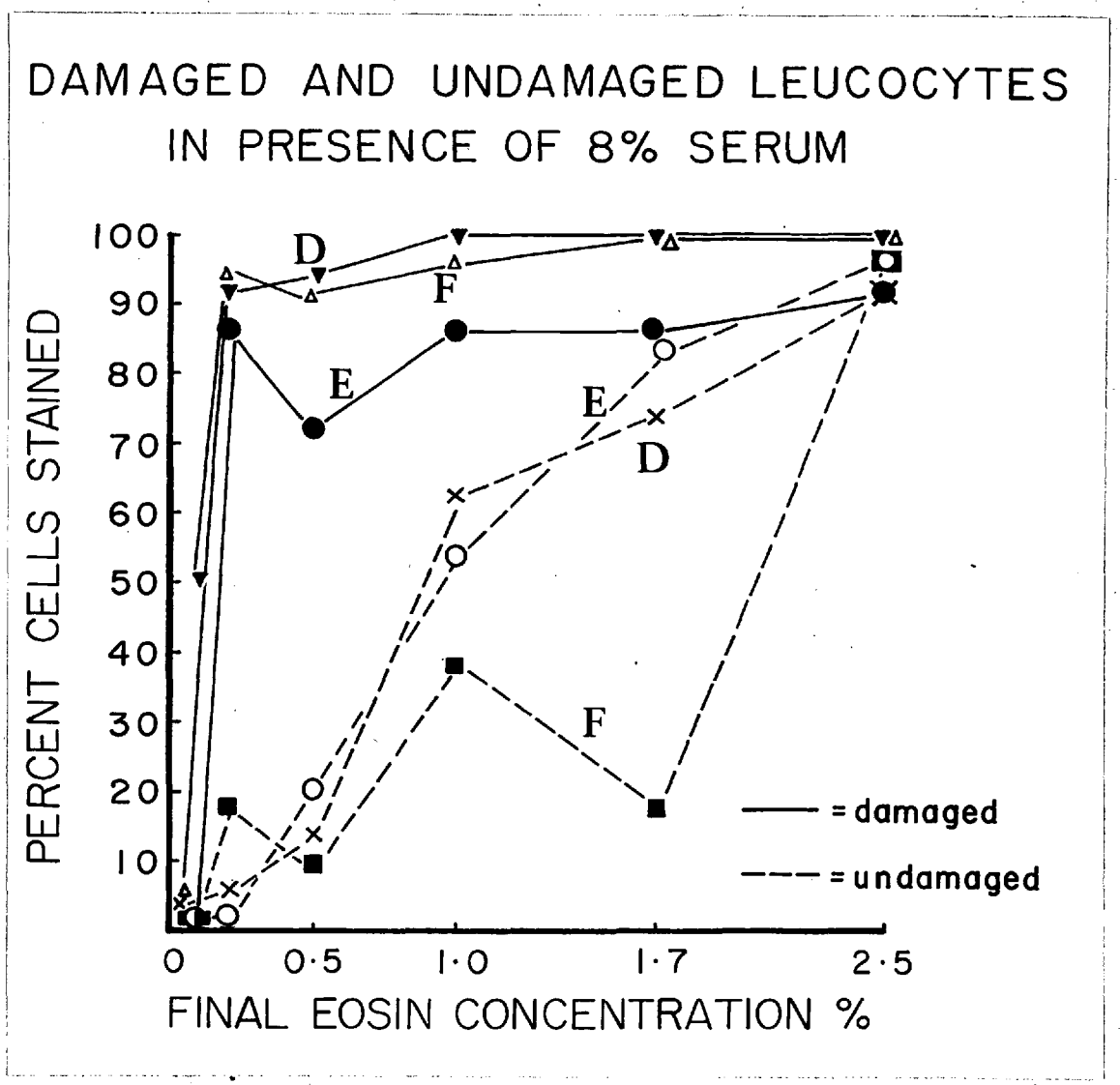


FIGURE 5

The effect of variation in the concentrations of eosin on the staining of rabbit leucocytes.
Lines marked with the same letter represent aliquots made from the same original suspension.
For clarity only 3 of the 4 sets of results are shown. (see Table III)

The proportion which stained in both damaged and undamaged suspensions approached 0% when the eosin concentrations were low, and approached 100% at high concentrations. At the intermediate dye concentrations more cells stained in damaged than in undamaged suspensions.

(ii) Undamaged rabbit leucocytes at various serum concentrations

TABLE IV (see Figure 6)

Final Concentrations: 0.7×10^6 cells/ml.
5 to 40% autologous serum
2 to 0.25% eosin Y

Serum Concentration:		5%	10%	20%	40%
Eosin Concentration 0.25%	i)	8	6	2	10
	ii)	8	6	0	2
	iii)	20	24	28	2
	Mean	12	12	10	5
0.5%	i)	8	4	0	0
	ii)	0	2	2	8
	iii)	56	48	44	20
	Mean	21	18	15	9
1%	i)	22	20	10	2
	ii)	28	7	10	2
	iii)	70	70	40	26
	Mean	40	30	20	10
2%	i)	90	44	46	8
	ii)	54	20	16	10
	iii)	90	92	58	40
	Mean	78	52	40	19

The whole experiment was repeated three times using cell-serum mixtures from different animals each time (i), ii) and iii) in Table IV), and the mean values have been plotted in Figure 6.

The proportions which stained increased when the stain concentrations were raised, and decreased when stain concentrations were lowered. This applied at each of the serum concentrations tested, but at any given eosin concentration a greater proportion stained at the lower serum concentrations.

Table V shows the results of a similar experiment using damaged rabbit leucocytes. Again, each experiment was repeated three times (i), ii) and iii)), using cell-serum mixtures from different animals each time and the mean values have been plotted in Figure 7.

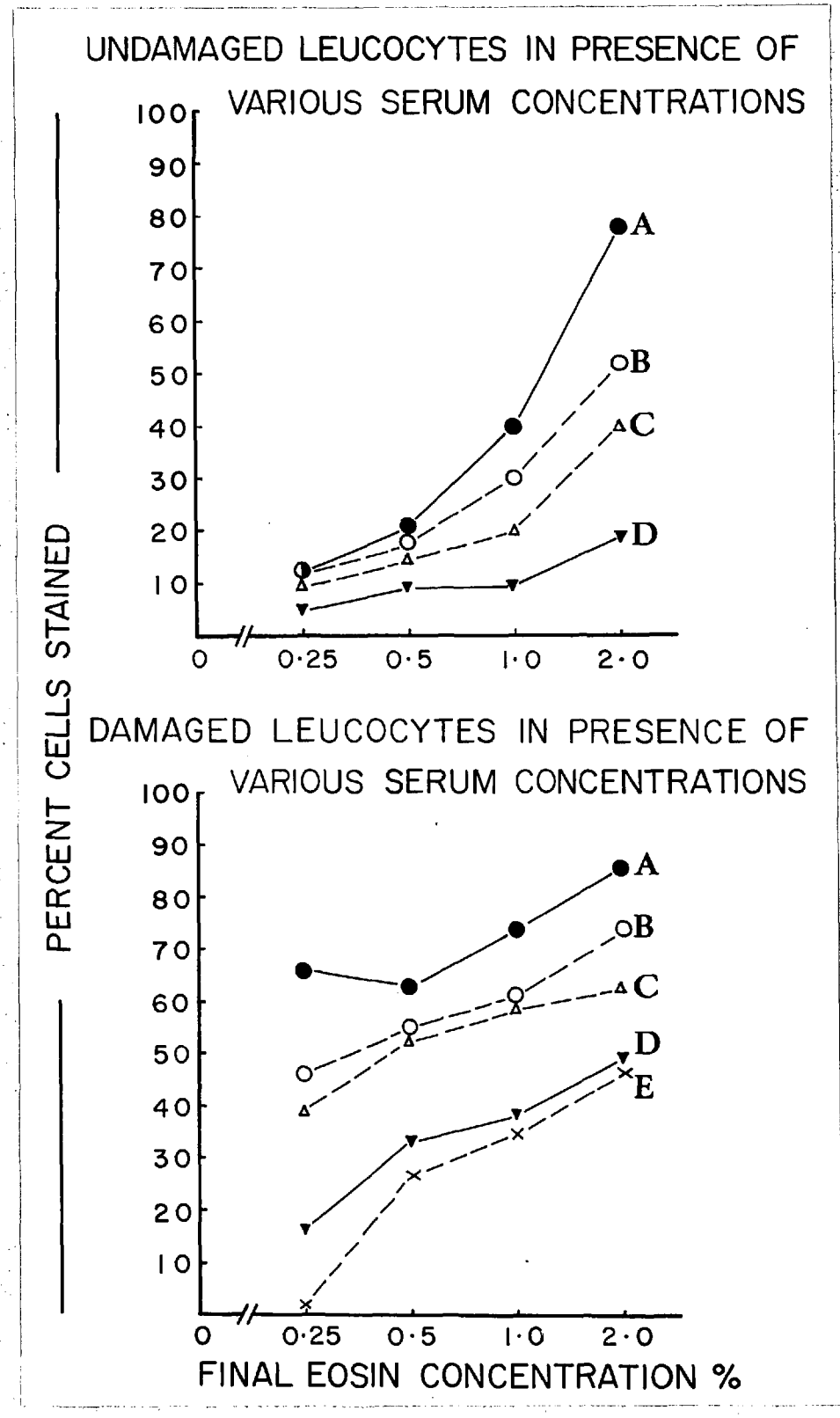
(iii) Damaged rabbit leucocytes at various serum concentrations

TABLE V (see Figure 7)

Final Concentrations: 0.7×10^6 cells/ml.
5 to 80% autologous serum
2 to 0.25% eosin Y

Serum Concentration:		5%	10%	20%	40%	80%
Eosin Concentration 0.25%	i)	63	48	36	0	-
	ii)	-	38	32	32	4
	iii)	69	51	49	-	0
	Mean	66	46	39	16	2
0.5%	i)	60	54	60	50	-
	ii)	-	41	32	16	24
	iii)	65	69	66	-	30
	Mean	63	55	53	33	27
1%	i)	65	64	65	52	-
	ii)	-	42	44	24	26
	iii)	82	76	72	-	44
	Mean	74	61	60	38	35
2%	i)	90	90	82	72	-
	ii)	-	60	38	26	20
	iii)	81	72	70	-	74
	Mean	86	74	63	49	47

In damaged suspensions, as in undamaged, a greater proportion of cells were stained at the higher eosin and lower serum concentrations. At corresponding concentrations greater proportions


UNDAMAGED LEUCOCYTES IN PRESENCE OF VARIOUS SERUM CONCENTRATIONS
DAMAGED LEUCOCYTES IN PRESENCE OF VARIOUS SERUM CONCENTRATIONS
PERCENT CELLS STAINED
100
90
80
70
60
50
40
30
20
10
0
0·25
0·5
1·0
2·0
A
B
C
D
E
FINAL EOSIN CONCENTRATION %

stained in damaged than undamaged suspensions and this was especially marked at the lower stain concentrations used (0.5% and 0.25%). (Compare Tables IV and V.)

(iv) Rabbit leucocytes in absence of serum

TABLE VI (see Figure 8)

Final Concentrations: 0.7×10^6 cells/ml.
0% serum
0.04 to 0.16% eosin Y

Stain Concentration %	% Cells Stained			
	D*	UD*	D	UD
0.04	48	24	36	8
0.063	62	30	56	20
0.083	53	36	62	44
0.125	58	38	60	48

* The adjacent columns show the results using damaged (D) and undamaged (UD) aliquots made from the same suspension. In the repeated experiment (the two right hand columns) the cell-serum mixture came from a different animal.

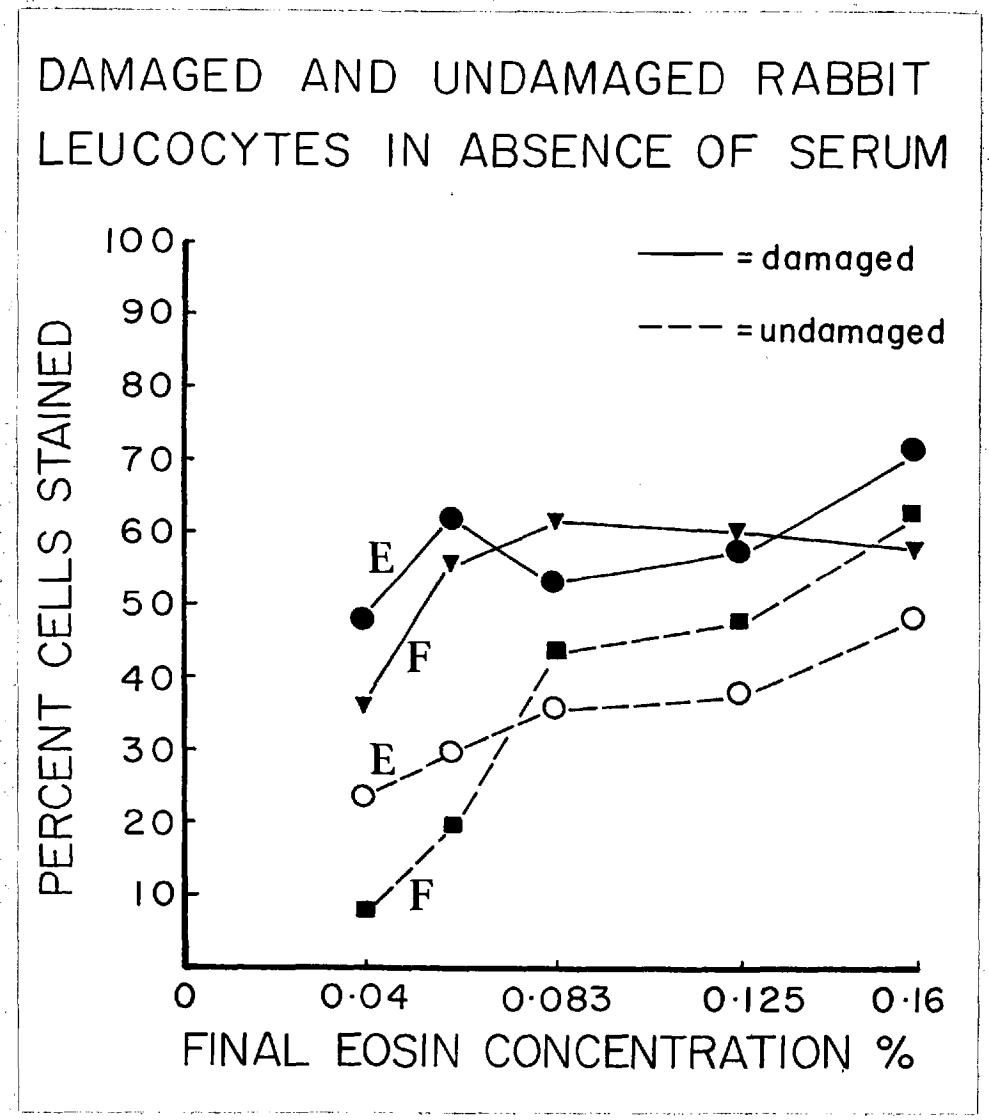


FIGURE 8

The effect of variation in the concentrations of eosin in absence of serum.
Lines marked with the same letter represent aliquots made from the same original suspension.
(see Table VI.)

When no serum was present 48% or more of the cells were stained in both damaged and undamaged suspensions at stain concentrations as low as 0.16%. On reducing the stain concentrations further the proportions of stained cells decreased and there was sometimes a greater difference between the damaged and undamaged suspensions. In order to differentiate between damaged and undamaged suspensions in absence of serum it was necessary to use low eosin concentrations.

(v) Damaged and undamaged dog leucocytes

TABLE VII (see Figure 9)

Final Concentrations: 0.7×10^6 cells/ml.
8% autologous serum
2.5 to 0.01% eosin Y

Eosin Concentration	% Cells Stained	
	Damaged	Undamaged
0.01	0	0
0.2	46	20
0.5	52	16
1.0	52	20
1.7	64	18
2.5	74	38

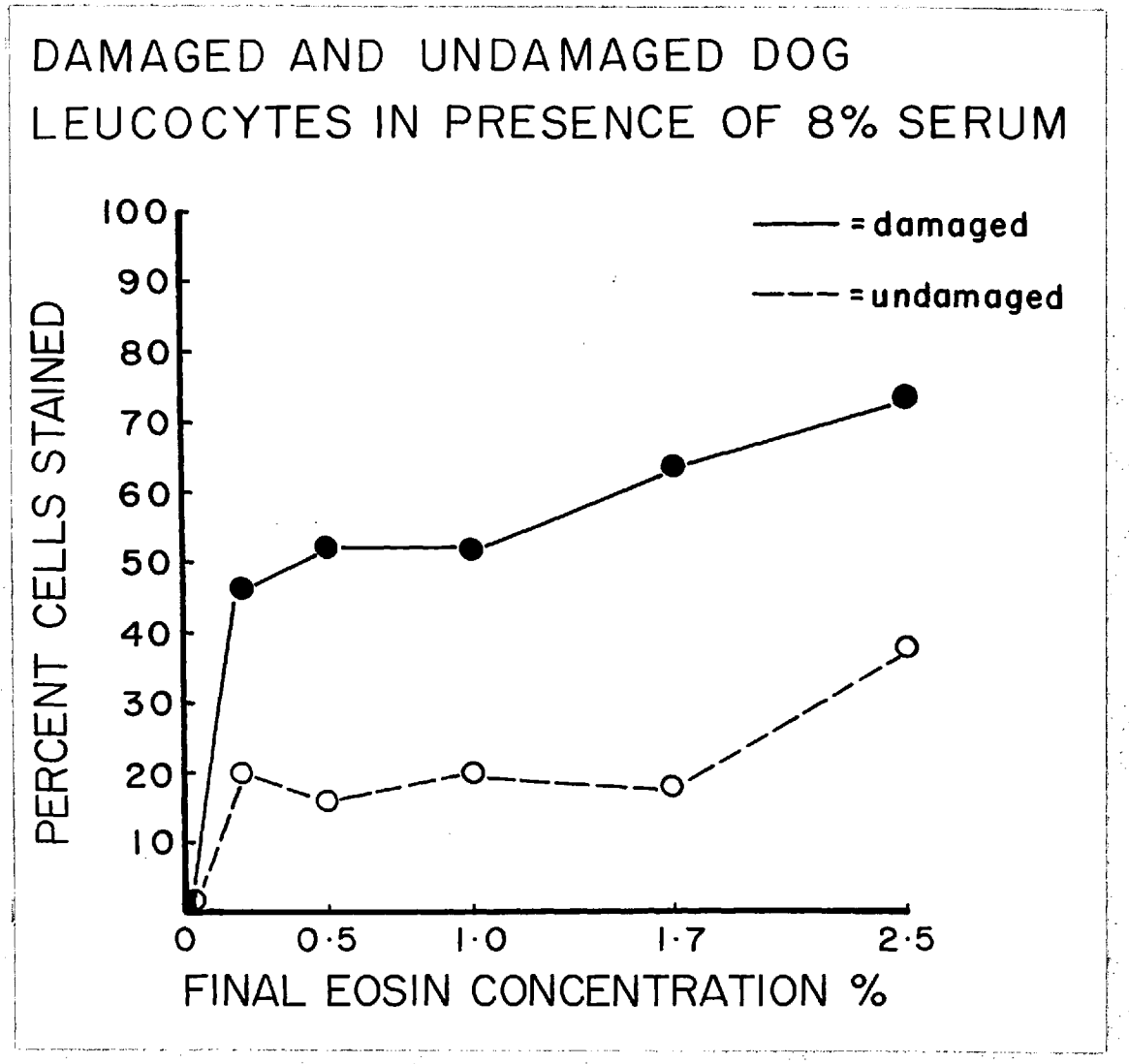


FIGURE 9

The effect of variation in the concentrations of eosin.
(see Table VII.)

The staining of damaged and undamaged dog leucocytes in 8% autologous serum was influenced by the stain concentration, as were the other cell suspensions considered. More cells stained at the higher dye concentrations and more were stained in damaged than undamaged suspensions.

CHAPTER VI

INTERRELATIONSHIP OF THE VARIABLES

The interrelationship of 3 variables, viz. stain concentration, serum concentration and cell damage, was investigated in the following way.

Undamaged rabbit leucocytes and mouse lymphoid cells were mixed with various concentrations of normal rabbit serum and eosin. The cells and serum were autologous when rabbit cells were being used. The serum is non-toxic for rabbit cells but toxic for mouse cells (Terasaki et al., 1961b). The interrelationship between the toxic action of the serum and its inhibitory effect on the uptake of dye could be observed by counting the stained mouse cells at each concentration. These counts were compared with those of the rabbit cells where serum toxicity did not play a role.

REAGENTS

0.2 ml. rabbit leucocytes or mouse lymphoid cells suspended in Hank's at 2.1×10^6 cells/ml.

0.2 ml. rabbit serum (autologous with the rabbit cells) or dilution in Hank's.

0.2 ml. eosin Y at various concentrations in Ringer's.

METHOD

The cell suspensions and serum were incubated together for 1 hour at 37°C and then the eosin solution was added. The mixtures were kept on ice for 1 hour before counting the cells. The experiment was repeated using different individuals as cell-serum donors.

RESULTS AND DISCUSSION

The results are shown in Tables VIIIa and VIIIb, IXa and IXb, and Figures 10, 11, 12 and 13. (Tables VIIIb and IXb, and Figures 11 and 13 are repeated experiments)

TABLE VIIIa (see Figure 10)

Final Concentrations: 0.7×10^6 mouse lymphoid cells/ml.
0 to 33.3% heterologous (rabbit) serum
0.4 to 0.05% eosin Y

Serum Concentration % :	% Cells Stained						
	0	1.0	2.1	4.2	8.3	16.7	33.3
Eosin Concentration % 0.05	58	37	37	29	12	0	0
0.1	87	54	55	65	71	71	11
0.2	85	47	53	60	75	83	66
0.4	94	54	77	71	75	81	81

TABLE VIIIb (see Figure 11)

Final Concentrations: 0.7×10^6 mouse lymphoid cells/ml.
0 to 33.3% heterologous (rabbit) serum
0.4 to 0.05% eosin Y

Serum Concentration % :		% Cells Stained						
		0	1.0	2.1	4.2	8.3	16.7	33.3
Eosin Concentration %	0.05	88	26	17	19	0	2	0
	0.1	64	27	26	25	27	19	0
	0.2	97	27	22	23	23	43	57
	0.4	94	23	22	20	20	49	52

TABLE IXa (see Figure 12)

Final Concentrations: 0.7×10^6 rabbit leucocytes/ml.
0 to 33.3% autologous serum
0.4 to 0.05 eosin Y

Serum Concentration% :		% Cells Stained						
		0	1.0	2.1	4.2	8.3	16.7	33.3
Eosin Concentration %	0.05	78	28	19	11	0	0	0
	0.1	95	65	33	30	17	15	0
	0.2	99	80	44	27	31	28	30
	0.4	99	93	68	56	38	32	32

FIGURE 10

The proportions of mouse lymphoid cells stained in presence of various concentrations of rabbit serum and eosin. The number on each line represents the final concentration of eosin (%) used to stain that series of preparations. (see Table VIIIa.)

FIGURE 11

An experiment similar to that above using other individuals as cell and serum donors. (see Table VIIIb.)

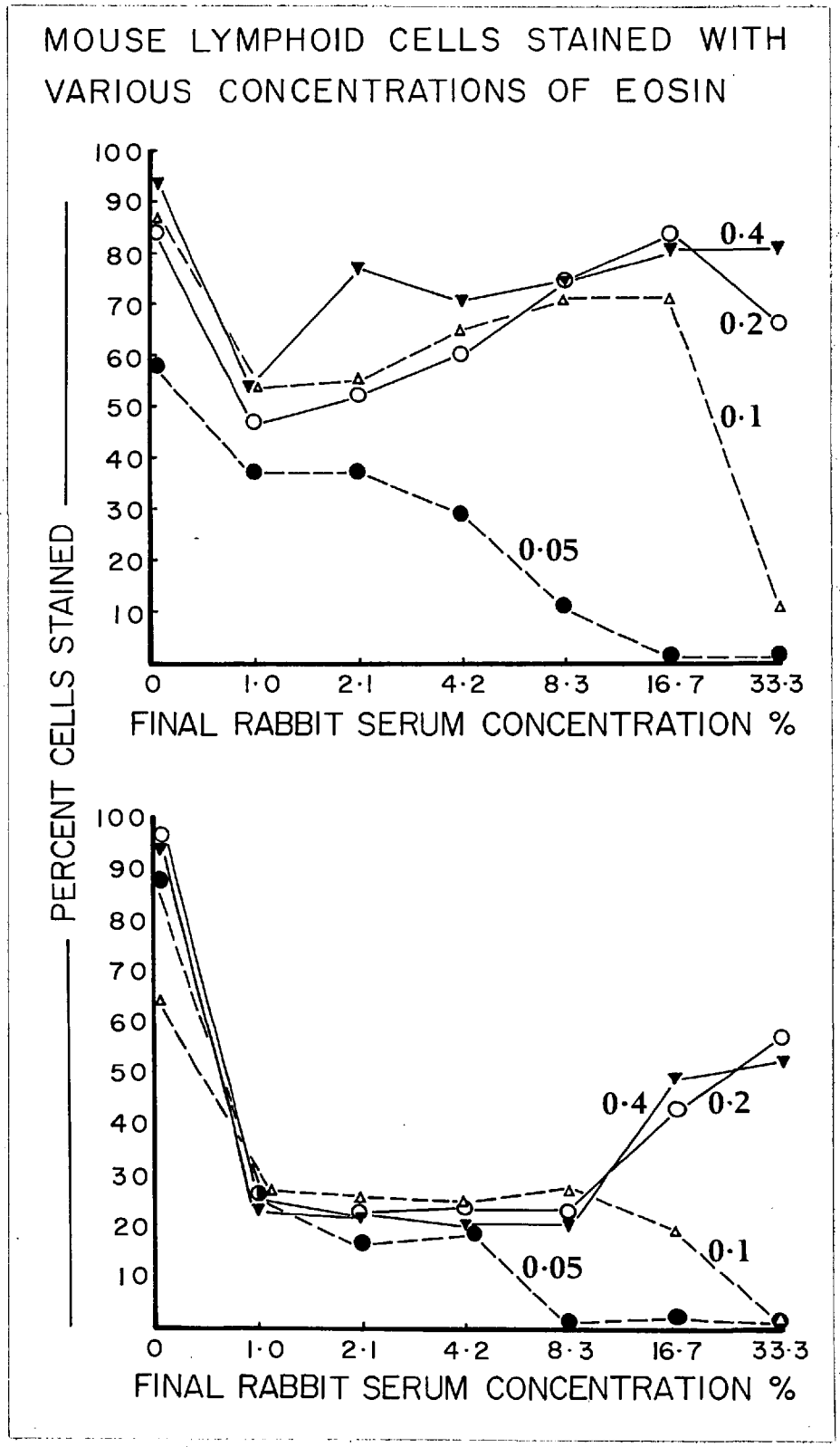

MOUSE LYMPHOID CELLS STAINED WITH VARIOUS CONCENTRATIONS OF EOSIN
PERCENT CELLS STAINED
100
90
80
70
60
50
40
30
20
10
0·4
0·2
0·1
0·05
0
1·0
2·1
4·2
8·3
16·7
33·3
FINAL RABBIT SERUM CONCENTRATION %
PERCENT CELLS STAINED
100
90
80
70
60
50
40
30
20
10
0·4
0·2
0·1
0·05
0
1·0
2·1
4·2
8·3
16·7
33·3
FINAL RABBIT SERUM CONCENTRATION %

FIGURE 12

The proportions of rabbit leucocytes stained in presence of various concentrations of autologous serum and eosin. The number on each line represents the final concentration of eosin (%) used to stain that series of preparations. (see Table IXa.)

FIGURE 13

An experiment similar to that above using other individuals as cell-serum donors. (see Table IXb.)

RABBIT LEUCOCYTES STAINED WITH VARIOUS CONCENTRATIONS OF EOSIN

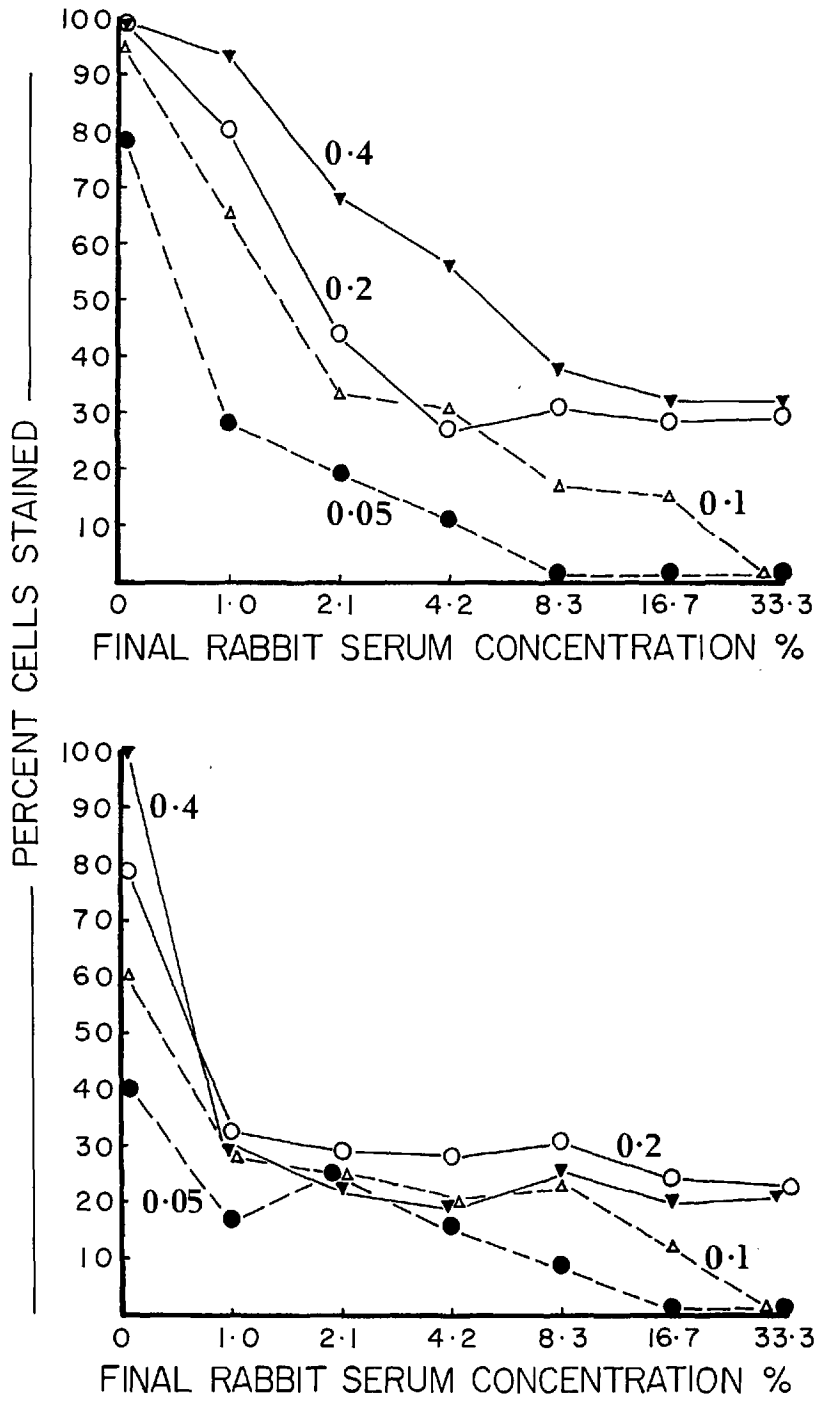


TABLE IXb (see Figure 13)

Final Concentrations: 0.7×10^6 rabbit leucocytes/ml.
0 to 33.3% autologous serum
0.4 to 0.05% eosin Y

Serum Concentration % :		% Cells Stained						
		0	1.0	2.1	4.2	8.3	16.7	33.3
Eosin Concentration %	0.05	40	17	25	16	9	0	0
	0.1	60	28	25	20	23	12	0
	0.2	79	32	29	28	31	24	22
	0.4	100	29	22	19	25	20	21

One possible interpretation of the results follows.

At high serum concentrations (33.3% and 16.7%). In the case of mouse cells (Figures 10 and 11), when the eosin concentrations were high (0.4% and 0.2%), a large proportion of cells stained because many cells were damaged by the serum and the eosin was sufficiently concentrated to stain these in spite of the inhibitory stain-binding effect of the serum. At low eosin concentrations (0.1% or 0.05%) the serum effectively blocked the staining in spite of the cell damage, and consequently low proportions of the cells were stained.

These results formed a contrast with those of the rabbit cells where the counts were relatively low at all the tested eosin concentrations (Figures 12 and 13). In these circumstances the different trends in the staining of rabbit and mouse cells may have been a manifestation of the toxicity of the serum for the latter.

When the serum concentrations were decreased (from 16% to 1%). In the case of mouse cells, the stained counts also decreased when the eosin concentrations were high (0.4% or 0.2%), (see Figures 10 and 11). Here, in presence of eosin excess the serum was relatively ineffective at binding the stain and consequently the diminishing stained counts were mainly a reflection of the decreasing toxicity of the serum.

At low eosin concentration (0.05%) the serum effectively inhibited staining (because of the limited quantities of dye available), and consequently as the serum concentrations diminished so the stained counts increased (Figures 10 and 11).

Again, the results formed a contrast with those of rabbit cells (Figures 12 and 13). The serum was not toxic here and the stained counts rose as the serum concentrations were decreased at each of the eosin concentrations tested.

CHAPTER VII

STAINING TIME AND "STANDING TIME"

An investigation was carried out into whether the proportion of cells which stained was influenced by

(i) the staining time, and

(ii) the "standing time", i.e. the period between completing the preparation of the cell suspension-serum mixtures and the beginning of the count of stained cells.

Rabbit or mouse cell suspensions were mixed with serum and handled in one of the following ways.

(a) Eosin solution was added immediately and the proportions of stained cells were counted after various intervals. Here staining time and standing time were concurrent.

(b) The mixtures were left to stand for various periods before adding eosin and then left to stain for a uniform period (1 hour) before counting the cells.

(c) The mixtures were left to stand for various periods before adding eosin and then left for various periods before counting the cells.

In any experiment the known variables other than staining and/or standing time were held constant. Some parameters such as the temperature, the species of the cell and serum donors, or the serum concentrations were changed from one experiment to another to ascertain whether the observations were generally applicable.

EXPERIMENT 1

Simultaneous variation of staining and standing time.

Reagents

0.1 ml. damaged or undamaged rabbit leucocytes suspended in Hank's containing 10% autologous serum at 8.5×10^6 cells/ml.

0.1 ml. autologous serum.

1 ml. 0.25 % eosin Y in Ringer's.

Method

The eosin solution was added immediately after mixing the cell suspensions and serum, and the cells were counted after various intervals during which the mixtures were kept on ice. The experiment was repeated six times using cell-serum mixtures from different animals.

Results and Discussion

The results are shown in Tables Xa - Xf and Figure 14. The undamaged rabbit leucocytes could be stained for at least 110 minutes without any marked increase in the proportions stained, but in damaged suspensions the proportion stained increased with the staining (and standing) times. The increase was especially rapid during the first hour.

The stained counts of damaged and undamaged suspensions diverged during the first 60 minutes after adding the dye. This suggests that it is desirable to carry out the counts after that staining period has elapsed in order to achieve the greatest contrast when such suspensions are oompared.

Furthermore, damaged suspensions which are counted after short staining periods are unlikely to give repeatable results because the stained counts change rapidly during this period.

TABLE X (see Figure 14)

Influence of simultaneous variation of staining and standing time.

Final Concentrations: 0.7×10^6 rabbit leucocytes/ml.
8% autologous serum
0.2% eosin Y

	Undamaged		Damaged	
	Staining Time	% Cells Stained	Staining Time	% Cells Stained
TABLE Xa	21 min	16	18 min	38
	50 "	10	50 "	36
	81 "	8	78 "	52
	115 "	28	113 "	56
	17hr 0 "	80	17hr 0 "	78
TABLE Xb	25 min	4	30 min	36
	50 "	2	55 "	56
	75 "	8	80 "	52
	115 "	6	120 "	60
	140 "	6	145 "	58
	18hr 30 "	6	18hr 40 "	86
TABLE Xc	26 min	8	30 min	38
	55 "	8	60 "	36
	90 "	16	95 "	54
	17hr 30 "	60	17hr 35 "	64
TABLE Xd	5 min	4	6 min	10
	14 "	2	15 "	22
	21 "	2	22 "	22
	31 "	6	35 "	30
	46 "	8	47 "	44
	57 "	4	57 "	34
	66 "	6	66 "	54
	76 "	6	78 "	46
TABLE Xe	25 min	20	30 min	26
	45 "	24	50 "	34
	75 "	16	80 "	42
	110 "	24	115 "	42
	20hr 0 "	46	20hr 5 "	50
TABLE Xf	30 min	2	35 min	40
	55 "	10	60 "	34
	95 "	10	100 "	52
	17hr 30 "	24	17hr 35 "	74

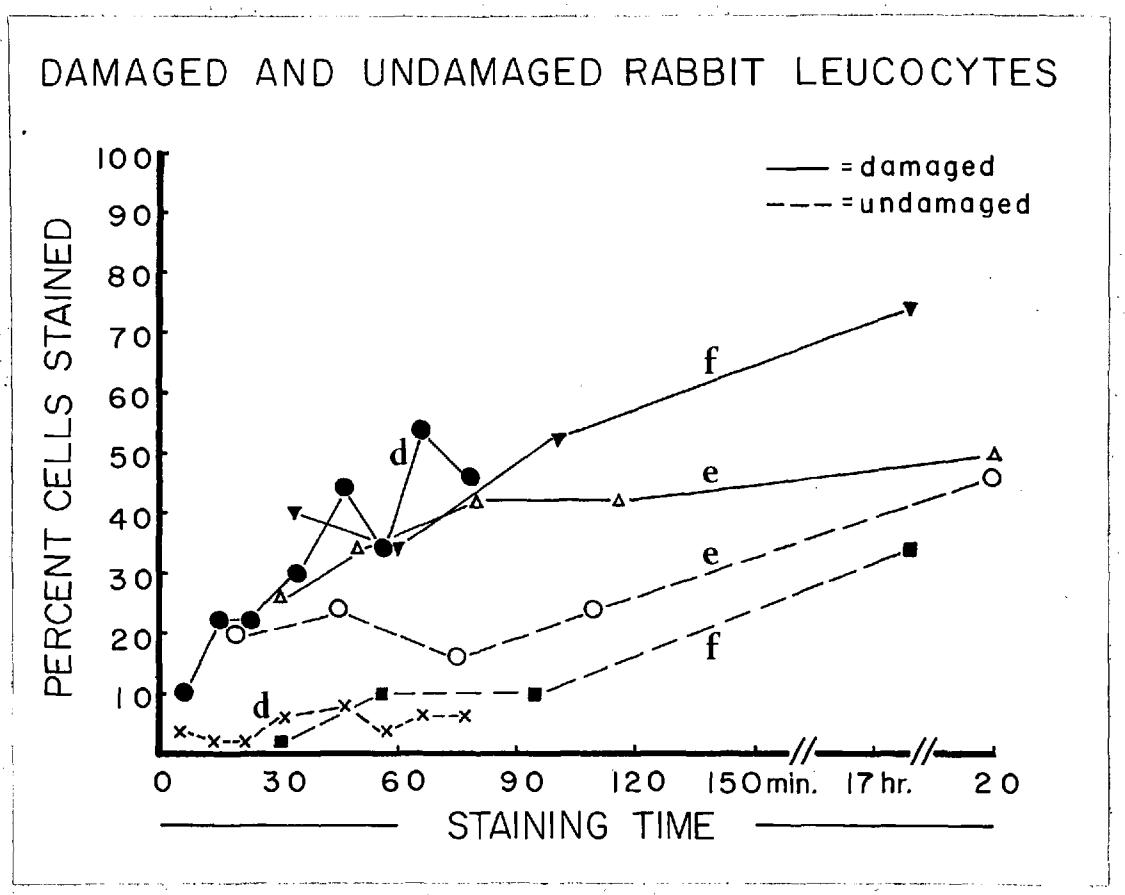


FIGURE 14

The effect of variation in the staining (and standing) times.
Lines marked with the same letter represent aliquots made from the same original suspension.
For clarity only 3 of the results are shown.
(see Table X.)

In the suspensions stained for long periods (17 to 20 hours) similar proportions were sometimes stained in the damaged and undamaged suspensions (see Tables Xa, Xc and Xe), but in other experiments the stained counts differed even after such protracted periods (see Tables Xb and Xf).

EXPERIMENT 2

Simultaneous variation of staining and standing time.

Reagents

0.2 ml. undamaged mouse lymphoid cells suspended in Hank's at concentration 2.1 x 10^6 cells/ml.

0.2 ml. 3% and 12% rabbit serum in Hank's.

0.2 ml. 0.15% eosin Y in Ringer's.

Method

The eosin solution was added immediately after mixing the cell suspensions and serum. The cells were counted after various intervals during which the mixtures were kept at room temperature or on ice.

TABLE XI (see Figure 15) Influence of simultaneous variation of staining and standing time.

Final Concentrations: 0.7×10^6 mouse lymphoid cells/ml.
4% or 1% rabbit serum
0.05% eosin Y

XIa

Serum Concentration: 4%				Serum Concentration: 1%			
Room Temperature		On Ice		Room Temperature		On Ice	
Staining Time	% Cells Stained	Staining Time	% Cells Stained	Staining Time	% Cells Stained	Staining Time	% Cells Stained
1 min	20	1 min	20	1 min	26	1 min	26
–	–	9 "	15	–	–	9 "	17
–	–	19 "	24	–	–	19 "	20
–	–	29 "	20	–	–	29 "	15
–	–	39 "	23	–	–	–	–
44 "	17	–	–	44 "	22	–	–
54 "	21	–	–	54 "	26	–	–
1hr 4 "	14	–	–	1hr 9 "	17	–	–
1 19 "	14	–	–	1 24 "	29	–	–
1 34 "	18	–	–	1 39 "	23	–	–
1 49 "	18	1hr54 "	25	1 54 "	27	1hr54 "	26
2 4 "	16	–	–	2 24 "	21	–	–
2 34 "	27	2 34 "	27	2 54 "	24	2 54 "	26
3 4 "	21	–	–	3 24 "	27	–	–
3 34 "	18	–	–	3 54 "	21	–	–
4 4 "	27	–	–	4 24 "	38	–	–
4 34 "	30	4 34 "	30	4 54 "	24	4 24 "	31
5 4 "	22	–	–	–	–	–	–
20 30 "	87	20 30 "	75	20 30 "	98	20 30 "	100

TABLE XIb

Final Concentrations: 0.7×10^6 mouse lymphoid cells/ml.
4% or 1% rabbit serum
0.05% eosin Y

Serum Concentration: 4%				Serum Concentration: 1%			
Room Temperature		On Ice		Room Temperature		On Ice	
Staining Time	% Cells Stained	Staining Time	% Cells Stained	Staining Time	% Cells Stained	Staining Time	% Cells Stained
1 min	12	1 min	16	5 min	7	1 min	14
15 "	14	25 "	9	20 "	19	-	-
30 "	19	-	-	35 "	19	30 "	18
45 "	17	40 "	12	-	-	-	-
60 "	12	55 "	13	50 "	14	-	-
1hr15 "	13	1hr10 "	17	1hr 5 "	24	-	-
1 30 "	11	1 25 "	18	1 20 "	17	1hr20 "	22
-	-	-	-	1 35 "	21	-	-
-	-	-	-	1 50 "	28	-	-
-	-	-	-	2 10 "	22	-	-
2 25 "	20	2 35 "	17	2 30 "	16	2 22 "	17
2 45 "	15	2 55 "	18	2 45 "	19	2 47 "	19
3 5 "	14	-	-	3 5 "	30	3 12 "	14
3 30 "	12	3 20 "	13	-	-	3 37 "	16
3 55 "	20	3 45 "	13	3 55 "	24	3 52 "	20
-	-	4 0 "	16	-	-	-	-
4 40 "	29	4 50 "	23	-	-	4 42 "	14
-	-	5 50 "	21	5 55 "	29	-	-
6 35 "	23	-	-	-	-	6 42 "	27
24 0 "	77	24 0 "	53	24 0 "	97	24 0 "	94

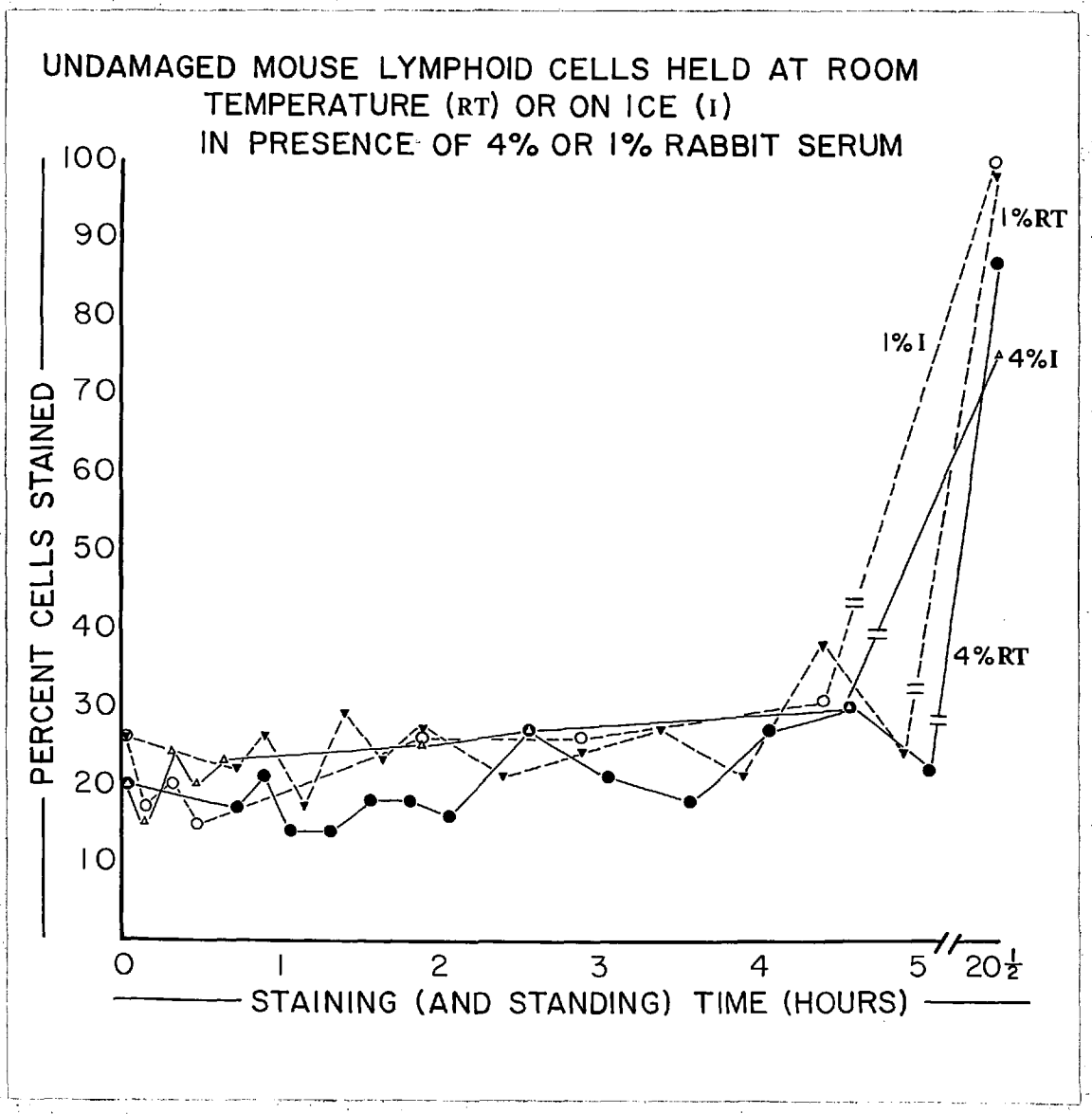


FIGURE 15

The effect of variation in the staining (and standing) times in the presence of different rabbit serum concentrations. The mixtures were kept at room temperature (RT) or on ice (I).
The numbers on the trend lines represent the final concentration of serum in each suspension. For clarity only two of the results are shown.
(see Table XIa.)

Results and Discussion

Tables XIa and XIb, and Figure 15, show that prolonged staining (and standing) times up to 4 hours hardly affected the counts of mouse lymphoid cells, originally undamaged. Counts carried out after 4 hours' staining were only slightly higher than counts done after only 1 minute's staining interval. After more than 20 hours in the dye most cells were stained, but more stained in the presence of 1% serum than in 4% serum.

EXPERIMENT 3

Variations in standing time where staining time was held constant.

Reagents

(i) 0.1 ml. damaged rabbit leucocytes suspended in Hank's containing 10% autologous serum at 8.5×10^6 cells/ml.

0.1 ml. autologous serum.

1 ml. 0.25% eosin Y in Ringer's.

or

(ii) 0.1 ml. cell suspension (as above).
0.5 ml. autologous serum.
0.6 ml. 2% eosin Y in Ringer's.

Method

The dye solution was added at various intervals after mixing the cell suspensions and serum, and the cells were counted 1 hour after adding the dye in each case. The preparations were kept on ice throughout.

Results and Discussion

Tables XIIa and XIIb, and Figure 16, show that the stained counts were not influenced by the standing times if the counts were carried out between 1 and 3 hours after separating the leucocytes, provided that a constant interval elapsed between adding the stain and counting.

TABLE XII (see Figure 16)

Variations in the standing time where the staining time was held constant.

Final Concentrations: 0.7×10^6 damaged rabbit leucocytes/ml.

8% or 42% serum

0.2% or 1% eosin Y

XIIa

Concentration:			
Autologous Serum 42% Eosin Solution 1%		Autologous Serum 8% Eosin Solution 0.2%	
Standing Time	% Cells Stained	Standing Time	% Cells Stained
65 min	28	65 min	53
75 "	32	80 "	55
85 "	28	-	-
95 "	32	95 "	62
105 "	25	110 "	55
115 "	26	-	-
125 "	32	125 "	52
135 "	21	-	-
145 "	37	140 "	68
155 "	38	159 "	56
165 "	45	-	-
175 "	48	170 "	52
185 "	34	185 "	34
197 "	34	191 "	48
-	-	207 "	61

TABLE XIIb

Final Concentrations: 0.7×10^6 damaged rabbit leucocytes/ml.
8% or 42% serum
0.2% or 1% eosin Y

Concentration:			
Autologous Serum 42% Eosin Solution 1%		Autologous Serum 8% Eosin Solution 0.2%	
Standing Time	% Cells Stained	Standing Time	% Cells Stained
65 min	22	-	-
80 "	17	75 min	62
95 "	20	95 "	55
110 "	20	110 "	52
125 "	11	125 "	68
140 "	16	144 "	56
155 "	24	155 "	52
170 "	19	170 "	44
-	-	176 "	48
185 "	16	-	-
-	-	192 "	61

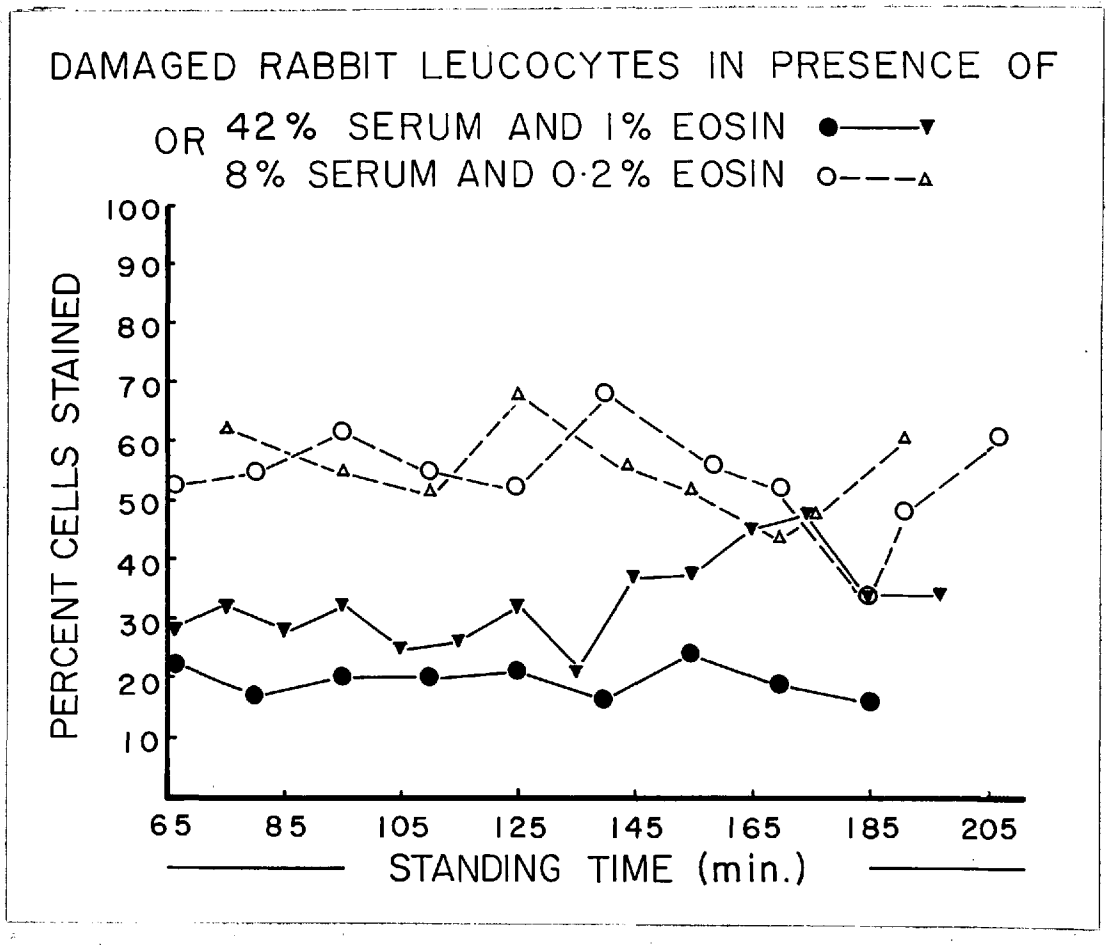


FIGURE 16

Effect of variation in the standing times when staining times were held constant at 1 hour. Rabbit leucocytes were stained with 1% or 0.2% eosin in present of 42% or 8% autologous serum. (see Table XII)

EXPERIMENT 4

Variations in standing and staining times.

Reagents

0.1 ml. damaged and undamaged rabbit lymph node cells suspended in Hank's containing 10% autologous serum at 8.5×10^6 cells/ml.

0.24 ml. autologous serum.

0.86 ml. 0.58% eosin Y in Ringer's.

Method

The mixtures of the cell suspensions and serum were incubated at 37°C for 1 hour and then placed on ice. At various intervals after incubation the eosin solution was added and the per cent stained cells were counted at various times after this.

Results and Discussion

Tables XIIIa and XIIIb, XIV, and Figures 17 and 18 show that in undamaged suspensions the counts were little affected by protracted staining and standing times. However, the counts were somewhat increased when the staining times reached 3 hours (corresponding to standing times of about 250 minutes).

TABLE XIII (see Figure 17)

The influence of standing and staining times after incubation.

Final Concentrations: 0.7×10^6 rabbit lymph node cells/ml.
20% serum
0.4% eosin Y

XIIIa

Undamaged Suspensions					
Period between end of incubation and adding eosin solution 5 mins		10 mins		40 mins	
Staining Time	% Cells Stained	Staining Time	% Cells Stained	Staining Time	% Cells Stained
-	-	5min (75)	28	5min (105)	8
-	-	-	-	10 " (110)	27
-	-	-	-	15 " (115)	24
30min (95)	28	30 " (100)	24	20 " (120)	24
60 " (125)	25	60 " (130)	26	52 " (152)	22
180 " (245)	38	180 " (250)	42	155 " (255)	27

Period between end of incubation and adding eosin solution 70 mins		105 mins		135 mins	
Staining Time	% Cells Stained	Staining Time	% Cells Stained	Staining Time	% Cells Stained
5min (135)	16	5min (170)	18	5min (200)	18
10 " (140)	21	10 " (175)	15	10 " (205)	20
15 " (145)	25	15 " (180)	25	15 " (210)	19
19 " (149)	24	-	-	20 " (215)	20
-	-	25 " (190)	27	25 " (220)	28
30 " (160)	22	30 " (195)	29	-	-
60 " (190)	19	60 " (225)	29	55 " (250)	33
-	-	100 " (265)	18	-	-
130 " (260)	27	104 " (269)	30	-	-

* Numbers in parenthesis indicate "standing time".

TABLE XIIIb Effect of standing and staining times after incubation.

Final Concentrations: 0.7×10^6 rabbit lymph node cells/ml.
20% serum
0.4% eosin Y

Undamaged Suspensions			
Period between end of incubation and adding eosin solution 26 mins		70 mins	
Staining Time	% Cells Stained	Staining Time	% Cells Stained
5 min (91)	15	5 min (135)	21
10 " (96)	26	11 " (141)	23
15 " (101)	17	17 " (147)	22
-	-	24 " (154)	26
40 " (126)	20	36 " (166)	21
50 " (136)	35	51 " (181)	27
72 " (158)	34	66 " (196)	26
80 " (166)	25	-	-
100 " (186)	33	-	-
125 " (211)	27	-	-
158 " (244)	23	-	-

TABLE XIV (see Figure 18)
Effect of standing and staining times after incubation.

Final Concentrations: as for Tables XIIIa and XIIIb.

Damaged Suspensions			
Period between end of incubation and adding eosin solution 60 mins		10 mins	
Staining Time	% Cells Stained	Staining Time	% Cells Stained
17 min (137)	14	20 min (90)	15
40 " (160)	36	42 " (112)	33
62 " (182)	50	63 " (133)	49
86 " (206)	54	89 " (159)	57
109 " (229)	45	110 " (180)	49
132 " (252)	55	135 " (205)	52

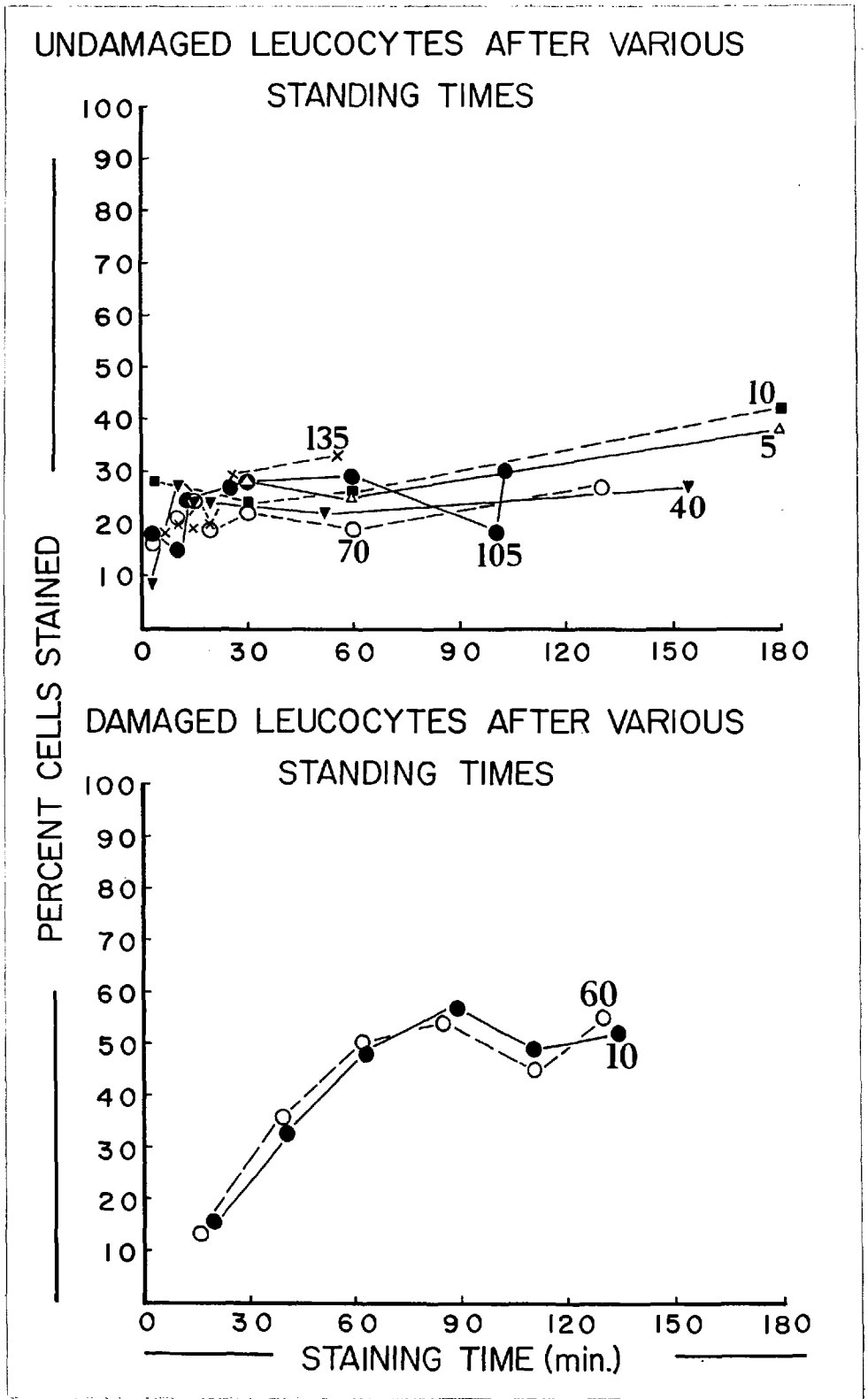
* Numbers in parenthesis indicate standing time.

FIGURE 17

Effect of variation in the staining times on undamaged rabbit leucocyte suspensions after various standing times.
To calculate the standing times in each case add the number on the trend line to 60 and the staining time.
(see Table XIIIa.)

FIGURE 18

As above, but using damaged rabbit leucocytes.
(see Table XIV.)


UNDAMAGED LEUCOCYTES AFTER VARIOUS STANDING TIMES
100
90
80
70
60
50
40
30
20
10
0
30
60
90
120
150
180
10
5
135
40
70
105
DAMAGED LEUCOCYTES AFTER VARIOUS STANDING TIMES
60
10
PERCENT CELLS STAINED
STAINING TIME (min.)

With damaged cells (Figure 18) the proportions stained increased markedly with the staining time. However, standing times made little difference and the trend lines were similar in suspensions which were held for 70 minutes and 120 minutes before adding eosin.

A consideration of standing times was of practical importance where a series of preparations which had been made up at one time was under investigation. As a uniform staining period was desirable it was necessary to add the stain to the wells at intervals and count the preparations in rotation. This resulted in a variation in the standing times. However, the results indicate that the standing times did not influence the stained counts in either damaged or undamaged suspensions, provided the staining time was held constant (see Figures 16, 17 and 18, and Tables XII, XIII and XIV).

CHAPTER VIII

INCUBATION

Two aspects of incubation were investigated:

(i) a comparison of the stained counts of incubated and unincubated suspensions. The stain was added after incubation was completed, or after a similar period in the case of the unincubated suspensions.

(ii) Whether the stained counts were affected by incubating the mixtures together with the eosin dye as opposed to adding the stain after incubation had been completed.

PART I

Reagents

0.1 ml. damaged or undamaged rabbit leucocytes suspended in Hank's containing 10% autologous serum at 8.5×10^6 cells/ml.

0.1 ml. autologous serum.

1 ml. 0.25% eosin Y in Ringer's.

Method

The cell suspensions and serum were mixed together and either incubated at 37°C or refrigerated at 4°C for 1 hour. The eosin solution was added and the proportion of stained cells counted after various intervals on ice. The experiment was repeated using cell-serum mixtures from different animals.

Reagents

0.1 ml. damaged rabbit lymph node cells suspended as above.
0.24 ml.autologous serum.
0.85 ml.0.57% eosin Y in Ringer's.

Method

Here the cell suspensions and serum were mixed and either incubated at 37°C or held at room temperature (21°C) for 1 hour. Further, they were handled as above.

Results and Discussion

The results are presented in Tables XVa and XVb (repeated experiments), XVc, and Figures 19, 20 and 21.

TABLE XVa (see Figure 19) Effect of incubation or refrigeration.

Final Concentrations: 0.7×10^6 leucocytes/ml.
8% autologous serum
0.2% eosin Y

Undamaged				Damaged			
Refrigerated 4°C		Incubated 37°C		Refrigerated 4°C		Incubated 37°C	
Staining Time	% Cells Stained	Staining Time	% Cells Stained	Staining Time	% Cells Stained	Staining Time	% Cells Stained
7 min	6	6 min	4	6 min	8	5 min	34
27 "	10	26 "	2	26 "	16	25 "	26
47 "	6	46 "	4	46 "	16	45 "	48
67 "	16	70 "	8	71 "	36	70 "	52
94 "	10	98 "	4	96 "	40	95 "	52

TABLE XVb (see Figure 20) Effect of incubation or refrigeration.

Final Concentrations: as for Table XVa.

Undamaged				Damaged			
Refrigerated 4°C		Incubated 37°C		Refrigerated 4°C		Incubated 37°C	
Staining Time	% Cells Stained	Staining Time	% Cells Stained	Staining Time	% Cells Stained	Staining Time	% Cells Stained
5 min	10	5 min	6	5 min	40	5 min	44
25 "	8	25 "	6	25 "	59	25 "	62
45 "	14	45 "	2	45 "	66	45 "	62
65 "	4	65 "	8	65 "	74	65 "	72
85 "	14	85 "	4	85 "	65	85 "	78

TABLE XVc (see Figure 21)

The effect of incubation or holding at room temperature.

Final Concentrations: 0.7×10^6 damaged lymph node cells/ml.

20% autologous serum

0.4% eosin Y

Held at 21°C for 1 hour		Incubated at 37°C for 1 hour	
Staining Time	% Cells Stained	Staining Time	% Cells Stained
5 min	34	3 min	35
10 "	38	12 "	46
17 "	37	18 "	54
21 "	31	25 "	53
55 "	49	51 "	73
70 "	48	-	-
87 "	55	86 "	74
103 "	66	103 "	71
-	-	118 "	76

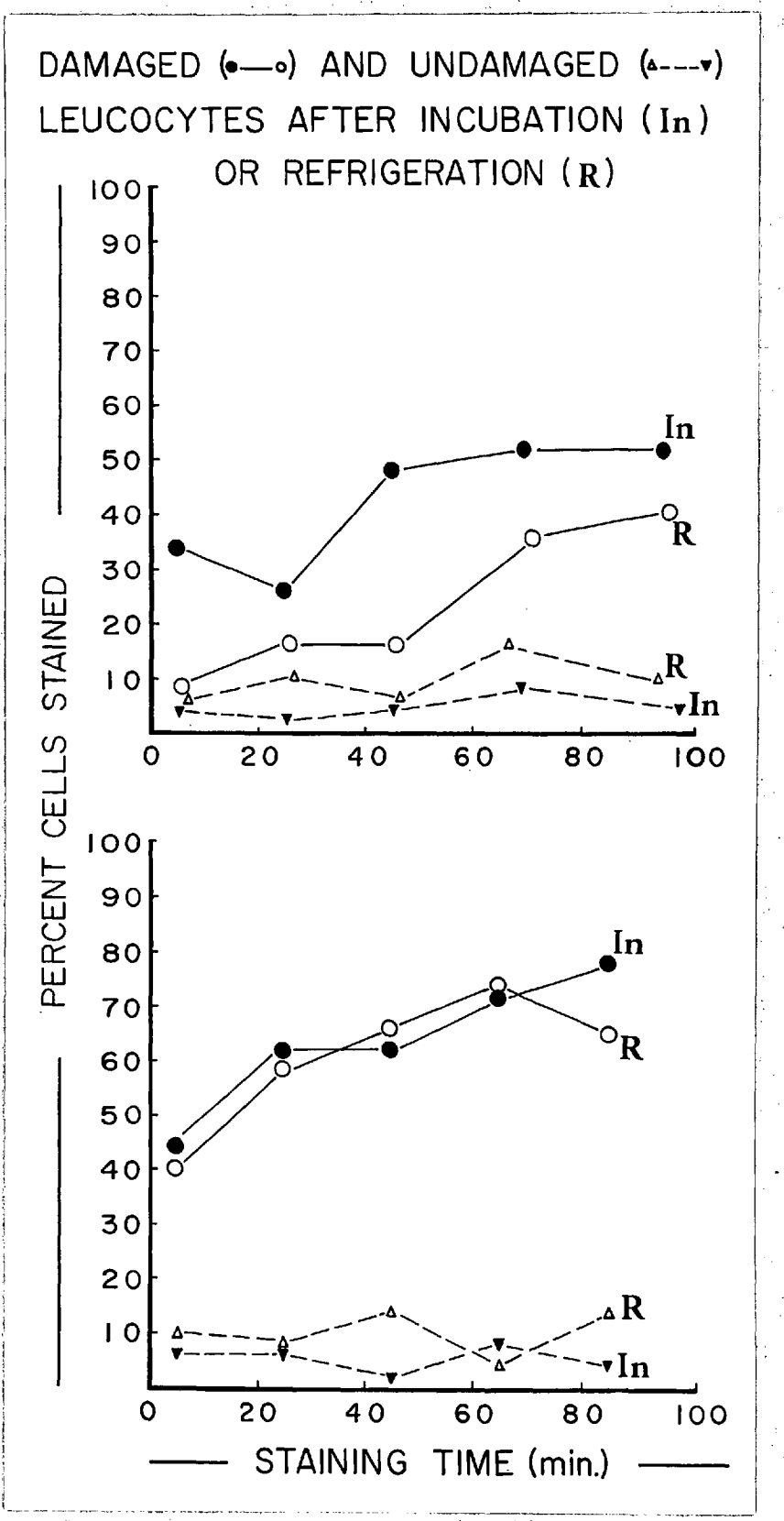
After some 90 minutes in the dye the stained counts in undamaged suspensions were low and were similar whether the suspensions had been refrigerated at 4°C or incubated at 37°C for 1 hour before staining (Figures 19 and 20). In the damaged suspensions the counts increased with the staining time and were sometimes similar in the suspensions which were incubated or refrigerated (Figure 20).

FIGURE 19

Effect of incubating at 37°C or holding at 4°C for 1 hour. Damaged and undamaged rabbit leucocytes stained for various periods. In each figure the 4 aliquots were prepared from the same original suspension. (see Table XVa.)

FIGURE 20

As above, but using different individual animals as cell and serum donors. (see Table XVb.)


DAMAGED (•—o) AND UNDAMAGED (▲---▼)
LEUCOCYTES AFTER INCUBATION (In)
OR REFRIGERATION (R)
PERCENT CELLS STAINED
100
90
80
70
60
50
40
30
20
10
0
20
40
60
80
100
In
R
R
In
In
R
R
In
STAINING TIME (min.)

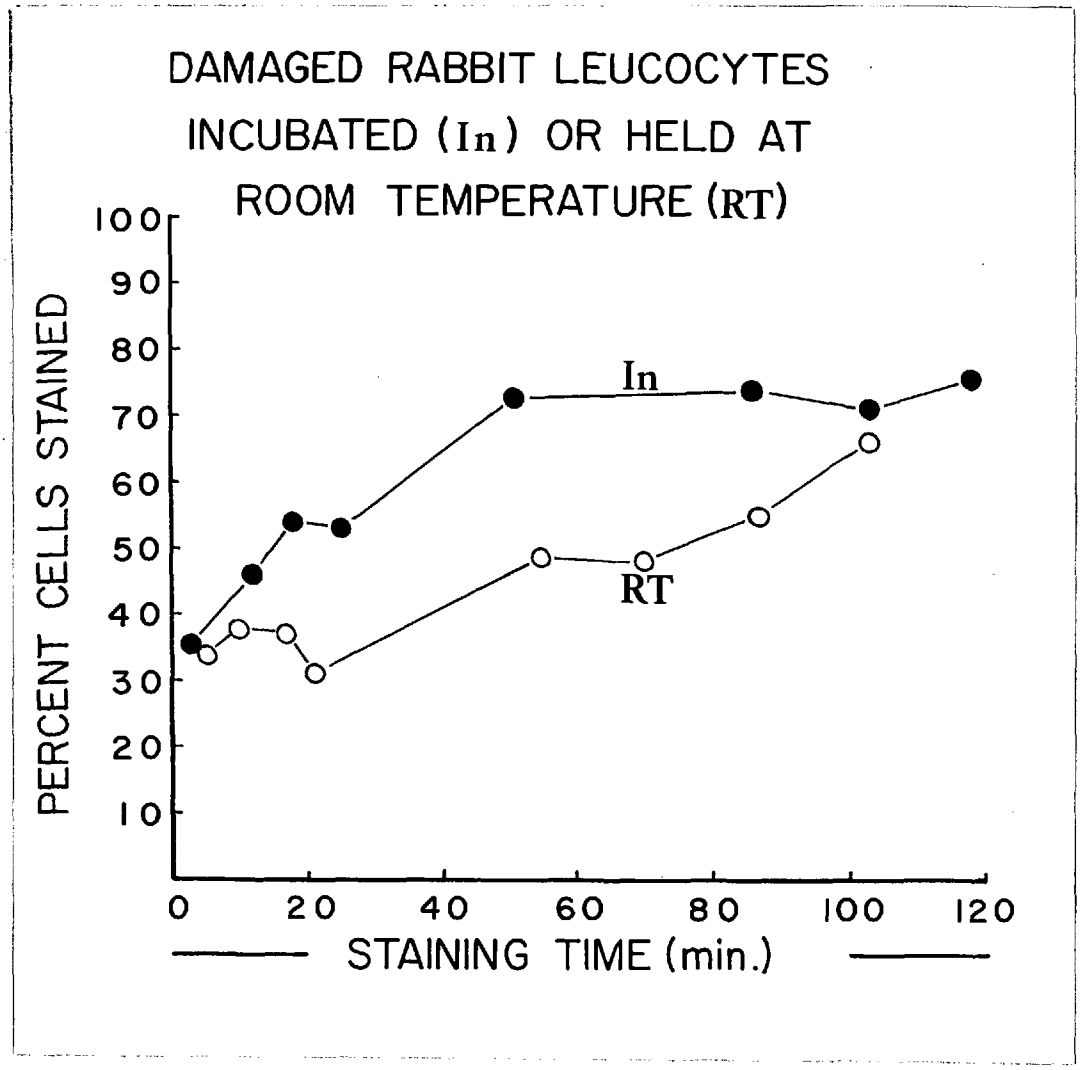


FIGURE 21

The effect of incubating or holding at room temperature for 1 hour.
Damaged rabbit leucocytes stained for various periods.
(see Table XVc.)

At other times however the counts were higher in the incubated than the refrigerated suspensions (Figure 19), or than those held at room temperature (Figure 21).

Because incubation sometimes increased the divergence between the stained counts in damaged and undamaged suspensions it is potentially capable of increasing the sensitivity of the dye exclusion test.

PART II

Reagents

0.1 ml. damaged rabbit lymph node cells suspended in Hank's containing 10% autologous serum at 8.5×10^6 cells/ml.

0.24 ml. autologous serum.

0.86 ml. 0.57% eosin Y in Ringer's.

Method

The eosin solution was added to the cell suspension-serum mixtures either before or after 1 hour's incubation at 37°C. The proportion of

stained cells was counted at various intervals after incubation. The mixtures were kept on ice during staining. The experiment was repeated using cell-serum mixtures from different animals.

Results and Discussion

The results are presented in Tables XVIa, XVIb and XVIc, and Figures 22a, 22b and 22c.

When the mixtures were incubated together with the dye, the proportions which stained were high at the initial counts which were carried out 2 to 11 minutes after incubation. These counts remained more or less unchanged for at least 130 minutes afterwards (= staining times up to 190 minutes).

When the dye was added after incubation the initial stained counts (14 to 20 minutes after incubation) were low. These rose steadily and at 50 to 60 minutes after incubation had usually attained the levels of their counterparts which had been incubated together with the dye.

TABLE XVIa (see Figure 22a)

Final Concentrations: 0.7×10^6 rabbit lymph node cells/ml.

20% autologous serum

0.4% eosin Y

Eosin incubated together with cell-serum mixture		Eosin added after cell-serum mixture had been incubated	
Counted at (Minutes after incubation)	% Cells Stained	Counted at (Minutes after incubation)	% Cells Stained
11	51	17	14
34	46	40	36
56	39	62	50
82	54	86	54
105	56	109	45
126	51	132	55

TABLE XVIb (see Figure 22b)

Final Concentrations: as for Table XVIa)

Eosin incubated together with cell-serum mixture		Eosin added after cell-serum mixture had been incubated	
Counted at (Minutes after incubation)	% cells Stained	Counted at (Minutes after incubation)	% Cells Stained
8	41	20	15
32	47	42	33
53	46	63	49
80	50	89	57
101	62	110	49
122	48	135	52

TABLE XVIc (see Figure 22c)

Final Concentrations: 0.7×10^6 rabbit lymph node cells/ml.

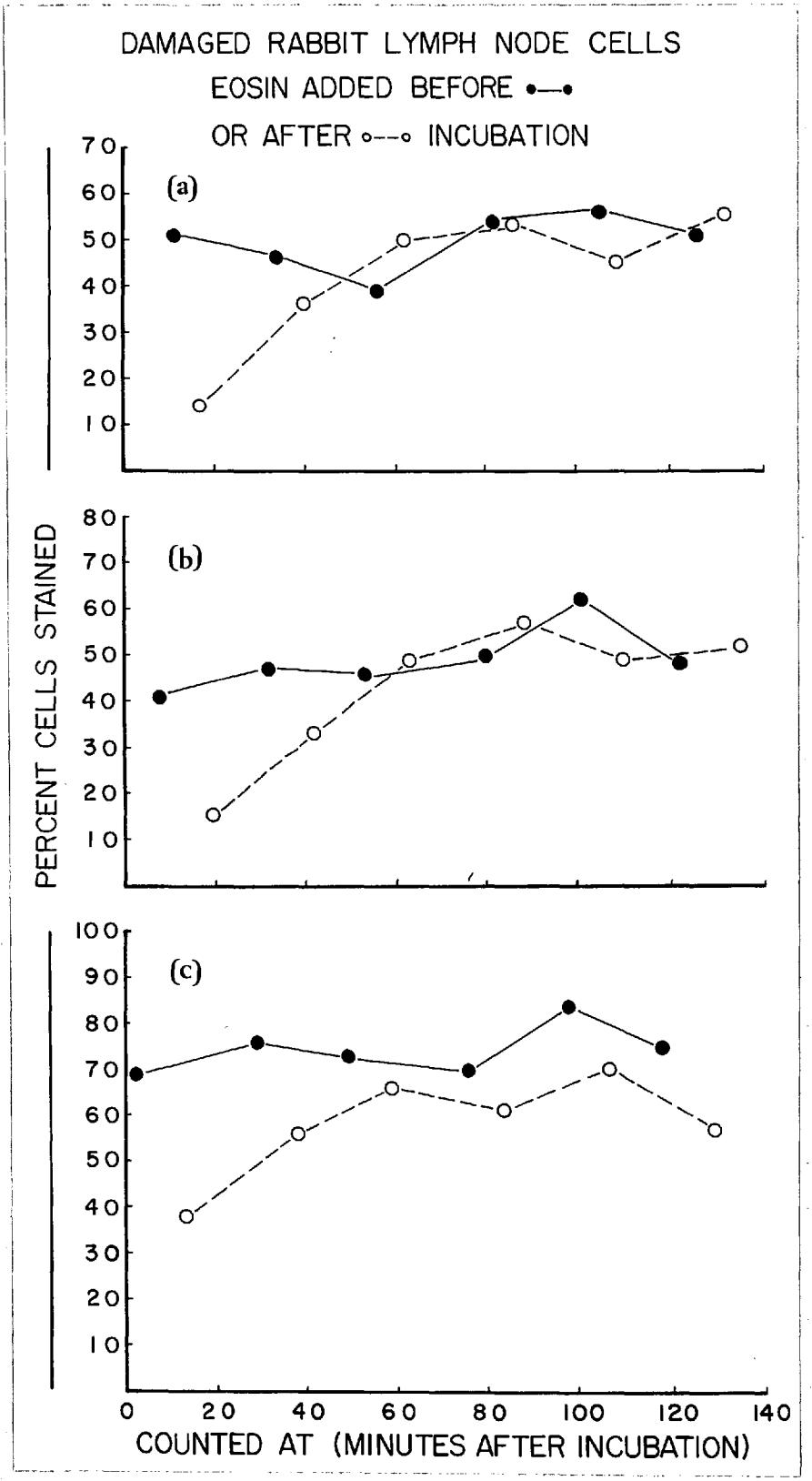
20% autologous serum

0.4% eosin Y

Eosin incubated together with cell-serum mixture		Eosin added after cell-serum mixture had been incubated	
Counted at (Minutes after incubation)	% Cells Stained	Counted at (Minutes after incubation)	% Cells Stained
2	69	14	38
29	76	38	56
49	73	59	66
76	70	84	61
98	84	107	70
118	75	129	57

FIGURES 22a, 22b and 22c

Effect of adding the eosin before or after incubation.
(see Tables XVIa, XVIb and XVIc.)


DAMAGED RABBIT LYMPH NODE CELLS
EOSIN ADDED BEFORE •—•
OR AFTER ○---○ INCUBATION
(a)
(b)
(c)
PERCENT CELLS STAINED
10
20
30
40
50
60
70
80
90
100
0
20
40
60
80
100
120
140
COUNTED AT (MINUTES AFTER INCUBATION)

CHAPTER IX

CELL CONCENTRATION

The influence of cell concentration was investigated by counting the proportion of cells which stained in a series of mixtures. Each mixture contained a different cell concentration, but was similar in respect of the other known variables.

REAGENTS

0.1 ml. damaged or undamaged rabbit leucocytes suspended in Hank's containing 10% autologous serum at concentration 6×10^6 cells/ml., or dilutions in the same medium.

0.1 ml. autologous serum.

1 ml. 0.25% eosin Y in Ringer's.

METHOD

The cell suspensions, serum and dye were mixed together and kept on ice for 1 hour before counting. The experiment was repeated using a different animal as cell-serum donor each time.

RESULTS AND DISCUSSION

The results are shown in Tables XVIIa and XVIIb, and Figure 23.

The stained counts were often raised when low cell concentrations were used (less than 0.1×10^6 cells/ml.) and this applied to both damaged and undamaged suspensions.

TABLE XVIIa (see Figure 23)

Final Concentrations: 0.5 to 0.01 x 10^6 rabbit leucocytes/ml.
8% autologous serum
0.2% eosin Y

Undamaged		Damaged	
Cell Concentration	% Cells Stained	Cell Concentration	% Cells Stained
0.5 x 10^6/ml.	15	0.4 x 10^6/ml.	50
0.25 "	14	0.2 "	64
0.125 "	27	0.1 "	54
		0.05 "	48
0.45 "	0	0.25 "	39
0.23 "	0		
0.11 "	0	0.17 "	37
0.05 "	16	0.088 "	32
		0.04 "	30
0.4 "	0	0.02 "	28
0.2 "	4	0.01 "	52
0.1 "	10		
0.05 "	0	0.4 "	60
0.025 "	30	0.2 "	63
		0.1 "	60
0.21 "	20	0.05 "	62
0.11 "	14	0.025 "	80
0.05 "	6	0.013 "	80
0.027 "	20		

TABLE XVIIb

Final Concentrations: 0.5 to 0.01 x 10^6 rabbit leucocytes/ml.

8% autologous serum

0.2% eosin Y

Undamaged		Damaged	
Cell Concentration	% Cells Stained	Cell Concentration	% Cells Stained
1.4 x 10^6/ml.	6	0.18 x 10^6/ml.	57
0.7 "	4	0.09 "	58
0.35 "	4	0.04 "	60
0.175 "	10	0.02 "	66
0.09 "	12		
		0.21 "	76
1.3 "	4	0.11 "	72
0.65 "	0	0.05 "	75
0.3 "	0	0.03 "	87
0.16 "	16		
0.08 "	10		
0.04 "	16		
		0.7 "	48
1.0 "	8	0.35 "	51
0.5 "	14	0.18 "	56
0.25 "	8	0.09 "	59
0.125 "	18	0.04 "	56
0.063 "	34	0.02 "	60
0.03 "	28		
0.18 "	6		
0.09 "	9		
0.04 "	6		
0.02 "	6		

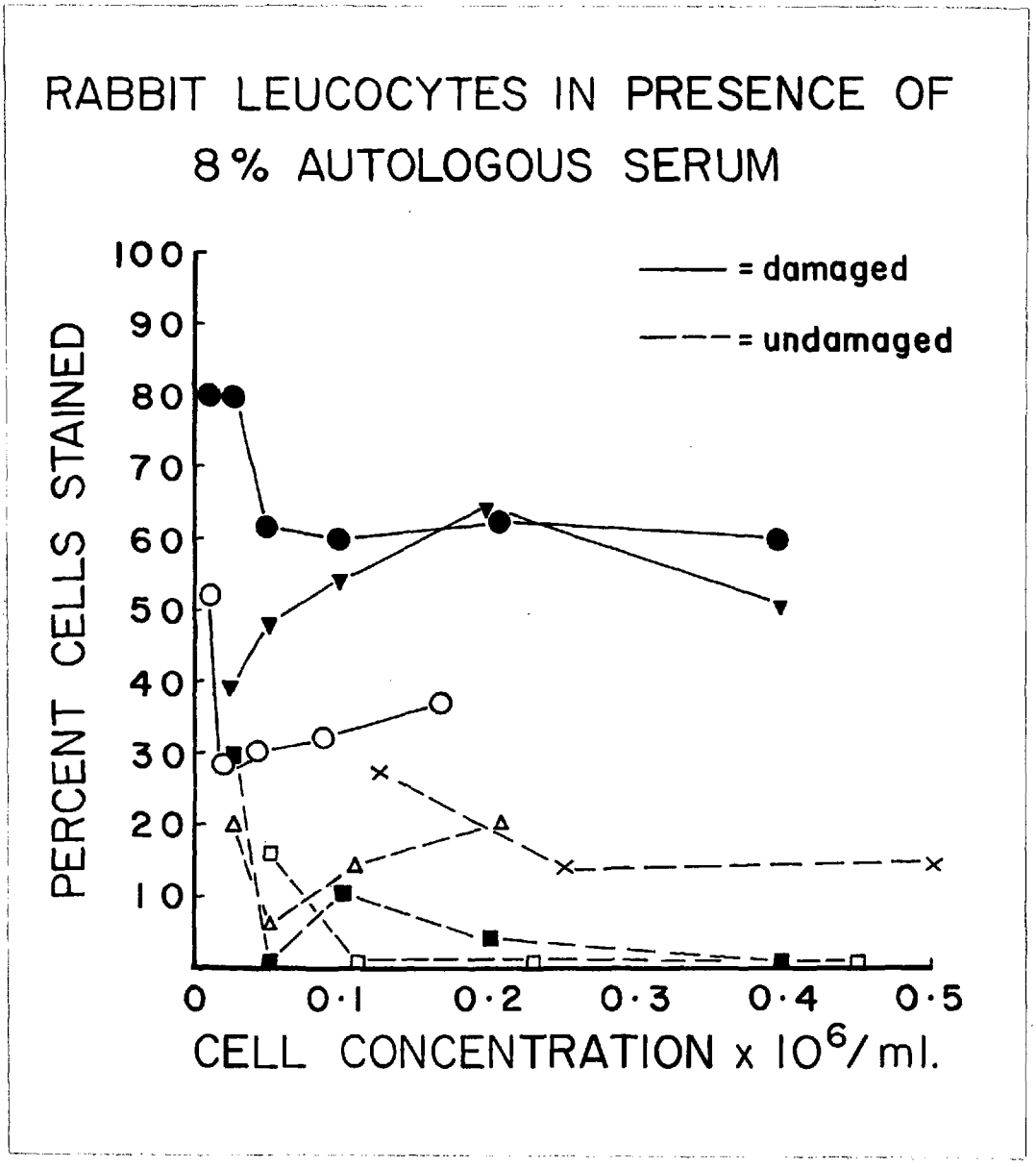


FIGURE 23

The effect of varying the concentration of cells using damaged or undamaged rabbit leucocytes in presence of 8% autologous serum.
(see Table XVIIa.)

CHAPTER X

CELL TYPE

Rabbit leucocytes were used in most of the experiments so far described. The following experiments were carried out to investigate whether other cell types were similarly affected by variables such as the serum and stain concentrations.

In the first lot of experiments the proportion of stained cells was counted in a series of suspensions of rabbit leucocytes, rabbit peritoneal macrophages or rabbit peritoneal polymorphs which were mixed with various concentrations of autologous serum and stained with eosin, the final concentration of which was held constant.

In the second lot, the concentrations of serum were held constant and damaged and undamaged rabbit leucocytes, dog leucocytes and rabbit peritoneal macrophages were stained with various concentrations of eosin.

PART I

Reagents

0.1 ml. rabbit leucocytes suspended in Hank's containing 10% autologous serum.

0.1 ml. rabbit peritoneal polymorphs suspended in 0.2% ethyldiaminotetra-acetic acid and 10% autologous serum in Hank's.

0.1 ml. rabbit peritoneal macrophages suspended as the polymorphs above.

Each suspension was undamaged and at concentration 8.5×10^6 cells/ml.

0.5 ml. autologous serum or dilution in Hank's.

0.6 ml. 0.4% eosin Y in Ringer's.

Method

The cell suspensions were each mixed with serum and stain and the proportions of stained cells were counted after 1 hour on ice. The experiment was repeated using a different animal as cell-serum donor each time.

Results and Discussion

The results are shown in Table XVIIIa, XVIIIb and XVIIIc, and Figure 24.

TABLE XVIII (see Figure 24)

The effect of various serum concentrations on different cell types.

Final Concentrations: 0.7×10^6 cells/ml. (undamaged rabbit macrophages, leucocytes or polymorphs)

42% to < 1% autologous serum

0.2% eosin Y

XVIIIa Macrophages

Serum Concentration %	42	34	16.7	12	8.4	4.2	< 1
% Cells Stained	–	14	–	32	–	31	100
	–	2	–	10	–	10	100
	–	22	–	34	–	40	100
	–	24	–	46	–	35	100
	–	22	–	40	–	53	100
	–	28	–	50	–	56	100
	15	–	20	–	22	12	100
	18	–	8	–	10	12	100
	–	53	58	–	59	64	100
Average	16.5	23.6	28.7	35.3	30	34.8	100

TABLE XVIIIb Blood Leucocytes

Final Concentrations: as for Table XVIIIa.

Serum Concentration %	42	34	16.7	12	8.4	4.2	< 1
% Cells Stained	-	0	-	-	18	-	100
	-	0	-	-	10	-	100
	-	0	-	-	2	-	100
	-	0	-	-	2	-	100
	-	9	-	-	3	-	100
	-	0	-	-	12	-	100
	-	2	5	-	10	-	100
	-	0	8	-	4	-	100
	-	0	-	4	-	28	100
	-	0	-	6	-	0	100
	-	0	-	6	-	16	100
	-	0	-	0	-	4	100
	0	-	0	4	-	0	100
	0	-	0	6	-	6	100
	1	-	0	-	-	3	100
Average	0.3	1	2.6	4.3	7.6	8.1	100

TABLE XVIIIc Polymorphs

Final Concentrations: as for Table XVIIIa.

Serum Concentration %	42	34	16.7	12	8.4	4.2	< 1
% Cells Stained	-	12	-	-	24	-	100
	-	6	-	-	16	-	100
	-	4	-	-	24	-	100
	-	6	-	-	36	-	100
	-	0	-	-	4	-	10
	-	0	-	-	6	-	4
	-	11	19	-	20	-	47
	-	6	12	-	20	-	83
	-	0	-	16	-	24	12
	-	2	-	7	-	12	16
	0	-	4	-	11	8	27
	0	-	2	-	2	4	11
Average	0	4.7	9.25	11.5	16.3	12	50.8

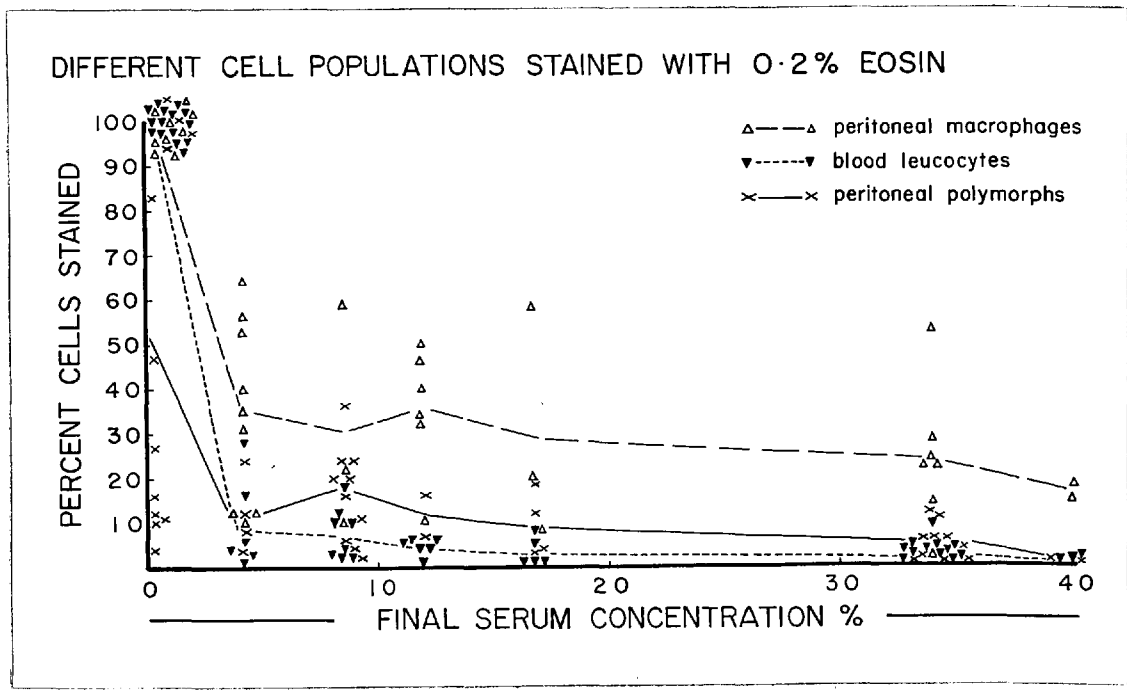


FIGURE 24

Effect of various serum concentrations on different rabbit cell populations stained with 0.2% eosin.
(see Tables XVIIIa, XVIIIb and XVIIIc.)

With all 3 cell types the proportions which stained were dependent upon the serum concentrations, and when the serum concentrations were lowered, the stained counts were raised. The proportions which stained were also dependent on the cell type. Rabbit leucocytes were most sensitive to variations in the serum concentration and when the concentrations were varied between $< 1\%$ and 42%, the stained counts fell from 100% to 0.3% on average. With a similar range in serum concentration the average stained macrophage count fell from 100% to 16.5% and the stained polymorphs from 50.8% to 0%. While 100% macrophages and leucocytes invariably stained at very low serum concentrations, an average of only 50.8% of the polymorphs were stained.

PART II

Reagents

0.1 ml. damaged or undamaged rabbit leucocytes, rabbit peritoneal macrophages suspended

as before, or dog leucocytes suspended in Hank's containing 10% autologous serum at 8.5×10^6 cells/ml.

0.1 ml. autologous serum.

1 ml. eosin Y at various concentrations.

Method

The cell suspensions, serum and stain were mixed together and kept on ice for 1 hour before counting.

Results and Discussion

The results are shown in Table XIX and Figures 25a, 25b and 25c.

In each case a greater proportion of cells was stained when the concentration of eosin was raised and a greater proportion of cells stained in the damaged than in the undamaged suspensions.

The proportions which stained were also dependent upon the cell type and the populations were affected differently by variations in the dye concentration (Figure 25). The rabbit leucocytes were most sensitive and the stained counts varied

TABLE XIX (see Figures 25a, 25b and 25c)

The effect of various stain concentrations on different cell types.

Final Concentrations: 0.7×10^6 cells/ml. (damaged or undamaged dog leucocytes, rabbit macrophages or rabbit leucocytes)

8% autologous serum

0.01% to 3.3% eosin Y

Eosin Concentration %	% Cells Stained							
	Dog Leucocytes		Rabbit Macrophages		Rabbit Leucocytes			
	UD*	D*	UD	D	UD	D	UD	D
0.01	0	0	0	2	0	0	0	50
0.02	-	-	0	54	-	-	-	-
0.05	-	-	0	51	-	-	-	-
0.1	-	-	18	58	-	-	-	-
0.2	20	46	12	74	0	86	6	92
0.5	16	52	12	87	20	72	14	94
1.0	20	52	14	81	54	86	62	100
1.7	18	64	-	-	84	86	74	100
2.5	38	74	-	-	98	92	92	100
3.3	-	-	48	93	-	-	-	-

* UD = undamaged

D = damaged

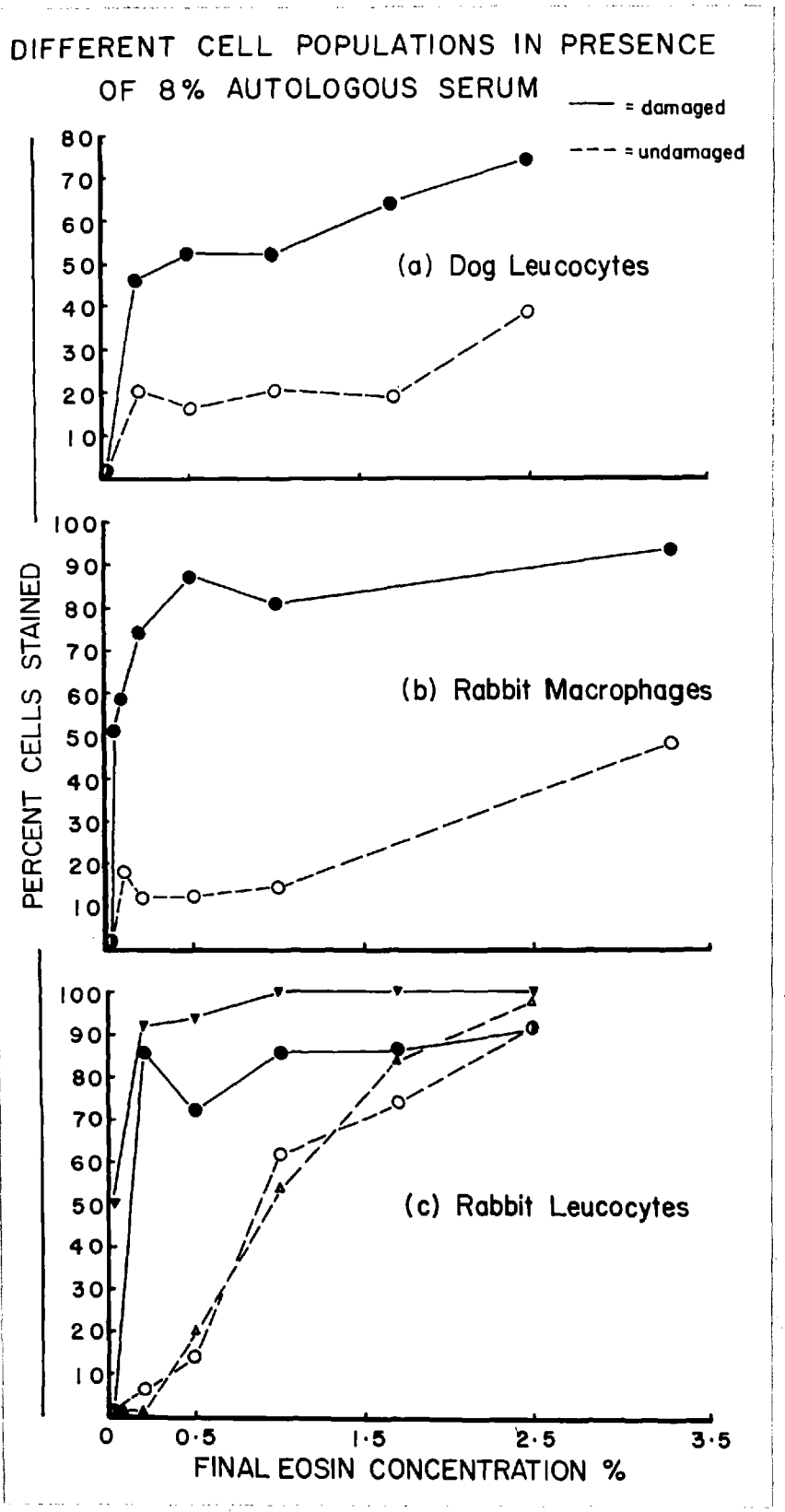
FIGURE 25

Effect of various stain concentrations on different cell populations in presence of 8% autologous serum.
(see Table XIX.)

(a) Dog Leucocytes.

(b) Rabbit Macrophages.

(c) Rabbit Leucocytes.


DIFFERENT CELL POPULATIONS IN PRESENCE OF 8% AUTOLOGOUS SERUM
= damaged
= undamaged
(a) Dog Leucocytes
(b) Rabbit Macrophages
(c) Rabbit Leucocytes
PERCENT CELLS STAINED
FINAL EOSIN CONCENTRATION %
0
0·5
1·5
2·5
3·5

from 0% to > 90% when the stain concentrations were raised from .01% to 2.5%. A similar range in dye concentrations resulted in less variation in the stained counts of the other two cell types.

The suspensions of different cell types were not strictly comparable because each was prepared in a different fashion and consequently the proportions damaged in each suspension may not have been similar. The stained counts at any given serum concentration may have been an indication of the extent of the damage and/or of the inherent ability of each cell type to exclude dye.

However, the fact that the suspensions differed in the degree to which they were influenced by the serum concentration suggests that variation in their staining susceptibilities was at least partly responsible for the observed differences in the stained counts.

Whether the cells differed in fragility or in their ability to exclude dye, the results suggest that some caution is necessary in interpreting experiments where the viability of different cell

types were compared according to their stained counts (Hellström, 1959).

The different cell types varied in the range of eosin concentrations at which damaged and undamaged suspensions could best be differentiated. For example, damaged dog leucocyte or rabbit macrophage suspensions could be distinguished from undamaged with almost equal efficiency at any stain concentration between 0.5% and 2.5% (Figures 25a and 25b). In the case of rabbit leucocytes, however, (Figure 25c) the stained counts converged when the eosin concentrations were raised to 1% and at 2.5% the damaged and undamaged suspensions were virtually indistinguishable.

CHAPTER XI

GENERAL DISCUSSION

Although the dye exclusion test is frequently used to indicate cell viability, relatively few papers have appeared which evaluate the technique (Pappenheimer, 1917; Schrek, 1936; Hoskins et al., 1956; Phillips and Terryberry, 1957; Hanks and Wallace, 1958; Tennant, 1960; Black and Berenbaum, 1964). Five of these (Schrek, 1936; Hoskins et al., 1956; Hanks and Wallace, 1958; Tennant, 1960; Black and Berenbaum, 1964) were concerned with eosin.

The factors which influence the results of this test might be of two main types,

(a) those which affect the dye uptake independent of the proportion of cells originally damaged, e.g. the dye, serum or cell concentrations, staining time, cell type, and

(b) those which affect the proportion of cells damaged, e.g. titre of the cytotoxic antiserum, complement and cell concentrations.

Any proportion from 0% to more than 90% of the cells in a suspension of rabbit leucocytes, damaged or not, could be stained by using appropriate concentrations of serum and eosin. Other cell types were influenced to varying degrees by these factors.

The stained counts were sometimes raised when damaged cells were incubated for 1 hour at 37°C, but in undamaged suspensions the stained counts were not influenced.

In damaged suspensions the counts increased rapidly with the staining time for up to 1 hour and then became more constant. On the other hand, in undamaged suspensions the counts were low and unchanged for at least the first 90 minutes of staining.

Both damaged and undamaged suspensions gave high counts at very low cell concentrations.

Other things being equal, a greater proportion of damaged than undamaged cells stained except at the extreme values of the variables. For example, the stained counts approached each other at high or low serum or stain concentrations and after long staining times.

The Manner in which the Variables affected the Counts

(i) Serum. The high and low stained counts at low and high serum concentrations might be explained by impairment of cell viability at the low serum concentrations. Tennant (1964) showed that serum protects cells from the toxicity of eosin.

However, in certain cases, mechanisms involving viability cannot be implicated, e.g. where few cells were stained in damaged suspensions in the presence of high serum concentrations. Berenbaum and Brown (1964) found that if cells which were stained in low protein media were resuspended in media of high protein concentration, stain was removed from the cells. The higher the serum concentration, the fewer the stained cells. Here one must postulate some interference with the staining mechanism itself. In serum-rich media osmotic pressures may have prevented the ingress of the dye into cells. Such a mechanism might be especially important in damaged cells where the energy dependent integrity of the cell membranes has been impaired and osmotic pressures have assumed the dominant role in controlling the flow across the

membranes (Green, Barrow and Goldberg, 1959; Green and Goldberg, 1960). High concentrations of serum were capable of inhibiting the swelling of cells (Shear, 1935) and in the present study the stained cells commonly appeared much larger than the unstained. At high serum concentrations most cells appeared small in damaged as well as undamaged suspensions.

The binding of stain to serum may be an important factor in the protection afforded by serum against the toxicity of eosin and/or in the inhibition of the staining mechanism itself. de Haan (1922) and Marshall and Vickers (1923) showed that certain dyes were strongly bound to plasma proteins, and other workers have confirmed these findings (Grollman, 1925). Bennhold (1932) and Bennhold, Kylin and Rusznyak (1938) quoted by Edsall (1947), showed that when aqueous solutions of various dyes were mixed with serum they diffused through a gelatin column at the same rate as did the serum. In most cases the dye binding was almost entirely due to the albumin fraction. However, there was a limit to the binding capacity and above a

critical concentration the dye solution moved with a different velocity to that of the albumin-dye complex.

Engelfriet (1965) demonstrated electrophoretically that trypan blue forms a complex with serum albumin and suggested that the dye concentration must exceed the "albumin binding capacity" before becoming available for cell staining.

(ii) Stain. The increased proportions of cells which stained when eosin concentration was raised might be the result of

(a) toxicity of the dye,

(b) increased quantity of free eosin available over and above that bound by the serum.

(iii) Staining Time. In damaged suspensions the increase in stained cells with time might result from

(a) the slow uptake of dye by cells originally damaged,

(b) the succumb of cells to deleterious influences with time, e.g. to the toxicity of eosin, after they had been rendered more vulnerable by the original damage.

(iv) Cell Concentration. At low cell concentrations the counts took longer and the suspensions were exposed to strong light during this time. This may have been responsible for the increased counts in both damaged and undamaged suspensions because Bolande and Wurz (1963) and Allison, Magnus and Young (1966) have shown that eosin is a photosensitising agent. They showed that cells were damaged to a greater extent in light than in darkness in presence of the dye.

Because the stained counts were influenced by several factors they were not indicative of the real proportions originally damaged in a suspension. Any proportion could be made to stain by adding appropriate concentrations of serum and/or dye.

This finding may support those of other workers who compared viability assessments based on different criteria and found that suspensions which

seemed viable according to dye exclusion test were incapable of growth *in vivo* or *in vitro* (Richter and MacDowell, 1933; Hewitt, 1953; Hoskins et al., 1956; Cassel, 1957; Eaton et al., 1959; Craven, 1960; Holmberg, 1961; Tennant, 1964), and that cells which stained were sometimes capable of metabolic activity (Kaltenbach et al., 1958), and sometimes incapable of it (Phillips and Terryberry, 1957; Eaton et al., 1959; Holmberg, 1961).

Nonetheless, the technique was capable of indicating damage between certain limits of the variables discussed and clearly distinguished damaged from undamaged suspensions within these limits.

It appears that stained counts are comparable only if all the salient variables are held constant.

S E C T I O N I I

The Action of Isoantisera

INTRODUCTION

The factors which influenced the proportion of cells damaged by specific isoantisera were investigated.

The investigations were concerned with:

1. The toxicity of normal allogeneic rabbit serum.
2. Whether allogeneic skin grafts or spleen cell injections gave rise to more potent cytotoxic antisera.
3. The time after stimulus when the isoantisera became demonstrable and when their potency reached a peak.
4. The effect of repeated stimuli.
5. The use of blood leucocytes as target cells for the cytotoxic action of isoantisera.
6. The effect of diluting the antisera.
7. The effect of varying the concentration of the target cells in the <u>in vitro</u> test.
8. The titration of complement.

CHAPTER XII

METHODS

A. Skin Grafting

The instruments used are shown in Figure 26. Clean but not sterile technique was employed. The instruments were boiled for 20 minutes before use and the linen was autoclaved.

(i) Anaesthesia: Nembutal (Abbott) was diluted 50:50 in 0.15 M saline and injected intravenously 30 mg./kg. body weight.

(ii) The Grafts: The donor's ear was shaved and cleaned with 1% Cetavlon (I.C.I.). Pieces of skin about 1.5 cm. in diameter were dissected from the dorsal ear surface and placed on moistened filter paper in a petri dish. The skin was scraped free of loose connective tissue. The dish was then covered and kept at 4°C while the recipient beds were prepared.

FIGURE 26

Instruments and apparatus used for skin grafting:

(a) Steriliser.

(b) Clippers.

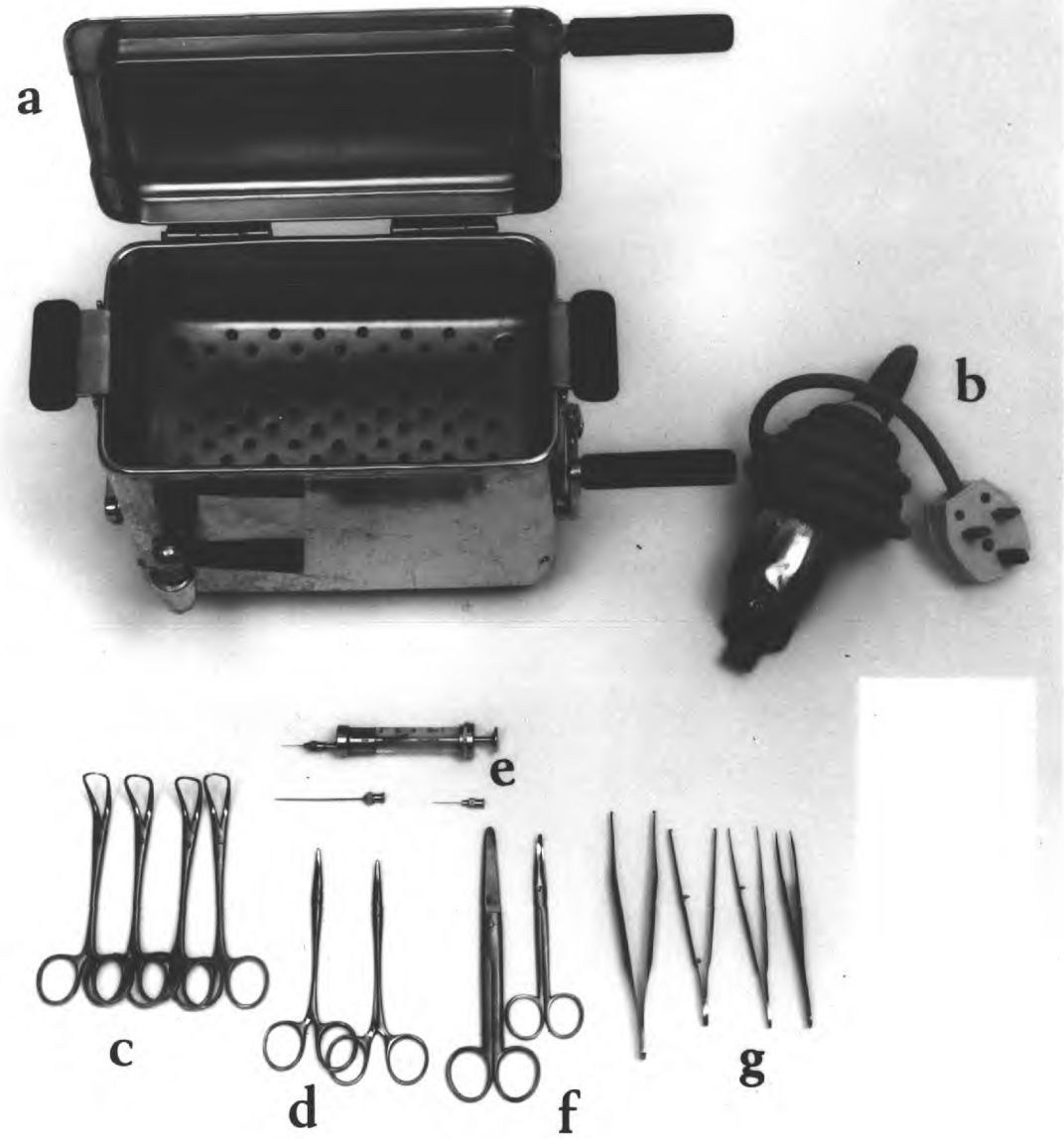
(c) Backhous' towel clips.

(d) Spencer Wells' artery forceps.

(e) 5 ml. syringe,
No. 1 serum needle and
No. 21 hypodermic needle.

(f) 5" straight and
4" curved scissors.

(g) Toothed and
untoothed dissecting forceps.


a
b
e
c
d
f
g

(iii) The Beds: The lateral chest wall was clipped, shaved and cleaned with 1% Cetavlon. Beds about 1.5 cm. in diameter were cut to the depth of the dermal blood vessels by taking away small slices of skin with 4-inch curved scissors. As far as was possible the vessels were kept intact, but if one was damaged haemostasis was accomplished before proceeding. Antibiotic aerosol (Polybactrin, Calmic Ltd.) was sprayed onto the beds.

(iv) Grafting: The grafts were blotted on a cotton swab and fitted to the beds. They were covered with a thick layer of vaseline and a gauze patch. This in turn was covered with a larger gauze patch which was stuck to the skin at the edges with Octaflex (Ward, Blenkinsop & Co. Ltd.). Gauze and plaster of Paris bandages completed the dressings. The grafts were first inspected at 5 to 8 days and the dressings were replaced after each daily inspection until 2 days after rejection was judged complete.

B. Spleen Cell and Adjuvant Inoculations

Under nembutal anaesthesia half the donor's spleen was excised and placed in 2 ml. Hank's. The spleen cells were teased out with forceps and any large cell clumps, pieces of fibrous tissue, etc., were removed. Two ml. Freund's complete adjuvant (Difco Laboratories) were added and the suspension was mixed by passing from one syringe to another 4 to 5 times through a 2 inch serum needle which connected the syringes together. The resulting suspension, which was milky pink, was injected in approximately equal quantities subcutaneously, intradermally and intraperitoneally.

C. The Cytotoxic Test

Leucocytes from the graft donor were separated from whole blood by the dextran sedimentation method described in Section I, page 29, and suspended in Hank's containing 10% serum from the cell donor. The cells were counted and diluted to contain 8.5×10^6 cells/ml. in the Hank's-serum medium. One tenth ml. of this suspension was mixed with 0.05 ml. fresh donor serum (as complement source) and

0.05 ml. antiserum. The preparations were covered with moist tissue paper and incubated for 1 hour at 37°C. One ml. 0.25% eosin Y in Ringer's was added and the proportion of stained cells in 50 leucocytes was counted after 1 hour on ice.

CHAPTER XIII

THE TOXICITY OF NORMAL ALLOGENEIC RABBIT SERUM

(i) Sera from 6 randomly selected normal rabbits were tested for toxicity against the leucocytes of 9 others. The stained counts were compared with those of the leucocytes tested against their own sera. The results are shown in Table XX.

TABLE XX Percent cells stained.

Final Concentrations: 0.7×10^6 leucocytes/ml.

8% serum

0.2% eosin Y

Allo-geneic Sera	Leucocytes								
	83	86	87	88	89	90	91	13	18
A	2	14	28	15	10	2	24	22	37
B	4	10	24	9	14	14	12	27	30
C	8	4	24	15	12	6	21	26	24
D	4	2	14	15	24	2	32	23	43
E	6	16	20	18	8	3	12	17	33
F	10	8	22	18	14	4	18	27	30
Mean and S.D.*	5.7 ±2.7	9 ±5	22 ±4.3	15 ±3	13.7 ±5.1	5.2 ±4.2	19.8 ±7	23.7 ±3.5	32.8 ±6
Auto-geneic Sera	4	6	20	9	14	6	23	20	35

* S.D. = standard deviation

The stained counts of cells in their own sera varied widely (4% to 35%). This may have reflected a difference in the fragility of cells from different individuals because the method of preparing the suspensions was similar in each case.

The stained counts of cells in normal allogeneic sera were of a similar magnitude to their counts in the autogeneic sera. In 7 of the 9 individuals tested the autogeneic counts fell within one standard deviation of the mean of the allogeneic counts, and in the remaining cases (leucocytes 88 and 13) the autogeneic counts fell within 2 standard deviations of the mean allogeneic counts. It would seem that the allogeneic sera were not demonstrably more toxic than were the autogeneic controls.

Normal allogeneic sera were also tested for toxicity in the following way.

(ii) The results obtained in Chapter VII indicate that the stained counts of damaged suspensions increased during the first 60 minutes of staining, while the counts remained more or less unchanged

during this period in undamaged suspensions. Applied in the present context the stained counts of leucocytes mixed with sera might be expected to increase with the staining time if the sera were toxic.

METHOD

Leucocyte suspensions from 3 rabbits were incubated for 1 hour at 37°C with 6 to 8 normal allogeneic sera. The test for cytotoxicity (Chapter XII, page 138) was modified in that the cells were counted at various intervals after adding the eosin.

RESULTS AND DISCUSSION

The results are presented in Tables XXIa, XXIb and XXIc, and Figure 27.

The stained counts were unaffected by variations in the staining time from 15 to 115 minutes (Figures 27a, 27b and 27c), indicating that the leucocyte suspensions were probably undamaged by any of the normal allogeneic sera.

TABLE XXIa (see Figure 27a)

Rabbit leucocytes exposed to normal allogeneic sera and stained for various periods.

Final Concentrations: 0.7×10^6 leucocytes/ml.
8% serum
0.2% eosin Y

Leucocytes from Rabbit No. 13

Normal Sera Nos.							
13 (autogeneic)		12		14		15	
Staining Time	%Cells Stained	Staining Time	%Cells Stained	Staining Time	%Cells Stained	Staining Time	%Cells Stained
15 min	19	13 min	22	15 min	19	17 min	18
30 "	24	28 "	17	28 "	26	30 "	18
60 "	27	58 "	24	83 "	33	45 "	29
80 "	19	78 "	16	95 "	24	65 "	18
115 "	22	98 "	17	112 "	28	77 "	21
127 "	13	128 "	30	127 "	28	100 "	21
-	-	-	-	-	-	135 "	14

Normal Sera Nos.							
16		18		19		20	
Staining Time	%Cells Stained	Staining Time	%Cells Stained	Staining Time	%Cells Stained	Staining Time	%Cells Stained
17 min	17	17 min	18	15 min	18	5 min	18
35 "	20	35 "	17	43 "	18	21 "	17
60 "	27	60 "	15	57 "	20	30 "	21
85 "	22	83 "	20	81 "	23	45 "	12

TABLE XXIb (see Figure 27b)

Final Concentrations: 0.7×10^6 leucocytes/ml.
8% serum
0.2% eosin Y

Leucocytes from Rabbit No. 17

Normal Serum Nos.					
17 (autogeneic)		12		14	
Staining Time	% Cells Stained	Staining Time	% Cells Stained	Staining Time	% Cells Stained
15 min	21	15 min	17	15 min	25
30 "	17	30 "	19	30 "	14
48 "	22	50 "	24	50 "	30
68 "	24	69 "	19	64 "	23
85 "	18	87 "	20	105 "	28
120 "	23	123 "	21	120 "	25

Normal Serum Nos.					
15		19		20	
Staining Time	% Cells Stained	Staining Time	% Cells Stained	Staining Time	% Cells Stained
20 min	18	15 min	14	15 min	16
40 "	27	30 "	26	45 "	11
57 "	20	53 "	17	62 "	15
93 "	27	75 "	19	-	-
110 "	15	-	-	-	-

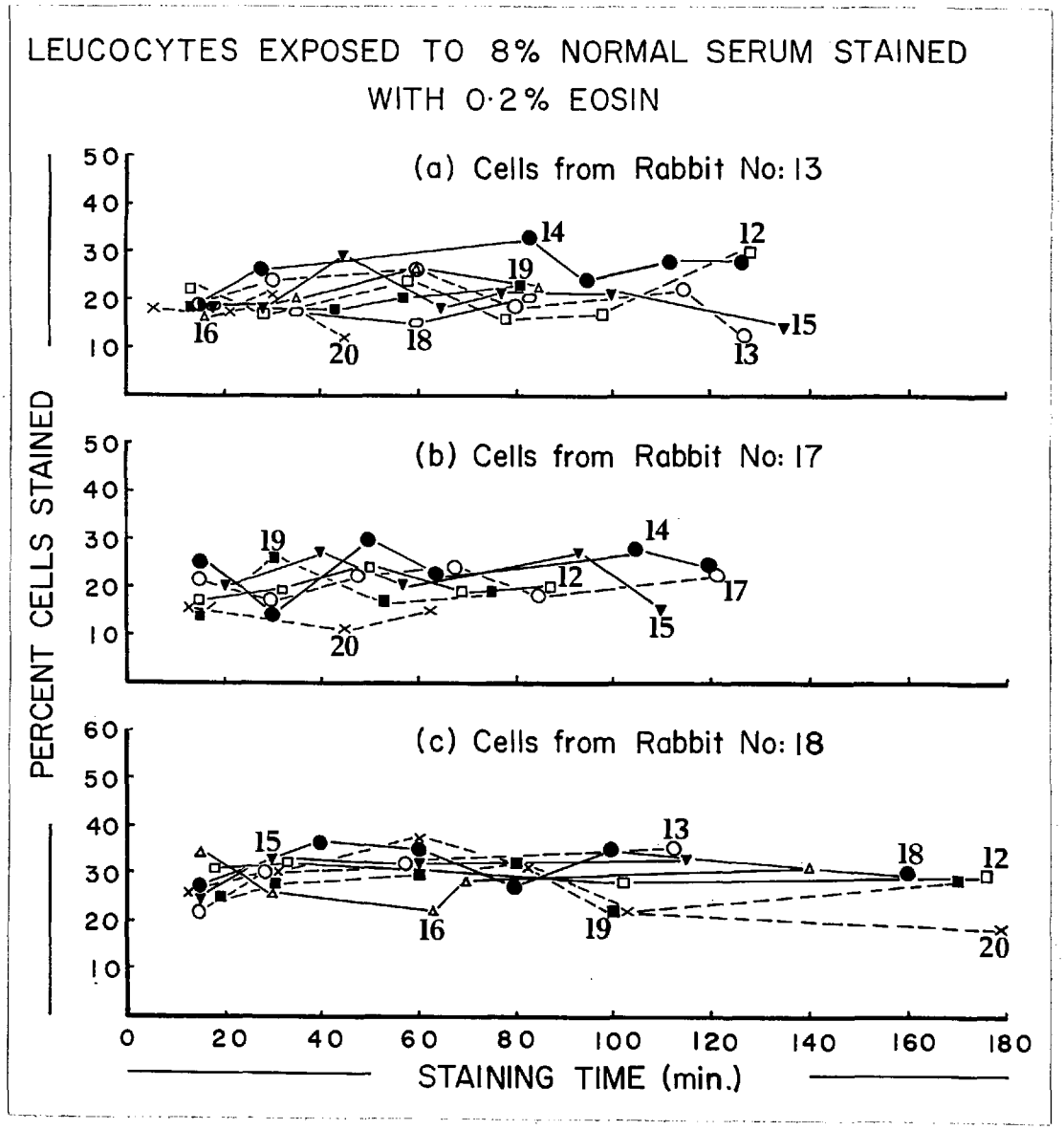
TABLE XXIc (see Figure 27c)

Final Concentrations: 0.7×10^6 leucocytes/ml.
8% serum
0.2% eosin Y

Leucocytes from Rabbit No. 18

Normal Serum Nos.							
18 (autogeneic)		20		19		16	
Staining Time	%Cells Stained	Staining Time	%Cells Stained	Staining Time	%Cells Stained	Staining Time	%Cells Stained
15 min	26	15 min	26	19 min	25	15 min	34
40 "	36	30 "	30	30 "	28	30 "	26
60 "	35	60 "	37	60 "	30	63 "	22
80 "	27	80 "	32	80 "	32	70 "	28
100 "	35	100 "	22	100 "	22	140 "	31
160 "	30	179 "	19	170 "	29	-	-

Normal Serum Nos.					
15		13		12	
Staining Time	% Cells Stained	Staining Time	% Cells Stained	Staining Time	% Cells Stained
15 min	24	15 min	22	18 min	31
30 "	33	29 "	30	33 "	32
60 "	31	58 "	32	102 "	28
115 "	33	113 "	35	175 "	30
215 "	32	197 "	22	-	-



FIGURES 27a, 27b and 27c

The effect of normal rabbit sera on leucocytes from rabbits 13, 17 and 18 respectively.
The number on each trend line is that of the serum.
(see Tables XXIa, XXIb and XXIc.)

CHAPTER XIV

HUMORAL RESPONSE TO ALLOGENEIC TISSUE

In 1937 Gorer first demonstrated *in vitro* a humoral response to allogeneic transplants and he showed that the haemagglutinins provoked by allogeneic sarcomas could be used to identify histocompatibility antigens in mice.

In rabbits, after the rejection of skin allografts the serum of the recipients was shown to be toxic for lymph node cells of the donors (Terasaki, Bold, Cannon and Longmire, 1961a). Puza, Rubinstein, Kasakura, Vlahovic and Ferrebee (1964) showed that in dogs, lymphoid cells were sometimes better stimulants of the humoral response than were skin grafts.

The present experiments were carried out to establish

(a) whether rabbit blood leucocytes were satisfactory target cells for cytotoxic isoantisera,

(b) whether skin grafts or spleen cells and adjuvant were more potent antibody stimulants,

(c) the time when toxicity becomes demonstrable and when its potency reaches a peak, and

(d) the effect of repeated stimuli from the same donor.

METHOD

Rabbits received 2 or 3 skin grafts or 1 or 2 injections of spleen cells and adjuvant at various intervals from the same donors. Serum was taken from the recipients at intervals of about 1 week, and stored in 0.8 ml. aliquots at -10°C. The sera were tested 2 or 3 times for toxicity against donor leucocytes and the results are shown in Tables XXII and XXIII. In Figures 28 and 29 the humoral immune responses to skin grafts and spleen cells are depicted, each point being the mean of the repeated tests.

TABLE XXIIa (see Figure 28)

Cytotoxicity of recipient serum for donor leucocytes at various intervals after skin grafting.

Final Concentrations: 0.7×10^6 leucocytes/ml.
8% serum
0.2% eosin Y

Donor 51 ——→ Recipient 52

Days after first graft	Toxicity of serum as indicated by % cells stained with eosin		
	Results of repeated tests		Average
0* ——→	14	17	16
9	18	22	20
14	5	11	8
21	12	20	16
24 ——→	-	-	-
28	12	8	10
34	18	30	24
42	68	60	64

XXIIb

Donor 36 ——→ Recipient 38

Days after first graft	Toxicity of serum as indicated by % cells stained with eosin		
	Results of repeated tests		Average
0 ——→	-	-	-
1	6	2	4
5	3	11	7
12	4	15	10
23	4	18	11
24 ——→	-	-	-
30	38	50	44
37	44	45	45
42 ——→	-	-	-
44	28	36	32
51	40	50	45
62	38	56	47

* ——→ indicate skin grafts.

TABLE XXIIc

Final Concentrations: as for Table XXIIa.

Donor 32 ⟶ Recipient 34

Days after first graft	Toxicity of serum as indicated by % cells stained with eosin		
	Results of repeated tests		Average
0 ⟶	-	-	-
1	6	2	4
7	2	4	3
12	8	16	12
21	16	2	9
30	22	14	18
36	10	14	12
47	22	8	15
52 ⟶	6	8	7
61	20	8	14
63	16	14	15
78	17	19	18
85	17	14	16
91	4	4	4
98	2	12	7
101 ⟶	-	-	-
105	12	6	9
112	9	8	7
126	12	20	16

XXIId

Donor 53 ⟶ Recipient 55

Days after first graft	Toxicity of serum as indicated by % cells stained with eosin			
	Results of repeated tests			Average
0 ⟶	2	8	5	5
7	8	4	6	6
9	6	15	0	7
14	0	6	9	5
20	8	4	9	7
24 ⟶	-	-	-	-
27	-	24	8	16
34	36	36	18	30
42 ⟶	38	25	24	29
48	36	41	43	40
56	49	62	58	56
61	35	24	40	33
75	32	33	27	31
78	29	24	28	27
88	24	11	19	18

TABLE XXIIe

Final Concentrations: as for Table XXIIa.

Donor 37 ⟶ Recipient 41

Days after first graft	Toxicity of serum as indicated by % cells stained with eosin		
	Results of repeated tests		Average
0 ⟶	18	4	11
7	6	2	4
14	7	3	5
21	0	10	5
24 ⟶	-	-	-
27	6	12	9
36	4	6	5
42 ⟶	16	14	15
49	20	20	20
57	20	30	25
64	13	18	16
71	13	14	14
78	8	20	14
84	9	9	9
92	10	17	14
98	12	4	8

TABLE XXIIIa (see Figure 29)

Cytotoxicity of recipient serum for donor leucocytes at various intervals after injection of spleen cells and adjuvant.

Final Concentrations: 0.7×10^6 leucocytes/ml.
8% serum
0.2% eosin Y

Donor 48 ⟶ Recipient 49

Days after first injection	Toxicity of serum as indicated by % cells stained with serum		
	Results of repeated tests		Average
0* ⟶	17	11	14
3	14	13	14
7	5	11	8
14	19	10	16
22	78	87	83
28	75	91	83
35	90	75	83
43	97	89	93
52	89	77	83

XXIIIb

Donor 47 ⟶ Recipient 45

Days after first injection	Toxicity of serum as indicated by % cells stained with serum		
	Results of repeated tests		Average
0 ⟶	12	8	10
1	16	22	18
7	15	14	15
14	52	30	41
21	21	26	24
28	28	34	31
35	38	26	32
43	16	24	20
49	24	25	25

* ⟶ indicate spleen cell and adjuvant injections.

TABLE XXIIIc

Final Concentrations: as for Table XXIIIa.

Donor 46 ⟶ Recipient 50

Days after first injection	Toxicity of serum as indicated by % cells stained with eosin		
	Results of repeated tests		Average
0 ⟶	9	9	9
2	4	8	6
7	10	32	21
14	10	8	9
22	12	12	12
28	16	16	16
35	14	10	12
43	24	10	17
51	20	14	17
57	14	15	15
59 ⟶	-	-	-
63	40	46	43
70	50	57	54
84	56	67	62
98	42	52	47

TABLE XXIIId

Donor 58 ⟶ Recipient 59

Days after first injection	Toxicity of serum as indicated by % cells stained with eosin			
	Results of repeated tests			Average
0 ⟶	18	2	13	11
5	28	18	10	19
8	20	34	20	23
14	23	23	24	23
21	60	60	40	53
28	38	48	34	40
35	36	60	30	42
39 ⟶	-	-	-	-
42	62	50	52	55
49	70	73	87	77
57	72	63	78	71
63	78	84	66	76
75	47	60	49	52
79	54	46	62	54
84	58	57	57	57
92	63	45	42	50

TABLE XXIIIe

Final Concentrations: as for Table XXIIIa.

Donor 56 ⟶ Recipient 57

Days after first injection	Toxicity of serum as indicated by % cells stained with eosin			
	Results of repeated tests			Average
0 ⟶	14	14	24	17
5	14	22	12	16
8	29	38	13	27
14	24	28	24	25
21	26	14	12	17
28	24	12	-	18
35	32	26	20	26
39 ⟶	-	-	-	-
42	56	20	50	42
49	49	69	71	63
56	58	38	51	49
63	65	49	57	57
74	62	56	45	54
77	63	42	45	50
84	25	14	27	22
93	27	25	31	28

TABLE XXIIIf

Donor 60 ⟶ Recipient 61

Days after first injection	Toxicity of serum as indicated by % cells stained with eosin			
	Results of repeated tests			Average
0 ⟶	6	2	15	8
5	19	12	8	13
9	8	14	2	9
14	12	12	8	11
21	15	17	0	11
28	16	5	5	9
35	4	8	8	7
38 ⟶	-	-	-	-
43	18	6	8	11
49	20	10	18	16
58	9	14	17	13
75	20	6	8	11
84	20	9	18	16
91	25	12	18	18

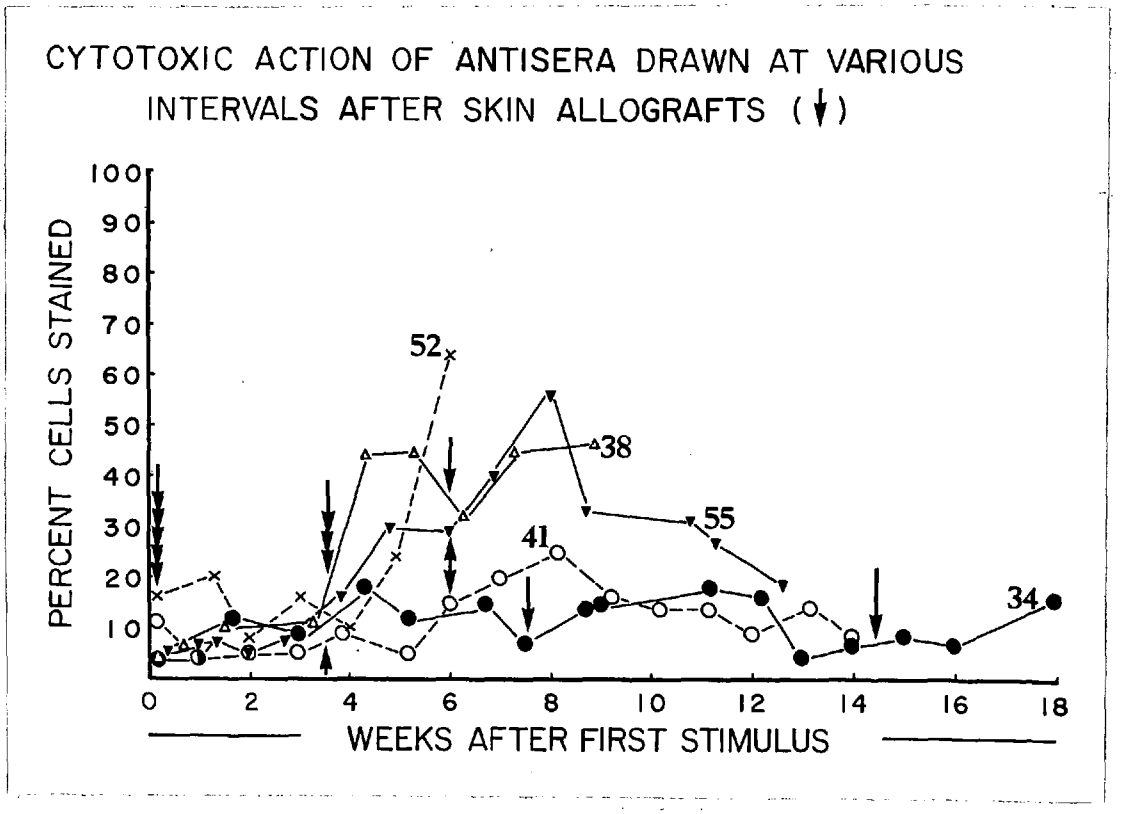


FIGURE 28

Toxicity of isoantisera provoked by skin grafts. The numbers on the trend lines are those of the graft recipients. (see Table XXII.)

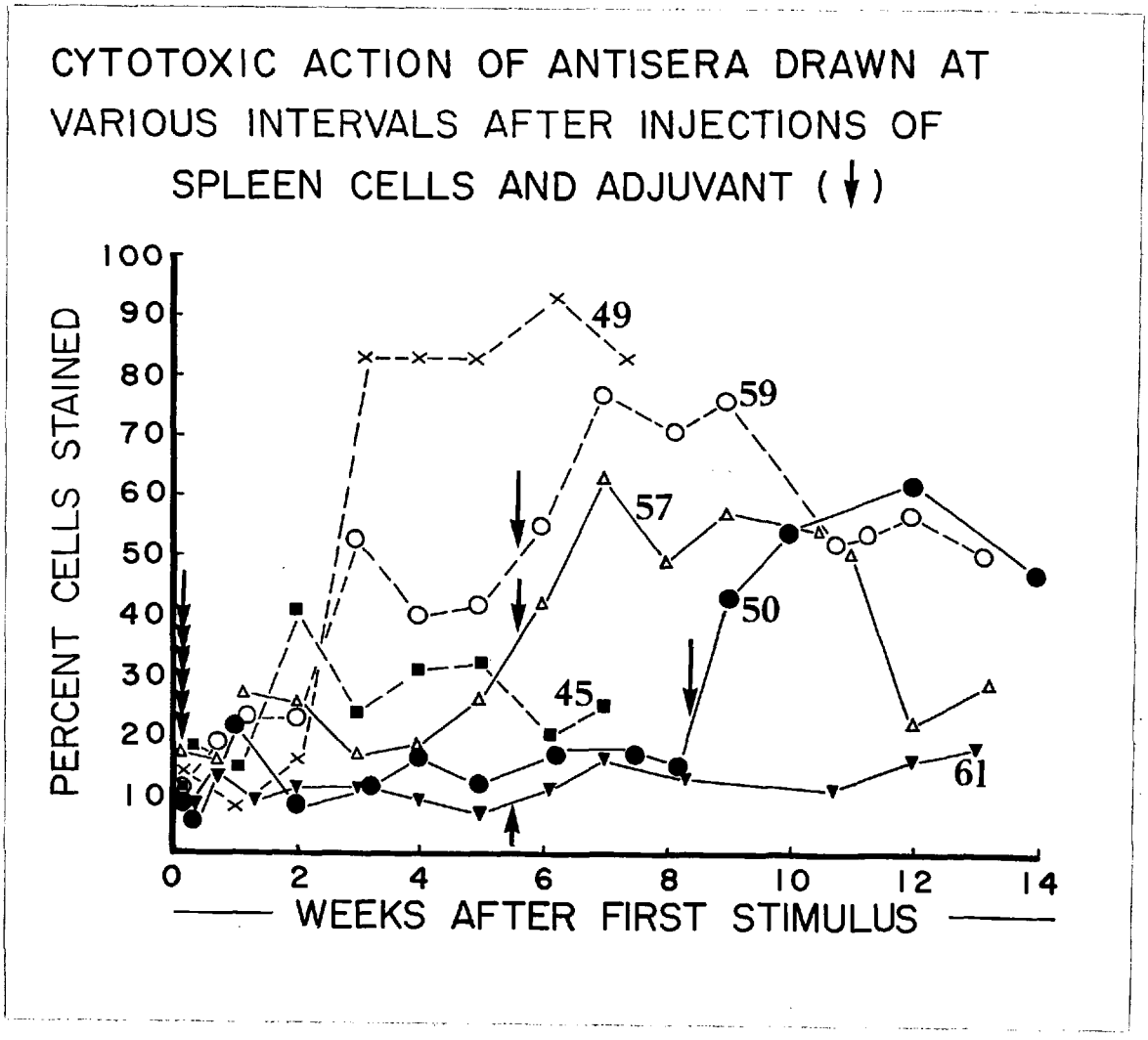


FIGURE 29

Toxicity of isoantisera provoked by injections of spleen cells and adjuvant.
The numbers on the trend lines are those of the spleen cell recipients.
(see Table XXIII.)

The results indicate that

(i) Blood leucocytes were satisfactory target cells for the *in vitro* demonstration of the cytotoxicity of rabbit isoantisera.

(ii) Spleen cells and adjuvant were better stimulants of antibody production than were skin grafts. (The potency of the spleen cell recipients' sera was greater than that of the skin allograft recipients' in spite of the fact that spleen cells were injected once or twice, whereas skin was sometimes grafted 3 times (see Figures 28 and 29).)

(iii) The toxicity of the recipients' sera became demonstrable 1 to 3 weeks after the primary stimulus in 4 of the 6 animals which received spleen cells and adjuvant (Figure 29). In a similar period no toxicity was demonstrable in any of the 5 which received a single skin graft (Figure 28).

A peak in toxicity usually occurred after 2 to 3 weeks in those recipients which responded to the primary stimulus and about 1 to 2 weeks after the repeated stimuli in most animals. High toxic levels were not long maintained and were usually on

the decline 4 to 5 weeks after reaching a plateau.

(iv) Repeated stimuli usually boosted the toxicity. The response was prompt and in 2 recipients (Nos. 57 and 59) the increased toxicity was demonstrated 3 days later. (The response may have occurred even earlier but the toxicity of the sera was not tested earlier than 3 days after the repeated transplants.)

Little cytotoxicity was demonstrable in 2 of the 5 skin graft recipients (Nos. 41 and 34), and in one of the 6 spleen cell recipients (No. 61), even after repeated transplants or injections. The lack of response in these 3 might indicate that
(a) cytotoxic antibodies were provoked by some histocompatibility antigens only, and these were not implicated in the 3 cases, or
(b) the cytotoxic test demonstrated only the antibodies exceeding a certain threshold activity.

For the purpose of the present investigation it is unimportant to differentiate between (a) and (b), i.e. between qualitative and quantitative failure in the humoral response provided that the antigens which were demonstrated were those

most important for histocompatibility. The ability of cytotoxic antisera to demonstrate the important histocompatibility antigens is further investigated in Section III.

In some recipients, (e.g. Nos. 52, 38 and 55), although no toxicity was demonstrable after a single graft, a repeated graft from the same donor provoked a prompt rise in toxicity. The first graft may have provoked the formation of humoral antibodies which were not detected because of the insensitivity of the test.

Some degree of insensitivity may not be disadvantageous because it might serve to eliminate from consideration the unimportant antigens which would otherwise complicate the histocompatibility test to be described.

The work of Walford, Martin, Troup and Goodwin (1966) suggests that, in humans, the failure to form lymphocytotoxins is correlated with close matching of the donor and host and with the prolonged survival of skin allografts.

CHAPTER XV

TITRATION OF ISOANTISERA

The toxicity of isoantisera was tested at various dilutions.

METHOD

Isoantisera were provoked by injecting spleen cells and adjuvant on 2 occasions with a 3-week interval and serum was taken from the recipients 1 week later. Doubling dilutions of this serum were prepared in fresh donor serum. One tenth ml. of each serum dilution was mixed with 0.1 ml. donor leucocyte suspension (8.5×10^6 cells/ml.). After 1 hour's incubation 1 ml. 0.25% eosin Y in Ringer's was added and the proportion of stained cells was counted after 1 hour on ice.

RESULTS AND DISCUSSION

The results are shown in Table XXIV. The proportion of cells which stained in the 1:2 antiserum dilution varied from 20% to 87% and, as the antisera were further diluted, so the counts decreased.

TABLE XXIV (see Figure 30)

Toxicity of isoantiserum at various dilutions. Total serum concentration held constant.

Final Concentrations: 0.7×10^6 leucocytes/ml.
8% serum (allogeneic and autogeneic)
0.2% eosin Y

		Ratio antiserum : total serum						
		1:2	1:4	1:8	1:16	1:32	1:64	0:1
% Cells Stained	(a)	58	42	56	42	18	22	2
	(b)	87	58	56	44	34	30	10
	(c)	36	18	21	11	-	-	14
	(d)	64	56	32	36	16	-	6
	(e)	20	30	16	2	12	-	14
	(f)	36	40	24	18	8	-	10
	(g)	20	18	8	6	-	-	4
	(h)	48	38	30	30	-	-	8
	(i)	20	16	12	10	14	-	6
	(j)	64	56	64	50	28	17	10
	(k)	34	36	14	10	-	-	17
	(l)	28	24	6	10	4	-	8

Where high proportions stained at 1:2 dilution the toxicity was demonstrable at greater dilutions. For example, when 87% stained at 1:2 (b), the toxicity was still demonstrable at 1:64 dilution, whereas when the count at 1:2 was 34% (k), the toxicity was no longer demonstrable at 1:8 dilution.

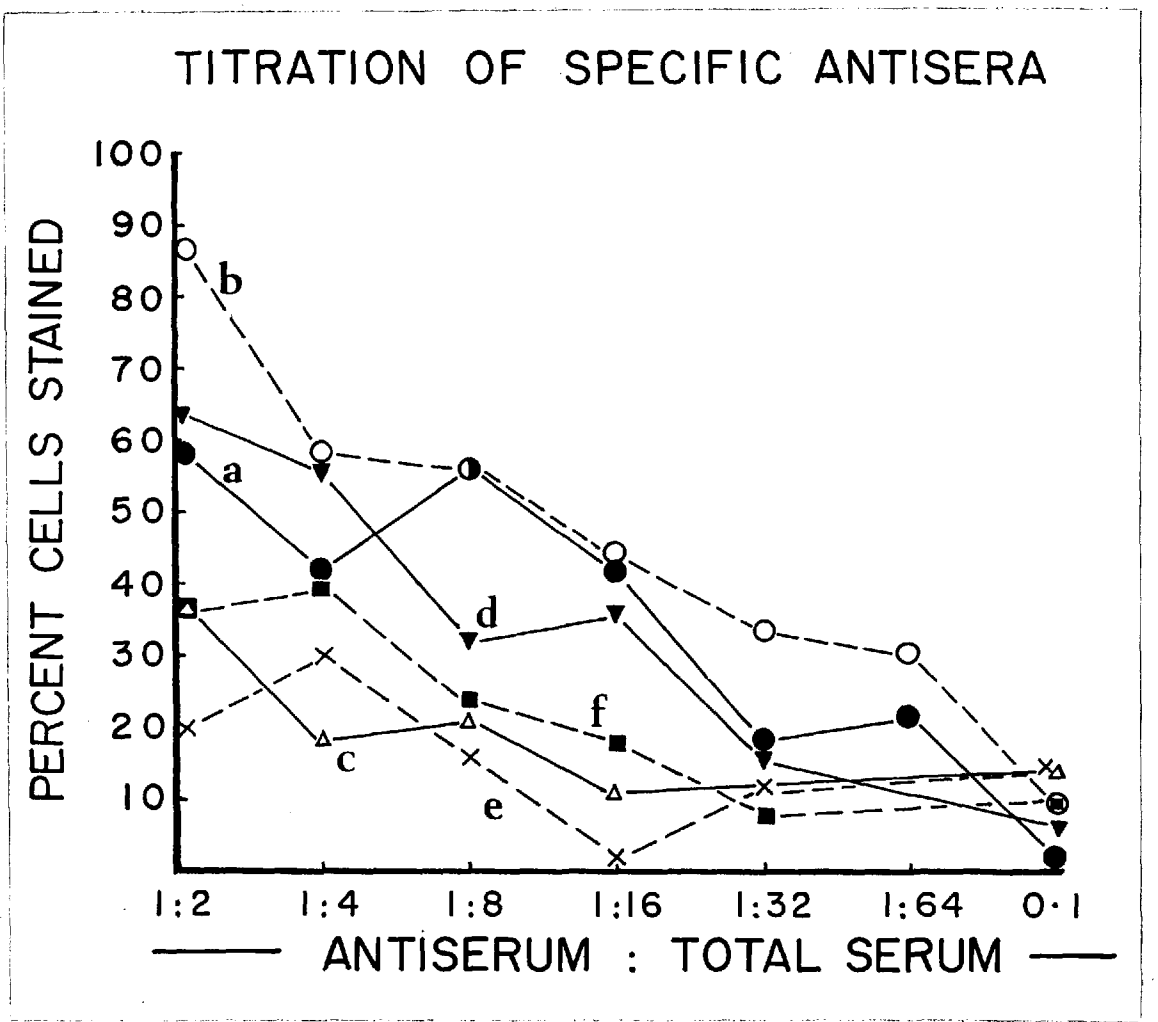


FIGURE 30

The effect of diluting toxic isoantiserum. The letter on each trend line is that of the experiment. (see Table XXIV)

The antisera were, in fact, diluted more that the titres in Table XXIV imply because further dilution occurred in the medium of the cell suspensions after mixing. Higher stained counts might have resulted in damaged suspensions if smaller volumes of concentrated cell suspension were used, thus reducing the antiserum dilution. However, this was undesirable because

(i) it would increase the total concentration of serum in the mixture and this in turn might inhibit staining (see Chapters IV and VI), and

(ii) the number of cells in each aliquot might vary considerably because of the difficulty of dispensing small volumes of concentrated suspensions accurately.

The importance of maintaining the cell concentration constant has been emphasised by the results of Boyse, Old and Stockert (1962), (see next chapter).

CHAPTER XVI

CELL CONCENTRATION

Boyse, Old and Stockert (1962) incubated various concentrations of mouse cells with specific isoantisera and used the dye exclusion test to estimate the damage to each suspension.

Figure 31 which is reproduced from their paper depicts the results.

The proportion which stained depended on the concentrations of cells and antiserum. When the concentration of cells was decreased the proportion which stained increased and at high cell concentrations the stained counts were low.

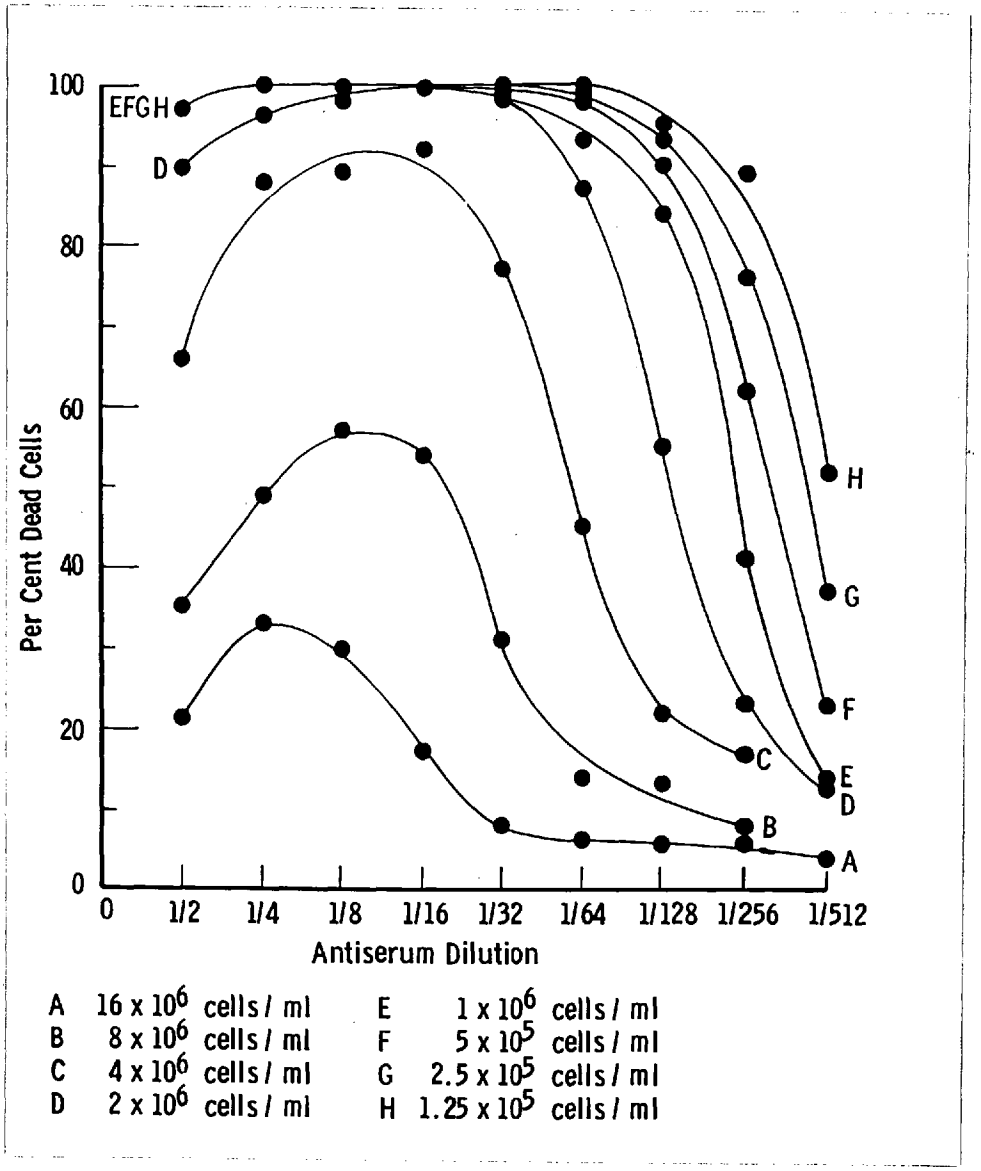


FIGURE 31

The influence of cell concentration when mouse ascites cells were mixed with various concentrations of toxic isoantiserum.

Reproduced from Boyse, Old and Stockert, 1962, by kind permission of the authors and the publishers of Annals of the New York Academy of Science.

CHAPTER XVII

COMPLEMENT

It is desirable to use the minimum volume of complement which is commensurate with full activity of the antiserum because

(i) high concentrations of serum inhibit the staining of leucocytes and impair the differentiation of damaged and undamaged suspensions (Chapter IV),

(ii) as the total serum in any preparation was made up of isoantiserum and complement source, the less complement in the mixture, the more antiserum which could be used while still maintaining the total serum concentration at a suitable level.

REAGENTS

0.1 ml. rabbit leucocyte suspension containing 8.5×10^6 cells/ml.

0.05 ml. toxic isoantiserum which had been inactivated by heating to 56°C for 30 minutes.

0.05 ml. fresh autologous serum, various proportions of which had been inactivated as above.

1 ml. 0.25% eosin Y in Ringer's.

METHOD

The cell suspension, antiserum and autologous serum were mixed together and incubated for 1 hour at 37°C before adding the stain. The counts were carried out after 1 hour on ice.

RESULTS AND DISCUSSION

The results are shown in Table XXV and Figure 32.

TABLE XXV (see Figure 32)

Complement titration.

Final Concentrations: 0.7×10^6 leucocytes/ml.

8% serum comprising
4% inactivated antiserum and
4% fresh autologous serum,
various proportions of which were inactivated.

0.2% eosin Y

Complement dilution*		1:1	1:2	1:4	1:8	1:16	0:1
% Cells Stained	(a)	18	17	8	4	0	7
	(b)	26	14	14	16	8	6
	(c)	14	10	16	8	6	2
	(d)	70	34	12	18	14	6
	(e)	58	28	12	6	-	10
	(f)	86	42	40	16	-	16

Figure 32 shows that complement was essential for the cytotoxic action of isoantisera on rabbit blood leucocytes.

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* unheated own serum : total autologous serum.

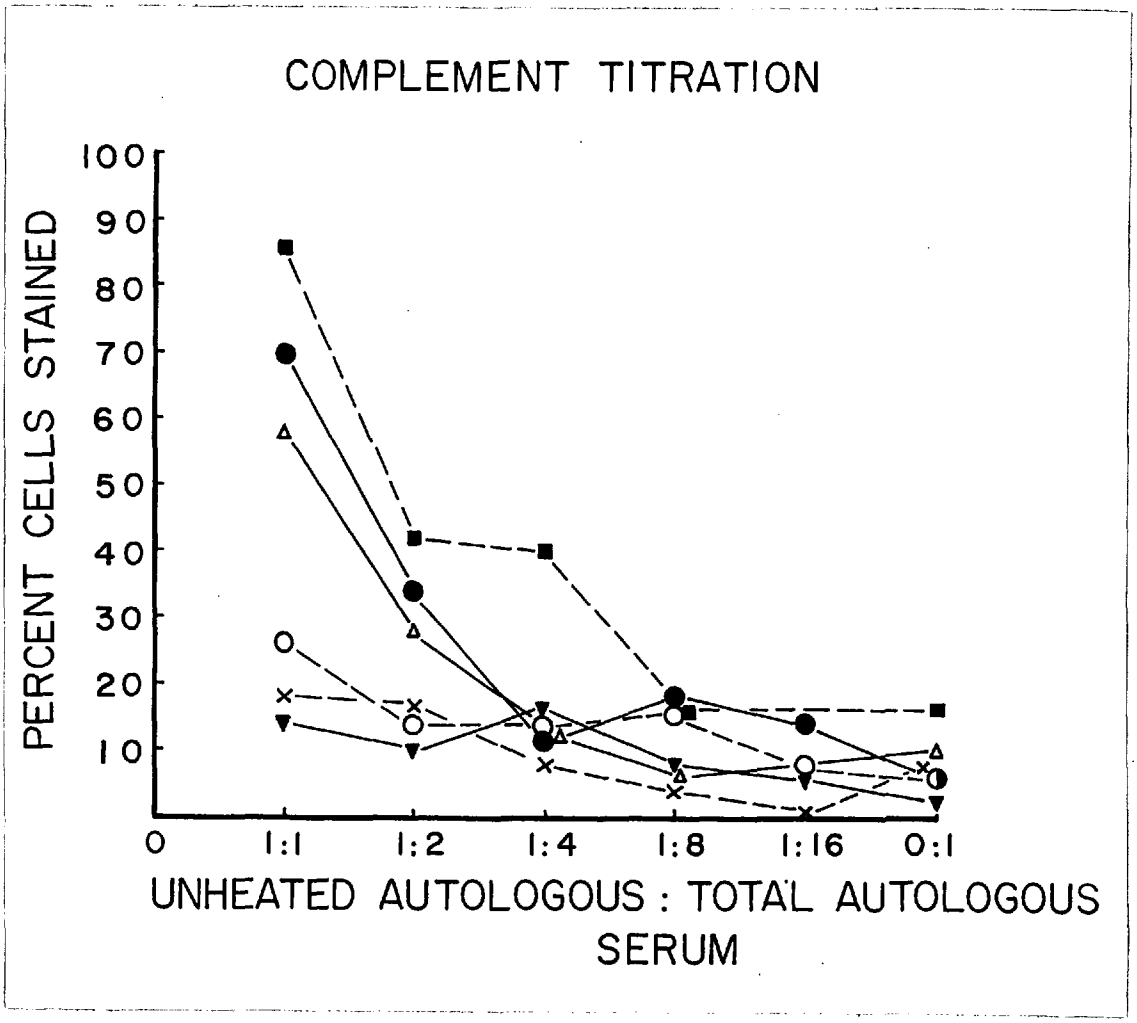


FIGURE 32

The influence of complement. The antisera were held at 56°C for 30 minutes to inactivate complement. Fresh autologous serum was added and the abscissa shows the proportions of this serum which were inactivated.
(see Table XXV.)

When the proportion of stained cells was high (e.g. (d), (e) and (f)), most of the complement which was present was needed for the reaction because twofold dilution of the complement source resulted in a marked decrease in the stained cell counts.

CHAPTER XVIII

GENERAL DISCUSSION

The cornerstone of the method to be described in the next section is the comparison of the damage caused by an antiserum to a number of different cell suspensions.

When the dye exclusion test is used to manifest the cytotoxicity of an antiserum, the stained counts may be influenced by many factors not all of which are directly associated with the damaging action of the antibodies.

In choosing a value at which to maintain each variable, the following factors had to be considered:

1. The test distinguished damaged and undamaged suspensions with optimal efficiency between certain ranges of each variable.
2. The variables were interdependent.
3. The factors sometimes exerted a complex influence and affected cell damage in one way and staining in another.

For instance, in selecting the appropriate serum concentration

(i) An intermediate total serum concentration was desirable because at the extremes the sensitivity of the test was diminished (Figure 2).

(ii) High concentrations of antiserum were desirable in order to exert a maximum toxic action (Figure 30).

(iii) An excess of complement was desirable to ensure that complement was not a limiting factor (Figure 32).

(iv) The total serum and stain concentrations were interdependent (Figures 6 and 7), and the antiserum and cell concentrations were interdependent (Figure 31).

There were also conflicting considerations with other variables. The proportions of stained cells increased when antiserum was mixed with decreasing numbers of target cells (Figure 31), and assuming that this was at least partly a manifestation of specific cytotoxic damage, low cell concentrations were desirable. On the other hand, at low concentrations greater proportions of cells were non-specifically stained (Figure 23).

It was not possible to satisfy all the requirements fully and the parameters used

for each variable represented a compromise, as those which gave satisfactory differentiation. However, it is possible that some other set of values might give as good or even more clearcut results.

S E C T I O N I I I

The Histocompatibility Test

INTRODUCTION

Four tests have been described as histocompatibility indicators, these are:

In Vivo

1. The normal lymphocyte test.
2. The third party test.

In Vitro

3. The mixed lymphocyte culture test.
4. The serological manifestation and matching of antigens.

None have been refined to a stage where accurate predictions can be expected. However, the results from the preliminary trials follow.

CHAPTER XIX

REVIEW

THE IN VIVO TESTS

1. Normal Lymphocyte Transfer (NLT)

(Brent and Medawar, 1963.) This involves the intradermal injection of lymphocytes from the prospective recipient into a number of potential donors. The intensity of the inflammatory reactions at the injection sites were closely correlated with the survival times of skin grafts in guinea pigs. The authors suggested that this test might be applied to donor selection in man.

They explained the inflammatory reactions as manifestations of the graft versus host (GVH) phenomenon, i.e. an expression of the reaction of the injected cells to the tissue antigens of the prospective graft donor which confronted them at the injection site. Hence the correlation with the rejection times when a similar confrontation occurred at skin grafting.

In rabbits the local reactions were predominantly graft-versus-host or host-versus-graft responses according to whether the injected cells had been presensitised against their hosts (Brent, Brown and Medawar, 1962; Dvorak, Kosunen and Waksman, 1963), or the hosts presensitised against the cells (Berg and Dausset, 1964). In the latter case, the reactions have been used to predict skin graft rejection orders.

In man the dermal reactions were to some extent a measure of the antigenic disparity between donor and host, and could be correlated with graft rejection orders if they were regarded as graft-versus-host reactions (Gray and Russel, 1963, 1965; Ceppelini, Curtoni, Leigheb, Mattiuz, Miggiano and Visetti, 1965b). The reactions were evaluated according to the areas of induration and erythema at 48 hours (Gray and Russel, 1965), or to the response to 3 different cell doses (Ceppelini et al., 1965b).

However, the results of the NLT test were often difficult to interpret in man. The local inflammations were at least partly host-versus-graft reactions (Gray and Russel, 1963; Amos, Nicks, Peacocke and Sieker, 1965; Harris, 1965), but the reactions were no more pronounced

in sensitised recipients of normal cells or normal recipients of sensitised cells than they were in normal recipients of normal cells (Amos et al., 1965). The reactivity of the subjects was altered by disease (Amos et al., 1965) and poor reactions were provoked when the injected cells were from uraemic subjects (Bridges, Nelson and McGeown, 1964; Gray and Russel, 1965; Harris, 1965). This is important because uraemic subjects might form a large proportion of those requiring a histocompatibility test.

Furthermore, the areas of erythema were influenced by non-specific factors (Harris, 1965; Harris, Clarke, Jones, Sheppard, Lehane, McCarthy, Lawler and Shatwell, 1966). They were greatly affected even by gentle rubbing, and were poorly reproducible after 10 weeks (Amos et al., 1965). Interpretation was especially difficult in darkly pigmented people (Amos et al., 1965). False negatives occurred when the cells were injected too deeply (Harris et al., 1966).

A weak correlation between the NLT test and the leucocyte agglutination reactions of a panel of isoantisera was noted by van Rood, van Leeuwen, Schippers, Vooys, Frederiks, Balner and Eernisse (1965), but none was noted by other workers (Goldsmith, 1964; Dausset (as quoted by

Dormont), 1965; Hamburger, Crosnier and Dormont, 1965; Harris et al., 1966). There was no correlation between the NLT test and A, B and O erythrocyte incompatibilities (Moorhead and Patel, 1964).

Gray and Russel (1965) concluded that the test was insensitive, but nonetheless it might be of use in excluding unsuitable donors.

Most authors mentioned the danger of transmitting pathogens and of sensitising the cell recipients to tissue antigens. In man, intradermally injected leucocytes sensitised the recipients against skin grafts (Friedman, Retan, Marshall, Henry and Merrill, 1961), but the sensitivity was no longer demonstrable 5 months later (Gray and Russel, 1965). Metzgar, Zmijewski and Amos (1965) showed that circulating leucoagglutinating and cytotoxic antibodies were also produced in response to intradermal leucocyte injections, and these were still demonstrable 15 months later (Amos, 1965).

Modifications have been suggested which might diminish some undesirable aspects of the NLT test.

Wolf-Jürgensen and Schwartz (1964) suggested that the skin window technique of Rebuck, Petz, Riddle,

Priest and Logrippo (1961) might provide a more reliable way of assessing the inflammatory reactions at the leucocyte injection sites.

Ramseier and Streilein (1965) and Streilein (1965) suggested that sensitisation of humans might be avoided by using the skin of animals as the sites of the dermal test. When the leucocytes from human donor and recipient pairs were mixed in vitro and injected into irradiated hamsters' skin, the intensity of the reactions was correlated with the survival times of grafts between the tested humans.

2. The Third Party Test

Antigens shared by the prospective donors and recipients were assessed according to the celerity with which skin grafts from each were rejected by a third party. Test grafts from the prospective donors were transferred to a third party which had previously rejected a sensitising graft from the prospective recipient.

The test graft rejected in the shortest time was regarded as from the best possible donor, and that which survived longest as from the worst. In other words, accelerated rejection of a test graft on the third

party was interpreted as evidence for the antigenic similarity of the sensitising and test grafts.

Matsukura, Méry, Amiel and Mathé (1963) compared the results of this test in rabbits with putative histocompatibility relationships, assuming that rabbits of the same breed were more histocompatible with one another than with individuals of other breeds, and they found a very high degree of correlation ($P < .00004$).

When the test was applied in man, Rapaport, Thomas, Converse and Lawrence (1961) found that the rejection times of skin grafts between 9 unrelated pairs were suggestive of correlation; Wilson, Henry and Merrill (1963) showed that the test grafts on the third party did not take at all, i.e. were "white grafts", when the prospective donor and recipient were identical twins. Using the third party test, Mathé, Méry Brézin, Sekiguchi, Vaubel and Amiel (1965) were able to predict correctly the rejection orders in 10 of 12 cases where there were clearcut differences between the survival times of 2 grafts placed on each recipient, and they concluded that the results were better correlated with graft rejection orders than were the results of the normal lymphocyte

transfer test, the mixed lymphocyte culture test, or the serological matching of antigens used by some other workers.

The test would seem to be limited because it presumes to measure the shared antigens and not the antigenic differences, and because only the antigens not carried by the third party are measured. However, the good results reported might indicate that the strong histocompatibility antigens in man are so few that the sharing of even some of them prolongs the graft survival times.

THE IN VITRO TESTS

3. <u>The Mixed Lymphocyte Culture (MLC) Test</u>

The transformation of lymphocytes in culture to cells resembling lymphoblasts is thought to be a response to contact with antigenic material (Elves, Roath and Israëls, 1963; Pearmain, Lycette and Fitzgerald, 1963). In a culture of mixed lymphocytes from two individuals the proportion of large dividing cells which are capable of incorporating H^3-thymidine may be an indication of the antigenic disparity between the lymphocytes and it has been suggested that a count of

these cells may provide a measure of their histocompatibility differences (Bain, Vas and Lowenstein, 1964; Bach and Hirschhorn, 1964).

A correlation has been demonstrated between the proportions of transformed cells and the familial relationship of the donors. There were few transformations in mixtures of cells from monozygotic twins, more in the case of dizygotic twins and there were many transformed cells in all the mixtures from unrelated individuals (Bain et al., 1964). Similar correlations have been reported after comparing blast counts in the cell mixtures from siblings with those from unrelated individuals (Bain, Lowenstein and MacLean, 1965); from identical twins with those from first degree relatives and unrelated individuals (Hirschhorn, Firschein and Bach, 1965), and those from siblings with parent-child mixtures and unrelated individuals (Ceppelini et al., 1965b).

Correlations have also been demonstrated between this test and the state of reactivity of the tested individuals, for example, in illness and in health or before, during and after allograft rejections. There was an increase in the MLC reactions following the rejection

of skin grafts in man (Bain et al., 1965) and in monkeys (Moynihan, Jackson and Hardy, 1965), and Oppenheim, Whang and Frei (1965) showed in 2 mixed cultures of human donor/recipient cells that the number of lymphoblasts increased at the time of primary skin graft rejection, fell afterwards, and increased again during the rejection of a second graft.

When the mixed lymphocytes were from the donor and recipient of a previous skin graft, the transformation to lymphoblasts was observed much earlier than in non-immunised individuals (Ceppelini, Celada and Zanalda, unpublished, reported by Hirschhorn et al., 1965), and this led to the suggestion that MLC reactions might represent <u>in vitro</u> equivalents of primary and secondary allograft rejection responses (Hirschhorn et al., 1965).

Bain et al. (1965) showed that in patients suffering from debilitating conditions, the MLC reactions were decreased and the allograft survival times increased. Hashem, Sedlis, Hirschhorn and Holt (1963), studying auto-immune disease, demonstrated an increased proportion of lymphoblasts when the lymphocytes from patients with infantile eczema were incubated with extracts of their

own skin and, to a lesser extent, with skin extracts of other individuals.

The MLC reactions were correlated with the third party test (Bach and Hirschhorn, 1964; Hirschhorn, Bach, Rapaport, Converse and Lawrence, 1964), and with certain serologically detected antigenic differences in man (Dausset, Ivanyi and Ivanyi, 1965b) and chimpanzees (de Witt, 1965).

Although the work cited above suggests that the MLC test can reflect broad antigenic differences, there is little evidence that the test is sufficiently sensitive where more subtle histocompatibility differences are concerned. Bain et al. (1965) found no correlation between graft survival times and the MLC reactions in 5 pairs of healthy individuals; and Ceppelini et al. (1965b) after 76 comparisons of skin graft survival times with the test, concluded that there was only a correlation in some circumstances. While strong activation of cultures was indicative of short graft survival there was a poor correlation with histocompatibility when the activation of cultures was weak. Moynihan et al. (1965) demonstrated a close correlation between the test and skin graft survival in monkeys.

Ceppelini et al. (1965b) and Bain and Lowenstein (1965) reported that the test was sometimes poorly reproducible, and Riddle and Berenbaum (1966) found that even minor surgical procedures altered the reactivity of lymphocytes in culture. Efforts have been made to achieve better reproducibility by standardising each of the variables which influence the technique (Bain and Lowenstein, 1965; Eijsvoogel and Schellekens, 1965).

The lymphoblasts may come from either of the lymphocyte populations and consequently the proportion of these cells probably represents an expression of a 2-way reaction, i.e. the response of A's cells against B's antigens, and B's cells against A's antigens. Some of the efforts to modify the test to indicate "one-way" reactions only are listed below. Lymphocytes in culture were stimulated to blastogenesis by the addition of cell-free medium which had been used for the culture of allogeneic lymphocytes (Kasakura and Lowenstein, 1965a, 1965b; Gordon and MacLean, 1965), and by the addition of allogeneic cells which were themselves incapable of transforming to lymphoblasts. These included freeze-thawed

lymphocytes (Bach and Hirschhorn, 1964; Hashem and Rosen, 1964; de Witt, 1965; Moynihan et al., 1965) or fractions thereof (Hashem and Rosen, 1964), irradiated lymphocytes (Ceppelini, Franceschini, Miggiano and Tridente, 1965a; Kasakura and Lowenstein, 1965c), lymphocytes treated with a nitrogen mustard (Cloramin^{R}) (Ceppelini et al., 1965a), and macrophages (Marshall, Rigo and Melman, 1966). However, some workers have found that frozen-thawed allogeneic cells and extracts were poor stimulants of the reaction (Ceppelini et al., 1965a; Kasakura and Lowenstein, 1965b), and erythrocytes, platelets and plasma were ineffective (Bain et al., 1964).

4. <u>The Serological Manifestation and Matching of Antigens</u>

The specificity of immune reactions makes it possible to use antisera to identify the tissues which carry certain antigens. Different techniques each show up different sets of antigenic determinants, but the extent to which any one method detects histocompatibility antigens has not yet been clearly established. The value of an immune reaction as an indicator of histocompatibility can be assessed only by comparing the results of each test with the results of grafting experiments because, so far,

there is no a priori method of knowing whether an antigen is concerned in histocompatibility.

Before assessing the value of the techniques separately, there are a number of general observations to be kept in mind.

(i) Any one technique may detect only some of the histocompatibility antigens. A combination of techniques may be necessary finally to demonstrate the full spectrum of these antigens. This has proved to be the case in mice where some H-antigens not detected by haemagglutination were demonstrable by using the leucocyte agglutination technique (Amos, 1953).

(ii) A multispecific serum may detect some H-antigens and some antigens unassociated with histocompatibility. Such sera in any histocompatibility testing panel would tend to confuse the results.

(iii) The survival times of allografts between different donor-host pairs may be similar despite the fact that the serological assessment of their antigens indicates that these pairs differ fairly markedly one from the other. Walford, Martin, Troup and Goodwin (1965) pointed out that the rejection time of a graft in a donor-host pair

which differ, for example at 10 histocompatibility loci, may be only slightly different from that in a pair which differ at, say, 3 or 4 loci and that marked prolongation of survival times can be expected only when the donor and host are almost completely matched. In outbred stock where strong histocompatibility differences are concerned, many degrees of incompatibility will appear similar when measured by so insensitive a criterion as the crude graft survival time.

Erythrocytes and Histocompatibility Antigens

In mice most of the H-antigens so far defined have been demonstrated on the erythrocytes by haemagglutination (see Snell, Hoecker, Amos and Stimpfling, 1964). Some histocompatibility antigens are probably represented on the erythrocytes in other animals too, e.g. rats (Breyere, 1959; Bogden and Aptekman, 1960; Palm, 1962, 1964), chickens (Schierman and Nordskog, 1961; Craig and McDermid, 1963), and dogs (Simonsen, 1955; Jacobson and Najarian, 1964).

Haemagglutinins were sometimes demonstrable after skin grafts in rabbits (Zotikov, 1958; Hancock and Mullan, 1962), but these did not coincide with the cytotoxins

which were also provoked in many of the recipients. The cytotoxins were sometimes absorbed by donor erythrocytes which suggested that some erythrocyte and skin antigens had common specificity (Kapitchnikov, Ballantyne and Stetson, 1962). This was confirmed by Cohen, DePalma, Colberg, Tissot and Hubay (1964) who showed that in rabbits the Hg incompatibilities were correlated with shorter graft survival but other erythrocyte incompatibilities were not. Erythrocyte injections provoked accelerated rejection of skin allografts on some occasions in rabbits (Colberg, Cohen and Hubay, 1964), but not on others (Medawar, 1946; Cohen et al., 1964), and white cells, tissue homogenates and spermatozoa failed to absorb rabbit haemagglutinating antisera (Cohen et al., 1964). In dogs, incompatible erythrocytes provoked the accelerated rejection of kidney allografts from the same donors (Jacobson and Najarian, 1964).

In man, kidney allografts sometimes survived well in spite of ABO erythrocyte incompatibilities (Woodruff and Allen, 1953; Woodruff, Robson, Nolan, Lambie, Wilson and Clark, 1963). Nonetheless, it seems possible that erythrocytes carry some histocompatibility

antigens. Ceppelini et al. (1965b) reported a significant correlation between ABO incompatibilities and a shorter graft survival after 95 skin allografts on 33 humans. Dausset et al. (1966) showed that incompatible erythrocytes sensitised the recipients to skin allografts from the same donor. Terasaki, Marchioro and Starzl (1965) showed that in 14 long surviving human kidney allografts there was matching of the donor-host pairs for A, B, N and e erythrocyte antigens. In 8 of the 11 cases where the grafts survived $\geqslant$ 6 months the donor-recipient pairs were mismatched for only one or none of the 13 erythrocyte antigens investigated. They concluded that multiple erythrocyte incompatibilities might be deleterious, but not critical, for graft survival.

Some support for these conclusions is to be found in the results of the <u>in vitro</u> experiments of Möller and Eklund (1963). These authors found that when human lymph node cells which carried receptors for A, B and Rh antibodies were exposed to anti-A, anti-B or anti-D serum

in vitro, only a small proportion of the cells were damaged when any antiserum acted alone (cell damage was assessed by the uptake of trypan blue dye); but progressively more cells were stained when the antisera were permitted to act 2 or 3 at a time. The authors suggested that erythrocyte isoantigens might act as histocompatibility antigens, although they need not necessarily create strong histocompatibility barriers. Such barriers might become stronger where the donor and host are mismatched for a multiplicity of erythrocyte isoantigens.

van Rood, van Leeuwen, Eernisse, Frederiks and Bosch (1964) showed that erythrocytes did not absorb any appreciable quantity of antileucocyte serum in man.

Leucocytes, Platelets and Histocompatibility Antigens

(a) Leucocytes. Leucocytes share antigens with other tissues and antileucocyte antibodies may be absorbed by homogenates made from various solid tissues (van Rood et al., 1964). Furthermore, leucocytes carry histocompatibility antigens because they are able to provoke the accelerated rejection of skin allografts in man (Friedman et al., 1961; Rapaport, Lawrence, Converse and

Mulholland, 1964) and animals (Medawar, 1946). Gray and Russel (1965) showed that this allograft immunity was demonstrable in man for up to 3 months after a single intradermal injection of 2.5 to 5 x 10^6 blood lymphocytes, but not when tested at 5 months. Metzgar et al. (1965) and Amos (1965) showed that in man leucocyte agglutinins and cytotoxins developed after multiple intradermal blood lymphocyte injections, and Amos (1965) found that such antibodies were sometimes still being produced 15 months after the last injection. Blood leucocytes were also able to induce tolerance to subsequent skin grafts in mice, rats and chickens (Billingham, Brent and Medawar, 1956; Billingham and Silvers, 1962), indicating that in these animals all the histocompatibility antigens which are present on skin are probably present on the leucocytes.

Granulocytes and lymphocytes from the same individual carry antigens of different specificities and are able to absorb cytotoxic antibodies independent of one another (Engelfriet, 1965). Allogeneic antisera sometimes react with one type of cell only (Walford, Anderson, Carter and Mihajlovik, 1962; Walford, Carter

and Sjaarda, 1963; Shulman, Marder, Hiller and Collier, 1964).

The significance of these findings for histocompatibility testing is unknown because the antigenic differences between various cells are only relevant if they happen to be transplant antigens, and at present this has not been determined.

(b) Platelets share some antigens with leucocytes (van Rood, van Leeuwen and Eernisse, 1959b; Dausset, Colin and Colombani, 1960; van Rood et al., 1964; Shulman et al., 1964; Lalezari and Bernard, 1965).

They have been shown to carry histocompatibility antigens in animals (Ebbe, Baldini and Dameshek, 1962a, 1962b; Wilson, 1963; Hager, Dupuy and Wallach, 1964), and in man (van Rood et al., 1964; Dausset and Rapaport, 1966).

However, large doses of platelets were necessary to provoke accelerated allograft rejection (Wilson, 1963; Dausset and Rapaport, 1966), indicating that they probably carry small quantities of the histocompatibility antigens.

Recently interest in leucocyte and platelet antigens has increased because of the observations that leucocyte and platelet antibodies are provoked by multiple

blood transfusions (Dausset, 1954; van Loghem, van der Hart and Borstel, 1957; Payne 1957b; van Rood, van Leeuwen and Eernisse, 1959a), or by pregnancy (Payne and Rolfs, 1958; van Rood et al., 1959b), and that such antibodies might be responsible for pathological conditions in man, e.g. febrile reactions after the transfusion of erythrocyte-compatible blood (Brittingham and Chaplin, 1957; Payne, 1957a), neonatal purpura (Moulinière, 1957), chronic agranulocytosis (Dausset and Nenna, 1952), neonatal agranulocytosis (Dausset and Malinvaud, 1964), or spontaneous abortion (van Rood et al., 1959b; Jensen, 1961).

Leucocyte agglutinating antisera from multiparious women or transfusion recipients have been used to define a number of individual leucocyte antigens in humans (Dausset, 1958; van Rood et al., 1959b; van Rood, 1962; van Rood and van Leeuwen, 1963; van Leeuwen, Eernisse and van Rood, 1964; Payne, Tripp, Weigle, Bodmer and Bodmer, 1964; van Rood and van Leeuwen, 1965; van Rood et al., 1965) and other techniques have also been used with success for this purpose, e.g. complement fixation, inhibition of complement fixation, mixed agglutination, antiglobulin consumption etc. This work is reviewed elsewhere (Shulman et al., 1964).

Attention has now turned toward establishing whether the leucocyte antigens which have been demonstrated are histocompatibility antigens and to the ability of different serological techniques to manifest histocompatibility.

To these ends graft survival times have been compared with the similarity of the donor and host according to the reaction patterns of their leucocytes after screening them against panels of agglutinating and/or cytotoxic antisera.

When the donor and host were selected by matching the reactions of the leucocyte agglutinogens the graft survival times were sometimes prolonged (Colombani, Dausset and Preaux, 1963; Colombani, Colombani and Dausset, 1964; van Rood et al., 1964; Dausset, Colombani, Feingold and Rapaport, 1965a; van Rood et al., 1965). For example, Colombani et al. (1964) screened the leucocytes of 247 individuals against a panel of 48 antisera and then grafted 12 recipients each with skin from the donors with the most similar and dissimilar reaction patterns. The mean survival time of the "most similar" grafts was 0.88 day more than the mean of the other grafts.

In a similar experiment van Rood et al. (1964) screened 100 individuals' leucocytes against a panel of 60 antisera. The most similar pairs were selected from the 9,900 possible combinations by using a computer for the comparisons. Although skin grafts between those assessed as most similar survived 2.4 days longer on average than did grafts between individuals selected at random from the general population the authors did not consider this to be significant. More recently van Rood et al. (1965) have shown clearly that their leuco-agglutinogens 6^b and 6^c were strong histocompatibility antigens.

Much less work has been done on the antigens which combine with cytotoxic antisera. Cytotoxic antibodies have been demonstrated <u>in vitro</u> after allografts in all the species tested, e.g. in man (Walford, Gallagher and Sjaarda, 1964; Batchelor, 1965b), rat (Schrek and Preston, 1956), rabbit (Terasaki et al., 1961a), mouse (Gorer and O'Gorman, 1956; Helström, 1959; Boyse et al., 1962), and dog (Kasakura, Thomas and Ferrebee, 1964; Altman and Simonsen, 1964), but as yet there is no clear indication that these antibodies react with histocompatibility antigens.

However, the results of Starzl, Marchioro, Terasaki, Porter, Faris, Herrman, Vredevoe, Hutt, Ogden and Waddell (1965), Terasaki et al. (1965) and Vredevoe, Terasaki, Mickey, Glossock, Merrill and Murray (1965) suggest that this may be the case. These workers used panels of 50 to 125 toxic isoantisera to screen the leucocytes of the donors and recipients in long-term surviving clinical kidney allografts. They found that the demonstrable cytotoxinogens (antigens which provoke cytotoxic antibodies) were mismatched on fewer occasions in these pairs than would have been the case with most other donors chosen randomly from the general population.

More recently Terasaki, Vredevoe, Porter, Mickey, Marchioro, Faris, Herrmann and Starzl (1966) concluded that the leucocyte antigens demonstrated by cytotoxic technique were probably histocompatibility antigens of intermediate strength. They found that of 36 human kidney allografts which survived for protracted periods, 21 did so in spite of one or two mismatched leucocyte groups. However, the results were clinically inferior in the mismatched grafts to those where the donor and host were more nicely matched.

Amos (1965) grouped the leucocytes of 81 healthy volunteers according to their reactions with a panel of

35 cytotoxic antisera. There was no overall correlation with the survival times of skin grafted from donors assessed as similar and dissimilar. However, a correlation was demonstrated between the reactions of certain antisera and "prolonged survival", taken arbitrarily at >13 days.

The most successful predictions of histocompatibility to date are probably those of Dausset, Rapaport, Ivanyi and Colombani (1965c) and Amos, Hattler, Hutchin, McCloskey and Zmijewski (1966). These were achieved by matching leucocyte antigens according to both their agglutination and cytotoxic reactions.

Dausset et al. (1965c) used 50 agglutinating and cytotoxic isoantisera to type 133 humans for 10 more or less defined leucocyte antigens (the Hu-1 system, Dausset et al., 1965b). Six recipients were then immunised with allogeneic tissues with which they were incompatible in respect of some of these recognised antigens. The association of the antigens with histocompatibility was established by grafting each recipient with skin from 4 to 6 donors, some of which carried the relevant antigens. In 93% of cases the grafts which were mismatched were rejected faster than the other grafts on that recipient.

Amos et al. (1966) typed the leucocytes of 10 members of a family against 65 agglutinating and 32 cytotoxic antisera and then exchanged skin grafts between 5 of them. In almost every case it proved possible to select the donor-host combinations which were more and less histocompatible. The authors pointed out that in this family there were probably many shared antigens because allografts survived for protracted periods even in the least compatible pairs.

In rhesus monkeys, cytotoxins or agglutinins were capable of indicating histocompatibility relationships. The serum from sensitised monkeys reacted most strongly with leucocytes from the animals which subsequently proved to be the worst skin donors (Balner and Dersjant, 1965).

Interspecies Relationships

The sera and tissues from animals may help to characterise the specificity of human cells in 2 ways. (i) Animal cells may be used to fractionate antisera containing a mixture of antibodies. For example, Shulman et al. (1964), Shulman (1965) used chimpanzee platelets to absorb some of the complement-fixing antibodies from human multispecific antisera; and Metzgar and Zmijewski (1966) used chimpanzee leucocytes and

platelets to absorb certain human leucocyte agglutinins. (ii) Antisera prepared in chimpanzees by the injection of human platelets or leucocytes may be used to recognise human isoantigens (Shulman et al., 1964; Shulman, Moor-Jankowski and Hiller, 1965; Metzgar and Zmijewski, 1966).

Chimpanzee tissues have also been grouped according to the reactions of their leucocytes with the isoantisera from chimpanzees and from multiparous women (de Witt, 1965).

As yet there is no proof that the antigens shared by human and animal tissues or the human antigens which react with xenogeneic antisera are histocompatibility antigens. However, the fairly long survival of some heterotransplants to humans suggests that important histocompatibility antigens may be among those shared. For example, a kidney heterotransplant from a chimpanzee survived more than 6 months (Reemstra, McCracken, Schlegel, Pearl, Pearce, de Witt, Smith, Hewitt, Flinner and Creech, 1964) and that from an East African Baboon survived 3 months (Starzl, Marchioro, Peters, Kirkpatrick, Wilson, Porter, Rifkind, Ogden, Hitchcock and Waddell, 1964a).

Only the histocompatibility tests which have been more or less extensively investigated have been mentioned here. However, sensitivity to isoantigens has been demonstrated by many other methods, for example, using antiglobulin consumption (Colombani et al., 1964), the inhibition of macrophage migration in mixed cultures (Al-Askari, David, Lawrence and Thomas, 1965), platelet agglutination (van der Weerdt, 1965), mixed agglutination (Abeyounis, Milgrom and Witebsky, 1964), mixed agglutination inhibition (Metzgar, 1965), complement fixation (Shulman et al., 1964; Shulman, 1965; Winn, 1965). These reactions show a degree of individual specificity and each may conceivably lend itself as a histocompatibility indicator if the antigens demonstrated prove to be concerned in histocompatibility.

CHAPTER XX

METHODS

The procedures followed in the serological test are shown diagrammatically in Figures 33, 34 and 35.

Briefly, a panel of antisera to allogeneic tissues was prepared (Figure 33), the leucocytes of the animals to be grouped were incubated with each antiserum in the presence of complement (Figure 34) and the cytotoxic actions of the sera assessed by the eosin dye exclusion method (Figure 35).

The possible donor-host pairs within batches of 7 to 12 animals were placed in order of similarity according to a comparison of the susceptibilities of their leucocytes to the cytotoxic action of the antiserum panel. Each rabbit was then grafted with skin from a number of others in the batch, some with leucocyte reaction patterns resembling the recipients' and some with dissimilar patterns.

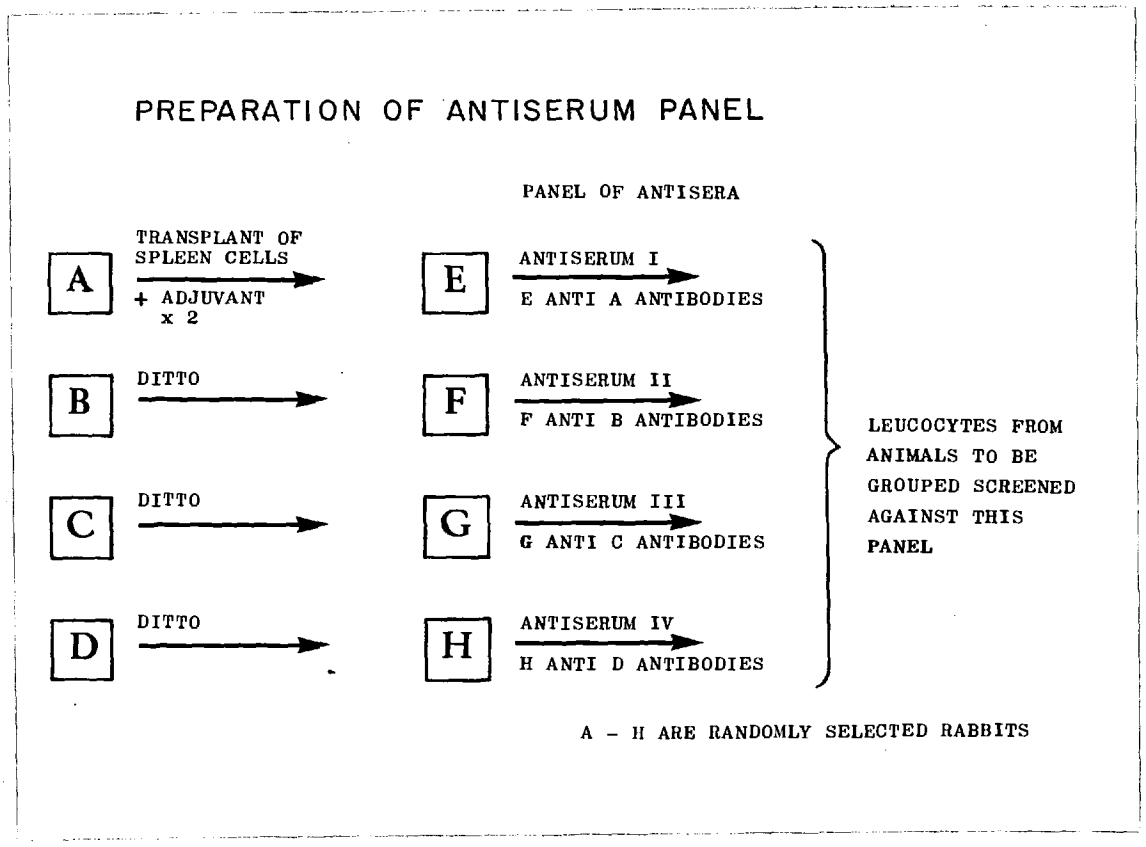

PREPARATION OF ANTISERUM PANEL
PANEL OF ANTISERA
A
TRANSPLANT OF SPLEEN CELLS
+ ADJUVANT x 2
E
ANTISERUM I
E ANTI A ANTIBODIES
B
DITTO
F
ANTISERUM II
F ANTI B ANTIBODIES
C
DITTO
G
ANTISERUM III
G ANTI C ANTIBODIES
D
DITTO
H
ANTISERUM IV
H ANTI D ANTIBODIES
LEUCOCYTES FROM ANIMALS TO BE GROUPED SCREENED AGAINST THIS PANEL
A - H ARE RANDOMLY SELECTED RABBITS

FIGURE 33

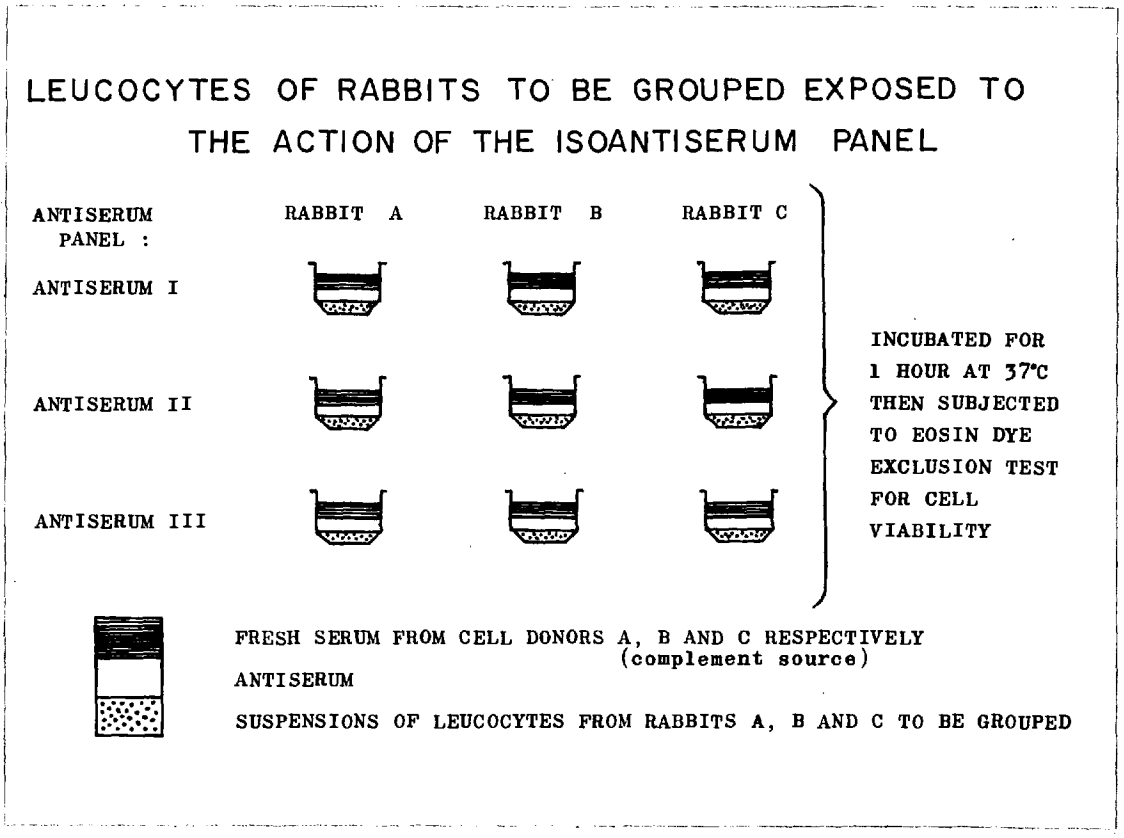

LEUCOCYTES OF RABBITS TO BE GROUPED EXPOSED TO THE ACTION OF THE ISOANTISERUM PANEL
ANTISERUM PANEL :
RABBIT A
RABBIT B
RABBIT C
ANTISERUM I
ANTISERUM II
ANTISERUM III
INCUBATED FOR 1 HOUR AT 37°C THEN SUBJECTED TO EOSIN DYE EXCLUSION TEST FOR CELL VIABILITY
FRESH SERUM FROM CELL DONORS A, B AND C RESPECTIVELY (complement source)
ANTISERUM
SUSPENSIONS OF LEUCOCYTES FROM RABBITS A, B AND C TO BE GROUPED

FIGURE 34

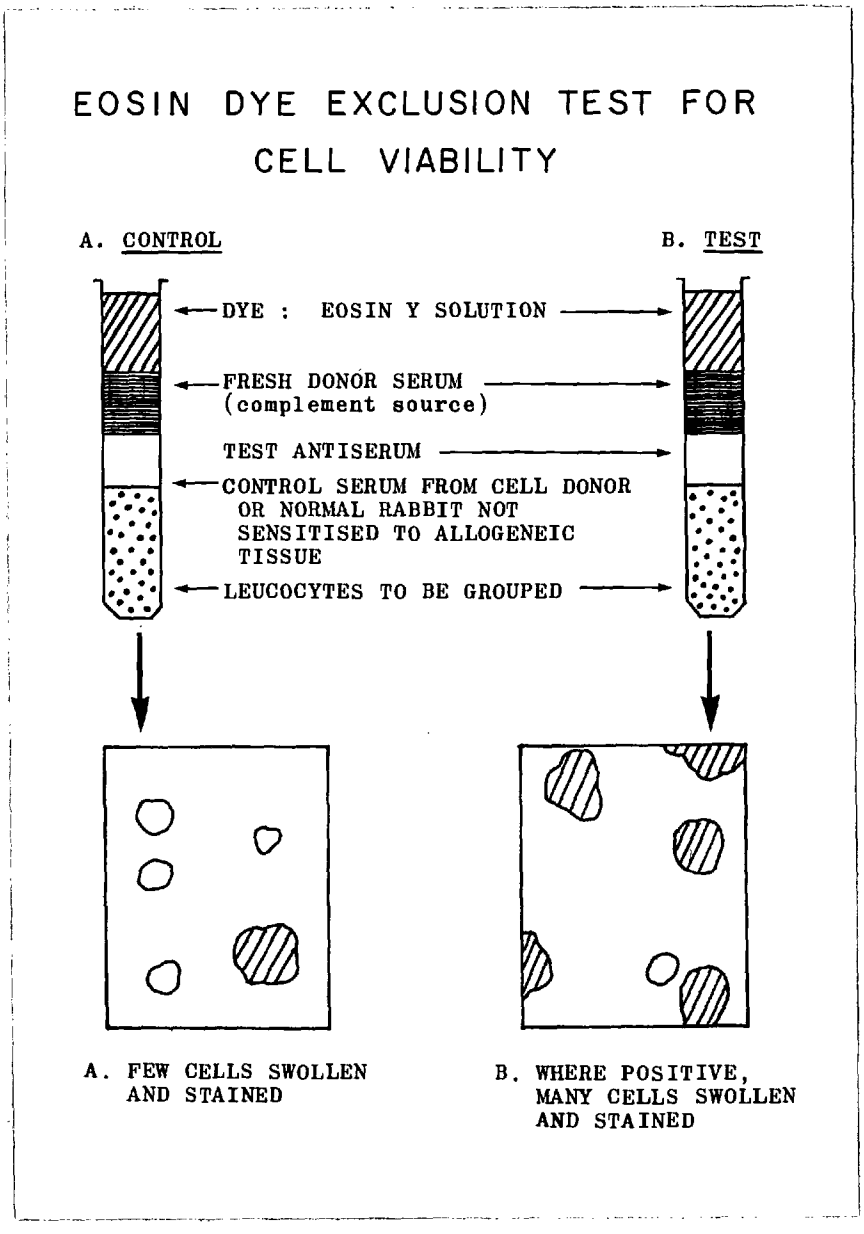

EOSIN DYE EXCLUSION TEST FOR CELL VIABILITY
A. CONTROL
B. TEST
DYE : EOSIN Y SOLUTION
FRESH DONOR SERUM (complement source)
TEST ANTISERUM
CONTROL SERUM FROM CELL DONOR OR NORMAL RABBIT NOT SENSITISED TO ALLOGENEIC TISSUE
LEUCOCYTES TO BE GROUPED
A. FEW CELLS SWOLLEN AND STAINED
B. WHERE POSITIVE, MANY CELLS SWOLLEN AND STAINED

FIGURE 35

The validity of this method as an indicator of histocompatibility was judged by comparing the serological assessments of similarity with the observed survival times.

1. The Isoantisera. Rabbits were immunised as described in Chapter XII, paragraph B, by injecting spleen cells and adjuvant suspension on 2 occasions at 3-week intervals and bleeding the recipients after a further week. Originally 17 antisera comprised the panel, but later this was increased to 26. The antisera were stored in 0.8 ml. aliquots at -10°C.

Table XXVI shows the donors and recipients used in the preparation of the antisera.

TABLE XXVI

Donor	Recipient	Donor	Recipient
80	32	69	72
44	33	70	73
32	34	81	74
37	41	67	75
78	45	12	93
76	49	108	94
46	50	7	95
53	55	4	97
77	56	82	98
60	61	22	2
66	68	8	3
72	69	95	7
4	71	3	8

2. The Leucocytes were separated from whole blood by the sedimentation method described on page 29, paragraph C(ii), and suspended at a concentration of 8.5 x 10^6 cells/ml. in Hank's containing 10% serum from the leucocyte donor.

Lymphocytes comprised 73.2 ± 11.3% of the leucocytes and polymorphs 21.6 ± 10.7%. Total yield was 0.75 to 2.8 x 10^6 leucocytes/ml. whole blood, i.e. 8 to 30% of the original content. There were 8 to 9 erythrocytes to each leucocyte and very few platelets.

3. The Serum from the Leucocyte Donors, prepared as described on page 31, paragraph G, was taken freshly on the day of each test.

4. The Cytotoxicity Test has been described on page 138, paragraph C. The leucocytes of 52 rabbits were screened against the isoantiserum panels. Each experiment was repeated 2 to 5 times to compensate for technical errors and the means of these counts were used in the calculations which follow.

5. Controls. In the first experimental batch (Nos. 83 to 91), one aliquot of each lot of cells separated was

exposed to autologous serum instead of antiserum. In subsequent batches 3 additional aliquots of each lot of cells were exposed to the sera of normal untreated rabbits instead of antiserum. These sera were sometimes pre-immunisation specimens from the rabbits used to produce the antisera of the panel. Other control normal sera came from rabbits not otherwise involved in this experiment.

Calculation of the Interrelationship of the Leucocyte Donors

The animals were divided into batches of 7 to 12 and the degree of incompatibility between each donor-host pair within a batch was expressed in the form of an immunogenic index which was calculated as follows:

The means of the repeated counts were converted to cytotoxic indices (C.I.) according to the formula

$$C.I. = 100 \frac{E - C}{100 - C}$$

where E = percentage of leucocytes stained after exposure to antiserum and C = the mean of the percentages of leucocytes stained after exposure to the control sera.

Regarding each rabbit as recipient in turn and all the others of the batch as donors, the values $C.I._d - C.I._r$ were calculated for each antiserum ('d' indicates donor and 'r' recipient). Values less than 10 and negative values were ignored. The immunogenic index (I.G.I.) for each donor-host pair was found by adding the $C.I._d - C.I._r$ values. The rabbits in each batch were placed in order of similarity according to their immunogenic indices, low I.G.I.'s indicating relative similarity.

Example:

	C.I.'s		
	Rabbit A	Rabbit B	Rabbit C
Antiserum I:	25	11	45
II:	73	50	22
III:	10	3	59
IV:	0	38	45

Regarding A as donor and B as recipient

I.G.I. = (25 - 11) + (73 - 50) = 37

(The result with serum III is ignored as the difference is less than 10, and so is that with serum IV because the difference is a negative quantity.)

Regarding C as donor and B as recipient

I.G.I. = (45 - 11) + (59 - 3) = 90

A would be predicted as the better donor to B than would C because A's tissues seem to carry less antigens foreign to B than do C's.

After testing the 52 rabbits in this way skin grafts were exchanged to test whether the serological assessments of similarity were correlated with the observed rejection orders.

Grafting Technique

In the earlier experiments (Batches 1, 2, 3 and 4) full thickness skin was grafted as described on page 134, paragraph A. In later work (Batches 5, 6 and 7) the technique was modified in that full thickness grafts were cut from the inner ear surface with a Schiller's dermatome (Figure 36). Here the hairs were clipped rather than shaved because the hair stubbles served to indicate the outer surface of the graft. After cleaning the skin as before, a layer of light grade liquid paraffin was applied and very thin grafts were cut with the dermatome at fine setting. The grafts were

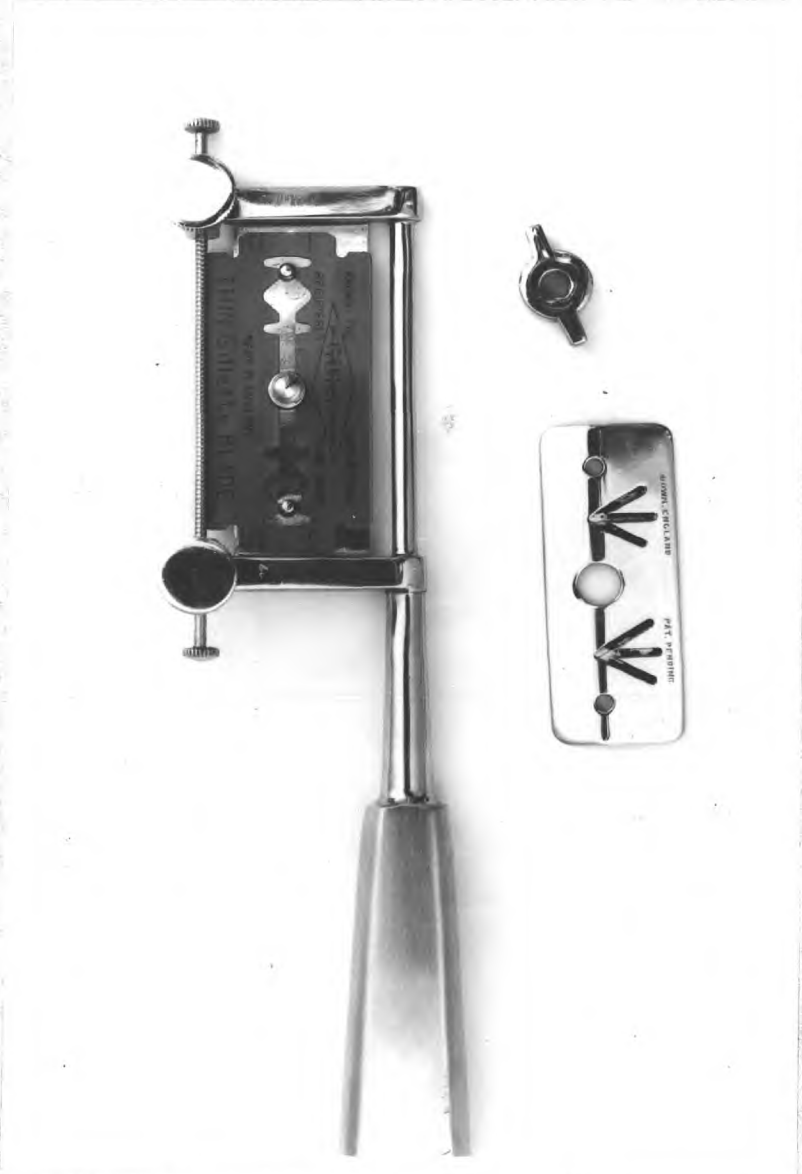


FIGURE 36

Schiller's dermatome.

floated in Ringer's until they were fitted to beds cut in the chest wall as before. (This technique is shown in Figures 37a to h.) All grafts were given code numbers and their arrangements on the recipients were randomised by an assistant to avoid bias. The grafts were sprayed with antibiotic aerosol and dressed and examined as before.

(a) Control Grafts. In order to ascertain the difference in rejection times which might be expected of grafts which were equally incompatible with their hosts, recipient rabbits received 4 skin grafts each. All 4 grafts on any one recipient were from the same donor.

(b) Test Grafts. (i) Where both donor and recipient were grouped, each animal bore 2 to 6 allografts from donors with high or low I.G.I.'s. Most animals also received autografts. (Three of the recipients were injected intramuscularly twice a week with 6-mercaptopurine 6 mg./kg. in 10% solution in 0.1 normal NaOH, starting at the time of grafting, as it was thought that immunosuppressive therapy might increase the scatter

FIGURE 37 : Skin grafting technique.



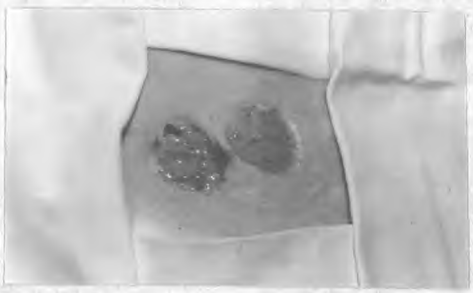
(a) Anaesthesia.



(b) Clipping the lateral chest wall.



(c) Cutting the beds.



(d) The beds completed.



(e) Cutting the grafts.



(f) Fitting the grafts.



(g) Vaseline dressing.



(h) Plaster of Paris bandage.

of rejection times. However, after 2 weeks one of the animals developed diarrhoea and the injections of all 3 were discontinued.)

(ii) In addition, ungrouped rabbits received skin allografts each from a pair of rabbits, one of which was termed a "good donor" with mostly low cytotoxic indices (either No. 28 or 31) and the other a "bad donor" with mostly high C.I.'s (either No. 27 or 29).

Criteria for Rejection

In some grafts rejection was acute. They became reddish-black overnight and the epithelium stripped away easily to reveal a moist necrotic layer beneath.

When rejection was slower, the onset and completion of rejection were recorded. Rejection usually began with reddening of parts of the graft. The red areas became larger and eventually coalesced. Rejection was regarded as complete when about 90% of a graft was discoloured. In other grafts the first signs were oedema, desquamation of the epithelium, or failure to blanch on pressure and rejection was regarded as complete when both desquamation and failure to blanch on pressure were observed. Figure 37(i) shows healthy and rejected grafts.

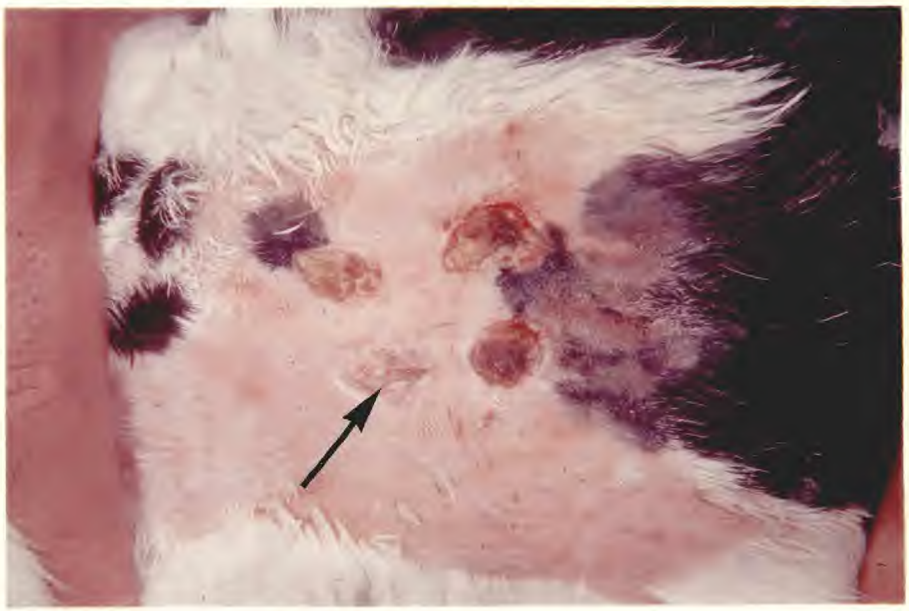


FIGURE 37(i)

The graft indicated by the arrow is viable.
The other three have been rejected.

RESULTS

A. The Serological Results

Table XXVII shows the proportions of cells stained after incubating the leucocytes of 52 rabbits with the antisera and with the control sera. The means of the repeated test and control counts are also shown, (E and C respectively).

Table XXVIII shows the cytotoxic indices calculated from the mean stained counts in Table XXVII according to the formula

$$C.I. = 100 \frac{C - E}{100 - C}$$

Figure 38 shows the incidence of high C.I.'s (30 or more). Four of the antisera (Nos. 34, 56, 61 and 69) gave no high C.I.'s with any of the leucocyte samples, while a further two (Nos. 32 and 33) reacted strongly with only one leucocyte sample each. It may be noted here that in certain sets of antisera there seemed a similarity in the reaction patterns. For example, the

antisera 41, 72 and 98 gave high C.I.'s with similar leucocyte samples and so did antisera 68, 73, 7 and 8, which suggested the antisera within each set probably recognised the same antigens. The significance of this will be discussed later.

Table XXIX shows the immunogenic indices for all the possible donor-host pairs in each of the 7 batches. These were calculated from the C.I.'s in Table XXVIII according to the methods detailed on pages 207, 208 and 209. The prediction of the graft rejection order on each recipient was based on the order of magnitude of the I.G.I.'s, the higher the I.G.I.'s the earlier the predicted rejection.

TABLE XXVII

Stained Cell Counts

BATCH 1

Antiserum	Rabbit No: 83			Rabbit No: 84				Rabbit No: 86			Rabbit No: 88			Rabbit No: 89			Rabbit No: 90			Rabbit No: 91			BATCH 2 Rabbit No: 96					Rabbit No: 99					Rabbit No: 1			
	a	b	E	a	b	c	E	a	b	E	a	b	E	a	b	E	a	b	E	a	b	E	a	b	c	d	E	a	b	c	d	e	E	a	b	c	E
32	14	21	18	20	16	12	16	26	40	33	88	36	62	16	38	27	30	12	21	58	38	48	3	8	3	-	5	6	8	8	15	8	9	2	8	4	5
33	28	12	20	8	24	22	18	34	20	27	81	39	60	36	44	40	26	26	26	36	42	39	38	21	27	23	27	16	12	8	10	2	10	25	21	23	23
34	12	8	10	4	24	8	12	28	50	39	51	30	41	14	30	22	16	12	14	52	44	48	11	25	11	4	13	16	6	9	6	10	9	2	0	0	1
41	0	12	6	54	66	54	58	63	88	76	46	21	34	22	32	27	38	9	24	50	62	56	6	14	17	16	13	8	4	12	10	16	10	2	0	2	1
45	56	54	55	28	64	30	41	54	78	66	51	31	41	28	38	33	14	18	16	50	70	60	19	25	7	12	16	18	42	16	30	22	26	4	2	2	3
49	24	9	17	52	54	30	45	49	65	57	24	12	18	20	22	21	28	28	28	64	56	60	25	9	13	17	16	6	20	22	12	12	14	6	0	1	2
50	26	10	18	30	16	20	22	36	58	47	70	36	53	22	30	26	14	12	13	22	34	28	25	24	12	30	23	6	12	40	6	20	17	4	6	4	5
55	38	20	29	26	18	24	23	41	37	39	39	21	30	54	56	55	18	20	19	54	42	48	12	28	20	12	18	10	20	10	0	10	10	4	8	4	5
56	15	21	18	6	24	4	12	12	14	13	27	25	26	14	34	24	18	0	9	58	54	56	13	17	15	15	15	12	2	4	10	12	8	14	3	3	7
61	8	4	6	0	14	20	12	27	20	24	18	12	15	20	38	29	20	8	14	38	50	44	13	21	8	11	13	24	16	4	9	2	11	8	7	4	6
68	58	34	46	46	24	32	34	42	23	33	51	21	36	38	60	49	38	18	28	60	52	56	34	52	47	63	49	12	16	8	6	18	12	24	24	14	21
69	14	8	11	28	14	14	19	44	36	40	21	12	17	18	40	29	6	18	12	28	46	38	32	28	17	15	23	6	8	14	9	12	10	6	6	0	4
71	11	26	19	24	28	20	24	42	28	35	55	32	44	41	24	33	38	22	30	34	42	38	9	21	22	40	23	10	4	6	6	0	5	27	23	23	24
72	20	8	14	52	62	54	56	50	78	64	74	42	58	28	22	25	24	18	21	46	50	48	17	11	15	8	13	8	14	42	16	18	20	6	2	2	3
73	58	38	48	30	46	38	38	34	44	39	47	26	37	68	44	56	38	20	29	30	46	38	75	46	42	56	55	14	14	12	2	12	11	35	24	22	27
74	54	31	43	52	30	62	48	44	62	53	33	48	41	52	36	44	12	28	20	36	52	44	40	30	48	62	45	16	22	10	14	18	16	24	20	14	19
75	38	26	32	28	24	30	24	38	26	32	58	30	44	54	38	46	26	36	31	48	70	59	15	16	34	34	25	24	10	22	18	32	21	38	24	32	31
93	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
94	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
95	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
97	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
98	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Controls	15(83)	10(83)		8(84)	6(84)	10(84)		30(86)	22(86)		30(88)	12(88)		26(89)	30(89)		14(90)	6(90)		36(91)	44(91)		14(96)	12(96)	18(96)	12(96)		10(99)	4(99)	12(99)	8(99)	10(99)		10(1)	0(1)	2(1)	
																							10(59)	6(59)	12(59)	16(59)		8(4)	0(4)	2(4)	2(4)	10(4)		8(44)	4(44)	0(44)	
																							4(6)	6(6)	18(6)	8(6)		16(5)	10(5)	2(5)	6(5)	12(5)		2(39)	8(39)	6(39)	
																							4(43)	14(43)	16(43)	20(43)		2(6)	2(6)	6(6)	8(6)	10(6)		4(38)	3(38)	0(38)	
C.	13			8				26			21			28			10			40			12					7						4			

Footnote: a, b, c ... = repeated counts.
E = mean of repeated counts.
C = mean of control counts.
Numbers in parenthesis are those of the control sera. These sera were from the leucocyte donors or from other non-immunised rabbits.

TABLE XXVII (cont.)

Stained Cell Counts

BATCH 2

Antiserum	Rabbit No: 10						Rabbit No: 11				Rabbit No: 12					Rabbit No: 13						Rabbit No: 9				
	a	b	c	d	e	E	a	b	c	E	a	b	c	d	E	a	b	c	d	e	E	a	b	c	d	E
32	18	12	30	10	28	18	10	10	6	9	12	9	18	9	12	10	14	12	10	18	13	6	14	26	18	16
33	33	30	52	12	4	26	6	9	10	8	24	22	20	18	21	28	38	24	10	20	24	16	20	24	12	18
34	16	16	28	24	21	21	6	2	14	7	10	8	24	6	12	20	8	10	8	20	13	6	24	18	16	16
41	20	28	52	18	8	25	4	10	18	11	18	18	22	21	20	14	10	18	22	20	17	14	12	20	14	15
45	10	10	36	12	28	19	9	15	4	9	12	0	8	6	7	16	12	16	20	10	15	16	12	24	26	20
49	16	14	22	30	20	20	20	6	12	13	14	6	24	12	14	20	8	18	14	14	15	14	28	14	20	19
50	46	30	30	12	34	30	24	16	36	25	54	54	50	66	56	44	36	18	24	32	31	36	48	58	40	46
55	30	24	54	32	36	35	12	12	12	12	54	24	26	32	34	16	12	28	8	16	16	60	81	84	78	76
56	20	14	34	28	8	21	8	10	6	8	12	10	23	12	14	26	12	18	20	20	19	12	16	24	10	16
61	18	24	22	18	24	21	4	0	8	4	6	10	20	12	12	10	20	16	12	12	14	14	10	18	18	15
68	50	58	62	66	54	58	84	14	72	57	60	44	44	16	41	20	14	16	16	20	17	16	12	16	18	16
69	30	12	36	26	15	24	0	14	10	8	18	0	10	14	11	20	24	10	12	20	17	10	22	16	16	16
71	10	12	40	14	16	18	10	4	16	10	8	6	16	9	10	24	6	10	10	14	13	10	12	12	12	12
72	22	8	16	26	18	18	10	4	30	15	6	16	12	10	11	42	12	28	18	26	25	12	19	18	18	17
73	60	52	80	54	52	60	68	48	70	62	78	48	34	42	51	36	32	22	18	34	28	12	26	24	30	23
74	92	60	62	80	78	74	70	34	62	55	74	28	44	39	46	38	16	16	22	26	24	22	20	26	18	22
75	24	40	42	18	20	29	2	14	24	13	20	18	18	10	17	20	20	18	14	26	20	14	22	30	14	20
93	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
94	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
95	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
97	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
98	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Controls	12(10)	18(10)	24(10)	12(10)	20(10)		6(11)	2(11)	12(11)		16(12)	10(12)	10(12)	8(12)		6(13)	4(13)	6(13)	20	(13)	10(13)	14(9)	10(9)	22(9)	8(9)	
	8(57)	20(57)	30(57)	16(57)	16(57)		2(4)	2(4)	16(4)		10(13)	8(13)	12(13)	10(13)		12(7)	0(7)	6(7)	14	(7)	10(7)	10(2)	14(2)	16(2)	8(2)	
	20(35)	10(35)	22(35)	18(35)	24(35)		8(6)	0(6)	10(6)		0(59)	14(59)	0(59)	4(59)		16(44)	18(44)	18(44)	14	(44)	10(44)	4(94)	18(94)	20(94)	18(94)	
	16(36)	16(36)	20(36)	10(36)	2(36)		8(5)	2(5)	2(5)		0(38)	22(38)	10(38)	8(38)		14(3)	10(3)	2(3)	8	(3)	14(3)	12(5)	20(5)	6(5)	22(5)	
C.	17						6				9					11						14				

BATCH 3

Antiserum	Rabbit No: 14				Rabbit No: 16					
	a	b	c	E	a	b	c	d	e	E
32	12	18	2	11	18	18	12	44	8	20
33	24	20	12	19	16	18	12	36	16	20
34	14	6	2	7	12	8	16	24	24	17
41	12	6	0	6	10	30	22	26	22	22
45	12	28	12	17	16	24	28	20	22	22
49	16	8	6	10	32	30	40	46	52	40
50	68	78	44	63	24	32	32	30	20	28
55	20	16	0	12	16	14	20	30	10	18
56	24	16	10	17	18	22	8	22	20	18
61	16	14	-	15	10	12	12	20	14	14
68	46	62	34	47	40	42	18	36	14	30
69	22	20	8	17	10	10	8	22	16	13
71	12	10	2	8	18	8	18	24	16	17
72	10	12	8	10	72	26	37	60	22	43
73	60	74	64	66	24	12	8	34	40	24
74	82	78	56	72	32	44	26	48	28	36
75	16	26	12	18	12	24	38	52	22	30
93	58	68	32	53	48	84	12	26	20	38
94	36	20	2	19	30	9	10	20	22	18
95	20	16	2	13	84	74	62	68	62	70
97	60	44	34	46	78	82	60	60	46	65
98	20	10	4	11	24	28	26	28	36	28
2	14	12	0	9	4	18	16	28	18	17
3	20	4	12	12	24	26	20	26	26	24
7	78	70	42	63	22	24	8	32	25	22
8	62	74	58	65	10	8	14	22	20	15
Controls	10(14)	18(14)	2(14)		18(16)	12(16)	14(16)	22(16)	6(16)	
	8(98)	10(98)	10(98)		12(95)	12(95)	10(7)	16(94)	8(94)	
	10(7)	8(95)	8(2)		26(3)	10(2)	14(3)	20(3)	16(3)	
	8(96)	10(96)	4(7)		16(97)	6(8)	10(2)	22(93)	18(93)	
C.	9				14					

Footnote: a, b, c ... = repeated counts.
E = mean of repeated counts.
C = mean of control counts.
Numbers in parenthesis are those of the control sera. These sera were from the leucocyte donors or from other non-immunised rabbits.

TABLE XXVII (cont.)

Stained Cell Counts

BATCH 3

Antiserum	Rabbit No: 17 a	b	c	E	Rabbit No: 18 a	b	c	E	Rabbit No: 19 a	b	c	E	Rabbit No: 20 a	b	c	E	Rabbit No: 21 a	b	c	d	e	E
32	8	10	10	9	14	10	16	13	12	10	30	17	18	8	32	19	22	12	16	16	8	15
33	16	25	20	20	8	12	16	12	18	18	26	21	28	20	26	25	40	14	28	30	36	30
34	0	0	6	2	9	12	4	8	14	18	32	21	2	4	16	7	18	6	16	14	14	14
41	92	90	68	83	20	18	10	16	20	12	42	25	12	8	16	12	16	6	20	10	14	13
45	80	18	44	47	12	14	14	13	18	20	30	23	4	4	20	9	12	10	8	16	20	13
49	60	46	38	48	12	14	8	11	22	14	38	25	8	14	10	11	22	6	18	16	12	15
50	42	18	36	32	16	34	29	26	26	12	28	22	34	46	22	34	22	24	30	16	12	21
55	6	0	12	6	19	72	18	36	12	22	32	22	68	20	14	34	56	10	36	38	24	33
56	6	8	16	10	10	8	18	12	22	10	34	22	4	12	24	13	10	8	14	24	56	22
61	2	3	0	2	7	20	16	14	16	10	34	20	8	18	10	12	16	14	8	22	34	19
68	14	20	26	20	96	36	64	65	16	8	32	19	30	26	14	23	58	40	64	34	42	48
69	8	10	18	12	18	10	16	15	6	6	26	13	10	48	30	29	24	2	12	12	12	12
71	8	4	10	7	12	20	16	16	24	10	28	21	6	4	16	9	30	48	34	16	34	32
72	80	86	78	81	18	10	18	15	24	24	38	29	6	8	16	10	22	8	4	20	14	14
73	10	10	20	13	78	78	68	75	24	12	34	23	36	32	34	34	62	72	80	60	44	64
74	66	60	84	70	84	76	65	75	18	14	44	25	64	36	14	38	58	46	8	12	16	28
75	10	20	36	22	28	12	22	21	14	18	30	21	18	16	24	19	34	30	40	48	34	41
93	12	18	22	17	90	52	68	70	10	18	32	20	26	8	18	17	66	24	28	16	12	29
94	4	4	8	5	88	48	62	66	16	10	30	19	30	16	46	31	32	34	34	21	36	31
95	46	44	36	42	8	12	4	8	26	8	38	24	2	14	32	16	34	12	26	16	22	22
97	20	30	24	25	46	58	62	55	26	24	28	26	80	68	32	60	48	54	52	34	27	43
98	96	88	68	84	10	12	8	10	28	42	64	45	8	10	14	11	22	6	8	12	16	13
2	42	52	38	44	14	12	23	16	20	10	32	21	6	6	18	10	48	28	34	30	46	37
3	26	24	29	26	18	6	24	16	18	16	40	25	4	4	20	9	20	12	2	20	20	15
7	14	22	8	15	82	82	76	80	40	34	52	42	6	10	16	11	58	60	12	25	12	33
8	44	28	22	31	72	44	52	56	10	18	38	22	26	20	24	23	50	42	26	14	12	29
Controls	12(17)	8(17)	10(17)		12(18)	16(18)	14(18)		14(19)	10(19)	30(19)		12(20)	6(20)	8(20)		26(21)	6(21)	6	(21)	14(21)	12(21)
	8(95)	0(7)	10(7)		18(2)	16(3)	20(3)		18(95)	18(95)	22(98)		16(95)	10(98)	6(95)		16(3)	6(3)	2	(3)	0(2)	16(7)
	4(3)	10(93)	4(98)		4(7)	14(94)	8(2)		20(3)	14(3)	26(93)		10(3)	2(94)	10(2)		18(93)	14(2)	6	(94)	6(7)	2(98)
	16(94)	6(8)	8(94)		14(94)	10(8)	12(97)		16(93)	12(98)	26(8)		10(97)	8(7)	8(7)		28(97)	14(97)	10	(8)	12(8)	10(93)
C.	8				13				19				9				11					

Antiserum	Rabbit No: 22 a	b	c	E	BATCH 4 Rabbit No: 15 a	b	c	E	Rabbit No: 23 a	b	c	d	E
32	18	42	26	29	16	22	10	16	14	30	32	12	22
33	18	6	12	12	38	28	20	29	12	30	42	22	27
34	12	26	14	17	6	14	12	11	10	14	28	8	15
41	94	96	92	94	28	14	8	17	14	22	12	28	19
45	66	58	68	64	6	12	14	11	6	20	14	36	19
49	86	86	68	80	10	14	8	11	6	26	30	24	22
50	34	82	48	55	56	20	38	38	16	30	42	27	29
55	20	26	14	20	2	12	6	7	30	36	52	30	37
56	10	12	26	16	22	22	14	19	4	20	27	16	17
61	8	18	10	12	18	16	8	14	8	28	14	26	19
68	34	34	32	33	51	54	42	49	12	26	30	30	25
69	16	6	13	12	20	26	8	18	8	20	24	32	21
71	12	10	14	12	10	10	16	12	8	28	30	22	22
72	94	98	94	95	10	12	10	11	8	30	24	14	19
73	4	26	16	15	80	60	38	59	10	28	37	20	24
74	64	100	90	85	20	14	20	18	14	44	38	40	34
75	30	16	12	19	24	30	18	24	12	18	42	36	27
93	10	20	16	15	50	52	24	42	8	14	33	14	17
94	8	12	10	10	52	26	30	36	8	26	16	16	17
95	20	28	16	21	10	34	20	21	4	38	18	20	20
97	18	48	24	30	44	34	24	34	30	30	34	36	33
98	96	100	80	92	6	14	4	8	20	18	34	24	24
2	30	66	48	48	6	12	16	11	14	28	34	10	22
3	12	25	12	16	18	26	8	17	2	22	33	9	17
7	88	92	63	81	56	34	68	53	8	34	40	22	26
8	46	68	44	53	45	52	38	45	18	24	36	22	25
Controls	8(22)	18(22)	0(22)		22(15)	10(15)	8(15)		10(23)	16(23)	26(23)		18(23)
	4(2)	22(3)	4(95)		8(3)	6(3)	12(2)		8(2)	20(7)	16(3)		14(3)
	12(94)	12(2)	4(7)		10(2)	0(2)	4(98)		4(98)	10(98)	16(7)		10(7)
	0(8)	12(7)	6(98)		16(93)	16(8)	8(94)		16(94)	18(94)	14(93)		12(93)
C.	9				10				14				

Footnote: a, b, c ... = repeated counts.
E = mean of repeated counts.
C = mean of control counts.
Numbers in parenthesis are those of the control sera. These sera were from the leucocyte donors or from other non-immunised rabbits.

TABLE XXVII (cont.)

Stained Cell Counts

BATCH 4

Antiserum	Rabbit No: 24				Rabbit No: 25		
	a	b	c	E	a	b	E
32	16	12	20	16	8	18	13
33	20	8	32	20	20	26	23
34	24	14	18	19	16	14	15
41	16	21	24	20	94	76	85
45	54	38	24	39	28	44	36
49	31	24	33	29	56	62	59
50	20	16	24	20	32	67	50
55	30	46	62	46	10	8	9
56	14	16	24	18	8	12	10
61	22	10	26	19	6	12	9
68	28	14	30	24	30	24	27
69	34	20	33	29	8	20	14
71	12	4	24	13	16	14	15
72	22	21	28	24	84	80	82
73	32	32	52	39	56	62	59
74	28	14	30	24	26	90	58
75	16	16	22	18	14	14	14
93	40	30	24	31	54	42	48
94	16	28	28	24	36	42	39
95	36	44	24	35	6	15	11
97	70	78	78	75	76	58	67
98	20	18	28	22	82	72	77
2	26	16	30	24	18	6	12
3	26	16	18	20	10	8	9
7	36	10	36	27	72	46	59
8	26	28	27	27	62	60	61
Controls	18(24)	10(24)	22(24)		12(25)	2(25)	
	20(7)	14(7)	16(3)		6(7)	0(7)	
	14(98)	18(98)	16(7)		12(98)	12(98)	
	20(94)	14(94)	18(93)		10(94)	10(94)	
C.	17				8		

Rabbit No: 26					Rabbit No: 27				
a	b	c	d	E	a	b	c	d	E
6	4	30	24	16	10	10	12	8	10
10	18	4	32	16	10	16	8	18	13
10	10	14	14	12	12	16	0	12	10
62	38	32	70	51	75	70	50	78	68
32	30	14	52	32	26	28	24	26	26
16	10	14	34	19	30	18	6	58	28
18	20	30	42	28	34	28	26	60	37
8	10	8	28	14	16	12	8	20	14
14	8	6	20	12	4	6	4	2	4
10	6	14	26	14	12	6	10	12	10
16	4	10	34	16	48	28	40	68	46
8	14	12	32	17	4	6	0	4	4
10	60	46	48	41	8	78	10	94	48
58	66	52	64	60	78	54	58	96	72
10	6	8	24	12	46	54	32	78	53
54	54	52	72	58	80	62	44	88	69
16	16	32	12	19	10	20	18	28	19
12	24	30	30	24	24	18	42	50	34
6	8	16	30	15	54	22	18	58	38
24	38	78	56	49	8	2	0	10	5
54	36	52	60	51	42	38	34	76	48
76	68	84	72	75	92	88	78	94	88
20	8	6	24	15	28	10	8	14	15
8	18	8	16	13	12	10	2	16	10
6	27	58	32	31	66	58	42	84	63
14	16	6	38	19	70	64	32	70	59
6(26)	2(26)	14(26)	18(26)		10(27)	10(27)	4(27)	12(27)	
4(7)	0(3)	12(3)	24(3)		16(3)	0(3)	8(2)	12(7)	
8(98)	10(7)	4(7)	16(7)		8(7)	4(98)	10(98)	10(98)	
6(94)	12(93)	10(93)	14(94)		14(93)	10(8)	2(93)	10(8)	
10					9				

BATCH 5

Rabbit No: 28			Rabbit No: 29			Rabbit No: 30			Rabbit No: 31				Rabbit No: 132			
a	b	E	a	b	E	a	b	E	a	b	c	E	a	b	c	E
6	0	3	8	4	6	8	12	10	14	14	8	12	10	12	8	10
28	22	25	16	10	13	16	8	12	6	12	14	11	10	8	12	10
6	14	10	2	0	1	6	3	5	6	4	8	6	4	4	10	6
14	22	18	70	72	71	8	12	10	10	14	12	12	24	36	54	38
6	10	8	18	30	24	12	22	17	14	14	8	12	14	12	20	15
2	6	4	24	30	27	8	15	12	8	22	12	14	6	22	18	15
10	10	10	54	38	46	6	0	3	10	12	8	10	10	8	30	16
32	48	40	10	8	9	2	4	3	30	16	6	17	4	10	8	7
6	14	10	6	8	7	8	16	12	6	18	14	13	12	14	10	12
2	6	4	4	2	3	8	8	8	18	10	2	10	6	6	14	9
6	15	11	4	10	7	12	8	10	12	12	12	12	2	14	22	13
2	6	4	0	0	0	6	6	6	12	14	4	10	6	10	16	11
8	10	9	68	70	69	18	8	13	8	28	22	19	6	74	84	55
8	9	9	48	68	58	0	6	3	12	12	18	14	22	34	74	43
12	20	16	15	18	17	4	6	5	16	16	10	14	10	20	32	21
4	8	6	74	64	69	8	10	9	18	14	6	13	28	54	62	48
6	22	14	8	14	11	16	10	13	22	18	8	16	8	18	20	15
22	14	18	16	28	22	12	24	18	2	12	4	6	10	38	62	37
18	18	18	8	8	8	6	14	10	6	12	14	11	4	14	18	12
16	12	14	88	40	64	48	32	40	12	16	10	13	2	10	14	9
24	30	27	50	38	44	36	22	29	6	0	12	6	8	14	34	19
8	16	12	66	90	78	8	8	8	20	54	22	32	46	66	64	59
8	12	10	16	10	13	20	18	19	10	12	12	11	4	2	6	4
6	12	9	12	2	7	12	24	18	12	6	12	10	10	6	26	14
26	9	18	20	16	18	10	18	14	20	18	22	20	18	36	38	31
0	18	9	34	28	31	4	12	8	6	28	30	21	24	38	30	31
12(28)	6(28)		8(29)	4(29)		8(30)	4(30)		10(31)	6(31)	6(31)		6(132)	8(132)	14(132)	
12(3)	2(3)		8(3)	0(2)		4(3)	0(2)		12(3)	0(3)	16(7)		2(3)	8(2)	12(7)	
8(98)	10(7)		10(7)	0(7)		10(7)	0(98)		12(7)	10(2)	8(98)		0(93)	6(94)	14(98)	
8(8)	8(8)		6(98)	2(98)		6(98)	10(93)		14(98)	8(98)	8(8)		4(97)	4(8)	16(8)	
8			5			7			9				8			

Footnote: a, b, c ... = repeated counts.
E = mean of repeated counts.
C = mean of control counts.
Numbers in parenthesis are those of the control sera. These sera were from the leucocyte donors or from other non-immunised rabbits.

TABLE XXVII (cont.)

Stained Cell Counts

BATCH 5

Antiserum	Rabbit No: 133 a	b	c	d	E	Rabbit No: 134 a	b	E
32	24	14	18	14	18	8	20	14
33	30	18	52	22	31	30	16	23
34	8	24	32	28	23	16	4	10
41	6	36	22	16	20	16	10	13
45	8	16	32	16	18	22	16	19
49	6	18	30	20	19	22	8	15
50	18	12	46	24	25	22	20	21
55	64	24	68	16	43	14	14	14
56	24	24	40	3	23	22	16	19
61	18	14	36	24	23	12	14	13
68	10	22	40	14	22	54	36	45
69	12	24	22	12	18	30	16	23
71	14	10	36	20	20	16	6	11
72	6	24	48	10	22	18	14	16
73	30	20	70	18	35	54	40	47
74	18	12	24	18	18	18	8	13
75	24	14	50	16	26	18	24	21
93	24	44	40	12	30	76	64	70
94	8	22	44	16	23	48	56	52
95	38	20	88	22	42	12	16	14
97	48	46	86	22	51	44	34	39
98	14	16	32	18	20	16	22	19
2	12	24	20	20	19	22	8	15
3	10	12	38	20	20	10	20	15
7	10	14	36	16	19	56	36	46
8	12	18	38	12	20	56	66	61
Controls	10(133)	14(133)	26(133)	12(133)		8(134)	14(134)	
	14(3)	16(2)	24(7)	10(7)		10(3)	8(2)	
	4(2)	20(7)	20(98)	26(98)		20(2)	12(7)	
	12(94)	12(98)	24(8)	24(8)		18(94)	4(98)	
C.	17					12		

BATCH 6

Rabbit No: 188 a	b	c	E	Rabbit No: 189 a	b	c	E	Rabbit No: 190 a	b	c	E	Rabbit No: 191 a	b	c	E	Rabbit No: 192 a	b	c	E	Rabbit No: 193 a	b	c	E
12	16	16	15	6	14	16	12	6	4	10	7	9	9	6	8	10	14	12	12	18	12	18	16
12	24	30	22	15	15	16	15	12	22	18	17	22	27	18	22	12	10	14	12	30	16	14	20
10	30	20	20	12	10	12	11	3	9	16	9	6	6	12	8	4	0	8	4	22	10	22	18
63	64	44	57	62	69	44	58	72	72	84	76	2	10	16	9	18	6	18	14	80	62	66	69
38	-	56	47	14	32	26	24	6	6	18	10	6	16	16	13	10	12	20	14	72	36	60	56
6	10	8	8	6	8	4	6	14	18	10	14	4	8	12	8	6	6	8	7	14	10	18	14
28	12	20	20	16	32	18	22	12	32	16	20	20	48	12	27	10	14	18	14	76	48	70	65
12	26	32	23	10	16	34	20	10	18	10	13	4	8	6	6	10	12	12	11	32	12	36	27
12	26	22	20	6	4	6	5	4	4	12	7	6	10	18	11	20	10	18	16	8	6	12	9
12	14	38	21	6	6	4	5	0	0	10	3	4	0	2	2	10	13	16	13	16	18	14	16
24	32	20	25	10	12	14	12	8	6	8	7	62	50	44	52	14	40	42	32	28	10	14	17
20	28	17	22	18	6	6	10	12	6	6	8	24	10	18	17	16	8	16	13	12	14	16	14
14	14	28	19	10	14	8	11	20	13	6	13	4	4	10	6	8	16	6	10	14	10	18	14
60	64	70	65	58	70	30	53	54	72	75	67	8	26	12	15	12	14	16	14	92	68	74	78
26	64	40	43	6	22	10	13	6	4	12	7	48	90	50	63	16	48	40	35	8	16	18	14
58	52	62	57	34	36	12	27	22	26	24	24	36	56	48	47	8	36	30	25	64	46	36	49
18	24	-	21	22	38	32	31	0	2	10	4	28	20	26	25	22	24	20	22	20	16	12	16
16	40	42	33	3	6	4	4	6	12	16	11	38	44	42	41	24	50	40	38	56	20	28	35
22	22	36	27	2	12	2	5	12	6	3	7	16	60	20	32	13	32	46	30	10	14	24	16
10	20	44	25	28	34	14	25	6	30	26	21	18	30	24	24	6	20	18	15	15	16	45	25
38	24	36	33	12	12	16	13	18	32	42	31	24	54	38	39	30	52	40	41	22	16	12	17
20	24	30	25	60	72	50	61	78	90	93	87	10	14	34	19	2	12	16	10	84	76	86	82
24	18	30	24	32	28	12	24	9	18	18	15	4	12	12	9	8	12	10	10	54	18	24	32
16	24	20	20	26	20	10	19	12	6	12	10	6	6	6	6	14	30	18	21	8	18	24	17
32	48	36	39	12	12	32	19	12	26	16	18	63	54	60	59	42	46	56	48	46	32	36	38
30	42	42	38	22	18	8	16	22	56	24	27	50	76	42	56	8	42	18	23	52	48	20	40
16(188)	18(188)	28(188)		8(189)	14(189)	6(189)		2(190)	4(190)	16(190)		6(191)	10(191)	10(191)		4(192)	6	(192)	8(192)	12(193)	14(193)	16(193)	
18(7)	10(2)	20(207)		8(2)	20(2)	10(207)		12(7)	6(2)	10(207)		0(2)	8(2)	2(205)		0(2)	8	(2)	8(204)	16(2)	8(209)	20(209)	
14(94)	10(94)	20(210)		12(94)	14(97)	8(210)		6(94)	2(97)	6(210)		4(94)	2(97)	2(209)		6(94)	12	(97)	16(206)	8(94)	12(211)	10(211)	
16(3)	14(97)	28(211)		4(95)	8(95)	0(211)		4(95)	0(95)	2(211)		6(95)	12(95)	2(211)		8(95)	14	(95)	16(208)	4(95)	12(213)	10(213)	
18				9				6				5				9				12			

Footnote: a, b, c ... = repeated counts.
E = mean of repeated counts.
C = mean of control counts.
Numbers in parenthesis are those of the control sera. These sera were from the leucocyte donors or from other non-immunised rabbits.

TABLE XXVII (cont.)

Stained Cell Counts

BATCH 6

Antiserum	Rabbit No: 194 a	b	c	E	Rabbit No: 195 a	b	c	E
32	14	2	14	10	10	6	8	8
33	14	6	16	12	8	4	10	7
34	6	6	18	10	4	4	2	3
41	16	20	10	15	24	12	30	22
45	28	18	20	22	8	20	10	13
49	10	0	14	8	18	4	20	14
50	4	4	10	6	20	12	16	16
55	8	6	14	9	8	16	8	11
56	2	12	14	9	24	4	4	11
61	10	6	16	11	14	12	10	12
68	62	42	38	47	24	26	28	26
69	20	0	18	13	10	6	8	8
71	8	10	22	15	20	8	8	12
72	20	24	8	17	36	26	52	38
73	46	20	30	32	18	26	10	18
74	38	24	40	34	20	8	20	16
75	16	14	22	17	10	26	42	26
93	44	16	28	29	30	12	8	17
94	30	32	18	27	9	12	6	9
95	8	6	12	9	20	24	44	29
97	20	30	34	28	6	20	26	17
98	14	18	34	22	32	20	62	38
2	20	10	8	13	52	12	44	36
3	14	18	16	16	44	50	52	49
7	60	45	45	50	12	20	34	22
8	28	36	50	38	18	15	12	15
Controls	12(194)	8(194)	8(194)		16(195)	6(195)	4(195)	
	14(2)	6(2)	8(211)		8(2)	4(2)	0(204)	
	4(94)	0(97)	4(205)		10(94)	2(210)	8(210)	
	18(95)	2(211)	4(210)		4(97)	4(211)	10(211)	
C.	7				6			

BATCH 7

Antiserum	Rabbit No: 179 a	b	c	d	E	Rabbit No: 180 a	b	c	E
32	18	14	10	14	14	20	20	38	26
33	8	12	18	24	16	18	12	44	25
34	12	10	18	20	15	6	12	32	17
41	16	14	26	56	28	72	68	88	76
45	20	26	54	54	39	42	38	60	47
49	4	20	6	14	11	24	32	52	36
50	8	22	8	16	14	30	70	80	60
55	26	32	44	72	44	22	24	60	33
56	10	12	12	22	14	26	28	46	27
61	9	9	12	9	10	24	16	40	58
68	10	24	28	35	24	32	70	72	26
69	10	18	18	28	19	16	20	42	35
71	12	20	20	10	16	28	28	50	72
72	22	12	-	54	29	60	72	84	68
73	16	18	20	42	24	50	64	90	74
74	14	36	24	70	36	66	72	84	23
75	28	24	45	38	34	14	18	36	49
93	8	26	30	32	24	40	40	68	61
94	10	10	12	36	17	40	52	90	25
95	14	30	32	55	33	20	18	38	64
97	26	30	60	62	45	56	46	90	87
98	14	24	42	32	28	86	84	91	30
2	18	20	36	50	31	30	16	44	23
3	24	36	56	62	45	16	16	36	68
7	15	21	32	36	26	54	76	74	79
8	6	18	44	34	26	80	70	86	
Controls	2(179)	6(179)	20(179)	24(179)		18(180)	24(180)	30(180)	
	12(7)	4(7)	12(2)	20(7)		20(7)	14(3)	34(2)	
	2(8)	2(8)	12(93)	30(3)		10(8)	14(97)	26(7)	
	0(98)	10(98)	18(94)	14(95)		10(98)	26(95)	30(97)	
C.	12					21			

Antiserum	Rabbit No: 182 a	b	c	E	Rabbit No: 183 a	b	c	E	Rabbit No: 184 a	b	c	E	Rabbit No: 185 a	b	E	Rabbit No: 186 a	b	c	E	Rabbit No: 187 a	b	E
32	6	24	18	16	30	18	48	32	10	12	24	15	0	0	0	6	10	20	12	28	20	24
33	6	14	6	9	48	28	50	42	14	34	24	24	22	26	24	10	10	10	10	28	10	16
34	16	12	16	15	12	16	18	15	14	8	26	16	6	10	8	16	10	20	15	8	6	7
41	54	74	90	73	26	38	18	27	22	54	63	46	2	4	3	80	68	78	75	90	82	86
45	8	48	26	27	16	18	8	14	22	48	48	39	16	6	11	12	8	22	14	48	36	42
49	10	20	18	16	16	22	18	19	24	18	12	18	2	0	1	12	10	36	19	26	18	22
50	18	46	70	45	54	64	68	62	8	18	32	19	18	22	20	4	0	12	5	22	46	34
55	18	14	15	16	72	78	80	77	8	10	14	11	62	82	72	12	14	6	11	36	18	27
56	10	20	12	14	42	26	24	31	10	10	24	15	6	10	8	8	8	20	12	8	14	23
61	10	18	20	16	42	16	24	27	12	12	10	11	6	12	9	12	10	10	11	20	22	21
68	18	62	75	52	78	90	84	84	8	14	26	16	44	54	49	50	42	30	34	24	20	22
69	12	14	16	14	36	16	22	25	14	10	22	15	14	16	15	8	10	12	10	22	12	17
71	16	12	14	14	26	22	20	23	12	6	24	14	8	8	8	44	12	14	23	12	16	14
72	48	68	90	69	30	20	28	26	16	86	40	62	8	14	11	82	48	56	62	76	80	78
73	20	52	68	47	90	90	78	86	16	34	40	30	64	62	63	12	10	24	15	14	28	21
74	48	76	60	61	24	28	34	29	38	50	38	42	2	6	4	54	68	60	61	96	74	85
75	12	14	14	13	40	30	28	33	12	24	18	18	20	22	21	38	28	50	39	28	32	30
93	32	78	76	62	68	90	70	76	10	26	24	20	12	42	27	12	12	10	11	16	12	14
94	28	50	80	53	70	98	74	81	12	4	22	13	50	20	35	16	6	6	9	24	24	24
95	14	16	24	18	36	54	22	31	12	8	24	15	8	16	12	84	93	90	89	44	24	34
97	22	50	88	53	66	74	89	76	10	28	30	23	46	46	46	14	8	20	14	22	20	21
98	76	80	100	85	22	28	32	27	90	68	76	78	2	6	4	46	24	54	41	84	64	74
2	10	22	16	16	26	12	18	19	14	12	16	14	0	6	3	60	84	75	75	68	48	58
3	10	16	12	13	34	28	22	28	10	12	18	13	14	6	10	93	84	96	91	28	24	26
7	28	66	56	50	70	82	76	76	26	36	64	42	44	62	53	24	14	34	24	34	40	37
8	56	66	88	70	62	68	80	70	12	36	54	34	30	26	28	8	6	16	10	28	18	23
Controls	10(182)	14(182)	18(182)		32(183)	20(183)	22(183)		10(184)	12(184)	16(184)		4(185)	4(185)		6(186)	8(186)	6(186)		18(187)	16(187)	
	4(7)	6(94)	14(7)		28(2)	16(95)	20(94)		8(2)	8(7)	6(7)		0(93)	12(3)		20(3)	0(2)	8(3)		24(93)	18(2)	
	6(8)	12(97)	22(3)		36(93)	28(7)	16(97)		8(3)	12(94)	12(94)		12(95)	10(95)		4(97)	14(95)	10(97)		28(94)	22(3)	
	4(98)	24(95)	10(95)		24(94)	32(3)	18(3)		4(95)	8(3)	14(3)		8(97)	8(8)		10(98)	8(7)	8(98)		12(95)	18(98)	
C.	12				24				10				7			9				20		

Footnote: a, b, c ... = repeated counts.
E = mean of repeated counts.
C = mean of control counts.
Numbers in parenthesis are those of the control sera. These sera were from the leucocyte donors or from other non-immunised rabbits.

TABLE XXVIII

Cytotoxic Indices (C.I.)

Rabbit No:

Serum No.	83	84	86	88	89	90	91	96	99	1	10	11	12	13	9	14	16	17	18	19	20	21	22	15	23	24	25	26	179	180	182	183	184	185	186	187	188	189	190	191	192	193	194	195	27	28	29	30	31	132	133	134
32	6	9	10	52	0	12	13	0	2	1	4	3	3	2	2	2	7	1	1	0	11	5	22	6	9	0	5	7	2	6	5	11	6	0	3	5	0	3	1	3	3	5	3	2	1	0	1	3	3	2	1	2
33	8	11	1	49	17	18	0	17	3	20	11	2	13	15	5	11	7	13	0	3	0	18	21	3	20	15	4	16	7	5	5	0	24	16	18	1	0	5	7	12	18	3	9	5	2	4	19	8	5	2	2	17	9
34	0	4	18	25	0	4	13	1	2	0	5	1	3	2	2	0	4	0	0	3	0	2	9	1	1	2	7	2	3	0	3	0	7	1	7	0	5	2	7	1	3	0	7	3	0	1	2	0	0	0	0	7	0
41	0	54	68	17	0	16	27	1	3	0	10	5	11	7	1	0	17	82	4	7	3	2	93	8	6	4	84	46	18	70	69	4	40	0	73	83	48	54	75	4	6	65	9	17	65	11	70	3	3	33	4	1
45	48	36	54	25	7	7	33	5	20	0	2	3	0	5	7	9	9	43	0	5	0	2	60	1	6	27	30	24	31	33	17	0	32	5	6	28	35	17	4	8	6	50	16	8	19	0	20	11	3	8	1	8
49	5	40	42	0	0	20	33	5	8	0	4	7	6	5	6	1	33	44	0	7	2	5	78	1	9	15	55	10	2	19	5	0	9	0	11	3	0	0	9	3	0	2	1	9	21	0	23	5	6	8	2	3
50	6	15	28	41	0	3	0	13	11	1	16	20	52	23	37	60	14	26	15	4	28	11	51	29	17	4	46	20	2	49	38	50	10	14	0	18	2	14	15	23	6	60	0	11	31	2	43	0	1	9	10	10
55	18	16	18	11	38	10	13	7	3	1	22	6	28	6	72	3	5	0	27	4	28	25	12	0	27	35	1	4	36	18	5	70	1	70	2	9	6	12	8	1	2	17	2	6	6	35	4	0	9	0	31	2
56	6	4	0	6	0	0	27	3	1	3	5	2	6	9	2	9	5	2	0	4	4	14	7	10	4	1	2	2	2	17	2	9	6	1	3	4	2	0	1	6	8	0	2	5	0	2	2	5	5	4	10	8
61	0	4	0	0	1	4	7	1	4	2	6	0	3	4	1	7	0	0	1	1	3	9	3	4	6	4	1	4	0	8	5	4	1	2	2	1	4	0	0	0	4	5	4	6	1	0	0	1	1	1	7	1
68	38	28	10	19	29	20	27	42	5	17	49	54	50	7	2	43	19	13	60	0	15	42	28	43	13	8	21	7	14	47	46	79	7	45	28	3	9	3	1	50	25	6	43	21	41	3	2	3	3	9	6	37
69	0	12	19	0	1	2	0	13	3	0	8	2	2	7	2	8	0	4	2	0	22	3	2	9	8	15	7	8	8	6	2	1	6	9	1	0	1	1	2	15	4	2	7	2	0	0	0	0	1	3	1	13
71	7	17	12	29	7	22	0	13	0	21	1	4	1	2	0	0	4	0	4	3	0	24	3	2	9	0	8	34	5	18	2	0	4	1	15	0	1	2	7	1	1	2	7	6	43	1	67	7	11	51	4	0
72	1	52	51	47	0	12	13	1	14	0	1	10	2	16	4	1	34	79	2	12	1	2	95	1	6	8	80	56	19	65	65	3	58	4	58	73	57	48	65	11	6	75	11	34	69	1	56	0	6	39	6	5
73	40	53	18	20	39	21	0	49	4	24	52	60	46	16	11	63	12	7	70	5	28	60	7	54	12	27	55	3	14	60	40	82	22	60	7	1	31	4	1	61	29	2	27	13	48	9	13	0	6	14	22	40
74	35	44	37	25	22	11	7	38	9	16	69	53	41	15	9	69	25	67	71	7	32	19	84	9	23	8	54	53	27	67	56	7	36	0	57	81	48	20	19	44	18	42	29	11	66	0	67	2	4	44	1	1
75	22	17	8	29	25	23	33	15	15	28	15	8	9	12	7	10	18	15	9	3	11	32	11	16	15	1	7	10	25	3	1	12	9	15	33	15	4	24	0	20	14	5	11	21	11	7	6	7	8	8	11	10
93	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	48	28	10	66	1	9	20	7	36	4	17	44	16	14	35	57	68	11	22	2	0	18	0	5	39	32	26	24	11	28	11	18	12	0	32	15	66
94	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	12	6	0	61	0	24	23	1	29	4	8	34	6	6	51	47	75	3	30	0	5	11	0	1	28	23	5	22	3	32	11	15	2	4	7	46
95	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4	65	37	0	6	8	12	13	12	7	22	3	43	24	5	7	9	6	5	88	18	9	18	16	20	7	16	2	25	0	7	62	36	4	1	30	2
97	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	41	59	18	48	9	56	36	23	27	22	70	64	46	38	54	47	68	14	42	6	1	18	6	27	36	35	6	23	13	43	21	41	24	0	12	41	31
98	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	16	83	0	32	2	2	91	0	12	6	75	72	18	84	83	4	76	0	35	68	7	57	86	15	1	80	16	34	87	4	77	1	25	55	4	8
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	4	39	4	3	1	29	43	1	9	8	4	6	22	11	5	0	4	0	70	48	7	17	10	4	1	23	7	32	7	2	8	13	3	0	4	3
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	12	20	4	7	0	5	8	8	6	4	1	3	38	3	1	5	3	3	90	8	2	11	4	1	13	6	10	46	2	1	2	12	1	7	4	3
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	60	9	8	77	28	2	25	79	48	14	12	55	23	16	60	43	68	36	50	17	21	26	11	13	57	43	30	46	17	59	11	14	8	12	25	2	39
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	61	1	25	49	4	15	20	48	39	13	12	58	10	16	73	66	61	27	23	1	4	24	8	23	54	15	32	33	10	54	1	27	1	13	25	4	56
Mean of Controls (C)	13	8	26	21	28	10	40	12	7	4	17	6	9	11	14	9	14	8	13	19	9	11	9	10	14	17	8	10	12	21	12	24	10	7	9	20	18	9	6	5	9	12	7	6	9	8	5	7	9	8	17	12

FIGURE 38

The incidence of high cytotoxic indices (>29).

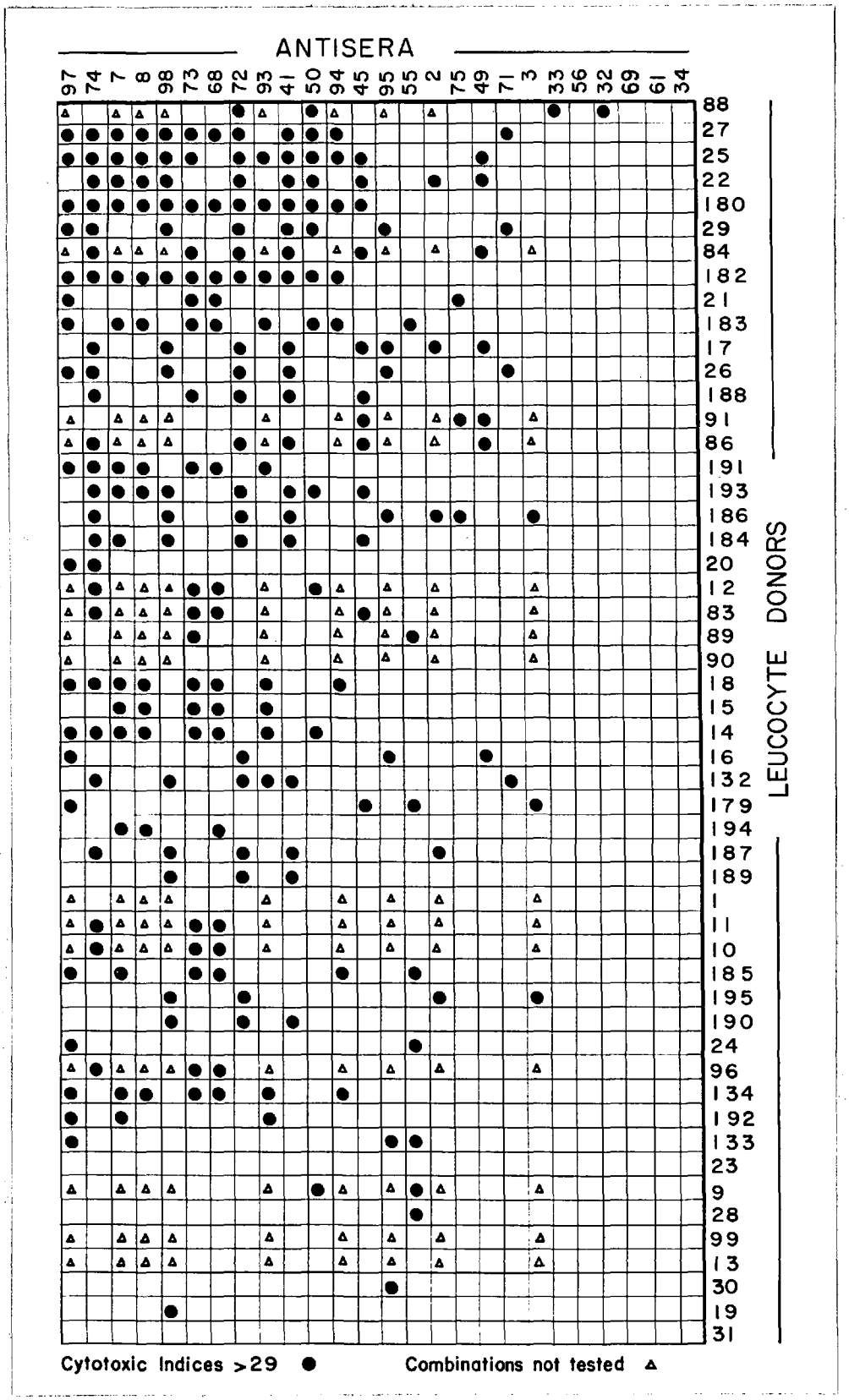

ANTISERA
97 74 7 8 98 73 68 72 93 41 50 94 45 95 55 2 75 49 71 3 33 56 32 69 61 34
88 27 25 22 180 29 84 182 21 183 17 26 188 91 86 191 193 186 184 20 12 83 89 90 18 15 14 16 132 179 194 187 189 1 11 10 185 195 190 24 96 134 192 133 23 9 28 99 13 30 19 31
LEUCOCYTE DONORS
Cytotoxic Indices >29 ●
Combinations not tested △

TABLE XXIX

Immunogenic Indices (I.G.I.)

BATCH 1

Recipients	Donors 83	84	86	88	89	90	91
83	-	162	214	232	20	67	112
84	22	-	59	152	22	0	39
86	54	43	-	141	93	52	69
88	72	132	153	-	56	20	64
89	54	233	297	254	-	75	152
90	102	194	242	197	57	-	87
91	94	191	207	242	96	61	-

BATCH 2

Recipients	Donors 96	99	1	9	10	11	12	13
96	-	28	13	89	46	38	70	25
99	148	-	83	95	171	149	195	36
1	97	44	-	107	159	139	169	38
9	143	23	85	-	148	145	140	22
10	12	31	33	71	-	0	36	15
11	26	17	55	83	32	-	65	13
12	23	32	39	44	28	26	-	14
13	102	15	45	80	148	129	150	-

TABLE XXIX (cont.)

Immunogenic Indices (I.G.I.)

BATCH 3

Recipients	Donors							
	14	16	17	18	19	20	21	22
14	-	174	406	125	40	66	118	500
16	286	-	335	377	35	118	216	575
17	281	87	-	377	20	129	228	262
18	68	170	437	-	42	61	115	533
19	399	256	486	482	-	241	319	677
20	275	180	455	329	67	-	176	660
21	203	165	431	257	40	69	-	622
22	154	109	46	254	0	128	188	-

BATCH 4

Recipients	Donors							
	179	180	182	183	184	185	186	187
179	-	558	446	484	161	194	329	265
180	105	-	22	196	11	77	259	87
182	140	133	-	272	31	117	117	116
183	178	360	272	-	224	0	486	363
184	146	412	296	490	-	225	345	155
185	190	514	400	276	233	-	502	385
186	118	484	382	581	152	310	-	122
187	156	439	346	577	99	316	234	-

TABLE XXIX (cont.)

Immunogenic Indices (I.G.I.)

BATCH 5

Recipients	Donors									
	14	15	16	18	19	21	23	24	25	26
15	151	-	182	244	43	101	53	128	431	314
23	327	201	182	397	34	199	-	112	586	295
24	316	221	176	373	42	212	53	-	544	283
25	51	22	84	168	0	123	26	53	-	66
26	272	197	82	359	0	211	23	79	353	-

BATCH 6

Recipients	Donors											
	188	189	190	191	192	193	194	195	27	29	180	183
188	-	92	119	261	97	208	65	141	387	317	481	506
189	145	-	103	340	163	231	186	79	460	316	558	580
190	126	37	-	301	145	175	184	117	371	251	467	561
191	117	163	196	-	12	304	0	128	284	356	384	265
192	152	204	213	195	-	383	72	154	447	446	562	432
193	41	19	31	244	106	-	112	82	280	203	339	462
194	123	186	219	163	12	336	-	146	394	408	505	420
195	150	74	178	309	105	318	148	-	488	375	591	538

TABLE XXIX (cont.)

Immunogenic Indices (I.G.I.)

BATCH 7

Recipients	Donors							
	27	28	29	30	31	132	133	134
27	-	44	98	46	0	0	78	65
28	620	-	505	62	43	264	56	261
29	208	42	-	10	0	25	27	220
30	618	49	467	-	36	284	92	280
31	611	75	495	89	-	242	135	291
132	350	52	266	60	0	-	104	204
133	575	0	458	10	31	264	-	240
134	397	53	457	44	28	207	67	-

B. The Grafting Results

(a) Controls

Figure 39 shows the survival times of the grafts on 10 rabbits where the 4 grafts on each recipient were from the same donor. There were 2 technical failures. Of the remaining 38 grafts, the difference between the rejection times on any one recipient was always less than 2 days whether the end point was the first sign of rejection or the last sign of viability.

(b) Tests

(i) Where both donor and recipient were grouped.

Of 52 rabbits serologically tested, 4 were not grafted (Nos. 1, 25, 27, 96) because the I.G.I.'s of all the donors in the batch were so similar as to preclude any prediction. Three died (Nos. 21, 133, 179) as the result of illness (diarrhoea and snuffles) and 3 (Nos. 20, 24, 193) as the result of accidental fractures before they could be grafted. In addition there were 9 operative and postoperative deaths (Nos. 10, 11, 12, 16, 18, 26, 29, 184, 186), most of which occurred in the early period when

inexperience resulted in a high anaesthetic mortality, and there were 5 technical failures (Nos. 17, 22, 28, 30, 31). There were 18 ungrouped recipients of which 3 were technical failures (Nos. 158, 172, 174).

The results used were therefore obtained from 43 recipients, which bore a total of 137 grafts.

Figure 40 shows the survival times of the grafts on 26 of the recipients which rejected their grafts over a period of 2 days or more, and Table XXX shows the survival times of the grafts on the remaining 17 where the grafts on each recipient were rejected within less than 2 days of each other.

Taking the last sign of viability as the end point the graft which survived as long or longer than any other on a recipient was from the donor that had been serologically assessed as the most compatible in 30 of 43 cases and was either from the donor selected as most compatible or second most compatible in all cases but one (recipient No. 180).

Considering the ungrouped recipients alone, 5 of the 15 rejected their grafts in accordance with the results of the serological test; on 8 both grafts were rejected on the same day, and on 2 the rejection orders were the reverse of those expected.

The autografts behaved typically except in 3 recipients (Nos. 17, 28, 31) which were therefore counted as technical failures and the results from these were discarded.

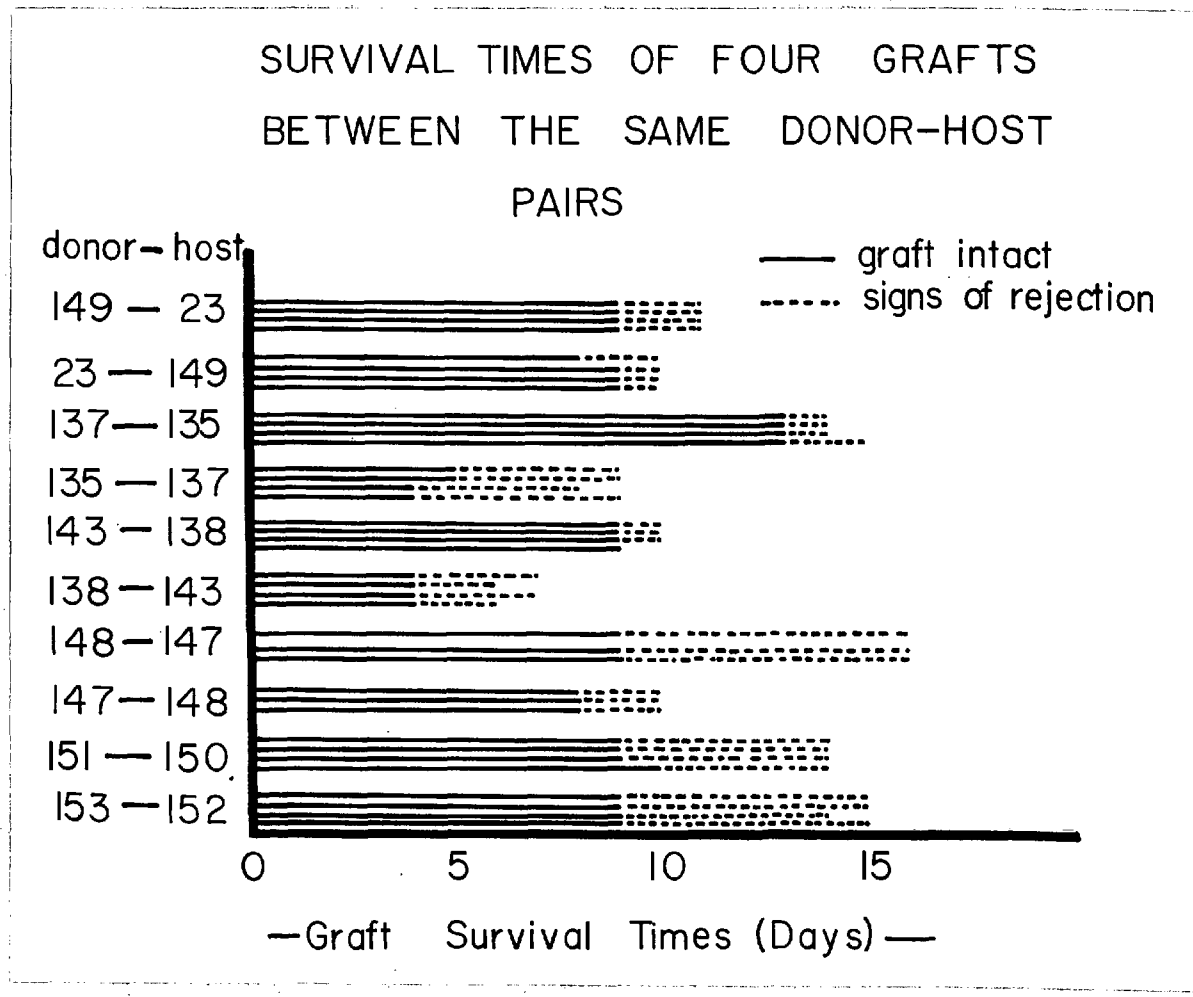


FIGURE 39

Survival times of skin allografts in rabbits. Each recipient received 4 grafts from the same donor.

FIGURE 40

The predicted rejection orders and observed survival times of skin allografts from donors serologically tested. The donors are arranged from top to bottom in each box according to their predicted rejection orders, those expected to survive shortest at the top.
Column A : recipients;
B : donors;
C : immunogenic indices;
D : observed survival times.
Recipient 99 was destroyed after a spinal injury on the 12th postoperative day with one graft still intact. Leucocytes of recipients 156, 162, 165 and 166 were not tested against the panel and received grafts from donors thought to be of high and low antigonicity (see text). Recipients 86, 88 and 89 were treated with 6-mercaptopurine.

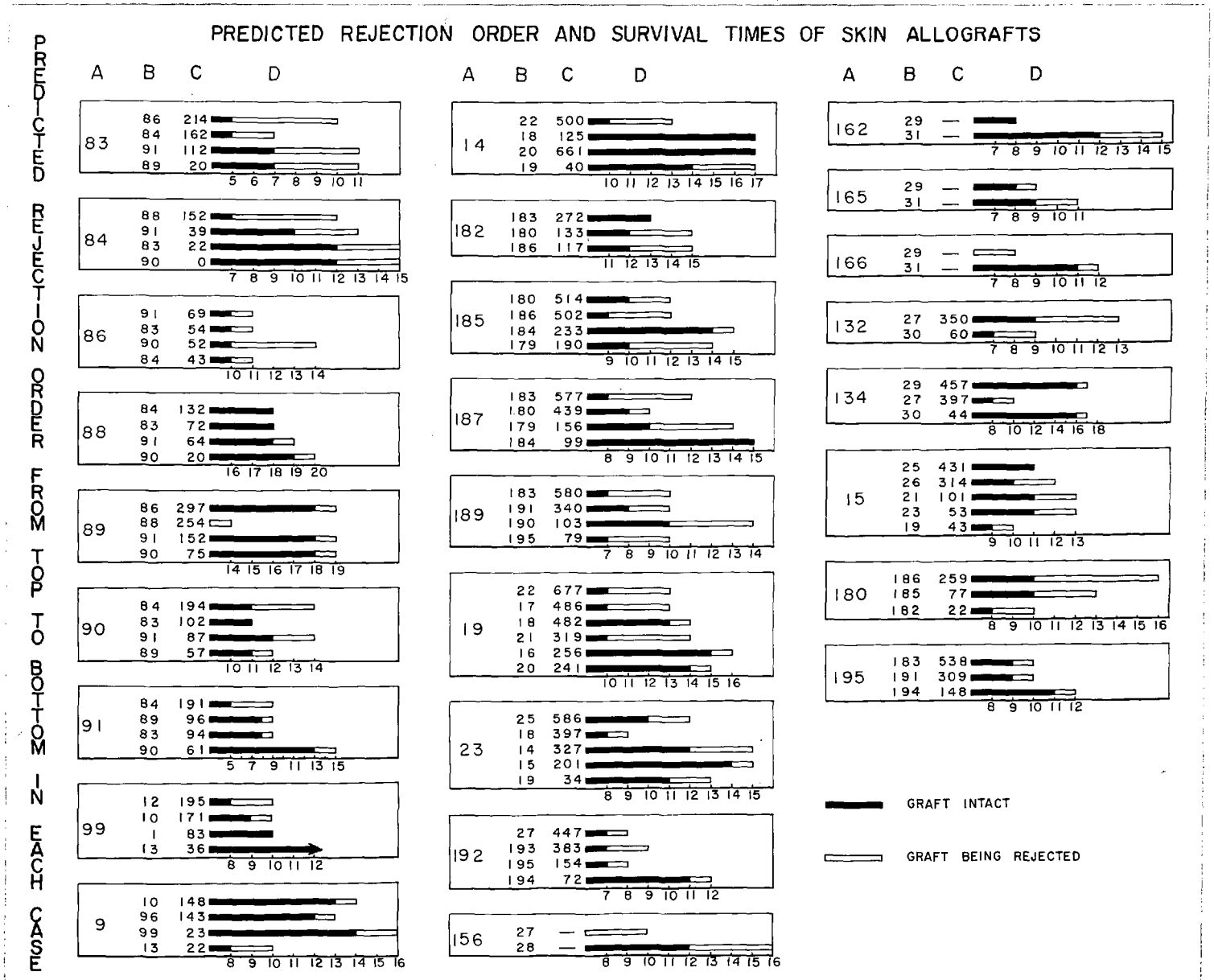

PREDICTED REJECTION ORDER AND SURVIVAL TIMES OF SKIN ALLOGRAFTS
PREDICTED REJECTION ORDER FROM TOP TO BOTTOM IN EACH CASE
A B C D
83 86 214 84 162 91 112 89 20 5 6 7 8 9 10 11
84 88 152 91 39 83 22 90 0 7 8 9 10 11 12 13 14 15
86 91 69 83 54 90 52 84 43 10 11 12 13 14
88 84 132 83 72 91 64 90 20 16 17 18 19 20
89 86 297 88 254 91 152 90 75 14 15 16 17 18 19
90 84 194 83 102 91 87 89 57 10 11 12 13 14
91 84 191 89 96 83 94 90 61 5 7 9 11 13 15
99 12 195 10 171 1 83 13 36 8 9 10 11 12
9 10 148 96 143 99 23 13 22 8 9 10 11 12 13 14 15 16
A B C D
14 22 500 18 125 20 661 19 40 10 11 12 13 14 15 16 17
182 183 272 180 133 186 117 11 12 13 14 15
185 180 514 186 502 184 233 179 190 9 10 11 12 13 14 15
187 183 577 180 439 179 156 184 99 8 9 10 11 12 13 14 15
189 183 580 191 340 190 103 195 79 7 8 9 10 11 12 13 14
19 22 677 17 486 18 482 21 319 16 256 20 241 10 11 12 13 14 15 16
23 25 586 18 397 14 327 15 201 19 34 8 9 10 11 12 13 14 15
192 27 447 193 383 195 154 194 72 7 8 9 10 11 12
156 27 — 28 — 8 9 10 11 12 13 14 15 16
A B C D
162 29 — 31 — 7 8 9 10 11 12 13 14 15
165 29 — 31 — 7 8 9 10 11
166 29 — 31 — 7 8 9 10 11 12
132 27 350 30 60 7 8 9 10 11 12 13
134 29 457 27 397 30 44 8 10 12 14 16 18
15 25 431 26 314 21 101 23 53 19 43 9 10 11 12 13
180 186 259 185 77 182 22 8 9 10 11 12 13 14 15 16
195 183 538 191 309 194 148 8 9 10 11 12
GRAFT INTACT
GRAFT BEING REJECTED

TABLE XXX

Graft survival times where the difference between the first and last rejected graft on a recipient was less than 2 days.

Recipient No.	Donor No.	I.G.I.	Graft Survival Time*
13	11	129	12 - 14
	1	45	13
	99	15	12 - 14
183	186	486	8 - 9
	179	178	7 - 9
	185	0	8 - 9
188	180	481	8 - 11
	190	119	9 - 11
	189	92	9 - 10
190	183	561	8
	191	301	8
	188	126	7 - 8
	189	37	8 - 9
191	29	356	6 - 7
	193	304	6 - 7
	192	12	6 - 7
	194	0	7 - 8
194	180	505	7 - 8
	193	336	8
	195	146	7 - 8
	192	12	8
157	27	-	9 - 11
	28	-	9 - 10
159	27	-	14 - 15
	28	-	14 - 15

* Days from the first sign of rejection to the last sign of viability.

TABLE XXX (cont.)

Recipient No.	Donor No.	I.G.I.	Graft Survival Time*
161	27	-	11 - 12
	31	-	11 - 12
163	29	-	11 - 12
	31	-	11 - 12
168	27	-	11
	31	-	12
171	27	-	11 - 12
	31	-	11
172	27	-	12
	31	-	12
175	29	-	6 - 7
	28	-	6 - 7
176	29	-	6
	28	-	6
177	29	-	6
	31	-	5 - 6
178	29	-	6
	31	-	6

C. Correlation between the Results of the Serological Test and Grafting

Kendall's Rank Correlation method (1955) was used to assess the agreement between the results of the serological test and the observed rejection orders.

Briefly, Kendall's 'S' was calculated as follows: The observed rejection rankings were placed in order of prediction from left to right so that a correctly predicted result would read

1, 2, 3, 4 ..... (a)

Here each figure has larger figures to the right of it. Where the observed rejection orders did not correspond with the predictions, for example

3, 1, 4, 2 ..... (b)

some of the digits would have smaller numbers to the right of them.

Considering each digit in turn a value +1 was assigned for each digit on the right of it which was higher and -1 for each digit on the right which was lower.

For example in ranking (a), the first number on the left has three numbers on the right of it which are larger (i.e. S = +3); the second number has two numbers on the right which are larger (S = +2) and the

third number has one number on the right which is larger (S = +1). The S for the whole ranking is therefore 3 + 2 + 1 = 6.

In ranking (b), the first number has two numbers on the right of it which are smaller and one which is larger (S = -1), and so on. Here S for the whole ranking is - 1 + 2 - 1 = 0.

In a sequence of random rankings these S values are asymptotically normally distributed.

In the case of ties, rankings intermediate between the relevant placings were given, e.g. two grafts which tied for third place were written $3\frac{1}{2}$, $3\frac{1}{2}$. The S value assigned for equal numbers was 0.

Applying this method, the S values were calculated for each recipient alternatively regarding the first and last signs of rejection as the graft end points. These are shown in Tables XXXIa, XXXIb, XXXIc and XXXId.

In Figure 41 the distribution of the S values is shown and a tendency toward a positively skewed distribution is noticeable, especially on the recipients bearing 4, 5 and 6 grafts with the last sign of rejection taken as the graft end points.

TABLE XXXIa

The calculation of S on the recipients which rejected their grafts over a period of 2 days and more (see Figure 40), taking the last signs of viability as the graft end point.

Recipient No.	No. of grafts per recipient	Ranking according to observed rejection orders. Predicted order from L to R.	'S'
83	4	2 1 3½ 3½	1+2+0 = 3
84	4	1 2 3½ 3½	3+2+0 = 5
86	4	2 2 4 2	1+1-1 = 1
88	4	1½ 1½ 3 4	2+2+1 = 5
89	4	3 1 3 3	-1+2+0 = 1
90	4	3½ 1 3½ 2	-2+2-1 = -1
91	4	2 2 2 4	1+1+1 = 3
99	4	2 2 2 4	1+1+1 = 3
9	4	3 2 4 1	-1+0-1 = -2
14	4	1 3 3 3	3+0+0 = 3
185	4	1½ 1½ 4 3	2+2-1 = 3
187	4	2 1 3 4	1+2+1 = 4
189	4	2 2 4 2	1+1-1 = 1
192	4	1½ 3 1½ 4	2+0+1 = 3
182	3	1 2½ 2½	2+0 = 2
134	3	2½ 1 2½	-1+1 = 0
180	3	3 2 1	-2-1 = -3
195	3	1½ 1½ 3	1+1 = 2
23	5	2 1 4½ 4½ 3	2+3-1-1 = 3
15	5	2 3 4½ 4½ 1	2+1-1-1 = 1
19	6	1½ 1½ 3½ 3½ 6 5	4+4+2+2-1 = 11
156	2	1 2	1
162	2	1 2	1
165	2	1 2	1
166	2	1 2	1
132	2	2 1	-1

TABLE XXXIb

The calculation of S on the recipients which rejected their grafts over a period less than 2 days (see Table XXX), taking the last sign of rejection as the end points.

Recipient No.	No. of grafts per recipient	Ranking according to observed rejection orders. Predicted order from L to R.	'S'	
190	4	2 2 2 4	1+1+1	= 3
191	4	2 2 2 4	1+1+1	= 3
194	4	2½ 2½ 2½ 2½		0
13	3	2½ 1 2½	-1+1	= 0
183	3	2 2 2		0
188	3	2½ 2½ 1	-1-1	= -2
157	2	2 1		= -1
159	2	1½ 1½		0
161	2	1½ 1½		0
163	2	1½ 1½		0
168	2	1 2		1
171	2	2 1		-1
172	2	1½ 1½		0
175	2	1½ 1½		0
176	2	1½ 1½		0
177	2	1½ 1½		0
178	2	1½ 1½		0

TABLE XXXIc

The calculation of S on the recipients which rejected their grafts over a period of 2 days and more, taking the first sign of rejection as the end points.

Recipient No.	No. of grafts per recipient	Ranking according to observed rejection orders. Predicted order from L to R.	'S'
83	4	1½ 1½ 3½ 3½	2+2+0 = 4
84	4	1 2 3½ 3½	3+2+0 = 5
88	4	2 2 2 4	1+1+1 = 3
89	4	3 1 3 3	-1+2+0 = 1
91	4	1 2½ 2½ 4	3+1+1 = 5
99	4	1 2 3 4	3+2+1 = 6
9	4	3 2 4 1	-1+0-1 = -2
14	4	1 3½ 3½ 2	3-1-1 = 1
185	4	2½ 1 4 2½	0+2-1 = 1
187	4	1 2 3 4	3+2+1 = 6
189	4	1½ 3 4 1½	2+0-1 = 1
192	4	2 2 2 4	1+1+1 = 3
134	3	2½ 1 2½	-1+1 = 0
180	3	2½ 2½ 1	-1-1 = -2
195	3	1½ 1½ 3	1+1 = 2
23	5	2 1 4 5 3	2+3+0-1 = 4
15	5	4 2 4 4 1	-2+1-1-1 = -3
19	6	3½ 1½ 3½ 1½ 6 5	0+2+1+2-1 = 5
156	2	1 2	1
162	2	1 2	1
166	2	1 2	1
132	2	2 1	-1

TABLE XXXId

The calculation of S on the recipients which rejected their grafts over a period of less than 2 days, taking the first sign of rejection as the end point.

Recipient No.	No. of grafts per recipient	Ranking according to observed rejection orders. Predicted order from L to R.	'S'	
86	4	2½ 2½ 2½ 2½		= 0
90	4	2 2 4 2	1+1−1	= 1
190	4	3 3 1 3	−1−1+1	= −1
191	4	2 2 2 4	1+1+1	= 3
194	4	1½ 3½ 1½ 3½	2−1+1	= 2
182	3	3 1½ 1½	−2+0	= −2
13	3	1½ 3 1½	1−1	= 0
183	3	2½ 1 2½	−1+1	= 0
188	3	1 2½ 2½	2+0	= 2
165	2	1 2		1
157	2	1½ 1½		0
159	2	1½ 1½		0
161	2	1½ 1½		0
163	2	1½ 1½		0
168	2	1 2		1
171	2	1½ 1½		0
172	2	1½ 1½		0
175	2	1½ 1½		0
176	2	1½ 1½		0
177	2	2 1		−1
178	2	1½ 1½		0

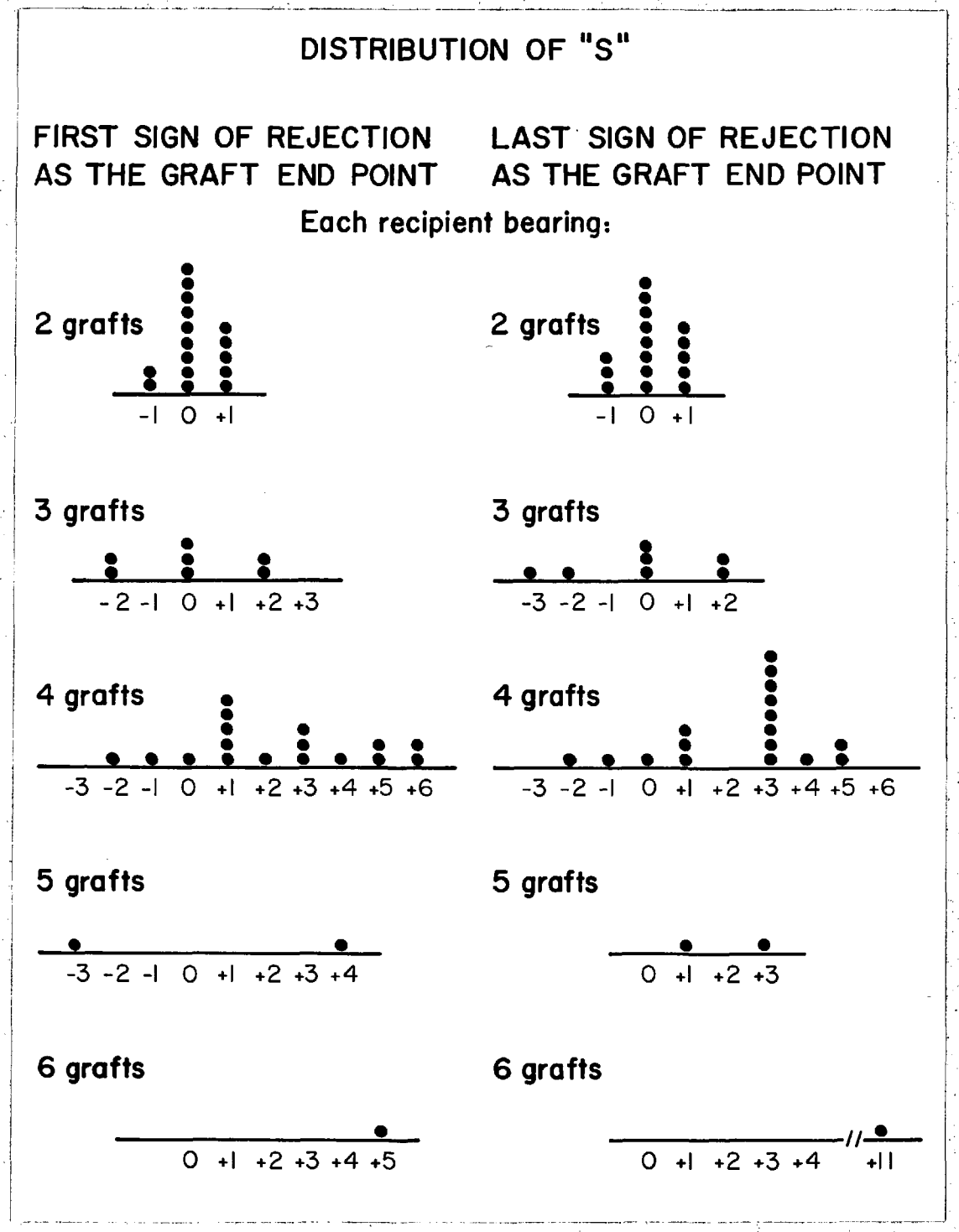

DISTRIBUTION OF "S"
FIRST SIGN OF REJECTION AS THE GRAFT END POINT
LAST SIGN OF REJECTION AS THE GRAFT END POINT
Each recipient bearing:
2 grafts
-1 0 +1
2 grafts
-1 0 +1
3 grafts
-2 -1 0 +1 +2 +3
3 grafts
-3 -2 -1 0 +1 +2
4 grafts
-3 -2 -1 0 +1 +2 +3 +4 +5 +6
4 grafts
-3 -2 -1 0 +1 +2 +3 +4 +5 +6
5 grafts
-3 -2 -1 0 +1 +2 +3 +4
5 grafts
0 +1 +2 +3
6 grafts
0 +1 +2 +3 +4 +5
6 grafts
0 +1 +2 +3 +4 +11

FIGURE 41

The significance of the observed distribution of 'S' was then calculated. The probability of 'X' occurring by chance was found by reference to normal distribution tables when

$$X = \frac{\frac{\sum S_6}{v_6} + \frac{\sum S_5}{v_5} + \frac{\sum S_4}{v_4} + \frac{\sum S_3}{v_3}}{\sqrt{\frac{m_6}{v_6} \quad \frac{m_5}{v_5} \quad \frac{m_4}{v_4} \quad \frac{m_3}{v_3}}}$$

and where m_6, m_5, m_4 and m_3 were the number of recipients with 6, 5, 4 and 3 grafts respectively, S_6, S_5, S_4 and S_3 their 'S' values in each case and v_6, v_5, v_4 and v_3 their variances. In this formula the sum of random variables with unequal variances has been weighted inversely according to the variance in each case.

The variance of 'S' was calculated according to the formula

$$\sigma^2 = \frac{n}{18}(n - 1)(2n + 5)$$

for the recipients bearing 2, 3, 4, 5 and 6 grafts.

The formula was not modified for tied ranks and this served to make the method conservative.

The test for statistical significance was applied considering the results in 4 different ways.

(i) The last signs of viability were taken as end points and the observed differences in rejection times on all the recipients bearing more than 2 grafts were taken into account.

$$\text{Here } \frac{\frac{\sum S_6}{v_6} + \frac{\sum S_5}{v_5} + \frac{\sum S_4}{v_4} + \frac{\sum S_3}{v_3}}{\sqrt{\frac{m_6}{v_6} \quad \frac{m_5}{v_5} \quad \frac{m_4}{v_4} \quad \frac{m_3}{v_3}}} = X = \frac{4.7399}{2.006} \text{ and } P = 0.018$$

(ii) As in (i), but considering the first signs of rejection as the end points

$$X = \frac{4.7365}{2.006} \text{ and } P = 0.018$$

(iii) The last signs of viability were taken as the end points, and all recipients with more than 2 grafts were taken into account. Tied ranks were assigned to the grafts on those recipients that rejected all their grafts within less than 2 days. The control grafts indicated that such small differences in rejection times might be due to factors other than histo-compatibility. It was thought that the consideration of these results at face value might increase the margin of error because survival time differences due to technical factors might

be ascribed to histocompatibility differences.

$$\text{Here } X = \frac{4.5932}{2.006} \text{ and } P = 0.022$$

(iv) As in (iii), but considering the first signs of rejection as the end points

$$X = \frac{3.8134}{2.006} \text{ and } P = 0.057$$

For details of these calculations see Table XXXII.

There was a significant positive correlation ($P \leq 0.02$) between the predicted and observed rejection orders in the first three calculations. When tied ranks were assigned to grafts on recipients that rejected all grafts within less than 2 days the correlation was not significant, but only just so ($P = 0.057$), when the first signs of rejection were taken as the end points.

With regard to (iv), it should be emphasised that the formula was not corrected for tied ranks. This made the test more stringent relative to (i) and (ii), because tied ranks were more frequent in the case of calculation (iv).

The results from the recipients bearing 2 grafts could not be included with the others because the variance is large ($\sigma^2 = 1$) relative to the range

of S (-1 to +1) and the normal approximation very poor with only two observations. Furthermore, most animals in this batch were not strictly comparable to the others because 15 of the 16 recipients were ungrouped. When the recipients with 2 grafts only were considered alone, there was a slight positive correlation which was not significant.

TABLE XXXII

(i) Last signs of rejection were taken as end points and the observed difference in rejection times on all the recipients bearing more than 2 grafts were taken into account.

No.of grafts per recipient (n)	No.of recipients with 'n' grafts (m)	$\sum$ 'S' from Tables XXXIa and XXXIb	Variance of 'S'(v) $\sigma^2 = \frac{n}{18}(n-1)(2n+5)$
2	16	2	1.
3	7	-1	3.667
4	17	38	8.667
5	2	4	16.667
6	1	11	28.333

$$X = \frac{\frac{\sum S_6}{v_6} + \frac{\sum S_5}{v_5} + \frac{\sum S_4}{v_4} + \frac{\sum S_3}{v_3}}{\sqrt{\frac{m_6}{v_6} + \frac{m_5}{v_5} + \frac{m_4}{v_4} + \frac{m_3}{v_3}}}$$

$$= \frac{\frac{11}{28.333} + \frac{4}{16.667} + \frac{38}{8.667} - \frac{1}{3.667}}{\sqrt{\frac{1}{28.333} + \frac{2}{16.667} + \frac{17}{8.667} + \frac{7}{3.667}}}$$

$$= \frac{4.7399}{2.006} = 2.363 \quad \text{and} \quad P = 0.018$$

TABLE XXXII (cont.)

(ii) As in (i), but considering the first signs of rejection as the end points.

No.of grafts per recipient (n)	No.of recipients with 'n' grafts (m)	$\sum$ 'S' from Tables XXXIc and XXXId	Variance of 'S'(v) $\sigma^2 = \frac{n}{18}(n-1)(2n+5)$
2	16	3	1.
3	7	0	3.667
4	17	39	8.667
5	2	1	16.667
6	1	5	28.333

$$X = \frac{\frac{5}{28.333} + \frac{1}{16.667} + \frac{39}{8.667} + \frac{0}{3.667}}{\sqrt{\frac{1}{28.333} + \frac{2}{16.667} + \frac{17}{8.667} + \frac{7}{3.667}}}$$

$$= \frac{4.7365}{2.006} = 2.361 \quad \text{and} \quad P = 0.018$$

TABLE XXXII (cont.)

(iii) Last signs of rejection were taken as the end points and the observed difference in rejection times was taken into account as in (i), except for those cases where all the grafts on a recipient were rejected within less than 2 days of one another (see Table XXXIb). These grafts were taken as being simultaneously rejected i.e. 'S' = 0.

No. of grafts per recipient (n)	No. of recipients with 'n' grafts (m)	$\sum$ 'S' from Table XXXIa	Variance of 'S'(v) $\sigma^2 = \frac{n}{18}(n-1)(2n+5)$
2	16	3	1.
3	7	1	3.667
4	17	32	8.667
5	2	4	16.667
6	1	11	28.333

$$X = \frac{\frac{1}{3.667} + \frac{32}{8.667} + \frac{4}{16.667} + \frac{11}{28.333}}{\sqrt{4.0259}}$$

$$= \frac{4.5932}{2.006} = 2.2898 \quad \text{and} \quad P = 0.02$$

TABLE XXXII (cont.)

(iv) As in (iii), but considering the first signs of rejection as the graft end points.

No.of grafts per recipient (n)	No.of recipients with 'n' grafts (m)	$\sum$ 'S' from Table XXXIc	Variance of 'S'(v) $\sigma^2 = \frac{n}{18}(n-1)(2n+5)$
2	16	2	1.
3	7	0	3.667
4	17	31	8.667
5	2	1	16.667
6	1	5	28.333

$$X = \frac{\frac{5}{28.333} + \frac{1}{16.667} + \frac{34}{8.667} + \frac{0}{3.667}}{\sqrt{\frac{1}{28.333} + \frac{2}{16.667} + \frac{17}{8.667} + \frac{7}{3.667}}}$$

$$= \frac{3.8134}{2.006} = 1.901 \quad \text{and} \quad P = 0.057$$

DISCUSSION

(a) The Number of Important Histocompatibility Antigens

Estimates of the minimum numbers of histocompatibility antigens have been made according to the proportion of allografts which survived for protracted periods in animals (Medawar, 1945; Snell, 1955; Barnes and Krohn, 1957; Prehn and Main, 1958; Bauer, 1960; Billingham, Hodge and Silvers, 1962) and man (Longmire, Stone, Daniel and Goon, 1947). Good (1952). Newth (1961), Lunghi (1965) and Ceppelini et al. (1965b) gave formulas for calculating the probability of compatibility in terms of the number of alleles at a given locus and Gilmour (1964) suggested a modification for taking into account sampling variations.

Simonsen (1965) applied Newth's formula to the calculation of the number of antigens which provoked reactions which could not be overcome by therapy in humans. He found that the proportion of long surviving ($\geq$ 6 months) kidney allografts reported up to 1964 (Murray, Gleason and Bartholomay, 1964) might fit Newth's formula if the strong histocompatibility antigens were controlled by 3 alleles of equal frequency situated at a single genetic locus.

In a subsequent paper (Simonsen, 1966) he noted that the grafts which survived for 1 to 2 years (Murray, Gleason and Bartholomay, 1965) no longer fitted the formulas and postulated that graft survival beyond 1 year might be influenced by factors such as the inhibiting influence of antigens present in the recipient and absent in the graft.

The serological results in the experiment described here suggest that the panel of antisera was not directed against a large number of antigens. If many antigens were involved one might expect that there would be few demonstrably duplicated antisera and that a large proportion of the antisera would be multispecific. In fact this was not the case.

The number of similar and dissimilar reactions in all the possible pairs of antisera were counted by comparing each horizontal line in Figure 38 with each of the others. Table XXXIII shows the number of reactions which were negative in both compared sera (a), negative in the first and positive in the second serum (b), positive in the first and negative in the second (c), and positive in both compared sera (d).

TABLE XXXIII

The number of reactions which were similar and dissimilar in each pair of antisera and the χ^2 as a measure of their associations.

1st Serum	2nd Serum	Reactions with the 1st and 2nd serum respectively				No. of Tests (K)	χ^2
		− − (a)	− + (b)	+ − (c)	+ + (d)		
32	56	51	-	1	-	52	-
	33	51	-	-	1	52	-
	3	34	3	-	-	37	-
	71	47	4	1	-	52	-
	49	44	7	1	-	52	-
	75	48	3	1	-	52	-
	2	32	5	-	-	37	-
	55	43	8	1	-	52	-
	95	30	7	-	-	37	-
	45	39	12	1	-	52	-
	94	29	8	-	-	37	-
	50	40	11	-	1	52	-
	41	33	18	1	-	52	-
	93	26	11	-	-	37	-
	72	31	20	-	1	52	-
	68	34	17	1	-	52	-
	73	31	20	1	-	52	-
	98	20	17	-	-	37	-
	8	24	13	-	-	37	-
	7	21	16	-	-	37	-
	74	26	25	1	-	52	-
	97	18	19	-	-	37	-

Footnote: χ^2 is necessarily positive.
A dash before the χ^2 values indicates a negative association, i.e. bc > ad.

TABLE XXXIII (cont.)

1st Serum	2nd Serum	Reactions with the 1st and 2nd serum respectively				No. of Tests (K)	χ^2
		− − (a)	− + (b)	+ − (c)	+ + (d)		
33	3	34	3	-	-	37	-
	71	47	4	1	-	52	-
	49	44	7	1	-	52	-
	75	48	3	1	-	52	-
	2	32	5	-	-	37	-
	55	43	8	1	-	52	-
	95	30	7	-	-	37	-
	45	39	12	1	-	52	-
	94	29	8	-	-	37	-
	50	40	11	-	1	52	-
	41	33	18	1	-	52	-
	93	26	11	-	-	37	-
	72	31	20	-	1	52	-
	68	34	17	-	1	52	-
	73	31	20	1	-	52	-
	98	20	17	-	-	37	-
	8	24	13	-	-	37	-
	7	21	16	-	-	37	-
	74	26	25	1	-	52	-
	97	18	19	-	-	37	-
3	71	30	4	3	-	37	-0.12
	49	29	5	3	-	37	-0.03
	75	33	1	2	1	37	0.81
	2	31	3	1	2	37	3.72
	55	29	5	2	1	37	0
	95	28	6	2	1	37	0
	45	27	7	2	1	37	0.05
	94	26	8	3	-	37	-0.05
	50	25	9	3	-	37	-0.1
	41	19	15	2	1	37	-0.06
	93	23	11	3	-	37	-0.27
	72	18	16	1	2	37	0
	68	22	12	3	-	37	-0.37
	73	21	13	3	-	37	-0.49
	98	19	15	1	2	37	0
	8	21	13	3	-	37	-0.49
	7	18	16	3	-	37	-0.94
	74	16	18	2	1	37	0
	97	16	18	2	1	37	0

TABLE XXXIII (cont.)

1st Serum	2nd Serum	Reactions with the 1st and 2nd serum respectively				No. of Tests (K)	χ^2
		- - (a)	- + (b)	+ - (c)	+ + (d)		
71	49	41	7	4	-	52	0
	75	45	3	4	-	52	-0.36
	2	28	5	4	-	37	0
	55	40	8	4	-	52	0
	95	28	5	2	2	37	1.0
	45	36	12	4	-	52	-0.27
	94	26	7	3	1	37	0.6
	50	38	10	2	2	52	0.5
	41	34	14	-	4	52	5.36
	93	23	10	3	1	37	-0.13
	72	31	17	-	4	52	4.0
	68	32	16	3	1	52	-0.05
	73	29	19	3	1	52	0
	98	20	13	-	4	37	3.12
	8	21	12	3	1	37	-0.01
	7	18	15	3	1	37	-0.06
	74	27	21	-	4	52	2.7
	97	17	16	1	3	37	0.21
49	75	43	2	6	1	52	0.03
	2	30	3	2	2	37	2.15
	55	37	8	7	-	52	-0.42
	95	28	5	2	2	37	1.0
	45	39	6	1	6	52	*14.03
	94	26	7	3	1	37	0.23
	50	35	10	5	2	52	0
	41	32	13	2	5	52	3.15
	93	23	10	3	1	37	-0.13
	72	30	15	1	6	52	4.9
	68	28	17	7	-	52	-2.4
	73	27	18	5	2	52	-0.03
	98	19	14	1	3	37	0.49
	8	22	11	2	2	37	0.01
	7	19	14	2	2	37	0.06
	74	25	20	2	5	52	0.85
	97	16	17	2	2	37	0

* $P < 0.01$

TABLE XXXIII (cont.)

1st Serum	2nd Serum	Reactions with the 1st and 2nd serum respectively				No. of Tests (K)	χ^2
		- - (a)	- + (b)	+ - (c)	+ + (d)		
75	2	31	4	1	1	37	0.24
	55	41	8	3	-	52	0
	95	29	6	1	1	37	0.05
	45	38	11	2	1	52	0.07
	94	27	8	2	-	37	0
	50	37	12	3	-	52	-0.07
	41	32	17	2	1	52	-0.3
	93	24	11	2	-	37	-0.02
	72	29	20	2	1	52	-0.1
	68	33	16	2	1	52	-0.37
	73	30	19	2	1	52	-0.18
	98	19	16	1	1	37	0.4
	8	22	13	2	-	37	-0.1
	7	19	16	2	-	37	-0.3
	74	25	24	2	1	52	0
	97	17	18	1	1	37	-0.47
2	55	26	6	5	-	37	-0.16
	95	27	5	3	2	37	0.46
	45	26	6	3	2	37	0.24
	94	24	8	5	-	37	-0.46
	50	24	8	4	1	37	-0.1
	41	20	12	1	4	37	1.69
	93	21	11	5	-	37	-1.08
	72	20	12	-	5	37	4.52
	68	20	12	5	-	37	-1.33
	73	19	13	5	-	37	-1.6
	98	20	12	-	5	37	4.52
	8	20	12	4	1	37	-0.07
	7	17	15	4	1	37	-0.4
	74	18	14	1	4	37	1.06
	97	13	19	5	-	37	-4

TABLE XXXIII (cont.)

1st Serum	2nd Serum	Reactions with the 1st and 2nd serum respectively − − (a)	− + (b)	+ − (c)	+ + (d)	No. of Tests (K)	χ^2
55	95	25	6	5	1	37	-0.17
	45	33	11	7	1	52	-0.1
	94	25	6	4	2	37	0.05
	50	34	10	6	2	52	0.15
	41	26	18	8	-	52	-3.36
	93	21	10	5	1	37	-0.08
	72	23	21	8	-	52	-4.58
	68	29	15	6	2	52	0
	73	27	17	5	3	52	-0.13
	98	14	17	6	-	37	-4.08
	8	19	12	5	1	37	-0.32
	7	17	14	4	2	37	0
	74	19	25	8	-	52	*-6.63
	97	17	14	1	5	37	1.6
95	45	23	7	6	1	37	0
	94	22	8	7	-	37	-1.07
	50	22	8	6	1	37	-0.04
	41	18	12	3	4	37	0.16
	93	19	11	7	-	37	-2.11
	72	17	13	2	5	37	0.85
	68	18	12	7	-	37	-2.52
	73	17	13	7	-	37	-2.97
	98	17	13	3	4	37	0.06
	8	17	13	7	-	37	-2.97
	7	14	16	7	-	37	-4.58
	74	16	14	3	4	37	0
	97	15	15	3	4	37	0
45	94	23	6	6	2	37	0.05
	50	32	8	8	4	52	0.33
	41	31	9	3	9	52	* 9.04
	93	20	9	6	2	37	-0.11
	72	28	12	3	9	52	6.01
	68	25	15	10	2	52	-1.0
	73	25	15	7	5	52	0
	98	18	11	2	6	37	2.14
	8	20	9	4	4	37	0.33
	7	18	11	3	5	37	0.83
	74	25	15	2	10	52	6.04
	97	13	16	5	3	37	-0.24

* $P < 0.01$

TABLE XXXIII (cont.)

1st Serum	2nd Serum	Reactions with the 1st and 2nd serum respectively − − (a)	− + (b)	+ − (c)	+ + (d)	No. of Tests (K)	χ^2
94	50	25	4	3	5	37	5.65
	41	17	12	4	4	37	0
	93	24	5	2	6	37	* 7.44
	72	15	14	4	4	37	0.1
	68	24	5	1	7	37	*11
	73	24	5	-	8	37	*15.4
	98	16	13	4	4	37	0.02
	8	23	6	1	7	37	* 9.52
	7	21	8	-	8	37	*16.89
	74	16	13	3	5	37	0.24
	97	18	11	-	8	37	* 7.35
50	41	29	11	5	7	52	2.64
	93	22	6	4	5	37	2.34
	72	27	13	4	8	52	5.07
	68	29	11	6	6	52	1.96
	73	27	13	5	7	52	2.6
	98	18	10	2	7	37	3.59
	8	23	5	1	8	37	*13.17
	7	20	8	1	8	37	* 7.79
	74	24	16	3	9	52	3.24
	97	16	12	2	7	37	2.07
41	93	14	7	12	4	37	-0.04
	72	31	3	-	18	52	*36.94
	68	20	14	15	3	52	-2.2
	73	20	14	12	6	52	-0.06
	98	19	2	1	15	37	*22.7
	8	14	7	10	6	37	0
	7	12	9	9	7	37	0.08
	74	25	9	2	16	52	*16.3
	97	8	13	10	6	37	-1.3

* $P < 0.01$

TABLE XXXIII (cont.)

1st Serum	2nd Serum	Reactions with the 1st and 2nd serum respectively				No. of Tests (K)	χ^2
		− − (a)	− + (b)	+ − (c)	+ + (d)		
93	72	12	14	7	4	37	-0.38
	68	22	4	3	8	37	* 9.13
	73	22	4	2	9	37	*12.2
	98	13	13	7	4	37	-0.16
	8	22	4	2	9	37	*12.2
	7	20	6	1	10	37	*11.85
	74	15	11	4	7	37	0.68
	97	16	10	2	9	37	4.2
72	68	17	14	18	3	52	-4.11
	73	17	14	15	6	52	-0.83
	98	18	1	2	16	37	*22.77
	8	12	7	12	6	37	-0.02
	7	10	9	11	7	37	0
	74	22	9	5	16	52	* 9.34
	97	7	12	11	7	37	-1.32
68	73	31	4	1	16	52	*29.65
	98	11	14	9	3	37	-2.01
	8	22	3	2	10	37	*15.11
	7	20	5	1	11	37	*14.17
	74	21	14	6	11	52	1.9
	97	16	9	2	10	37	5.8
73	98	11	13	9	4	37	-1.04
	8	21	3	3	10	37	*12.66
	7	19	5	2	11	37	*11.5
	74	21	11	6	14	52	4.9
	97	16	8	2	11	37	* 6.9
98	8	13	7	11	6	37	0.18
	7	11	9	10	7	37	-0.01
	74	15	5	4	13	37	* 7.8
	97	7	13	11	6	37	-2.17
8	7	21	3	-	13	37	*22.86
	74	15	9	4	9	37	2.24
	97	14	10	4	9	37	1.58
7	74	13	8	6	10	37	1.29
	97	13	8	5	11	37	2.3
74	97	10	9	8	10	37	0

* $P < 0.01$

Where appropriate, Fisher's 2 x 2 test was used to calculate the association between the antiserum pairs using the formula

$$\chi^2 = \frac{(|\ ad - bc\ | - \frac{1}{2}K)^2\ K}{e\ f\ g\ h}$$

where $K = a + b + c + d$

$e = a + c$

$f = b + d$

$g = a + b$

$h = c + d$

The results suggest that many antisera detected similar antigens. The reactions of sera 41, 72, 98, 45, 71 and 2 were usually positively correlated with one another and so were those of sera 68, 73, 93, 94, 97, 7 and 8. Furthermore, there was usually a negative correlation between the reactions of the sera in one set and those in the other and the antigens recognised by each set were probably controlled by alleles.

It is proposed to call these alleles 1a and 1b and refer to the antisera as rabbit leucocytotoxins (RLC 1a and 1b). Table XXXIV shows the χ^2 association between the relevant antisera.

TABLE XXXIV

Fisher's 2 x 2 test as a measure of association between RLC 1a and 1b sera.

	68	73	93	94	97	7	8	2	98	72	71	45	41
68	-												
73	29.7	-											
93	9.1	12.2	-										
94	11.0	15.4	7.4	-									
97	5.8	6.9	4.2	7.4	-								
7	14.2	11.5	11.9	16.9	4.2	-							
8	15.1	12.7	12.2	9.5	1.6	22.9	-						
2	*1.3	*1.6	*1.1	*0.46	*4.0	*0.4	*0.07	-					
98	*2.0	*1.0	*0.16	0.02	*2.2	*0.01	0.18	4.5	-				
72	*4.1	*0.83	*0.38	0.1	*1.3	0	*0.02	4.5	22.8	-			
71	*0.05	0	*0.13	0.6	0.21	*0.06	*0.01	0	3.12	4.0	-		
45	*1.0	0	*0.11	0.05	*0.24	0.83	0.33	0.24	2.1	6.0	*0.27	-	
41	*2.2	*0.06	*0.04	0	*1.3	0.08	0	1.69	22.7	26.9	5.4	9.0	-

RLC 1b RLC 1a

χ^2 values calculated with Yates' correction.

* Negative association.

Although often used (Payne et al., 1961; van Rood, 1962; Dausset et al., 1965b), the 2 x 2 test is not ideal in the present context because when negative reactions occurred in both the compared antisera these added weight to the positive associations and decreased the significance of the negative ones. This probably accounts for the fact that in Table XXXIV the χ^2 for positive associations are sometimes very high while those for the negative ones are not significant. In these calculations reduced significance should be attached to the occurrence of double negatives especially as the number of alleles which may occupy any locus is unknown.

Figure 42 shows the relationship between the antisera of the panel more clearly. The sera and leucocytes have been arranged using the χ^2 values as a guide, so that those which reacted similarly were placed together.

It becomes evident that most of the antisera of the panel detected antigens controlled at one genetic locus only and that this locus was probably occupied by three or more alleles.

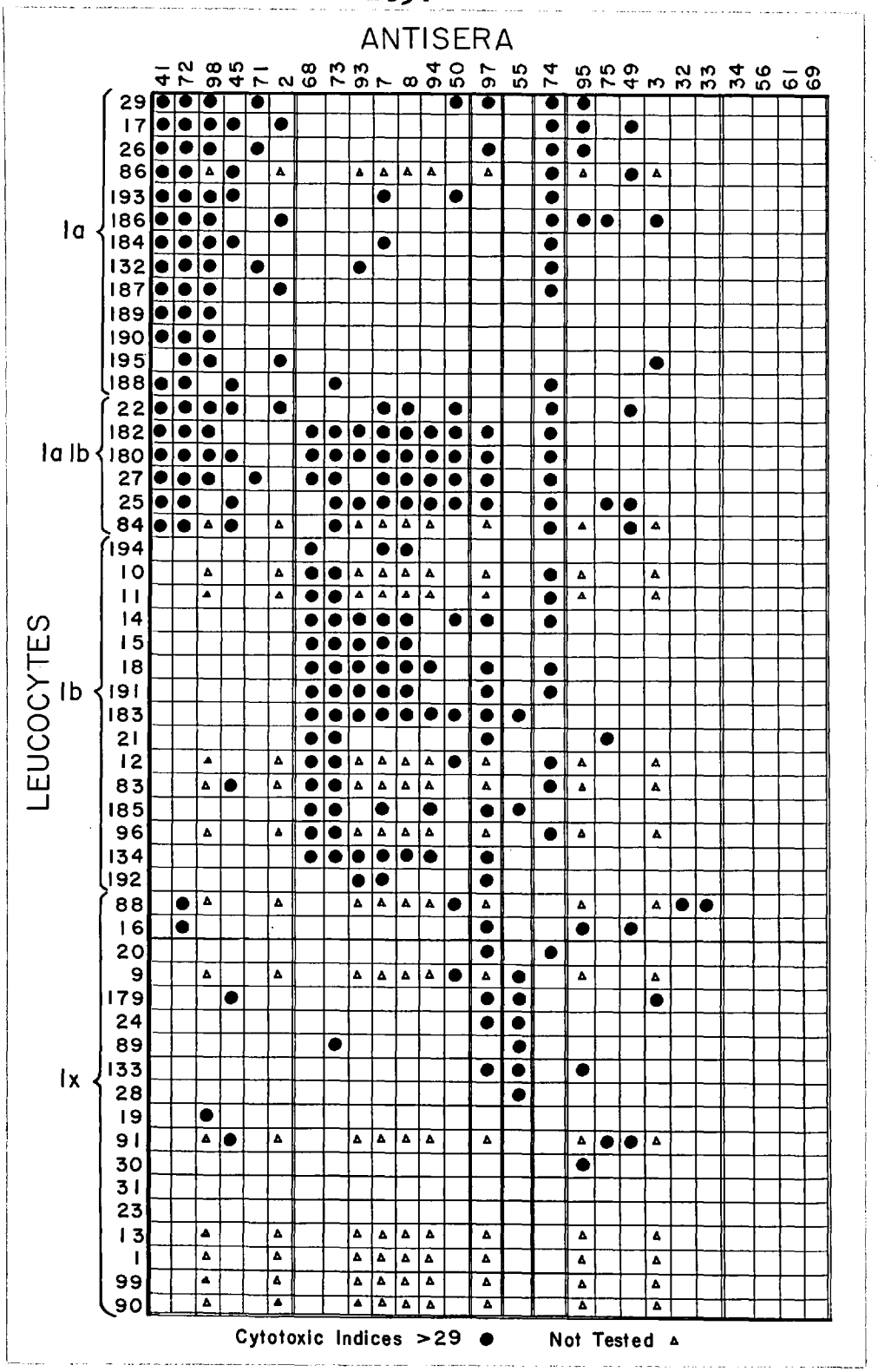

ANTISERA
41 72 98 45 71 2 68 73 93 7 8 94 50 97 55 74 95 75 49 3 32 33 34 56 61 69
LEUCOCYTES
Ia
Ia Ib
Ib
Ix
29 17 26 86 193 186 184 132 187 189 190 195 188
22 182 180 27 25 84
194 10 11 14 15 18 191 183 21 12 83 185 96 134 192
88 16 20 9 179 24 89 133 28 19 91 30 31 23 13 1 99 90
Cytotoxic Indices >29 ●
Not Tested △

FIGURE 42

It is suggested that the 13 leucocyte samples at the top of Figure 42 (29 to 188 inclusive) carry 1a antigens, the next 6 samples (22 to 84) carry 1a and 1b, and the next 15 samples (194 to 192) carry 1b antigens. The leucocytes of a further 18 individuals usually failed to react with either the RLC 1a or 1b sera and so it would seem that this locus can be occupied by other alleles. One of these "other" alleles may be detected by serum 55 because the reactions of this serum were negatively correlated with those of both RLC 1a and 1b.

It may be relevant that the reactions of serum 74, which was directed against both 1a and 1b antigens, showed a strong negative association with those of serum 55 ($\chi^2 = 6.3$). Serum 97 may have been directed against the antigens demonstrated by 55 as well as the 1b antigens.

van Rood et al. (1965) have stressed the difficulty of establishing that antisera are monospecific and this is especially so where no absorption or population studies are undertaken. However, the fact that the reactions conformed to a recognisable pattern (see Figure 42) suggests that if the antisera were not monospecific then the antigens recognised by each set of sera often

occurred as a unit. The reactions which did not conform to the general pattern were attributable in about half the cases to C.I.'s which fell close to 30, the value taken as the threshold of positivity.

The skin grafting results were then re-examined to see whether they supported the hypothesis that 1a and 1b were strong histocompatibility antigens. Table XXXV shows the putative phenotypes of the donors and recipients according to the alleles at this locus only. The leucocytes were phenotyped with reference to Figure 42 and those which reacted strongly with neither RLC 1a nor 1b were designated as 1x.

A comparison between compatible and incompatible grafts (according to phenotypes) was possible in 20 of the recipients (marked with one asterisk in Table XXXV), and the period between the rejection of the first and last graft was 2 days or more on 14 of these (marked with a second asterisk in Table XXXV). On 13 of the 14 recipients the graft surviving as long or longer than any other was of phenotype compatible with the host. The 14 recipients bore 60 grafts altogether and in only 6 cases an incompatible graft survived longer than a compatible one on the same recipient (see Recipients 89, 90, 9 and 23).

TABLE XXXV

Graft survival times in relation to the putative phenotypes.
In each box the donors are arranged from top to bottom in their predicted rejection orders according to the I.G.I.'s.

Recipient and Putative Phenotype	Donor and Putative Phenotype	Survival Time (Days from first to last sign of rejection)
83 1b	86 1a	5 - 10
	84 1a1b	5 - 7
	91 1x	7 - 11
	89 1x	7 - 11
** 84 1a1b	88 1x	7 - 12
	91 1x	10 - 13
	83 1b	12 - 15
	90 1x	12 - 15
86 1a	91 1x	10 - 11
	83 1b	10 - 11
	90 1x	10 - 14
	84 1a1b	10 - 11
** 88 1x	84 1a1b	18
	83 1b	18
	91 1x	18 - 19
	90 1x	19 - 20
** 89 1x	86 1a	18 - 19
	88 1x	14
	91 1x	18 - 19
	90 1x	18 - 19
** 90 1x	84 1a1b	11 - 14
	83 1b	11
	91 1x	12 - 14
	89 1x	11 - 12

* Recipients upon which compatible and incompatible grafts could be compared, and

** those which also rejected their grafts over a period of 2 days or more.

Recipient and Putative Phenotype	Donor and Putative Phenotype	Survival Time (Days from first to last sign of rejection)
** 91 1x	84 1a1b	5 - 9
	89 1x	8 - 9
	83 1b	8 - 9
	90 1x	13 - 15
** 99 1x	12 1b	8 - 10
	10 1b	9 - 10
	1 1x	10
	13 1x	12
** 9 1x	10 1b	13 - 14
	96 1x	12 - 13
	99 1x	14 - 15
	13 1x	8 - 10
** 14 1b	22 1a1b	10 - 13
	18 1b	17
	20 1x	17
	19 1x	14 - 17
182 1a1b	183 1b	13
	180 1a1b	12 - 15
	186 1a	12 - 15
185 1b	180 1a1b	10 - 12
	186 1a	9 - 12
	184 1a	14 - 15
	179 1x	10 - 14
** 187 1a	183 1b	8 - 12
	180 1a1b	9 - 10
	179 1x	10 - 14
	184 1a	15
** 189 1a	183 1b	7 - 10
	191 1b	8 - 10
	190 1a	10 - 14
	195 1a	7 - 10
** 19 1x	22 1a1b	13
	17 1a	10 - 13
	18 1b	13 - 14
	21 1b	10 - 14
	16 1x	15 - 16
	20 1x	14 - 15

Recipient and Putative Phenotype	Donor and Putative Phenotype	Survival Time (Days from first to last sign of rejection)
** 23 1x	25 1a	10 - 12
	18 1b	8 - 9
	14 1b	12 - 15
	15 1b	14 - 15
	19 1x	11 - 13
** 192 1b	27 1a1b	7 - 8
	193 1a	7 - 9
	195 1a	7 - 8
	194 1b	11 - 12
156 (?)	27 1a1b	10
	28 1x	12 - 16
165 (?)	29 1a	8 - 9
	31 1x	9 - 11
162 (?)	29 1a	8
	31 1x	12 - 15
166 (?)	29 1a	8
	31 1x	11 - 12
132 1a	27 1a1b	9 - 13
	30 1x	7 - 9
134 1b	29 1a	16 - 17
	27 1a1b	8 - 10
	30 1x	16 - 17
** 15 1b	25 1a1b	11
	26 1a	10 - 12
	21 1b	11 - 13
	23 1x	11 - 13
	19 1x	9 - 10
180 1a1b	186 1a	10 - 16
	185 1b	10 - 13
	182 1a1b	8 - 10
195 1a	183 1b	9 - 10
	191 1b	9 - 10
	194 1b	11 - 12

Recipient and Putative Phenotype	Donor and Putative Phenotype	Survival Time (Days from first to last sign of rejection)
* 13 1x	11 1b 1 1x 99 1x	12 - 14 13 12 - 14
* 183 1b	186 1a 179 1x 185 1b	8 - 9 7 - 9 8 - 9
* 188 1a	180 1a1b 190 1a 189 1a	8 - 11 9 - 11 9 - 10
* 190 1a	183 1b 191 1b 188 1a 189 1a	8 8 7 - 8 8 - 9
* 191 1b	29 1a 193 1a 192 1b 194 1b	6 - 7 6 - 7 6 - 7 7 - 8
* 194 1b	180 1a1b 193 1a 195 1a 192 1b	7 - 8 8 7 - 8 8
157 (?)	27 1a1b 28 1x	9 - 11 9 - 10
159 (?)	27 1a1b 28 1x	14 - 15 14 - 15
161 (?)	27 1a1b 31 1x	11 - 12 11 - 12
163 (?)	29 1a 31 1x	11 - 12 11 - 12
168 (?)	27 1a1b 31 1x	11 12
171 (?)	27 1a1b 31 1x	11 - 12 11

Recipient and Putative Phenotype	Donor and Putative Phenotype	Survival Time (Days from first to last sign of rejection)
172 (?)	27 1alb 31 1x	12 12
175 (?)	29 1a 28 1x	6 - 7 6 - 7
176 (?)	29 1a 28 1x	6 6
177 (?)	29 1a 31 1x	6 5 - 6
178 (?)	29 1a 31 1x	6 6

The sera which reacted similarly (41, 72, 98, 45, 71, 2 and 68, 73, 93, 94, 97, 7, 8) were probably of narrow specificity because the chance of recognising duplications decreases with an increase in the frequency of the shared components (Payne et al., 1964). There were a further 6 antisera (34, 61, 69, 32, 56, 33) which reacted with one or none of the 52 leucocyte samples. Thus, in 19 out of 26 pairs of rabbits in which the antisera were prepared, the donors and recipients differed in respect of few antigens which were capable of provoking cytotoxicity. These were probably the more important transplantation antigens because they

were significantly successful as indicators of the graft rejection orders.

The 4 inert antisera (34, 56, 61 and 69) reduced the effective size of the panels to 13 and 22 in the cases of the 17 and 26 antiserum panels respectively. The success of these relatively small panels in reflecting the graft rejection orders also suggests that the number of strong histocompatibility antigens is not great and/or that the cytotoxic test shows up the most important of them.

(b) The I.G.I. as an Index of Interrelationship

Other workers (Colombani et al., 1964; Dausset et al., 1965b; Amos et al., 1966) have calculated interrelationships according to the number of antisera with which the donor and recipient leucocytes reacted positively or negatively. However, the classification of the cytotoxic indices as 'positive' or 'negative' involves an arbitrary decision as to the level of positivity. The calculation of an index according to the numerical difference between the C.I.'s in the case of each antiserum avoids this difficulty and takes account of both the number and

magnitude of the donor-recipient differences. A high I.G.I. indicates that the antigenicity of the donor, as measured by cytotoxic indices, greatly exceeded that of the recipient and Walford et al. (1965) have pointed out that the prospects of allograft survival are poorest in these circumstances. Vredevoe et al. (1965) have used a semi-quantitative method for donor-host comparisons which is similar in some respects to the method used here.

Although some antisera were recognised as being duplicated, none were eliminated for the calculation of the I.G.I.'s. Assuming that each antibody has an equal chance of duplication, to omit some sera might serve to compound inaccuracies of the I.G.I.'s because it might result in the exclusion from the test of antibodies easily recognised as duplicated and leave the duplicated antibodies not easily recognised as such (e.g. those in the antisera of broad specificity).

(c) <u>The Grafts from Grouped Donors to Ungrouped Recipients</u>

The leucocytes of some animals gave many more strongly positive reactions than others (Table XXVIII, Nos. 27, 29, 180 and 183) and consequently their immunogenic indices

were usually high when they were regarded as donors (Table XXIX, Batch 6). One might expect that, if the I.G.I.'s reflected histocompatibility differences, these animals would usually be bad donors when their tissues were grafted to randomly selected animals from the general population, and likewise that animals with few high C.I.'s (e.g. Nos. 28 and 31) would usually be good donors. This suggested that donors might be classified as being of high or low antigenicity on the basis of the I.G.I.'s obtained in one batch of animals and that the results of grafting further (ungrouped) recipients from these donors might accord with the results in the grouped recipients.

However, when this was put to the test the results were inconclusive. In 8 of the 15 ungrouped recipients the grafts from the donors of high and low antigenicity were rejected on the same day and in 3 recipients there was only a one day difference in rejection times. Some importance may be attached to the fact that in the four recipients in which there was a greater difference in rejection times the order of rejection was that which had been predicted.

(d) Accuracy of Serological Assessments

The repeatability of the stained counts was only fair. The range of C.I.'s for repeated counts was less than 11 in 41% of cases, less than 21 in 73% of cases and less than 31 in 85% of cases. This was partly attributable to the dye exclusion method which was not ideal as an indicator of cell damage. The counts were time consuming and the results subject to numerous variables (Boyse, Old and Stockert, 1962; Black and Berenbaum, 1964). Terasaki and Rich (1964) and Terasaki et al. (1964) have described the use of an automatic counting machine (Coulter counter) for the measurement of cytotoxicity. The methods of Klein and Perlman (1963) and Sanderson (1965) might give more reproducible measurements. In their experiments cell damage was measured by the escape of radioactivity from labelled nucleated cells and the results paralleled those obtained with the dye exclusion method (Klein and Perlman, 1963). In mice most of the reactions were in accordance with known H_2 antigens of the strains used (Sanderson, 1965).

The reproducibility in the present experiments was also better with the leucocytes of certain individuals

and this is similar to the observations made by Payne et al. (1964) when working with human leuco-agglutinins.

The measures taken to offset technical inaccuracies included

(i) the repetition of each test up to 5 times and the calculation of the average values,

(ii) the conversion of stained counts to cytotoxic indices, which took into account the effects of technical variables according to their actions on the control preparations,

(iii) the consideration only of the donor-host C.I. differences which exceeded 10 during calculation of the I.G.I.'s, and

(iv) carrying out test grafts mainly on those pairs of rabbits in which the I.G.I.'s differed markedly.

The last two measures, (iii) and (iv), had the effect of precluding from consideration those donor-host pairs which differed least serologically and in which technical variables might make the greatest difference to the predicted orders of rejection.

The Grafting Results

Although the predicted and observed orders of graft rejection were positively correlated, the immunogenic indices were not correlated with the absolute survival times. On the one hand graft survival was not always markedly prolonged in donor-host pairs with very low I.G.I.'s. For example, in the donor-host pair 90-84 the I.G.I. was zero, suggesting that the donor possessed no antigens not present in the recipient, but the graft was rejected in 15 days. Such discrepancies were probably due to incomplete coverage of transplantation antigens by the panel so that certain antigens failed to be detected. Conversely, very high I.G.I.'s did not always indicate very short survival times. This could have been due to duplication in the panel, some antigens being detected by more than one of the sera. These defects may possibly be remedied by enlarging the panel so that it covers more histocompatibility antigens and by narrowing the specificity of the individual sera, for instance by selective absorption.

It may also be noted that the finding of widely different I.G.I.'s for different donors relative to the

one recipient did not always indicate a wide range of rejection times in practice. In some cases (e.g. recipients 183 and 194) all grafts were rejected on the same day in spite of considerable differences in I.G.I.'s. Although the serological test suggested that the tissue of one donor was more antigenic than that of another, the grafted tissues of both may have been sufficiently antigenic to be rejected in similar times. Alternatively, this discrepancy may have been due to omissions or duplications in the panel.

Graff, Silvers, Billingham, Hildemann and Snell, (1966) have shown that in inbred mouse strains there was a cumulative effect on the median skin graft survival times as the number of histocompatibility differences between the donor and host increased, but there was a limit to this cumulative effect. When a maximum was reached on a given host the addition of immunogens did not further accelerate graft rejection.

The fact that the method described was able to reflect graft survival with success in spite of the

technical shortcomings indicates that cytotoxic antisera are able to detect the major histocompatibility factors in the rabbit, or factors associated with them and that non-histocompatibility antigens were demonstrated to a lesser degree.

Applicability to Man

The efficiency of a histocompatibility test based on the serological matching of antigens depends upon the number of important H-factors in the species under investigation. In man the number is unknown but the fact that the survival times of allografts often vary widely suggests that the number may not be prohibitively large.

The source of antisera which react with isoantigens presents no major problem because these occur fairly frequently in humans after multiple pregnancies (Metzgar et al., 1965; Dausset et al., 1958, 1965b; van Rood et al., 1962, 1965; Shulman, 1965) and may be provoked by skin allografts (Batchelor, 1965b), multiple blood transfusions (Shulman et al., 1965), intradermal leucocyte injections (Amos et al., 1965),

or skin grafts followed by intradermal lymphocyte injection (Amos, 1965; Walford et al., 1965).

Recently it has been shown that antisera from some primates can also react with individual specific human antigens (Metzgar et al., 1965) and these might be useful in that they circumvent the necessity of immunising humans to transplantation antigens.

The work cited by Dausset, Starzl, Colombani, Terasaki, Cepellini, Amos, van Rood, Walford, Balner, Batchelor, Goldsmith, Engelfriet, van Leeuwen, and van Loghem suggests that in man too serological methods may be used to determine histocompatibility relationships.

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FACTORS AFFECTING THE DYE EXCLUSION TEST FOR CELL VIABILITY

L. BLACK and M. C. BERENBAUM

Surgical Unit, St. Mary's Hospital, London, England

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A commonly used method for estimating the proportion of viable cells in a suspension is to mix it with a solution of a dye such as eosin or trypan blue [1, 3, 4, 6]. Stained cells are assumed to be damaged and those unstained to be undamaged.

In using this method to study damage to rabbit leucocytes by immune sera we found the results to be erratic and apparently in disagreement with much published work. It appeared possible that this could be due to variables in the staining procedure, e.g. concentration of dye, serum and cells and staining time. These might affect the proportion of cells staining independently of the proportions initially damaged or undamaged. We therefore investigated this possibility.

MATERIALS AND METHODS

Preparation of leucocyte suspensions.—Fresh rabbit venous blood, 20 ml, was mixed with 4 ml 6 per cent dextran in 0.9 per cent saline (Dextraven, Benger Laboratories) and 1000 units heparin (Heparin Injection B.P., 5000 units per ml, Boots Pure Drug Co.). The mixture was allowed to settle in an upright 15 cm by 1.5 cm tube in a 37°C incubator for 1½ hr. The supernatant fluid was drawn off together with the topmost layer of cell deposit, and centrifuged at 950 *g* in a swing-out head for 20 min at 18–20°C. A cell suspension was made by mixing the deposit from this centrifugation with 0.5 ml of the supernatant.

An albumin solution with a density gradient was prepared by layering 0.7 ml of the supernatant over 0.7 ml of a concentrated bovine plasma albumin (Fraction V, Armour Laboratories) solution [7].

The cell suspension was layered over the albumin solution and the whole centrifuged at 950 *g* for 15 min at 18°C. The leucocytes, which lay in a band above the packed red cells, were drawn off, washed twice in 0.15 *M* saline and suspended in Hank's balanced salt solution.

Aliquots of these suspensions were damaged by heating at 45°C for 30 min in a water bath.

[1] Revised version received January 2, 1964.

Staining and examination of leucocytes.—In each experiment one of the factors under investigation was varied and the others kept constant; both damaged and undamaged cells were examined. Cell suspensions of various concentrations and dilutions of fresh autologous serum in Hanks' solution and of eosin Y in Ringer's solution were mixed in WHO agglutination trays. The trays were kept on ice and, after varying times, the cell suspensions were examined in a haemocytometer cham-

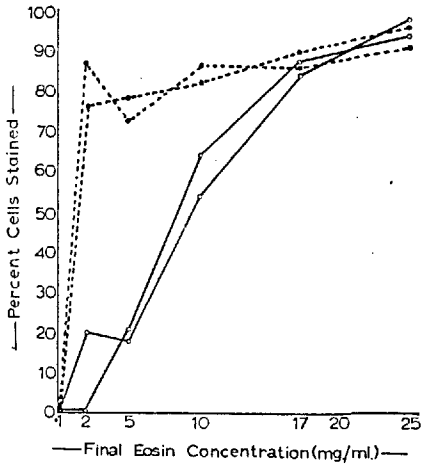


Fig. 1.

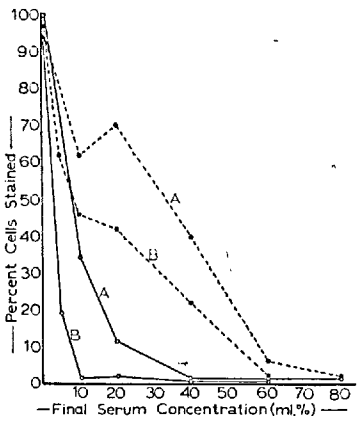


Fig. 2.

Fig. 1.—Effect of dye concentration on the percentage cells stained. Final serum concentration 8 %. Final cell concentration 0.5×10^6 leucocytes/ml. Staining time: 60 min. Solid lines represent undamaged suspensions. Broken lines represent damaged suspensions.

Fig. 2.—Effect of serum concentration on the percentage cells stained. Final dye concentration: eosin Y: 2.1 mg/ml. Final cell concentration: 0.5×10^6 leucocytes/ml. Staining time: 60 min. Solid lines represent undamaged suspensions. Broken lines represent damaged suspensions. Lines marked with the same letter represent aliquots made from the same original suspension.

ber 0.1 mm deep at 400 × magnification. The number of stained and unstained cells in 50 leucocytes were counted. The wells were given code numbers to avoid bias during counting.

RESULTS

For both damaged and undamaged cells the proportion that stained was dependent to a high degree on the final concentrations of stain and serum. Raising the concentration of stain or lowering the concentration of serum both increased the stained cell count (Figs. 1, 2, 3). A greater proportion of damaged than undamaged cells was stained but at the extremes of dye and serum concentrations the stained cell counts approached each other.

Staining time could be varied between 5 and 90 min without affecting the proportion of cells staining in undamaged suspensions. In damaged

suspensions, however, the stained cell count rose rapidly during the first 30–40 min after adding the stain and, with some suspensions, continued to do so even after this period (Fig. 4).

The proportion of stained cells was often raised when very low concentrations of cells, (less than 10^5/ml), were used (Fig. 5).

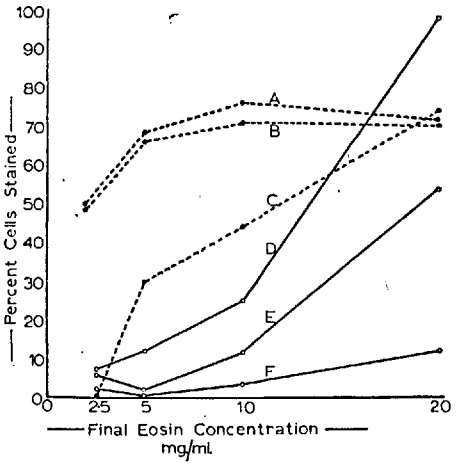


Fig. 3.—Effect of simultaneous variation of dye and serum concentrations. Staining time: 50 min. Final cell concentration: 0.8×10^6 leukocytes/ml. Final serum concentrations: *A*, 10 %; *B*, 20 %; *C*, 80 %; *D*, 5 %; *E*, 20 %; *F*, 40 %. Solid lines represent undamaged suspensions. Broken lines represent damaged suspensions.

DISCUSSION

The dependence of the stained-cell count on serum concentration in the dye-exclusion test was first described by Pappenheimer [4] and has been noted occasionally by other workers [9, 10]. Factors that may be relevant are impaired cell viability in media of low protein concentration [8], the inhibition of cell swelling by macromolecules [2, 6] and, where complement-mediated cell damage is concerned, the anticomplementary effect of certain sera [11].

However, it is likely that binding of stain by serum proteins is also an important factor, and this phenomenon would explain many of the results reported here. In unpublished experiments with I. N. Brown we have found that, if suspensions of cells stained in low-protein media were centrifuged and the deposits resuspended in media of high protein concentration, stain was removed from the cells; the higher the protein concentration, the more stain was removed. This finding cannot be explained on the basis of a protective effect of serum.

It is clear that one and the same cell suspension may give a stained cell

count varying from 0 per cent to more than 90 per cent, whether it is initially damaged or not, depending on the concentrations of stain and serum chosen. Further, in damaged cell suspensions the percentage of stained cells depends on the time between adding the stain and performing the count. This dependence is specially marked when short staining times are used.

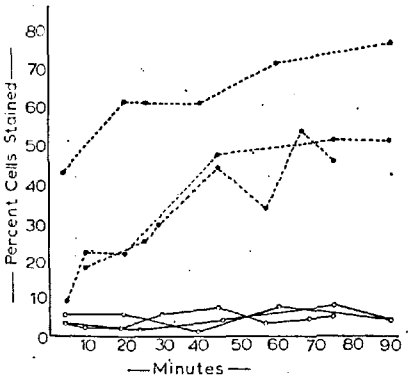


Fig. 4.

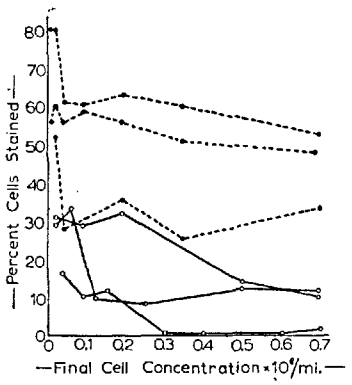


Fig. 5.

Fig. 4.—Effect of staining time on the percentage stained cells. Final dye concentration: eosin Y: 2.1 mg/ml. Final serum concentration: 8 %. Final cell concentration: 0.5×10^6 leucocytes/ml. Solid lines represent undamaged suspensions. Broken lines represent damaged suspensions.

Fig. 5.—Effect of cell concentration on the percentage stained cells. Final dye concentration: eosin Y: 2.1 mg/ml. Final serum concentration: 8 %. Staining time: 60 min. Solid lines represent undamaged suspensions. Broken lines represent damaged suspensions.

It is only within certain limits of variation that this test distinguishes damaged from undamaged cells with optimal efficiency. It is possible that these limits vary with the type of cell used, its species of origin, the nature of the suspending medium and the stain. The limits may be relatively wide for some cell types. For instance, undamaged ascites tumour cells are readily distinguishable from damaged cells by the dye-exclusion test even in the absence of serum. Possibly the dye-binding capacity of undamaged ascites cells is very low or their viability is relatively unimpaired in the absence of serum. With such cells the results of the test do not depend critically on technical minutiae. For other cell-types, however, the limits may be relatively narrow, as in the experiments reported here. Our results suggest that it is advisable to establish what these limits are for the particular material under investigation before using the dye-exclusion test.

SUMMARY

The influence of four factors on the dye exclusion test for cell viability was investigated. Eosin concentration, serum concentration, cell concentration and staining time were each varied in turn to assess their effects on the number of rabbit leucocytes staining in damaged and undamaged suspensions.

Stained cell counts tended to be high when high concentrations of stain and/or low concentrations of serum were used, and *vice versa*, irrespective of whether suspensions were initially damaged or not. The stained cell counts of undamaged suspensions were not affected by staining times up to 90 min. In damaged suspensions the proportion of stained cells increased with the staining time. Stained cell counts tended to be high when very low concentrations of cells were used.

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