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EFFECTS OF RADIATION ON THE IMMUNE RESPONSE AT THE CELLULAR LEVEL

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ABSTRACT

Utilizing the Jerne plaque technique, supplemented by titration of circulating haemolysin and haemagglutinin, the effects of X-irradiation on the primary immune response of mice to sheep erythrocytes were investigated.

The doses of radiation and their timing were varied widely, and where necessary the responses were followed over an extended period. In some experiments non-specific physiological effects were excluded by using isolated spleen cells "cultured" in irradiated recipients.

Normally, 19S plaque-forming cells rose rapidly from the pre-immunization level to a sharp peak on the 4th day after injection. The titre of haemolysin also rose correspondingly, although that of haem-agglutinin followed a different course. Two peaks of 7S PFC were also detected. PFC irradiated *in vitro* were much less sensitive than were their precursors, whether irradiated *in vitro* or *in vivo*; Nitrogen had a protective effect, the oxygen-enhancement ratio being in the order of 2.4.

Irradiation of intact mice before immunization slightly depressed the numbers of "natural-PFC" but markedly inhibited the immune response in three ways:-

It was retarded and the rate slowed, and the ultimate level was reduced, probably due to the death of antigen-responsive cells. The effect, which was dose-dependent, was reduced if irradiation followed immunization, especially in the case of circulating antibodies, but large doses caused

the numbers of PFC to fall to the background level with a mean halving-time of less than 8 hours. However, the response continued after 3-4 days following sublethal doses.

Recovery from the effects of radiation was slow, and not detectable for about 10 days, both in intact mice and with transferred spleen cells. Conversely, split-dose irradiation showed a rapid sparing effect on a subsequent response. Spontaneous nodules produced during repopulation of the spleens appeared to be relatively deficient in responsive cells.

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Part I

INTRODUCTION

INTRODUCTION

(i) Radioimmunological Observations

The effects of ionizing radiation upon living organisms were noticed very soon after the discovery of X-rays by Roentgen in 1895. The first radiation accidents to operators of X-ray devices occurred in 1896, and also in that year Genoud used local doses of X-rays in the unsuccessful treatment of tuberculosis.

However, it was not until 1903-1905 that a comprehensive series of experiments on animals was reported by Heineke. He observed the rapid disappearance of blood leucocytes and severe depletion of the lymphoid organs, including the lymph nodes and thymus. The bone marrow also showed marked destruction, especially in the leucopoietic tissues, but the erythropoietic foci were less affected, and mature erythrocytes were not affected at first. However, these tissues also showed remarkable powers of regeneration, and recovery was complete within 3-4 weeks.

In 1908 Benjamin and Sluka published the first account of the effects of X-irradiation on antibody production. Using rabbits injected with bovine serum proteins, they followed the disappearance of the proteins and the appearance of precipitins in the blood of the rabbits. When the rabbits were irradiated two or three days before the immunization the appearance of antibodies was very much inhibited and delayed, but the same dose given 4 days after immunization had much less effect, despite severe damage to the haematopoietic organs. Therefore it was not possible to correlate the antibody-forming

capacity with the overall survival of the lymphoid tissues, since it appeared that once antibody-formation had begun it could maintain itself despite the damaging effects of the radiation.

This apparent change in the radiosensitivity of the immune mechanism, from highly sensitive in its initial stages to highly resistant during the remainder of the response, has been confirmed by many investigators using rabbits and a wide range of antigens (e.g. Clemmesen and Andersen, 1948 Craddock and Lawrence, 1948; and Dixon, Talmage & Maurer, 1952).

The latter workers studying the clearance of ^{131}I-labelled bovine gamma globulin ([^{131}I]BGG) and the appearance of circulating anti-BGG antibody, showed that maximum suppression of the immune response occurred when the rabbits were irradiated with 400 r. 48 hours before the injection of the antigen. When the antigen was injected 5 hours after irradiation the response was unaffected. Even when the dose was increased to 800 r., which caused severe sickness, when given 3 days after immunization it did not depress the production of antibody, but only delayed the time of the peak titre.

The secondary response also had a lower radiosensitivity. 800 r. two days before a re-injection of antigen into immunized rabbits caused only a slight delay in the subsequent rise of circulating antibody.

Dixon et al concluded that there was an initial phase in the immune response, lasting less than 12 hours, which was relatively radiosensitive, but that the remainder of the response and the whole of the secondary response were much less radiosensitive. In the secondary

response it appeared that the first stage was not necessary. The very shortness of this sensitive phase excluded the possibility of antibody synthesis being completed before the more resistant phase. However they produced no direct evidence for the existence of any process unique to the earliest part of the response, and other explanations are possible. For example, the number of antigen-sensitive cells may increase sufficiently for the surviving fraction to exceed the threshold level to give a normal response.

In rats there is a similar apparent change in radiosensitivity, for instance in the production of haemagglutinins and haemolysins to foreign erythrocytes (Hektoen, 1915). However, more careful study has shown that in rats and mice the response is still sensitive up to three days after the injection of the antigen, and radiation doses of 350-600 r. given 4, 5 or more days after immunization cause a distinct slowing of the response or an actual decline in the antibody titre. Despite this, the production of antibody recovers and the overall titre is little affected compared to unirradiated controls (Kohn, 1951; Fitch, Wissler, La Via & Barker, 1956; Gengozian and Makinodan, 1958; Simić, Šljivić, Petrović & Cirković, 1965).

Also despite the severe depletion of circulating lymphocytes and of the lymphoid organs, X-irradiation appears to have little effect on the uptake of particulate antigens. Simonds and Jones (1915) incubated leucocytes from irradiated rabbits with bacilli <u>in vitro</u> and showed the phagocytic index to be the same as for normal leucocytes, even though such animals had a markedly reduced ability to form agglutinins. This

finding has been repeated by many workers, e.g. Brecher, Endicott, Gump and Broner (1948), Barrow, Tullis & Chambers (1951), Wish, Furth, Sheppard & Storey (1952). The complement-fixing ability of the serum was also unaffected by the radiation.

While most studies have shown only inhibitory effects of radiation on antibody synthesis, there are some well-documented (and many poorly controlled) instances of enhanced antibody production occurring in irradiated animals. Small doses, or locally applied radiation, sometimes have this effect. See, for instance, Fried (1925), Holzknecht (1926), and review by Taliaferro and Taliaferro (1951). Under certain circumstances, however, large doses can produce enhancement.

Hektoen (1920) irradiated dogs with two large doses of X-rays 15 and 16 days before injecting them with foreign erythrocytes, and observed an increase in the overall production of haemagglutinins. He believed lymphocytes to be involved in antibody production and suggested the increased output to be correlated with hyper-regeneration of the lymphoid tissues, which by this time may have occurred.

However, a completely different type of enhancement has been demonstrated in rabbits by Taliaferro, Taliaferro & Janssen (1952), Taliaferro and Taliaferro (1954a, b), and Dixon and McConahey (1963):-

Rabbits irradiated with 300-700 r. 1-4 hours after a single intravenous injection of sheep erythrocytes (SRC) showed a delayed immune response, as determined by the appearance of haemolysin in the serum. However, although the rate of production of antibody was slowed, the peak titre eventually reached was usually greater than that of

unirradiated rabbits, despite considerable depopulation of the lymphoid tissues, and the total quantity of antibody produced also seemed to be increased. (See review by Taliaferro, Taliaferro and Jaroslow, 1964).

Using [^{131}I] BGG, limpet haemocyanin, [^{131}I] BSA plus anti-BSA (to speed its clearance), or, especially, heat-aggregated [^{131}I] BGG, Dixon and McConahey were able to demonstrate distinct, and sometimes substantial, enhancement of the subsequent antibody titres, when the rabbits were irradiated at appropriate intervals after the injections. X-ray doses of 400-600 r. were necessary and the interval between injections and irradiation had also to be adjusted to coincide with the time of most rapid elimination of the antigen from the serum. For BGG or BSA-anti-BSA this interval had to be 1-3 days, but for aggregated BGG and haemocyanin, which were cleared more rapidly, the time was shorter. At these times a vigorous proliferative response had already been initiated. Therefore it seems probable that the number of specific antigen-responsive cells had increased, and possible that the already-stimulated survivors of the irradiation were in a suitable physiological environment to continue proliferating even more rapidly.

Probably the actual amount of antibody synthesized is increased under these circumstances, as Maurer, Dixon & Talmage (1953) and Dixon and McConahey (1963) showed that the antibody against BGG or BSA produced by rabbits irradiated with 400 r. or 600 r. had the same immunochemical properties as that from unirradiated animals. There is no evidence that the antibody produced by irradiated animals has a longer half-life or higher affinity. Rohn (1951) found the rate of

disappearance of haemolysin passively injected into rats irradiated with 600 r. 8 days previously was the same as in normal rats, although the production of antibodies and the "immune clearance" antigen were both inhibited.

However enhancement by a factor of two or more, despite severe radiation-induced depletion of the lymphoid tissues, has only been observed in rabbits, so this may not be a general phenomenon.

(ii) Irradiation and Phagocytosis

As has been mentioned above, the uptake of foreign particles by phagocytes has been shown repeatedly to be little, if at all, affected by doses of radiation which normally have a marked inhibitory effect on the production of antibodies. This has been well demonstrated with whole-body doses of 500-800 r. given to rabbits and mice. (Barrow, Tullis & Chambers, 1951; Wish et al., 1952; Esplin, Marcus & Donaldson, 1953) and many others.)

However, the immune response may be influenced indirectly by effects of radiation on the phagocytic process. Due to the killing of the precursors of phagocytes, which Virolainen (1968) has shown to be radiosensitive, the number of phagocytes declines following irradiation and this may significantly reduce the overall phagocytic capability of animals. This effect would probably take several days to become apparent, and would last for some time, since Balner (1963) has shown that peritoneal macrophages have a mean turnover period of 5-6 weeks. Indeed,

Kornfeld and Greenman (1966) demonstrated that the numbers of peritoneal macrophages in mice were depressed 14 days after 600 r. and then recovered gradually. Studying the numbers of phagocytic cells entering water-in-oil mixtures injected subcutaneously into mice, Muramatsu, Morita & Sohmura (1965) found that they were reduced 10-100-fold between 2 and 15 days following 500 r. whole-body irradiation. The uptake of BCG from the emulsion by individual cells was enhanced by about 10 times by irradiation, but due to the reduction in total cells, the overall amount of antigen phagocytosed was depressed about 10-fold.

In the case of those antigens where phagocytosis plays an important role in initiating the response, the reduced uptake of antigen would be expected to have an inhibitory effect.

Doses of radiation in the range 100-800 r. given either <u>in vivo</u> or <u>in vitro</u> also appear to have a direct effect upon the processing of antigens by phagocytes which might, in some cases at least, have a considerable effect on the immunogenic stimulus. Although such doses have little effect upon uptake of antigenic particles, they have been shown to impair the ability of phagocytes to kill some kinds of bacteria, which survive and proliferate intracellularly (Gordon, Cooper & Miller, 1955; Nelson and Becker, 1959; and others). Survival is inversely proportional to the dose of radiation. Similarly, other foreign cell types are not destroyed at the same rate by irradiated phagocytes as by normal ones (Donaldson, Marcus & Gyi, 1954; Donaldson, Marcus, Gyi & Perkins, 1956).

The results of different laboratories, using different species of bacteria and other antigens, are not strictly comparable, but it would appear that radiation has a deleterious effect on the degradation of those phagocytosed cells, such as erythrocytes, which are sufficiently sensitive to the enzymes of unirradiated phagocytes to be destroyed without the mediation of specific antibody (Möller, 1969). If the phagocytes are irradiated their catabolic capability may be reduced below that required for lysis.

The mechanism of such a change is obscure. It may be relatively small, since Balner (1963) showed that the activity and properties of peritoneal macrophages of mice irradiated with a dose of 910 r. were near to normal. However, Shechmeister and Fishman (1955) showed that the migration of blood leucocytes in slide chambers was depressed if the donor rats or rabbits had been irradiated with 500-800 r. 2-5 days previously. Although this effect was temporary, it indicated a physiological change within irradiated phagocytes.

McIntyre, Rowley & Jenkin (1967), using a microdrop technique, showed that the presence of specific antibody was necessary for the phagocytosis and killing of Salmonella and Listeria by mouse peritoneal macrophages. Probably it is the combination of antibody plus the enzyme-rich intracellular environment of phagocytes which actually brings about the destruction of bacterial cells (Mackaness and Hill, 1969; Mackaness, 1969).

The presence of specific antiserum may in some cases be sufficient to raise the threshold of activity sufficiently to compensate for the

effects of the radiation, although irradiated non-immune rabbit phagocytes were still unable to destroy intracellular Candida, or chicken red blood cells, even in the presence of specific antiserum (Donaldson et al., 1956). However, irradiated phagocytes from previously immunized mice were still able to destroy ingested Pseudomonas (Nelson and Becker, 1959).

Radiation-induced inhibition of antigen uptake or "processing" has been suggested as a major cause of immunological inactivation of mice against Shigella antigen (Gallily and Feldman, 1967) and rabbits against BGG (Pribnow and Silverman, 1967). Lymphoid cells were also essential for antibody synthesis. However in the anti-Shigella response these seemed less radiosensitive than the macrophages, since host mice irradiated with 550 r. and injected with an almost pure suspension of sensitized macrophages could still synthesize significant quantities of antibody, while recipients which were irradiated with 900 r. could not. In the anti-BGG response, on the other hand, 450 r. was sufficient to inactivate the recipients and normal lymphocytes had to be injected together with the sensitized macrophages. Species differences may also have played a part in producing these different results. In both cases, however, the macrophages were radiosensitive, in that the response induced by macrophages irradiated with 450 r. or 550 r. was less than that induced by unirradiated macrophages.

In the response to Salmonella the macrophages of rats appear less radiosensitive than the lymphoid tissues. Fitch, Barker, Soules & Wissler, (1953) showed that while phagocytosis, tissue localization and

disappearance *in vivo* of formalin-killed, ^{131}I-labelled cells were unaffected by 500 r. whole-body irradiation, the histological reaction of the lymphoid tissues normally associated with antibody production, was inhibited.

In the case of certain other antigens, in which macrophages are able to transfer the antigenic stimulus, the uptake of antigen and transfer is unaffected by sublethal doses of radiation. Unanue and Askonas (1968) incubated mouse peritoneal exudate cells in the presence of ^{131}I-MSH (*Maia squinado* haemocyanin) and injected them into normal or previously immunized recipients. An antibody response followed whether or not the macrophage donors had been irradiated with 700 rads 1-2 days before the exudate was obtained. The same dose given to the recipients abolished their response. Thus the response of the lymphoid cells appears to be the more radiosensitive in this system. Unanue and Askonas suggested that the macrophage was relatively unimportant in the case of highly immunogenic antigens, such as MSH or SRC. Gershon and Feldman (1968) agreed with this. It would seem therefore, that radiation acting upon phagocytic cells is not a universal cause of a depressed immune response, though it may be a contributory factor in some types of response, probably to weak antigens.

In any case, no satisfactory mechanism by which radiation may reduce the activity of mature macrophages has been proposed. If the transfer of the immunological stimulus is mediated by a specific type of RNA, e.g. similar to "messenger RNA", its synthesis might well be radiosensitive. However the evidence for such a messenger is meagre,

and even if RNA is involved at all, it seems more likely that it is a non-specific type, already present in the cell before contact with the antigen. Therefore this would be unlikely to account for the immediate effects of irradiation.

A more likely explanation may be that macrophages, by partially destroying resistant antigenic particles, or antigens with low immunogenicity, expose, release or concentrate highly immunogenic determinants, thereby enhancing the response of antibody-forming cells. If irradiation inhibits this catabolic activity the subsequent response will be lessened.

(iii) Irradiation and the Production of Antibody-forming Cells

Perhaps the best studied immune response is that of mice or rabbits to SRC. Macrophages are able to transfer the antigenic stimulus very effectively, e.g. if they are incubated with SRC before being transferred either to unimmunized mice (Argyris, 1967) or to cultures of spleen cells from immunized rabbits (Harris and Littleton, 1966). It also appears that they take part in, and increase the efficiency of, a primary response *in vitro* by mouse spleen cells (Mosier and Coppleson, 1968). The latter workers also obtained indirect evidence that two types of lymphoid cell are also involved. This agreed well with the earlier results of Claman, Chaperon and Triplett, 1966a, b), which led to the theory that cells arising in the thymus co-operated with cells from the bone marrow in the response to SRC in irradiated recipient mice.

It seems probable that the thymus-derived cells interact with the antigen, perhaps on the surface of macrophages or perhaps directly, and initiate the differentiation of the bone marrow-derived precursors of PFC to become active antibody-forming cells (Mitchell and Miller, 1968a, b; Nossal, Cunningham, Mitchell & Miller, 1968). The latter cells appear to have antigen-specific immunoglobulin receptor sites in their membranes (Raff, 1970), which enable them to take up SRC. They may then be detected and isolated as "rosette-forming cells" (Brody, 1970). However, without the synergism of the thymus-derived cells they either fail to react to the antigenic stimulus, or respond very slowly. Playfair (1970) has suggested that there may be two distinct classes of bone marrow-derived cells, one of which interacts with stimulated thymus-derived cells, while the other may respond directly to SRC, though less efficiently. Indeed, Tyan, Herzenberg & Gibbs (1969) obtained evidence of a response to SRC which took place in the absence of any thymic influence.

Kennedy, Till, Siminovitch & McCulloch (1965) were among the first to use Jerne's plaque technique for the direct study of the effects of irradiation on antibody-forming cells. They showed that while the development of the immune response in mice injected with SRC was relatively sensitive to γ-rays, mature PFC were much less radiosensitive, and appeared unaffected by doses of less than 2000 rads in air. Their determination of the D_o (that increment of dose of radiation sufficient to reduce the number of surviving cells to 1/e [i.e. to about 37%] of its previous value on the exponential part of a survival curve) of the

in vivo response was obtained by comparing the numbers of splenic PFC present on the 4th day of the response. However, as will be discussed in more detail later (see page 90), this estimate may be biased if the onset of the response is delayed or if its rate is reduced by the larger doses of radiation. Therefore their estimate of approximately 80 rads was probably rather low, as various factors may partially compensate for the initial radiation-induced damage.

An earlier estimate of the radiosensitivity of the primary immune response was obtained using another method by Makinodan, Kastenbaum & Peterson (1962). These workers injected spleen cells from unimmunized mice into immunologically-inert, lethally irradiated isologous recipients following various doses of radiation in vitro, and then tested their ability to respond to an injection of SRC given to the hosts. By determining the titre of haemagglutinin produced, they estimated the D_0 to be approximately 70 r. However, the use of transferred cells and irradiated recipients introduced different factors, so this figure must be interpreted with caution.

The work of Kennedy et al (1965) confirmed that the most radiosensitive step in the response to SRC occurred before the maturation of the antibody-forming cells. Macrophage mediation in this response is either unaffected by doses of radiation up to 900 rads, since a response may be obtained in irradiated mice which receive only thoracic duct lymphocytes and few, if any, macrophages (Mitchell and Miller, 1968a, b), or non-essential (Gershon and Feldman, 1968; Unanue and Askonas, 1968). Roseman (1969) has shown (using the method of Mishell and Dutton, 1966).

that in the primary response to SRC *in vitro*, cells adherent to plastic, consisting mainly of macrophages, are essential, but remain functional for at least 2 days following X-ray doses of up to 1000 r. Therefore the most sensitive cells involved are probably lymphoid cells; either the bone marrow-derived precursors of the PFC, or the auxiliary thymus-derived lymphocytes, or both of these cell-types.

Although the mechanism of the response to polymerized *Salmonella* flagellin is probably somewhat different from that to SRC, in that it appears to be thymus-independent (Armstrong, Diener & Shellam, 1969), there is evidence that it is mediated by small lymphocyte-like cells, with antigen-specific receptors, which are radiosensitive. Using a mouse spleen cell transfer system, Ada and Byrt (1969) found that cells which took up ^{125}I-labelled flagellin *in vitro* were specifically damaged by the β- and γ-emissions. After about 20 hours at 0-4° the cells were injected into irradiated recipients where their responses to the same or an unrelated flagellar antigen were tested. The response to the antigen corresponding to the radioactive one was inhibited, but that to the other one was unaffected. If macrophages were involved in the response they were not affected by the radioactivity of the antigen, since this would also inhibit their participation in the responses to other antigens. Russell and Diener (1970) suggested that these lymphoid cells may be potential antibody-forming cells.

The latter workers showed that although a minority of antibody-forming cells appeared to arise by direct differentiation, without cell-division, in this response, the majority were the daughters of thymidine-

incorporating precursors. Using their *in vitro* culture system, Dutton and Mishell (1967a) showed that in the immune response to SRC by mouse spleen cells, under suitable conditions, the proportion of PFC could be made to increase to 20% of the total surviving cell population. Even allowing for differential survival of the various types of spleen cell, this could only be achieved by multiplication of their precursors. Claflin and Smithies (1967) have claimed to have observed active PFC pass through mitosis once, or even twice, under the microscope, while PFC in arrested metaphase have been used for chromosome analysis by Nossal et al (1968). However, PFC and their precursors are not the only cells which proliferate, since the number of cells incorporating thymidine into their nuclei is always greater than that actually releasing antibody. Harris (1968) has estimated that in the secondary immune response to SRC *in vitro* less than 10% of rabbit spleen cells which incorporate ^{3}H -thymidine were PFC.

Davies et al (Davies, Leuchars, Wallis & Koller, 1966; Davies, Leuchars, Wallis, Marchant & Elliott, 1967; Koller, Davies, Leuchars & Wallis, 1968; Leuchars, Wallis & Davies, 1968) have shown from the study of thymectomized mice that most of the cells which divide in response to the antigenic stimulus are of thymic origin. If the thymus-derived cells are destroyed, e.g. by radiation or anti-thymocyte serum, both the proliferative response and antibody synthesis are inhibited. Since it is the bone marrow-derived cells which synthesize antibody (Nossal et al, 1968), it would appear that the proliferation of the thymus-derived cells is a part of the synergistic process

involving these two cell-types.

Dutton and Mishell (1967b) showed that the addition of ^{3}H-thymidine of high specific activity to their cultures markedly inhibited the appearance of PFC, provided it was present at least 24 hours after the addition of the antigen. Thymidine added before this was not incorporated to a large extent, since the main proliferative response only began after a lag phase of 24-32 hours. Proliferating cells were therefore subjected to the largest doses of radiation (probably in excess of 1000 rads per cell). If the response took place independently of cell proliferation, the addition of thymidine at this time would not be expected to have any great effect. Hunter and Monro (1970) modified this experiment, using a protein antigen (rabbit Fab'_2) instead of SRC, but obtained similar results.

(iv) Irradiation and the Continuation of the Response

Assuming the proliferative phase in the production of antibody-forming cells to be the most radiosensitive event in the immune response, this would explain the rapid disappearance of PFC observed by Berenbaum (1966a), when mice were irradiated with a dose of 450 r. at various times during the response to SRC. PFC continue to incorporate [^{3}H]-thymidine throughout the period of exponential increase in their numbers (Krisch, 1969), indicating a continued proliferative phase. Although this may become less active after the response has passed its peak, it would appear likely that there is a continuous turnover of PFC during the

decline of the response, since Krisch detected 4% of PFC labelling with 1 hour pulses of thymidine on the 13th day, compared to 39% on the 3rd day.

Irradiation on the 3rd, 6th and the 20th days of the response caused similar rates of disappearance of the PFC (Berenbaum, 1966a), and this has been confirmed by the present studies (see page 85). This would not be expected if the continuation of the response after the 5th day were due only to the persistence of long-lived 19S PFC which no longer proliferated, since as has already been described, mature PFC are not significantly affected by doses of radiation in this order of magnitude. Moreover, despite a decline in the numbers of PFC by a factor of 100-1000 following 450 or 500 rads, after 3 or 4 days they began to recover, and increased steadily, though at a slower rate than in the normal response. This secondary rise may also have been due mainly to cell proliferation.

Previous investigators, for instance Taliaferro and Taliaferro (1954), have shown that in rabbits immunised with SRC the response appears to vary in radiosensitivity at different times in relation to the injection of the antigen, as indicated by the titres of haemolysin. However, Berenbaum (1966a) suggested that the radiosensitivity of the responding cells may in fact be constant, but due to their proliferation following immunization, the number of potential survivors may increase. This would allow the synthesis of antibody to continue at a rate which may be detectable by serum titration methods. If the animals were irradiated before antigenic stimulation the number of surviving immunocompetent cells might be too low to produce detectable amounts of anti-

body. Although there were some discrepancies, his results supported this hypothesis, at least up to the 20th day of the response. This led to the conclusion that the relatively long half-life of antibody molecules, compared to the lifetime of the PFC had previously obscured the true radiosensitivity of the immune response once it had been initiated.

Proliferation is probably not essential to the continued synthesis of antibody by individual cells. Makinodan, Nettesheim, Morita & Chadwick (1966) irradiated spleen cells taken from immunized mice in vitro with a dose of 10,000 r., and then cultured them in diffusion chambers in irradiated recipients. Despite this dose, antibody synthesis continued almost unaffected by the radiation. The same investigators (1967) found that a dose of 5000 r. given during a secondary response to SRC in the chambers would stop further development if it was given before the peak of the response, the antibody titre on the 19th day being proportional to the number of antibody-forming cells present at the time of irradiation. The majority of the cells in the cultures were killed, but plasma cells were least affected and came to constitute 80% of the total survivors. RNA synthesis was markedly suppressed, and DNA synthesis reduced still more.

Sado (1969) extended the diffusion chamber experiments. He showed that 10,000 r. reduced the numbers of active 19S and 7S PFC to a half, while total nucleated cells fell to one-sixth of the number in control

cultures. The surviving PFC had half-lives of about 5 days, whereas in unirradiated cultures they underwent a rapid turnover, as *in vivo*. It appeared that *in vitro* the radiation not only inhibited further proliferation of antibody-forming cells, but also arrested PFC in the synthetic phase.

It is unlikely that antibody-induced repression of the response is important in terminating the activity of irradiated PFC *in vivo*, since they ceased functioning at the same rate when they were irradiated on the 3rd day of the response, when the antibody levels were still low, as on the 6th and 20th days, when the levels were much higher (Berenbaum, 1966a). Moreover, Vann (1969), again using the diffusion chamber method, showed that after 7 days in culture the antibody-forming cells were insensitive to the feed-back inhibition of anti-SRC antibody, whether or not they had been irradiated. This was in accord with the widely accepted hypothesis that antibody-induced suppression of the immune response probably acts at its initiation, on the lymphocytes, rather than on the antibody-synthesizing cells (see, for example, Lang, Nase & Rajewsky, 1969). The latter cells were resistant both to irradiation and antibody inhibition.

Therefore it is probably the positive proliferative stimulus which *in vivo* causes irradiated PFC to enter mitosis, whereas *in vitro* the stimulus itself is also affected by the radiation, and the cells remain in interphase. Perhaps the intimate contact between the synergistic clusters of thymus-derived and bone marrow-derived cells which is thought to be essential for the response to SRC *in vitro* (Mosier, 1969)

is broken down following irradiation, which destroys the mediation of the proliferative stimulus, but does not stop the synthesis of antibody by the already-active PFC. In vivo presumably conditions would be more favourable for the reformation of such cellular unions, and the lethal mitotic stimulus is restored.

Nevertheless, if the dose of radiation is sublethal, a fraction of irradiated immunocompetent cells may survive and continue proliferating in response to the antigen. In the mouse their response seems limited (Berenbaum, 1966a, and also in these studies, see page 96), but in the rabbit sublethal irradiation at suitable times after immunization appears to lead to excessive proliferation of antibody-forming cells and correspondingly high antibody titres (Dixon and McConahey, 1963). Why this should occur is not known at present, but possibly the later 7S antibody response is still inhibited so that the normal feed-back regulation is delayed.

(v) Recovery of Immunocompetence following Irradiation

The recovery of the immune mechanism, as a whole, varies according to the antigen tested. In the case of the response to SRC, recovery from the effects of radiation takes some considerable time, and is thymus-dependent (Miller, 1962). Nettesheim and Hanna (1966) found that the primary immune response of mice to SRC required 2-3 months before it returned to the normal level following a dose of 400-600 r. Moreover, the cells which were regenerated were "immunologically virgin", in that only a

primary-type response could be obtained, even in mice which had been immunised before being irradiated. Presumably the immunological "memory" cells were destroyed, and a completely new population had to be built up. Results described in Section 8 of this thesis are compatible with these findings, although the rate of recovery of the strain of mice used (T0) appeared to be slightly slower.

Probably surviving lymphopoietic stem cells in the bone marrow supply new immunocompetent lymphocytes. In the case of the "bone marrow-derived" cells, such repopulation may take place within a few days, and be complete by about the 20th day, but the "processing" of thymus-derived cells would presumably take much longer. From the studies of Harris and Ford (1963, 1964), who made use of the T6 chromosome as a cellular "marker", and Tyan, Cole & Nowell (1966), investigating the recovery of graft-versus-host reactivity in sublethally irradiated mice, it would seem probable that this process requires 30-40 days to complete. It may take somewhat longer than this, since the thymus itself is damaged by radiation (Takeda, Huang & Ambrus, 1969).

Conversely, since Armstrong et al (1969) showed that the bone marrow contains cells which are able to respond to Salmonella flagellin without the assistance of thymus cells in irradiated hosts, it would be expected that the ability to respond to this antigen should recover more rapidly.

(vi) Aims of This Study

This investigation was undertaken in the hope of providing more detailed information on the effects of ionizing radiation on the initiation, development and continuation of an immune response to a particulate antigen, on the activity of antibody-forming cells, and on the recovery of the immune mechanism. The antigen chosen was the sheep erythrocyte, because of its high immunogenicity, ready availability and suitability for cellular studies, using the haemolytic plaque technique of Jerne (Jerne & Nordin, 1963; Jerne, Nordin & Henry, 1963) as the main assay. Mice were used partly because of their suitability for radiation and plaque-forming cell studies, and partly because they have been used by many other workers in this field.

Part II

MATERIALS AND METHODS

PART II MATERIALS AND METHODS

Animals

Throughout these investigations female Tyler's Ordinary (T.O.) white mice were used. Most were obtained from A. Tuck & Son Ltd., Laboratory Animal Breeding Station, Rayleigh, Essex, and were maintained in the animal house in the Medical Research Council's Cyclotron Building. They were fed on "Diet 41B" animal food, supplied by E. Dixon Ltd., Ware, Hertfordshire, and provided with ordinary tap water. The mice varied in age from 6 to 24 weeks at the beginning of each experiment, but were generally less than 15 weeks old. Their average weight was about 28 gm.

While these mice are from a common breeding stock, they are not strictly inbred, and cannot be considered isologous. No test of their mutual histocompatibility was made, but it was assumed that any graft-versus-host reaction in transfer experiments would be slight.

Two New Zealand white rabbits, obtained from Purdey's Farm, Roehford, Essex, were used to prepare antimouse serum.

Antigen and Immunization Procedures

The experimental antigen used was sheep erythrocytes (SRC) obtained from Wellcome Reagents Ltd., Beckenham, Kent. These were supplied in Alsever's solution. Before use they were washed by

centrifugation and resuspended in sterile isotonic saline, usually to a concentration of 1 x 10^8 SRC per ml. The mice were immunised by an intraperitoneal injection of 1 ml. of the final suspension.

For plating, the SRC were finally resuspended in T.C. Medium 199 (see below), to a concentration of approximately 10^8 per 0.1 ml. For serum haemolysin titrations they were suspended in veronal buffer (see below) and the concentration adjusted to 5 x 10^8 per ml. by photometric determination.

Media and Chemicals

Glass-distilled water was used in the preparation of all aqueous solutions.

Saline was usually obtained sterile and free from pyrogens at a concentration of 0.9% w/v from the Dispensary at Hammersmith Hospital.

Phosphate-buffered saline (PBS) was prepared from "Analar" grade chemicals in the laboratory:-

8 gm. NaCl + 3.78 gm. $Na_2HPO_4.12\,H_2O$ + 0.2 gm. KH_2PO_4 + 0.2 gm. KCl per litre, giving a pH of 7.4.

The solution was sterilized by autoclaving at 15 lb/sq. in. for 15 minutes, and usually stored frozen until required.

Veronal buffer was first prepared as a concentrated stock solution:-

85.0 gm. NaCl + 3.75 gm. Na 5,5-diethylbarbiturate + about 1400 ml. distilled water, dissolved and autoclaved. 5.75 gm. 5,5-diethyl-barbituric acid + about 500 ml. distilled water heated to dissolve it. The two solutions were mixed and the volume adjusted to

exactly 2000 ml. with sterile distilled water. For use, a five-fold dilution was made with distilled water and 0.5 gm. of gelatin was added per 500 ml. The final pH was 7.3

Citrate-saline inhibitor was prepared by dissolving 4.41 gm. sodium citrate dihydrate and 7.02 gm. sodium chloride per litre in distilled water.

"Wellcome" brand Tissue Culture Medium 199 (Morton, Morgan and Parker, 1950), 10 times concentrated, and guinea pig serum ("Complement"), preserved by Richardson's method, were obtained from Wellcome Reagents Ltd.

For use, the T.C. Medium 199 was diluted with glass-distilled water and the pH adjusted to approximately 7.2 by the addition of 1.1% sodium bicarbonate solution. Serum and antibiotics were not added, since the experiments involved only relatively short incubation periods.

Complement was prepared immediately before use by diluting the guinea pig serum. For plaque development on agar plates it was diluted 10 times with glass-distilled water, while for serum haemolysin titration it was diluted 7.5 times in veronal buffer.

Difco "Bacto-Agar" (Difco Laboratories, Detroit, Michigan, U.S.A.) was supplied by Baird and Tatlock (London) Ltd., Chadwell Heath, Essex.

Diethylaminoethyl-(DEAE-)Dextran was obtained from Pharmacia (Gt. Britain) Ltd., Ealing, London, W.5. This was dissolved in glass-distilled water to a concentration of 1% (w/v) and autoclaved, before adding it to the agar.

Benzidine was supplied in a partially purified form by Hopkin and Williams Ltd., Chadwell Heath, Essex. For staining plates 2 gm. was dissolved per 100 ml. glacial acetic acid, and 80 ml. hydrogen peroxide and 820 ml. isotonic saline (non-sterile) added to give a final concentration of 0.2%. The solution was used at once.

Silicone Antifoam Emulsion R.D. was obtained from Hopkin and Williams Ltd., and used at 0.2% concentration. However, this amount appeared to be inadequate and should be increased.

Trypan Blue dye was obtained from G.T. Gurr Ltd., London, N.W.9.

Other chemicals used were generally of "Analar" grade and were obtained from various chemical manufacturers.

Special Apparatus

Disposable plastic Petri dishes, about 9 cm. in diameter and sterilized by gamma-irradiation, were used throughout the experiments. They were supplied through the Medical Research Council's Central Store, Colindale Avenue, London, N.W.9., from various manufacturers.

Sterile, disposable plastic hypodermic syringes and disposable needles were used for all injections. These were obtained from Southern Syringe Services Ltd., Enfield, Middx.; Gillette Industries Ltd., (Surgical Div.), Isleworth, Middx.; and B. Lipmann (Plastics) Ltd., London, N.8.

Cells were counted in a Hawksley "Cristalite" Improved Neubauer counting chamber (Hawksley and Sons Ltd., Lancing, England).

Sterile procedures were performed inside a glass-fibre cabinet fitted with a Philips T.U.V. 30 watt ultra-violet tube, as well as normal fluorescent tubes, a small Bunsen burner and a glass front.

Mice were injected intravenously while they were inside zinc gauze cylinders 2.5-2.7 x 8 cm., from which their tails protruded. These were held in a heavy clamp in a warm room at 37°, with additional lighting provided by an articulated table lamp.

Treatment of Apparatus

Glassware and other apparatus were washed in "Decon 75" detergent, obtained from Medical Pharmaceutical Developments Ltd., Brighton, Sussex, and were rinsed in metal-distilled water. Glassware was routinely sterilized by heating overnight in an oven, and plastic centrifuge tubes and bottles were sterilized when necessary by flaming in alcohol.

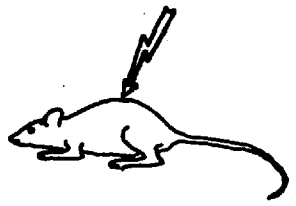
Preparation of Rabbit Anti-mouse Serum

Ascitic fluid was obtained from mice infected with Ehrlich ascites tumour. The cells were removed by centrifugation and potassium alum and sodium hydroxide were added to the supernatant to form a flocculent precipitate. This was used to immunize rabbits by a 4-week course of injections, starting with 1 ml. per injection, and finishing with 5-10 ml. per injection. In all, 13-16 injections were given, totalling about 60 ml. per rabbit and the rabbits were bled 5 or 6 days after the last injection. Sera from two rabbits, which were bled several times, were pooled, absorbed with SRC, and aliquots frozen at -20° until required. The initial batch of antiserum thus prepared appeared to retain its full activity for over 3 years, and was used in all the experiments. Throughout the remainder of this account it will be referred to as "Anti-mouse Serum" or AMS. No attempt was made to prepare antiserum to purified mouse gamma-globulin, since this general antiserum was found to be satisfactory in developing "indirect" plaques. The AMS was diluted 1:100 with glass-distilled water immediately before use.

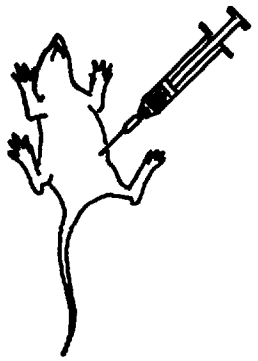
As a control, normal rabbit serum (NRS), obtained from heated, naturally clotted whole blood of unimmunized rabbits (supplied by Wellcome Reagents Ltd.) was added to some plates, but was found to have little effect on the plaque number.

EXPERIMENTAL PROCEDURE

① Whole-Body X-Irradiation



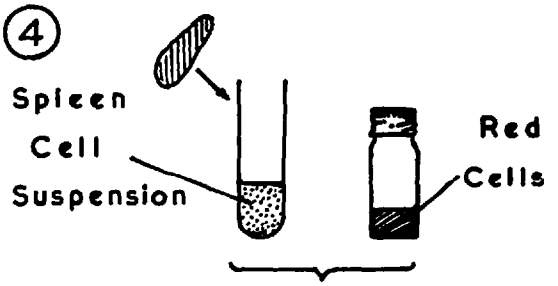
② Injection with Sheep Red Blood Cells



③ Antibody-forming Cells appear in Spleen

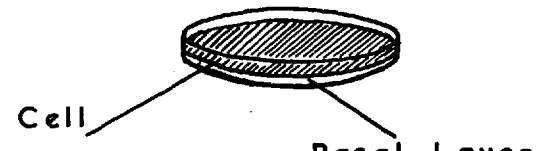


④



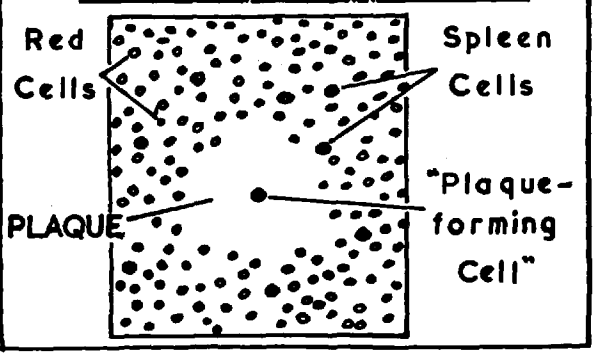
Mixed in 0.8% agar and poured onto Petri Dish:-

⑤



Dishes incubated with Complement at 37° to allow release of Antibody and lysis of Red Cells.

⑥ Microscopic Appearance



Preparation of Spleen Cell Suspensions

The basic procedure in the experiments is summarized diagrammatically in Fig. 1. Mice were killed by cervical dislocation and their spleens removed with sterile surgical instruments. When necessary the whole preparation procedure was carried out completely aseptically, but for short-term incubation, strict sterility was usually dispensed with. No contamination was observed in any of the experiments.

The spleens of mice from each group (between 3 and 8) were placed together in a small beaker on ice, and covered with ice-cold medium until all the groups to be tested had been killed. The spleens of the first group were then transferred to a second beaker and chopped into small fragments with scissors. These were further broken up by transferring the pooled tissue into a sterile stainless steel gauze cylinder, about 1.6 x 7.0 cm., in a larger beaker containing fresh medium and agitated vigorously by means of a glass syringe barrel. This produced a mainly single-cell suspension which was passed through the sieve again to remove any clumps which had been forced through. Usually the agitation process was repeated twice in order to obtain the maximum yield of cells. Although detailed morphological studies were not made, microscopic observation of the suspensions at a magnification of x400 revealed apparently intact cells. In one experiment in which trypan blue dye was

used, only about 6 - 8% cells took up the stain, indicating damage to their membranes. Therefore this process did not seem to damage many of the cells.

After sieving, the cell suspensions were centrifuged in polypropylene tubes on an M.S.E. "Major" refrigerated centrifuge at 800 rev./min. (160 x g.) for 10 minutes at 4°, and the supernant medium discarded. The cells were then resuspended to the desired volume in fresh cold medium.

Usually the volume was first adjusted to yield about 5-10 x 10^6 cells per 0.1 ml., and a 0.1 ml. sample taken for a cell count. The cells were added to a tube containing 0.9 ml. gentian violet in 1% acetic acid which lysed the erythrocytes and stained the lymphoid cells, ready for microscope-counting. When few antibody-forming cells were expected, and the suspension exceeded a total volume of 3-4 ml., it was recentrifuged and resuspended to about 0.8 - 1.0 ml. and further samples plated on Petri dishes. When a high proportion of antibody-forming cells was expected, the suspension was usually diluted until it contained an estimated 500-1000 antibody-forming cells per 0.1 ml. before the samples were plated. In some cases cells were plated at two different dilutions.

Estimation of Antibody-forming Cells

In these studies the numbers of haemolysin-producing cells were estimated directly by the localized haemolysis-in-gel technique of Jerne and Nordin. (For original description see Jerne, Nordin and Henry, 1963). This detects cells producing "direct" haemolytic plaques in agar containing SRC mixed with lymphoid cells. The modification of Dresser and Wortis (1965) was also applied in several experiments to enable the visualization of "indirect" plaques.

It is generally accepted that "direct" plaques are usually produced by cells releasing γM (macroglobulin) haemolytic antibodies, which have a high hameolytic efficiency, while "indirect" plaques are usually produced by cells releasing γM or γG antibodies, which have low efficiencies, and require the addition of heterologous anti-mouse or anti-mouse-globulin serum. In these experiments AMS was used to develop the plaques. γM immunoglobulin (IgM) has a sedimentation coefficient of about 19 Svedberg units. Therefore the cells producing "direct" plaques will be referred to as "19S plaque-forming cells" or 19S PFC. Most forms of γA or γG (IgA and IgG) have a lower molecular weight and a sedimentation coefficient of 7S. Therefore the cells producing "indirect" plaques will be called 7S PFC. However, it should be noted that there are exceptions to the above generalizations. Germfree piglets have been shown to synthesize

a 19S IgG, distinct from IgM (Kim, Bradley and Watson, 1968), while Plotz, Talal and Asofkay (1968) have produced evidence that some types of mouse IgM form only indirect plaques and possibly some 7S immunoglobulins can produce direct haemolytic plaques.

The Petri dishes were first filled with 12-15 ml. 1.4% agar dissolved in phosphate-buffered saline (PBS), pH 7.2, containing 50 mg./ml. DEAE-dextran, which formed a basal layer. The DEAE-dextran was added to bind anti-complementary sulphated polysaccharides in the agar, and so to increase the efficiency of the system (see Liebhaber and Takemoto, 1961). Plates could be kept for several days (occasionally for up to two weeks) before use, provided they were enclosed and stored at 4°.

For the assay of plaque-forming cells, 0.1 ml. samples of the cells were added to test tubes containing about 1×10^{9} SRC, suspended in 2.1 ml. of 0.8% agar dissolved in medium, which also contained DEAE-dextran. The agar was maintained as a liquid by placing the tubes in a water bath at 45°. After the cells had been added, the contents of the tubes were mixed and poured onto the agar-containing plates where the agar was allowed to set to a gel. The plates were then incubated in a warm room at 37°, for a total of 140 minutes. About 40 minutes from the end of the incubation 1.5 ml. complement was added to each plate. If it was desired to detect

indirect plaques, 1.5 ml. AMS was added after the first 60 minutes, and the complement also added after 100 minutes. Plaques began to appear within a few minutes of adding the complement, and were fully developed by the end of the incubation.

At the end of the incubation period the plates were transferred to a cold room at about 4° and left overnight before being stained with benzidine, which turned them a dark blue colour and made it easier to count the plaques, as well as preserving the plates from infection. If necessary plates could be sealed in plastic bags and kept for up to a week before staining, and when stained could be stored almost indefinitely before the plaques were counted.

Plaques were counted with a modified bacterial colony counter, fitted with a circular fluorescent light tube to provide indirect rear lighting, a large low-power magnifying lens, and an electric scoring device.

Plaques varied in size from almost invisible up to 1 mm. or more in diameter, and it appeared that the frequency was approximately inversely proportional to the size. This meant that a high proportion of plaques were near the lower limit of resolution under the magnification used, which besides making the counting more tedious, probably introduced the greatest source of error into the method. Thus, a slight inhibitory influence in a particular plate might well reduce

the average output of antibody molecules from the PFC and so adversely affect the development of plaques. Many near the lower limit of resolution might then become invisible, resulting in a relatively large reduction in the total count per plate. This could only be countered by making the plating conditions as uniform as possible.

Variation between plaque counts on different plates within the same group was inversely related to the mean count, but even when this exceeded 200 per plate the standard error was quite frequently higher than 7% for groups of 6 plates. As far as possible, at least 6 plates were prepared from each individual cell sample in order to reduce pipetting and other errors, but occasionally shortage of time or cells made it necessary to reduce the group size to 4 or 3. When indirect as well as direct plaques were to be tested for the group size was usually doubled, and half the plates were treated with AMS.

Sometimes areas of lysis were formed around clumps of tissue which had not been separated during the sieving process, but these were not scored as plaques. Microscopic examination showed that the great majority of plaques had a single isolated lymphoid cell near the centre, or at most a few cells scattered within the area of haemolysis. All of these which were visible on the counter were scored, irrespective of their size. Human error was reduced by scoring the plates in a random order, with only a number for identification. In a large experiment the person counting therefore

had no indication as to its group or what kind of treatment it had received.

No plaques developed without incubation at 37°, nor in the absence of complement. AMS-developed plaques appeared similar to direct plaques, except that they were often rather cloudy and less well-defined.

Detection of Serum Antibody

As a subsidiary assay, blood was obtained from the mice in some experiments. Immediately they were dead their heads were severed and blood was collected in tubes. Since the amount of blood obtained from each mouse was very small, all the mice in each group were bled into the same tube. The blood was allowed to form a natural clot at 37° for about an hour, then the clot was freed from the glass walls, and the tubes kept overnight at 4° for the clots to contract. The serum was separated by centrifugation, and frozen in small tubes until required.

In most experiments the antibody titre was determined by the 50% haemolysis method of Taliaferro (Taliaferro and Taliaferro, 1950; see also Kabat and Mayer, "Experimental Immunochemistry", 2nd Edition (1961).

Veronal buffer containing 0.1% gelatin was used as the diluent. The serum samples were thawed and diluted with buffer to a suitable

starting concentration. (In many cases the titre was very low, so the highest possible concentration was used). A series of two-fold dilutions were then made and 0.5 ml. was taken from each and placed in a corresponding set of small reaction tubes.

These were transferred to a water bath maintained at 37° in the same order as that for initiating the haemolytic reaction, 20 minutes later. 0.5 ml. aliquots of a standardized SRC suspension in veronal buffer were added in the same sequence. The suspension was adjusted so that the equivalent of a 12.5-times dilution in distilled water (actually a 25-times dilution of a double-strength suspension) gave an optical density of 0.420 as measured at a wavelength of 543 mμ in a "Unicam" spectrophotometer, with a 1 cm. light-path. (The hypotonic shock lysed the erythrocytes). This was equivalent to about 2.5×10^8 SRC per 0.5 ml.

The tubes were mixed well to sensitize the SRC and after a further 20 minutes 0.25 ml. volumes of complement, diluted 7.5 times in buffer, were added in sequence at carefully timed intervals. The total reaction volume was 1.25 ml. per test tube. Finally, after a reaction time of exactly 15 minutes ice-cold citrate-saline inhibitor was added, 2.5 ml. per tube, to stop further haemolysis. The tubes were removed and cooled to room temperature in cold water.

The mean interval between additions of reaction media to the tubes was 15 seconds, so that 56 tubes could be conveniently set up

during each test run. Appropriate serum, complement and cell blanks, and 100% haemolysis controls were also included in order to determine the percentage of antibody-produced haemolysis.

Unhaemolysed erythrocytes were centrifuged down and the optical densities of the supernatants were measured. Date were evaluated using the von Krogh equation (or probit function):-

$$y' = \frac{\text{O.D. of tube}}{(\text{O.D. of 100\% lysis}) - (\text{O.D. of tube})}$$

(Therefore $y' = 1$ when 50% of the SRC in the tube have been haemolysed). Values of y' obtained for a series of antiserum dilutions are plotted on a double-logarithmic graph, when they should normally fall on a straight line. From this the dilution producing 50% haemolysis can readily be determined. The reciprocal of this dilution was taken as the antibody titre of the serum sample.

In later experiments a microtitre modification of this method was used, employing a Cooke "Microdiluter" (Cooke Engineering Company, Virginia, U.S.A.; supplied by Flow Laboratories Ltd., Irvine, Scotland). This required much smaller amounts of antiserum and other reagents and was possibly more sensitive. Reaction constituents were 0.035 ml. antiserum, 0.035 ml. SRC and 0.0175 ml. complement, totalling 0.0875 ml.

Both the serum dilutions and the reaction were performed using lucite plates with 80 wells, a calibrated dropper (0.0175 ml.) and

microdilutors (0.025 ml.) The reaction media were added in sequence until all the complement had been added. The plates were then sealed with "Sellotape" to prevent evaporation, and placed in the Warm Room (37°) for 15 minutes. After incubating 0.15 ml. volumes of citrate-saline were added with conventional pipettes in the same sequence to terminate any residual reaction. Therefore the period between adding complement and inhibitor to each well was approximately constant, and all were incubated for the same length of time, although the relative times at room temperature before and after incubation varied slightly from one well to another.

The degree of haemolysis was determined visually, using a graded series of blanks.

The simpler method of Uyeki and Klassen (1968) was also used in later experiments and gave similar results. Antisera were also diluted using the microtiter apparatus, but the assay was performed on Jerne-type agar dishes. These contained the usual basal layer of 1.4% agar. Onto this was poured 2.1 ml. 0.8% agar containing SRC which was allowed to set. (No lymphoid cells were present).

Approximately 1 microlitre volumes of diluted antisera were spotted on the upper gel using a wire loop. These were spaced at 5-10 mm. intervals and were absorbed by the agar. The plates were then incubated at 37° for 135 minutes, complement being added after

90 minutes. Spots of serum containing 19S haemolysin became visible as areas of lysis, similar to cell-produced plaques. The titre was estimated by the highest dilution producing definite lysis.

The haemagglutinin titre of the same samples could also be determined by adding 0.035 ml. of the standard SRC suspension to wells containing serial dilutions of antiserum (or to the wells from which microlitre samples had been taken for plating, since this only slightly affected the volume). The red cells were well mixed and then the dishes sealed and placed at 4° overnight. The following day they were agitated and the maximum dilutions producing agglutination visible to the naked eye were taken as the titres of the sera.

X-Irradiation

Two 250-kilovolt X-ray sets were used during these investigations, an industrial model by Marconi Instruments Ltd. and a Type 11645 Therapy Apparatus of Phillips Electrical Ltd. Both were operated at maximum voltage and current (15 mA) with added filtration by 0.5 or 0.25 mm. copper and 1.0 mm. aluminium, to produce transmitted X-rays with a half-value layer of approximately 1.4 mm. copper. In some experiments, in which spleen cells were irradiated, no extra filtration was added so that the half-value layer was only about 0.45 mm., but the dose-rate much higher.

Dosimetry was performed mainly with a Farmer Sub-Standard X-ray Dosimeter, Mark 2. (Baldwin Instrument Co. Ltd., Dartford, Kent.) The results from this instrument (which uses an ionisation chamber) were in good agreement with results from lithium fluoride thermo-fluorescence capsules placed inside a perspex "phantom mouse".

For irradiation mice were placed inside 15 cm.-square, 2.5 cm. deep, perforated perspex boxes. These were positioned vertically under the X-ray beam, 60.2 cm. below the X-ray head (about 70 cm. from the target) where they were irradiated at a dose-rate of 63.5-69.7 rads per minute. Usually 7-10 mice were placed in each box.

Spleen cells were irradiated in different ways, though they were usually suspended in sterile PBS during irradiation. In one experiment the cell suspensions were placed in sterile polythene centrifuge tubes arranged concentrically around the beam, 75 cm. below the X-ray head, and supported and partially covered by ice. The dose rate was about 40 rads per minute. In other experiments suspensions were irradiated in sterile plastic Petri dishes about 20 cm. below the head, at a dose-rate of about 200 rads per minute with normal added filtration, or 350 rads per minute at a distance of 35 cm. with no extra filtration.

However, cells irradiated in tubes or plates had an uncontrolled atmosphere and may have become somewhat anoxic during the delivery of

large doses. Therefore in one experiment, in which particularly large doses were required, the cell suspensions were placed in glass irradiation-vessels containing sintered glass filters through which either air or nitrogen was bubbled. (Howard-Flanders and Alper, 1957). The vessels were attached by means of a special mounting directly onto the X-ray head, which was pointed horizontally. This allowed the relatively high dose-rate of 1300 rads per minute (without added filtration) to be achieved.

In order to control the build-up of bubbles in these vessels, a silicone anti-foam agent was added to a concentration of about 0.2%. This reduced the surface tension of the PBS. Approximately 1 ml. samples of the suspension were removed at intervals by closing the main outlet from the vessel, thereby forcing fluid out through the secondary gas inlet which was opened for the purpose. Duplicate vessels containing cells served as unirradiated controls to determine the effect of bubbling on PFC.

In one experiment agar plates already containing spleen cells and SRC were irradiated immediately before incubation was commenced. These were placed 23.5 cm. below the head, and received a dose of about 200 rads per minute, with normal added filtration.

Transfer of Spleen Cells

Several experiments were performed in which spleen cells from donor mice were injected into lethally-irradiated recipient mice.

Donor spleen cell suspensions were prepared as previously described, except that they were suspended in sterile PBS instead of medium, and sterile procedures were strictly adhered to. In some experiments the cells were specially treated in some way, e.g. irradiated, in vitro.

The recipient mice were irradiated with a dose of 850 rads before they received either donor cells or SRC. This dose was chosen as being adequate to inhibit any significant primary immune response either to the SRC or against the injected cells, but not so great that it caused too many deaths before they could be tested.

After adjusting their concentration, the donor spleen cells were injected into these immunologically inert recipients, usually via the lateral tail veins. The mice were kept immobile inside the special injection cages, and their veins made to dilate by placing them in the warm room for at least 10 minutes before injecting them. In contrast, the spleen cells to be injected were kept in ice until required, and then taken up in 2 ml. sterile syringes. Each recipient received 0.4 - 0.6 ml. of the suspension, the exact quantity varying between experiments, so that each syringe was sufficient to inject 3 to 5 mice.

The number of spleen cells was varied widely, according to the experimental requirements, but the greatest number which could

be satisfactorily injected in a volume of 0.5 ml. was 180 x 10^6. Higher cell numbers had no harmful effects provided the donors were normal. However, when irradiated donor mice were used, the number of cells transferred had to be reduced considerably.

SRC were injected separately into the recipient mice (10^9 SRC per mouse, intraperitoneally). In some experiments the antigen was injected immediately after the donor spleen cells, but it was found that the same results were obtained when they were injected up to 2 days previously, provided the mice had been irradiated first.

Statistical Treatment of Cell and Plaque Counts

Many statistical analyses depend for their reliability on the data conforming to a normal distribution. Where it follows another type of distribution, e.g. binomial or Poisson, it is necessary first to transform it mathematically in an appropriate way to give a normal type of distribution. Therefore for the analysis of the cell and plaque count data it was important to investigate the kind of frequency distribution which they followed.

Since the process of plating and incubating potential plaque-forming cells probably introduced extra errors, it was thought that cell samples placed in stain-containing tubes, instead of blood-agar-containing ones, should give the best indication of the underlying distribution of spleen cells in the suspension. Of these cells, a very small proportion, approximately 1 - 1000 per million, should be PFC, unless these tend to congregate locally. Workers who have tried to separate PFC from other spleen cells have had only limited success, suggesting that such segregation is unlikely.

18 0.1 ml. samples were therefore taken from a single cell suspension, prepared from the spleens of three mice in the usual way.

Sub-samples from each tube were placed on a haemocytometer slide and cells counted in 10 areas of 0.1 x 1.0 mm., giving 180 counts in all. The mean number of cells per unit area was 85.97, with a variance of 76.07.

"Student" (1907) showed that yeast cells counted in a similar way followed a distribution which closely fitted one expected from a Poisson distribution (Poisson, 1837). Since it is a characteristic of the Poisson distribution that the variance is equal to the mean, whereas with a normal distribution these parameters are independent, the spleen cell counts obtained were also compatible with a Poisson distribution in this respect. However, as the mean of a Poisson distribution is increased, so the distribution approximates to normality. Therefore with a mean count as high as 85.97 no significant difference was expected; this value was chosen because it was typical of plaque counts per Petri dish.

Both normal and Poisson distribution curves were fitted to the data and chi-squared tests made. The values of χ^2 so obtained were 19.52 and 19.43, which were not significantly different, and were both well within acceptable limits ($P = 0.500 - 0.750$; 21 degrees of freedom).

Another cell suspension was also tested in a similar way, except that 160 areas of only 0.05 $mm.^2$ were counted, thereby

reducing the mean to only 2.42 cells per square. The variance was found to be similarly reduced, to 2.585, again in close agreement. This time the frequency distribution was distinctly skewed, and gave a close fit to a Poisson distribution (χ^2 = 1.035 with 6 degrees of freedom; P = 0.975 - 0.990) whereas the fit to a normal distribution was less good (χ^2 = 3.2908; P = 0.750 - 0.900). Therefore it was concluded that the counts of spleen cells followed a Poisson distribution, as would be expected if the cells were randomly distributed in suspension.

Since the number of plaques per plate was as low as 2.42 exceedingly infrequently, and was often greater than 86, it was decided that no transformation was necessary to normalize the data for statistical analysis. However, the variance of the cell counts could be taken as being the same as the mean so that a single count was sufficient to provide an estimate of both the mean and the variance.

Although it appeared that total spleen cells were randomly distributed and their frequencies in small samples followed a Poisson distribution, it seemed that the development of plaques during the incubation process introduced significant additional variation into the plaque counts, as had been expected. While

variances of groups of plates (5 or 6 per group) were often in the same order as their mean plaque counts, very high variances were also obtained quite frequently, and the range of variances was much greater than expected.

In order to estimate the standard errors of the means of the PFC per spleen as accurately as possible, it was necessary to allow for the proportion of each spleen cell suspension actually plated. This was varied from over 0.75 when a small number of PFC was expected, to less than 0.002 when many PFC were expected, in order to obtain countable numbers of plaques on each plate. This was in contrast to the extremely small proportion (always less than 10^{-4}) of suspensions actually required for total spleen cell counts.

When most of the cell suspension was assayed for PFC the standard error of the sample was close to that of the entire cell population. When only a small proportion was required the sample error was considerably less than the population error. The following corrections were therefore applied:-

Standard Error of the estimated mean for an infinite population $= \frac{S}{\sqrt{n}} = S \times \sqrt{\frac{1}{n}}$ where S = sample standard deviation and n = number of observations in sample.

Standard Error of population with N individuals =

$$S \times \sqrt{\frac{1}{n} - \frac{1}{N}}$$

Approximate Standard Error of PFC in suspension, volume VP =

$$VP \times S \times \sqrt{\frac{1}{n} - \frac{1}{VP}}$$

where n = number of samples (volume 0.1 ml.) plated, VP = total volume in units of 0.1 ml., and S = standard deviation of plaque counts on plates.

Approximate Standard Error of PFC per spleen =

$$\frac{VP}{mice} \times S \times \sqrt{\frac{1}{n} - \frac{1}{VP}} = \frac{VP}{mice} \times \sqrt{\left(\frac{\sum(y_i - \bar{y})^2}{n-1}\right)\left(\frac{1}{n} - \frac{1}{VP}\right)}$$

where mice = number of spleens used to prepare cell suspension, y_i = number of plaques on i^{th} plate and $\bar{y}$ = mean number of plaques per plate.

$$\text{Mean PFC per } 10^6 \text{ spleen cells} = \frac{VP \times \bar{y} \times X \times 10^6}{C \times VC}$$

where C = cell count, X = actual volume of suspension used for cell count and VC = total volume of suspension from which sample taken for cell count (both in units of 0.1 ml.).

Approximate Standard Error of PFC per 10^6 cells =

$$\frac{VP \times \bar{y} \times X \times 10^6}{C \times VC} \times \sqrt{\frac{S^2\left(\frac{1}{n} - \frac{1}{VP}\right)}{\bar{y}^2} + \frac{1}{C}}$$

The basic data were fed into an ICL 4100 Series electronic computer belonging to the Royal Postgraduate Medical School of London.

Part III

EXPERIMENTAL PROCEDURE AND RESULTS

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Section 1 : Kinetics of the Normal Primary Immune Response

The development of plaque-forming cells (PFC) in the spleens of normal mice following the injection of 10^9 sheep red blood cells (SRC) is shown in Fig. 2, and the appearance of circulating haemolysin and haemagglutinin in Fig. 3.

Although circulating haemolytic antibody was undetectable by the titration methods until the third day after immunization, small numbers of direct PFC were always detected in the spleens of unimmunized mice. These "natural plaque-forming cells" (n-PFC) averaged about 120 per spleen, and were inhibited by the addition of anti-mouse serum (AMS) during the plaque test.

24 hours after immunization the number of PFC had increased to about 300 above the background. Thereafter they increased approximately exponentially to reach a peak of about 330,000 between days 4 and 5. Despite this increase in the numbers of PFC, the total spleen cell number remained approximately constant at about 3.10×10^8, variations being statistically insignificant. Initially n-PFC represented approximately 0.46 cells per million spleen cells; by the peak of the response PFC accounted for about 1000 cells per million. Between 18 and 96 hours after immunization the mean observed doubling time of the PFC was 9.0 hours.

DEVELOPMENT OF PLAQUE-FORMING CELLS IN SPLEENS OF NORMAL MICE IN RESPONSE TO SRC.

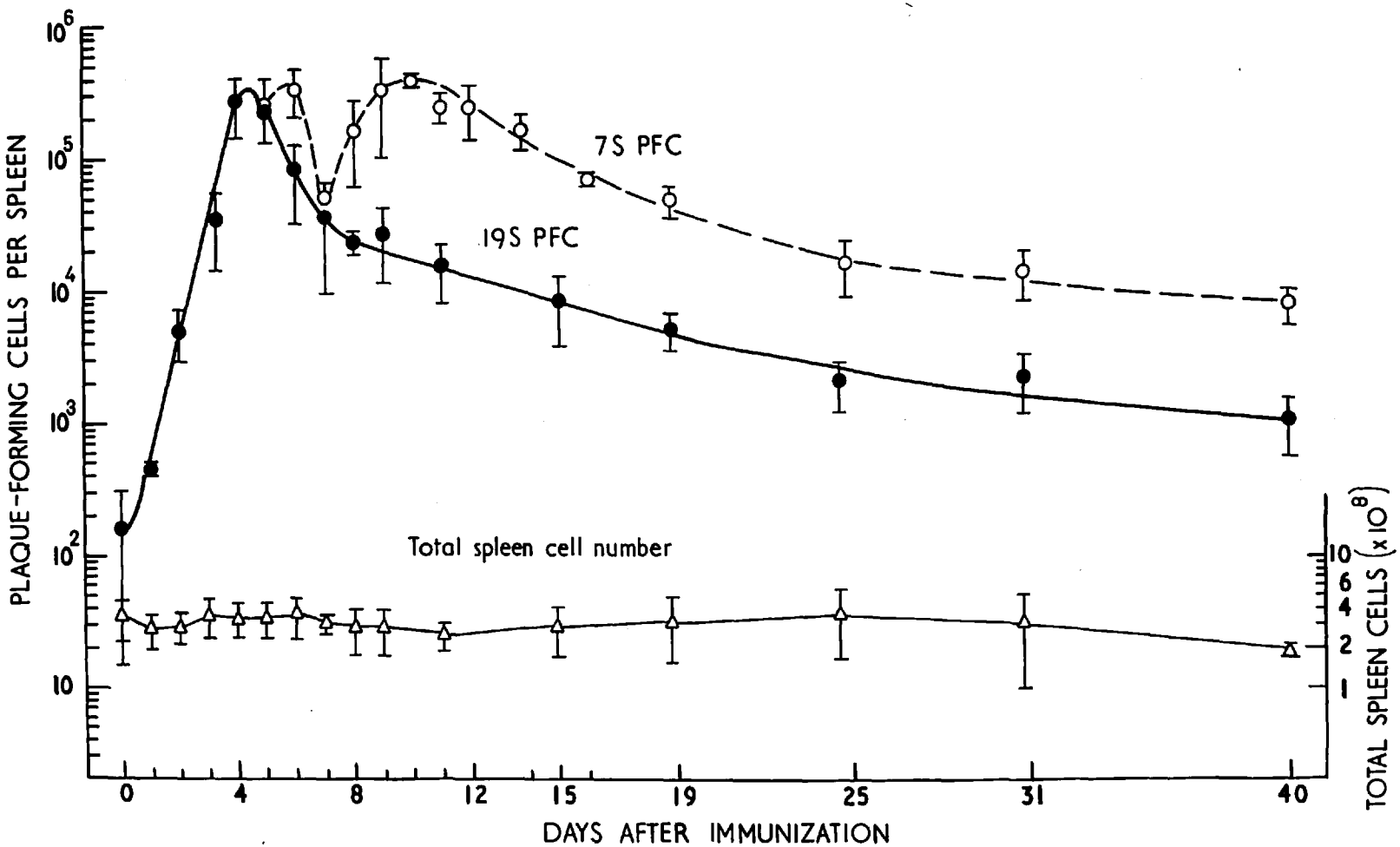


FIGURE 2

Up to the 4th day of the response AMS was consistently inhibitory to the development of plaques, so that only 19S PFC could be detected. However, by the 5th day 7S PFC were also detectable. By this time they were already numerous, and still increasing, and reached a peak of 340,000 per spleen, or 1000 per million cells, on day 6. Thereafter they were the predominant type of haemolysin-producing cell.

After reaching their peak, the numbers of 19S PFC decreased again, rapidly at first and then at a progressively slower rate. Between days 5 and 7 the mean halving time was 17.5 hours, between days 8 and 24 it had increased to 4.8 days, and between days 25 and 40 it was about 14 days.

It was not possible to estimate the doubling time of the 7S PFC in the first peak, but between days 7 and 9 they appeared to double in number in a mean time of 17.7 hours, to reach a second peak of about 390,000 per spleen, or 1470 per million cells, on day 10. The 7S PFC of the first peak seemed to fall in number very rapidly, possibly having a mean half-life as short as 9 hours. After the second peak, however, the number of PFC fell more gradually, again at a progressively decreasing rate. Between days 10 and 19 the mean halving time was 2.8 days, while between days 25 and 40 it was about 14 days, similar to that of the 19S PFC.

APPEARANCE OF HAEMOLYSIN AND HAEMAGGLUTININ IN SERA OF NORMAL MICE

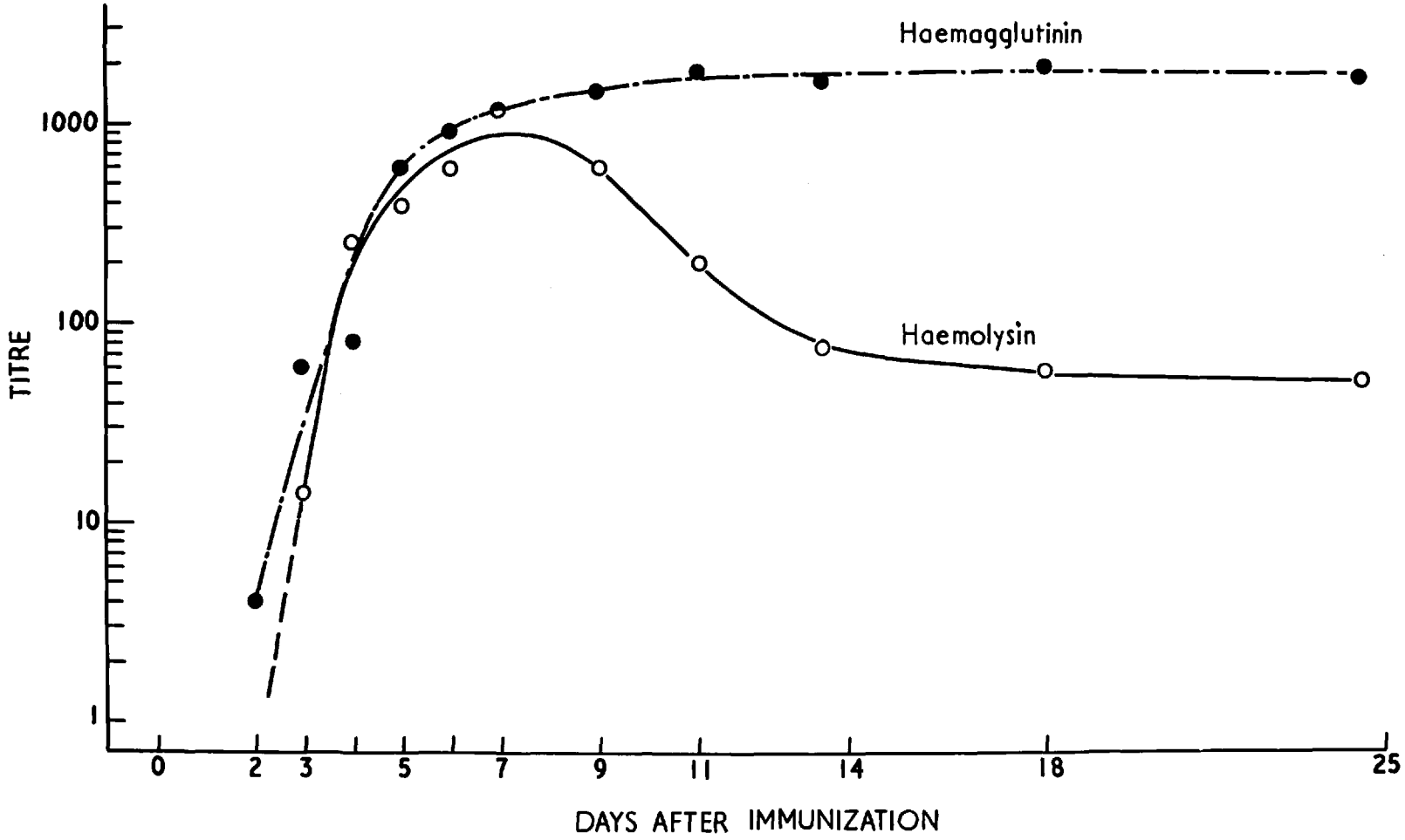


FIGURE 3

The concentration of haemolysin in the blood (see Fig. 3) roughly paralleled the rise and fall in the number of splenic 19S PFC, but the method of estimation was less precise. Between days 3 and 5 the titre doubled approximately every 9-10 hours, and then increased more slowly, to reach a peak of about 880 on the 7th day, i.e. approximately 60 hours after the 19S PFC reached their maximum number. The antibody titre then began to decline, though less steeply than did the PFC. Between days 7 and 14 the overall half-life of the serum antibody was approximately 36 hours, disregarding the counter-effect of continued synthesis.

Haemagglutinin was already detectable on the second day of the response, and also increased in titre rapidly between days 2 and 4, with a mean doubling time of 8-9 hours. After this the net rate of accumulation progressively decreased, resulting in a broad plateau on the graph from day 11 onwards, with a titre of approximately 1800, or double the maximum haemolysin titre.

Section 2 : Effect of X-Irradiation on Mature PFC *in vitro*

Kennedy et al (1965) obtained a survival curve for the ability of PFC taken from immunized mice and irradiated *in vitro* to release antibody. This showed mature 19S PFC to be insignificantly affected by doses of radiation sufficient to inhibit the development of a detectable response by the 4th day, if the mice were irradiated *in toto* prior to the injection of antigen. Three experiments were performed to check this finding, and also to determine the oxygen-enhancement ratio for the effectiveness of doses of radiation sufficient to inhibit the activity of the PFC.

In the first experiment spleen cells were obtained from three mice 4 days after immunization, when the spleens contained many active 19S PFC. The cells were suspended to medium and 20 ml. samples were taken. Two were irradiated to plastic Petri dishes, one on a cold water bath which kept the temperature at 7-8°, the other on warm water at 40-42°. The dose given was 1000 rads, which, as will be discussed in Section 6, would have a profound effect on the development of the response, at a dose-rate of 330 rads per minute, with no added filtration. Two similar samples served as unirradiated temperature controls.

Following treatment, subsamples were plated and incubated normally. However, extra plates were also prepared from the original

cell suspension, allowed to set and then irradiated either at room temperature, or after having been cooled to about 4° in a refrigerator. Again the dose was 1000 rads, but the dose-rate was reduced by added filtration to 200 rads per minute. (It was necessary to use a different X-ray set for these irradiations). All the plates were incubated immediately after irradiation.

It was found that a dose of 1000 rads, whether given to PFC in liquid suspension, or while suspended in agar, had no significant effect. Cells subjected to the higher temperatures were less active than those which were kept in the cold throughout, but were also unaffected by the radiation. Whether temperature affects the radiosensitivity of PFC was not shown by this experiment.

In the second experiment, which is described below, the doses given were much increased, and at the same time the cells were irradiated in bubbling-vessels (Howard-Flanders and Alper, 1957) so that their state of oxygenation could be controlled. Irradiation was performed at room temperature at a dose-rate of 1300 rads per minute.

Spleen cells were obtained from five mice 5 days after immunization. They were suspended in phosphate-buffered saline (PBS), and samples transferred to 4 irradiation vessels, which received the following treatments:-

1. Air bubbling only.
2. Nitrogen bubbling only.
3. X-Irradiation in air.
4. X-Irradiation in nitrogen.

At various intervals samples of approximately 1 ml. were obtained and placed in test tubes. At the end of the irradiation 0.1 ml. sub-samples were plated out to test the activity of the PFC, while cell counts were also obtained. The results are included in Table 1 and shown diagrammatically in Fig. 4.

Although silicone anti-foam agent was added to the spleen cell suspension, the concentration used in this experiment appeared to be insufficient to prevent the formation of a layer of foam on its surface, and the progressive loss of cells from suspension. However, by expressing the results in terms of PFC per million cells, this change in cell number was compensated for, except that the statistical errors increased with increasing doses of radiation.

An estimate was also made of the damage to the cells caused by this treatment by means of the trypan blue dye-exclusion method. The results are also included in Table 1. This shows that the fraction of cells staining with trypan blue increased with time, but was not significantly affected by irradiation. Since there was no corresponding fall in the activity of the control suspensions, it appeared that uptake of the stain was unconnected with the release of antibody.

Table 1. Recovery of Spleen Cells and Effect of Irradiation on their Activity

Dose (Krads) and gas phase		% cells recovered	% cells "viable"	PFC/10^6 cells	Ratio & 95% conf. limits
0	(A)	100	91.7	1098	1.00 ± 0.16
0	(N_2)	100	81	837	1.00 ± 0.17
0.325	(A)	N.D.	N.D.	*1150	*1.04 ± 0.14
0.65	(A)	N.D.	N.D.	*1037	*0.94 ± 0.13
0.65	(N_2)	N.D.	N.D.	* 791	*0.94 ± 0.17
1.3	(A)	N.D.	N.D.	*1094	*0.99 ± 0.17
1.3	(N_2)	N.D.	N.D.	* 801	*0.95 ± 0.11
2.6	(A)	N.D.	N.D.	*1173	*1.06 ± 0.31
2.6	(N_2)	N.D.	N.D.	* 836	*0.99 ± 0.15
5.2	(A)	N.D.	N.D.	*1053	*0.95 ± 0.32
5.2	(N_2)	N.D.	N.D.	* 863	*1.03 ± 0.18
9.75	(A)	59	90	759	0.69 ± 0.11
9.75	(N_2)	N.D.	N.D.	* 965	*1.15 ± 0.20
19.5	(A)	42	76.5	599	0.54 ± 0.17
19.5	(N_2)	49	64.5	640	0.76 ± 0.11
39	(A)	28	79.4	324	0.29 ± 0.09
39	(N_2)	23	48	525	0.62 ± 0.37

A = Air N_2 = Nitrogen N.D. = Not Determined * Estimated Value

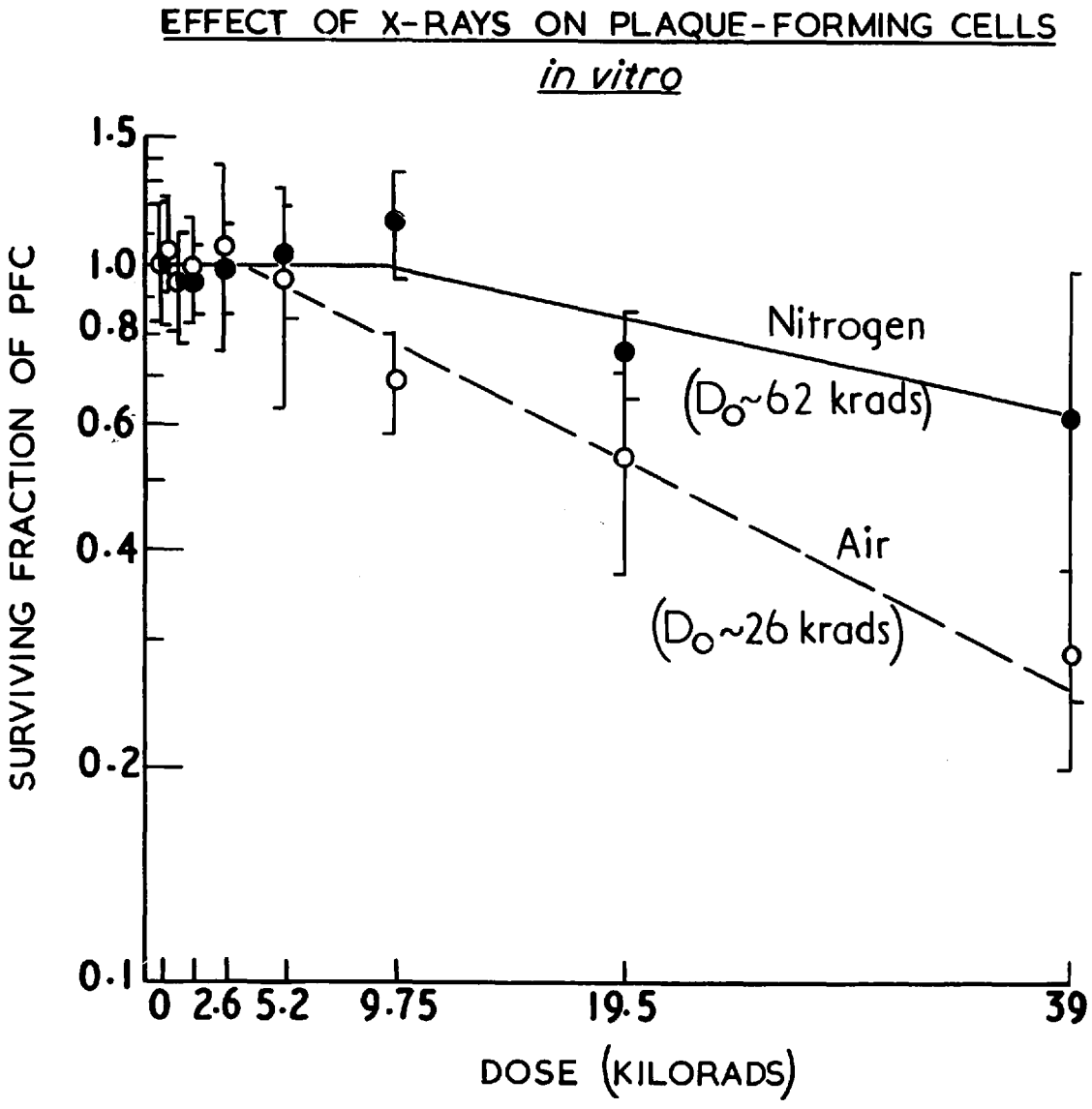

EFFECT OF X-RAYS ON PLAQUE-FORMING CELLS
in vitro
SURVIVING FRACTION OF PFC
1.5
1.0
0.8
0.6
0.4
0.2
0.1
Nitrogen
(D0~62 krads)
Air
(D0~26 krads)
0
2.6
5.2
9.75
19.5
39
DOSE (KILORADS)

FIGURE 4

Doses of radiation of 5.2 kilorads or less had no significant effect in either gas, but a dose of 9.75 kilorads significantly depressed the specific activity of the PFC in air, although it had no effect on those in nitrogen. 19.5 and 39 kilorads reduced the activity of PFC, whether in air or nitrogen, but the effect was greater in air. Because the maximum reduction in the "surviving fraction" was only by 70%, with most of the points in the shoulder region of the "survival curve", it was not possible to determine its parameters with accuracy. Estimates are as follows:- D_o values of 26 and 62 kilorads, respectively, for aerobic and anaerobic irradiations, giving an estimated oxygen-enhancement ratio of about 2.4; extrapolation number not greater than 1.5.

Section 3 : Effect of Irradiation on the Total Spleen Cell Number

Fig. 5 shows the changes in total spleen cell numbers following irradiation with a range of doses from 200 to 1500 rads. In all cases the effect was to produce an immediate depletion of cells from the spleens, the rate and the maximum degree of depletion being proportional to the dose.

It is well known that, for mice, the LD_{50} at 30 days is in the order of 700 rads (due to haemopoietic death), and that with doses over 1000 rads. animals begin to die at 4 to 5 days as a result of the intestinal syndrome. With the two larger doses used, therefore, spleen cell counts could not be followed for more than 7 days.

50 and 100 rads caused only a slight and transient loss of cells and have not been included in Fig. 5 for the sake of clarity. 1000 and 1500 rads, on the other hand, resulted in dramatic falls, to about one 30th of the normal spleen cell number, followed by death. With 700 rads the cell number fell by a factor of about 12 and between days 3 and 9 remained approximately constant. Thereafter there was a progressive recovery until the 23rd day, when it reached the control level again. 900 rads caused the loss of 7/8ths of the cell population, and recovery began after 3 days. 350 and 200 rads had similar effects to 500 rads, except the initial fall in the cell number was correspondingly less. In all groups of mice which received 500 rads or less the cell number had returned to near-normal levels by 16-23 days after irradiation. However, it tended to remain somewhat less than in the controls for

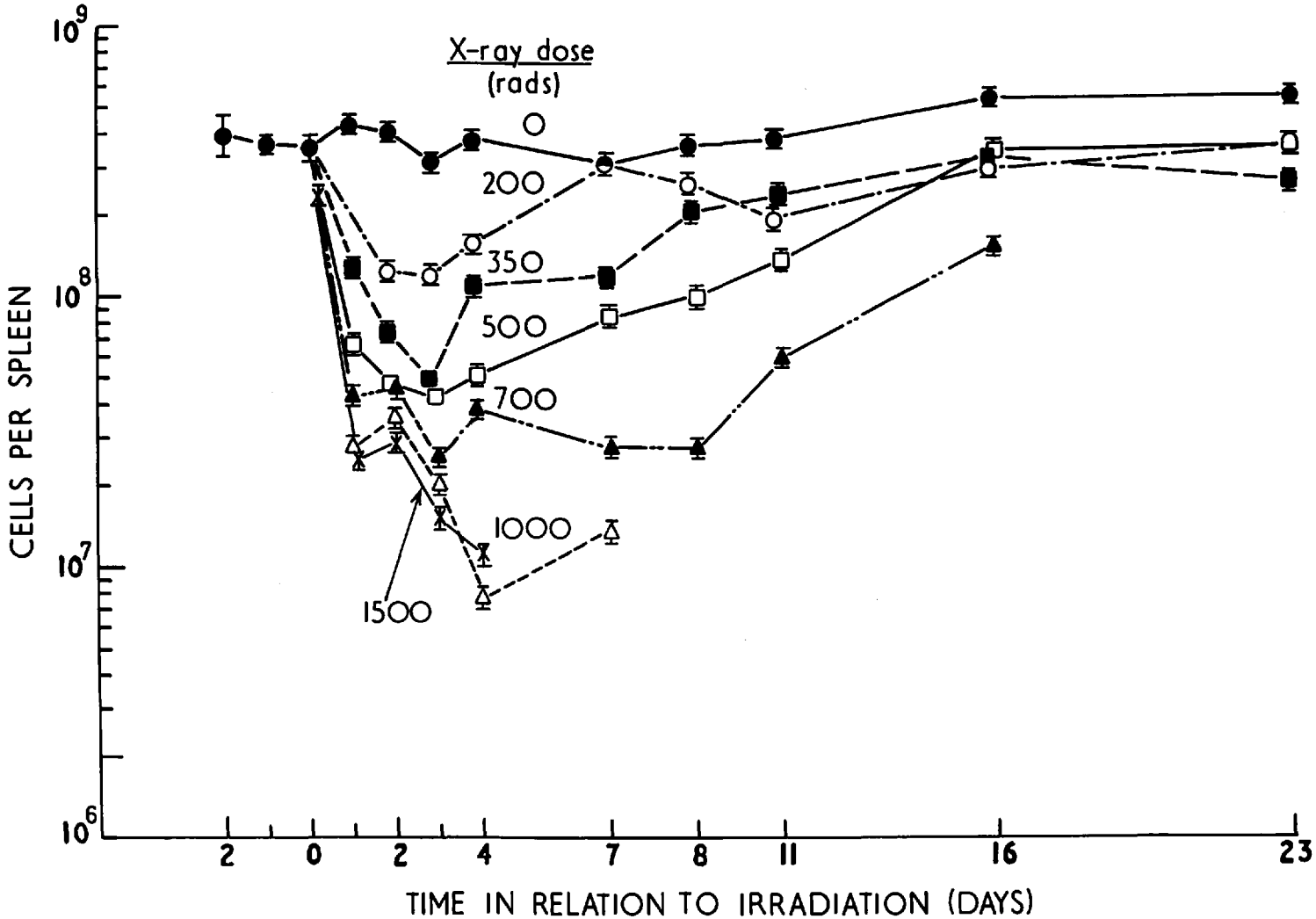

EFFECT OF RADIATION ON TOTAL SPLEEN CELL NUMBER
X-ray dose
(rads)
0
200
350
500
700
1000
1500
CELLS PER SPLEEN
10^9
10^8
10^7
10^6
2
0
2
4
7
8
11
16
23
TIME IN RELATION TO IRRADIATION (DAYS)

FIGURE 5

some time.

Results from another experiment plotted in Fig. 6 show the effect of 500 rads on the spleen cell number in more detail. In this diagram the vertical bars represent the means of several groups of mice plus or minus the standard error. The overall pattern is the same as that illustrated in Fig. 5.

Although migration into or out of the spleens may have partially accounted for the changes in their cell numbers following irradiation, presumably cell-killing was the main cause of depletion. Since the spleen cell population is very heterogeneous, consisting of lymphocytes (small, medium and large), plasma cells, blast cells, polymorphonuclear leucocytes, granulocytes, macrophages, etc., with different rates of turnover in the unirradiated mouse, it is not possible to determine their radiosensitivities from total cell counts.

The smaller doses were relatively more effective in reducing the cell numbers which were observed about the third day after irradiation than were the larger doses. This is indicative of the heterogeneity of the spleen cell population: the majority of the more sensitive and/or most rapidly dividing cells were killed by doses up to 700 rads, leaving the spleens relatively rich in slowly-dividing or less sensitive cells, which persisted for at least 3 days even following 1000 or 1500 rads. After this time surviving stem cells were beginning to repopulate the tissues.

Macroscopic examination showed that recovery of the spleens tended to be centred in a variable number of "nodules" which appeared to

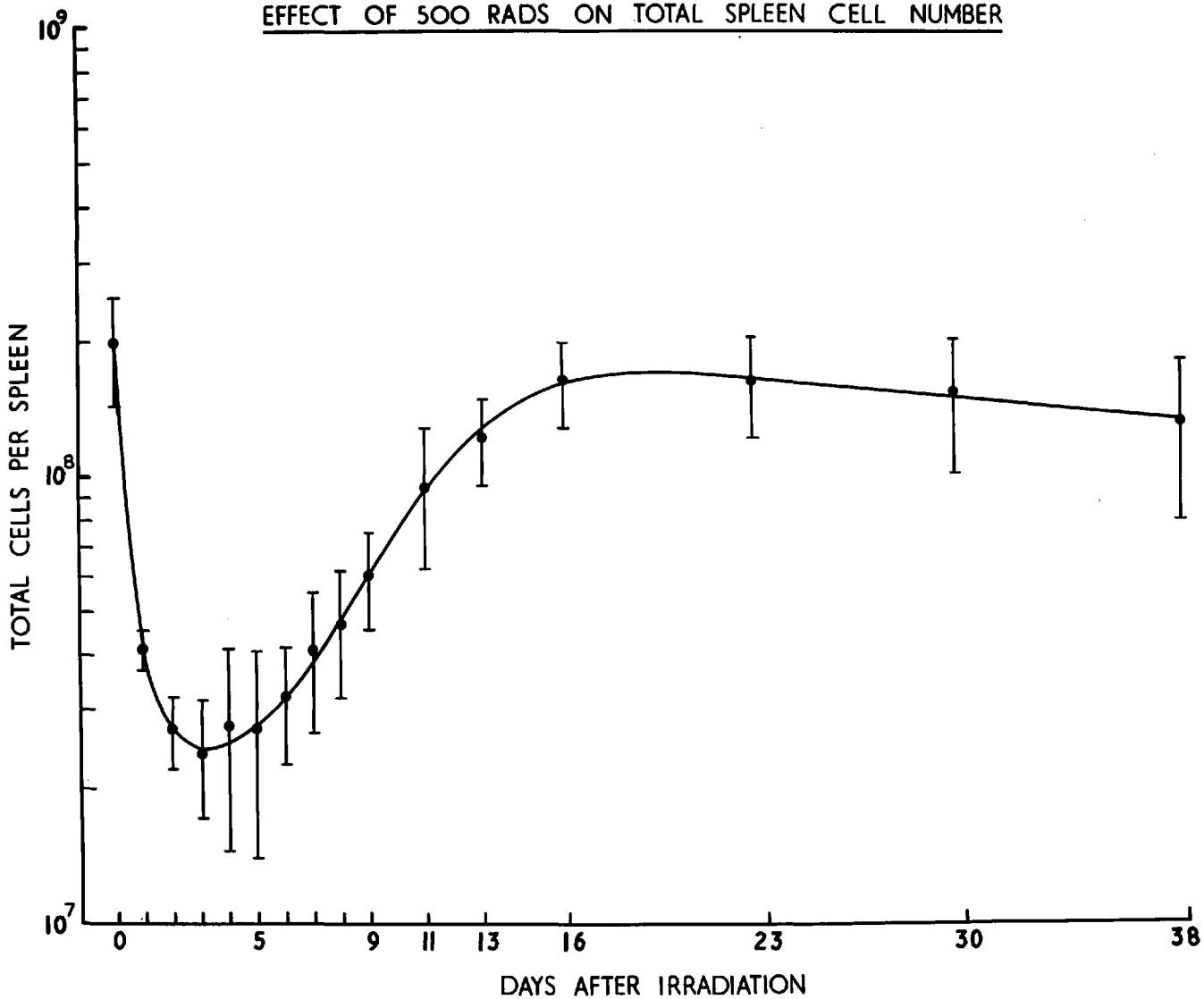

EFFECT OF 500 RADS ON TOTAL SPLEEN CELL NUMBER
TOTAL CELLS PER SPLEEN
10^9
10^8
10^7
0
5
9
11
13
16
23
30
38
DAYS AFTER IRRADIATION

FIGURE 9

consist of rapidly dividing cells, and which increased in size until they coalesced about 15 days after irradiation (with a dose of 500 rads). These nodules were probably produced by the proliferation of the surviving stem cells, either from within the spleen or which had immigrated from other tissues, e.g. the bone marrow. Probably each nodule was derived from a single clone of cells plus other cells which entered it at a later stage.

Section 4: Effects of Irradiation on "Natural Plaque-forming Cells"

The normal "background" of n-PFC is affected by irradiation. The effects of 500 rads are shown in Fig. 7. The number of n-PFC fell to about half its normal value within 5 days and then began to recover again steadily with a mean doubling time of 10-11 days. By about day 20 it had reached the normal range, but continued to increase until about 50 days after irradiation. This resulted in some "over-compensation" for the initial loss of PFC, with about 5 times the normal number being present. However, there was a gradual fall back towards the control level after the 50th day.

Figures 6 and 7 show that there is a similar pattern of response for total spleen cells and n- PFC. Following irradiation cells were lost from the spleen, but were replaced after a few days, presumably by non-specific proliferation. However, the number of n-PFC showed a smaller and slightly slower initial dealine than did the overall spleen cell number, and a similar, though delayed recovery afterwards. In both cases the number of cells increased about 10 times during the initial recovery period, although the n-PFC were initially only depressed by a factor of 2, compared to one of 8 in the case of total cells.

EFFECT OF 500 RADS ON NUMBERS OF "NATURAL PLAQUE-FORMING CELLS"

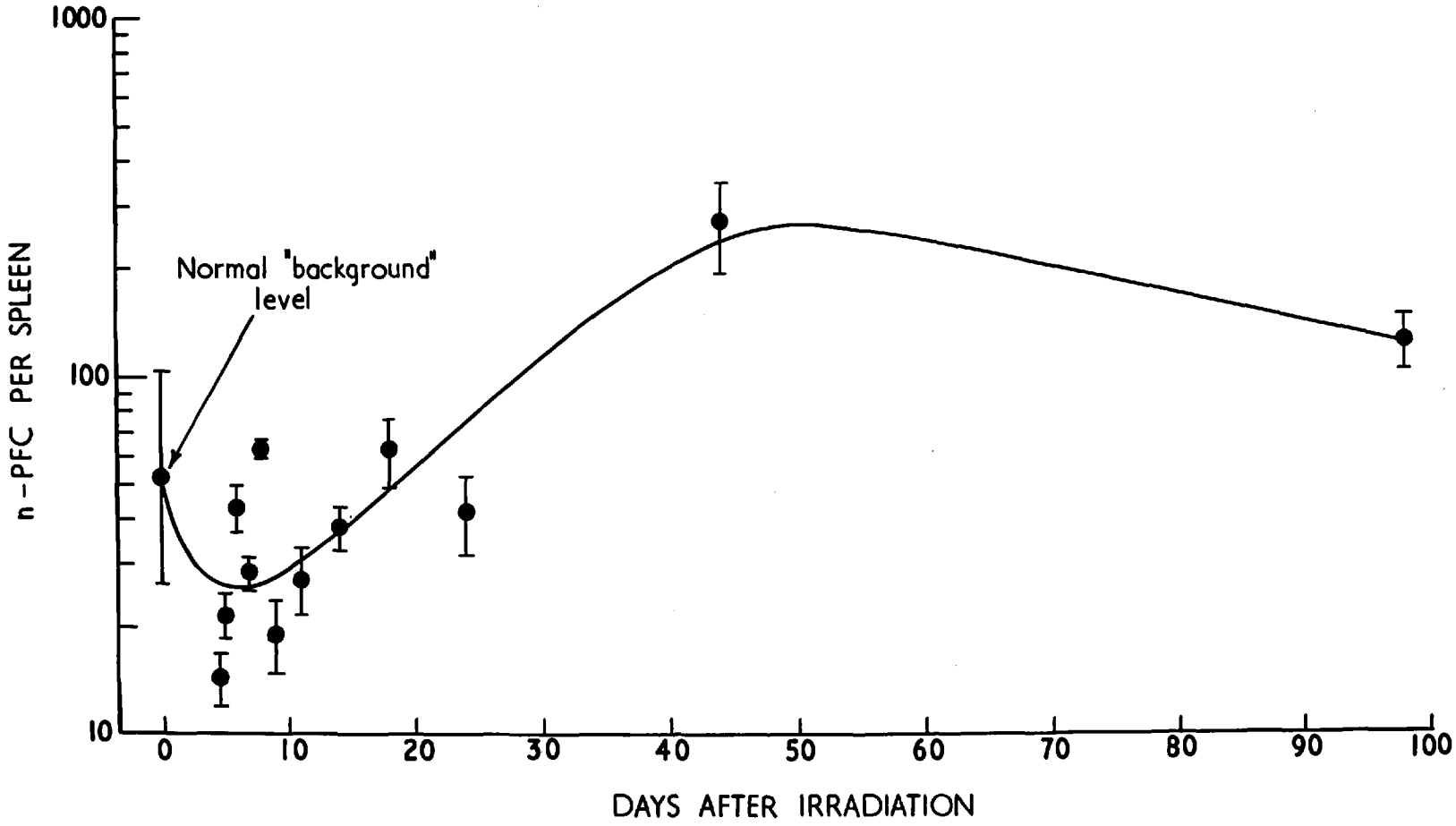


FIGURE 7

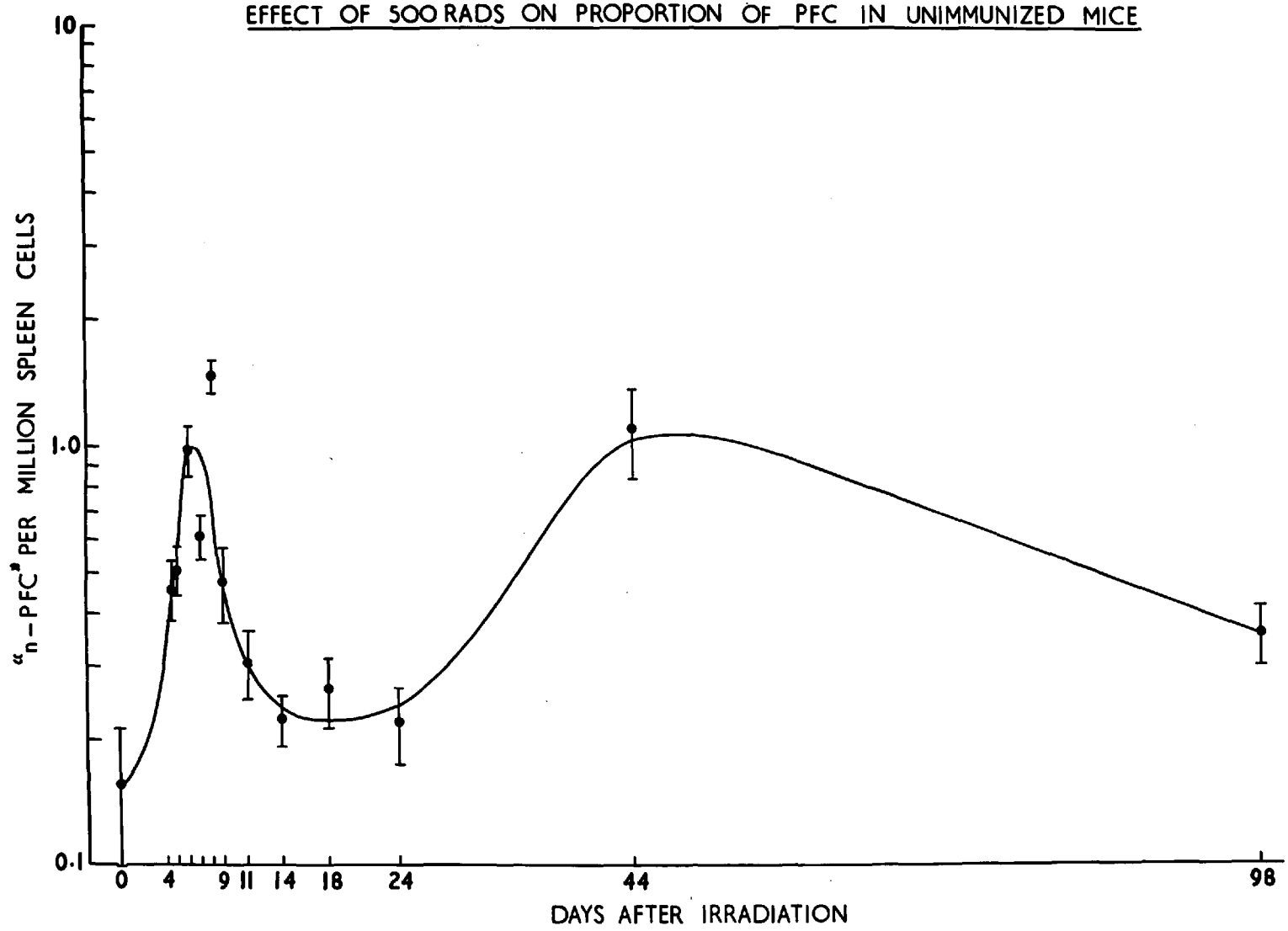

EFFECT OF 500 RADS ON PROPORTION OF PFC IN UNIMMUNIZED MICE
10
1·0
0·1
"n-PFC" PER MILLION SPLEEN CELLS
0
4
9
11
14
18
24
44
98
DAYS AFTER IRRADIATION

FIGURE 8

The net effect of cellular depletion of the spleens on the fraction of n-PFC is shown in Fig. 8. Since n-PFC seemed initially less affected by the radiation, their proportion in the spleen rose at first to a maximum at about 7 days after 500 rads. General splenic repopulation then caused the proportion to fall again to a minimum about day 18, but then the continued increase in the number of n-PFC again raised the proportion to a second peak 45-50 days after irradiation. There was finally a gradual fall.

Section 5: Comparative Effects of a Single Dose of Radiation Given at Different Times in Relation to the Immune Response

Preliminary experiments showed that the development or maintenance of the response of mice to SRC was severely inhibited by a single dose of 500 rads of X-rays. This dose usually produced a death-rate of approximately 10% within a 30-day period, but had no effect on the release of antibody by mature PFC irradiated *in vitro*, as discussed in Section 2.

A large-scale experiment was carried out to show whether or not the effect of this dose varied when mice were irradiated at different times before immunization or during the course of the immune response. The following times were chosen:-

22 hours before immunization with a single injection of SRC:- "minus 1 day" group.

6½ hours, 24½ hours, 3 days, or 8 days after immunization:- "¼ day", "1 day", "3 day" and "8 day" groups, respectively.

Immunized, but unirradiated mice served as controls.

Mice from each group were sacrificed at frequent intervals after irradiation, and the levels of their immunity were estimated by the determination of the numbers of splenic PFC, both 7S and 19S, and their serum haemolysin titres. The results are shown in Fig. 9, 10 and 11, and further details summarised in tables 2, 3 and 4.

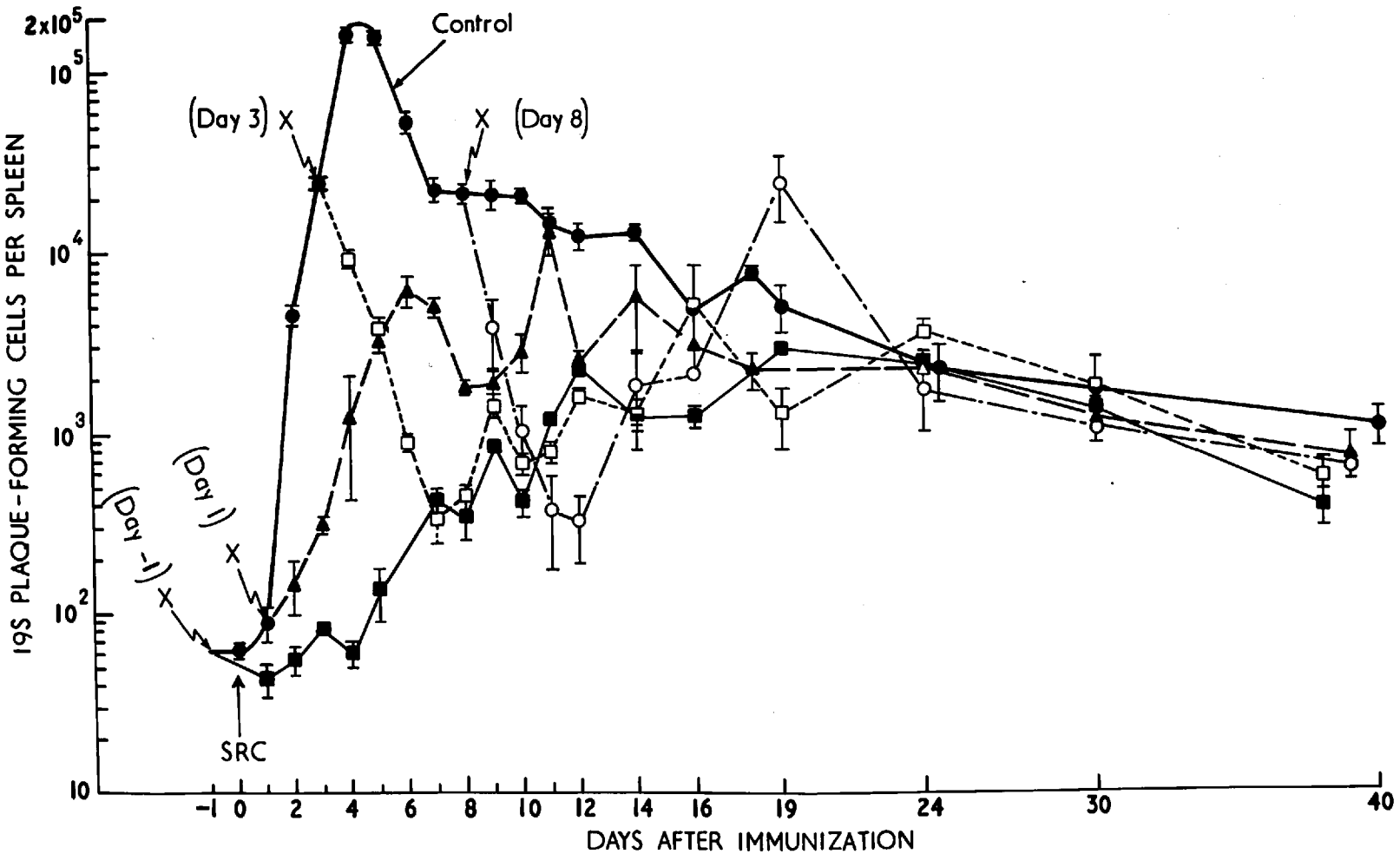

EFFECT OF IRRADIATION AT DIFFERENT TIMES DURING RESPONSE
Control
(Day 3)
(Day 8)
(Day 1)
(Day -1)
SRC
19S PLAQUE-FORMING CELLS PER SPLEEN
DAYS AFTER IMMUNIZATION
2x10^5
10^5
10^4
10^3
10^2
10
-1 0 2 4 6 8 10 12 14 16 19 24 30 40

FIGURE 9

The data show that the immune response was affected by radiation with all the regimes chosen for investigation. However there appeared to be some overall differences in the radio-sensitivities of the different groups. All three figures show that the lowest response to the antigen was obtained in the "minus 1 day" group, i.e. those mice irradiated before they received SRC. The next lowest response was given by the "$\frac{1}{4}$ day" group, which was irradiated before any significant change in the number of PFC would be expected to have occurred following the injection of SRC.

First, the differences between the responses in the "minus 1 day" group and in the controls will be described, and then the responses of the five irradiated groups will be compared.

Two days after irradiation, one day after immunisation, while the number of 19S PFC in the controls had increased slightly above background (see Fig. 9), the number in the "minus 1 day" group of mice was slightly depressed below the normal background level. During the next 3 days PFC in the controls increased rapidly, with a mean doubling time of only 6.6 hours, whereas those in the irradiated mice only doubled once in 46 hours (see table 3). Therefore the difference between these two groups widened rapidly from 46 PFC per spleen on day 1 to approximately 165,000 on day 4.

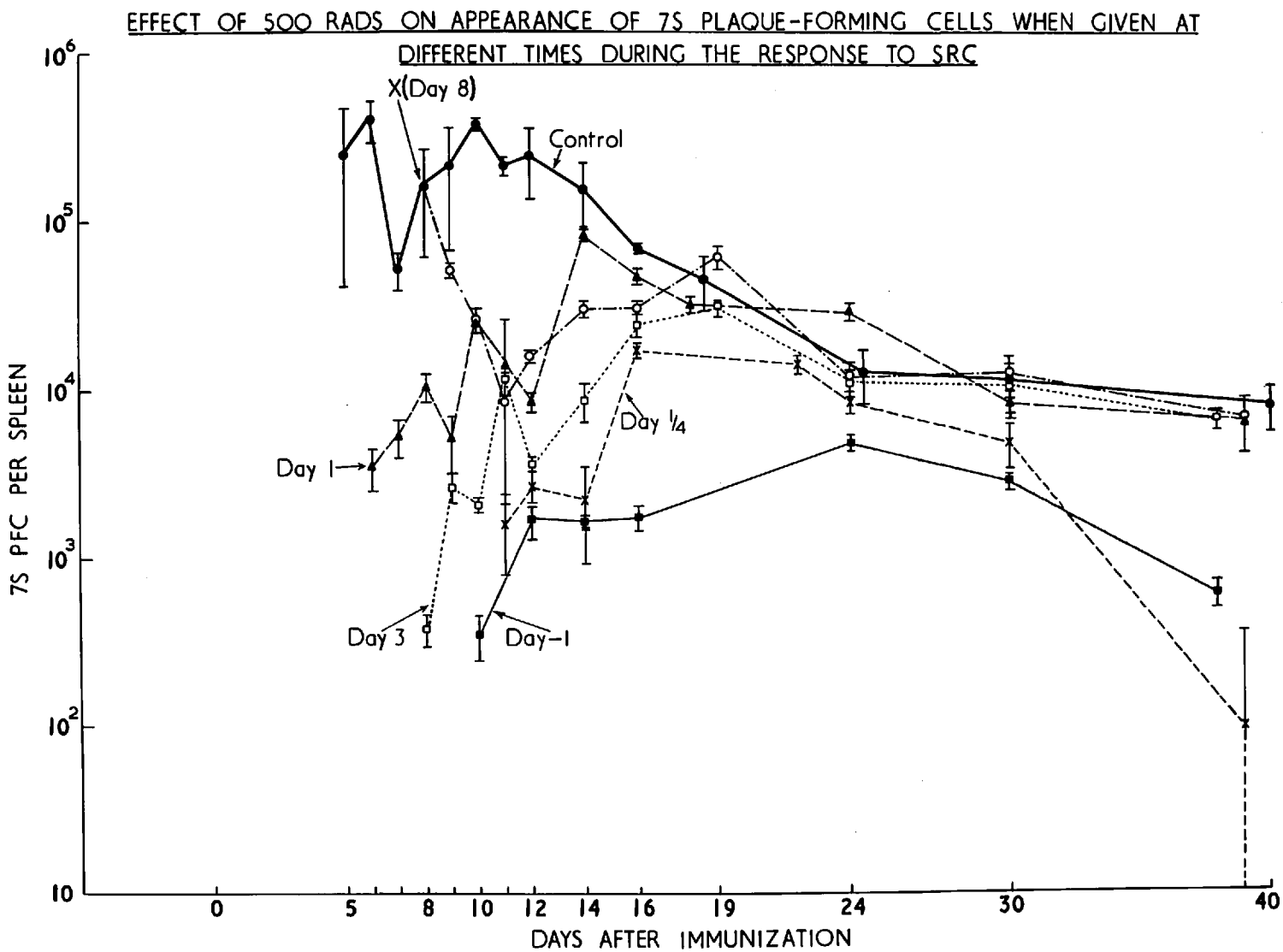

EFFECT OF 500 RADS ON APPEARANCE OF 7S PLAQUE-FORMING CELLS WHEN GIVEN AT DIFFERENT TIMES DURING THE RESPONSE TO SRC
7S PFC PER SPLEEN
10^6
10^5
10^4
10^3
10^2
10
X(Day 8)
Control
Day 1
Day 1/4
Day 3
Day -1
0
5
8
10
12
14
16
19
24
30
40
DAYS AFTER IMMUNIZATION

FIGURE 10

However, after day 5 the number of PFC in the controls passed its peak and began to fall again, while the response continued to develop in the irradiated mice. By day 12 the controls had a mean of only 12,300 PFC per spleen, and this number was still declining with a mean halving-time of 5 days. At this time the "minus 1 day" mice had reached a plateau, with a mean of approximately 2,300 PFC per spleen. By day 24 both groups had about 2,400 PFC per spleen, but thereafter the numbers declined in each, though slightly faster in the irradiated mice.

This prolonged rise in the number of splenic 19S PFC following irradiation allowed some degree of compensation for the initial inhibitory effects of the radiation, but since the number never exceeded that in the controls, this compensation was only partial and the response was very much depressed overall.

There was a similar, or even greater, depression of the production of 7S PFC (see Fig. 10). It should be pointed out that "indirect" plaques could not be detected early in the response with the AMS used for their development, but suddenly became detectable during the response. Their numbers may by then have already been high. Radiation delayed their appearance, but since each group of mice was not always tested at daily intervals, slight inconsistencies may have

HAEMOLYSIN TITRES OF MICE IRRADIATED WITH 500 RADS AT DIFFERENT TIMES DURING RESPONSE

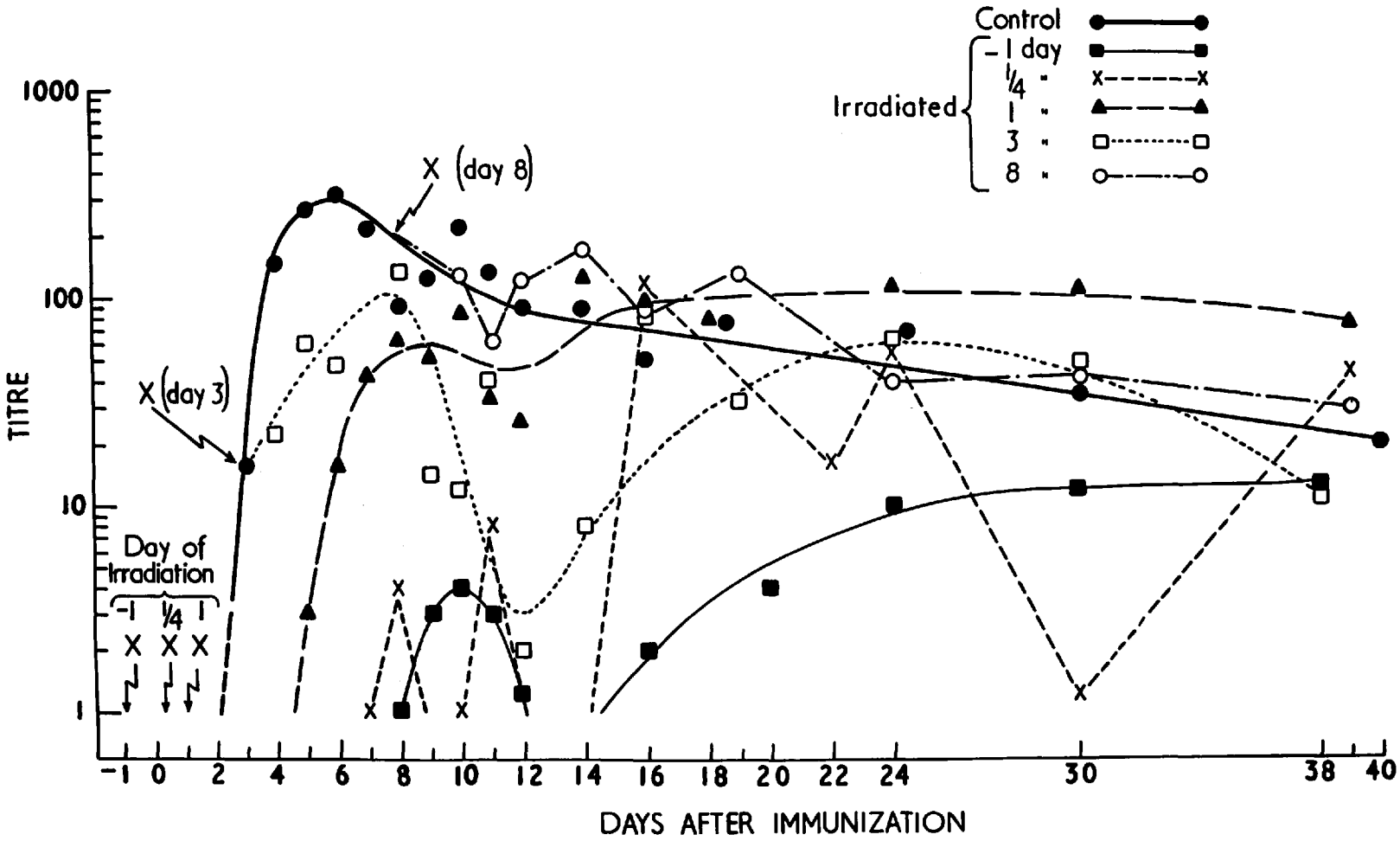


FIGURE 11

arisen, e.g. in the "$\frac{1}{4}$ day" group 7S PFC were not detected on the 9th day after immunization, and the mice were not tested again until day 11. Conversely, the "minus 1 day" group were tested on the 10th day, when 7S PFC were detected.

The two well-defined peaks (400,000 on day 6, and 390,000 on day 10 - see table 2) which were detected in the controls were delayed and reduced to only about 17,600 on day 12 and 4,900 on day 24 in the "minus 1 day" group of mice. Even in the later part of the experiment 7S PFC were well below the control level, unlike the 19S PFC. Since the two peaks of the response were so poorly defined, only an overall doubling time could be calculated with any accuracy. This was about 88 hours, compared to a second peak doubling time of 25 hours in the controls.

Haemolysin was undetectable in the sera of the mice in the "minus 1 day" group until day 8, nor was any antibody activity detected in mice killed on day 14. From day 16 onwards, however, the titre rose slowly, but steadily (see Fig. 11). Compared to this, the rate of haemolysin production in the controls was very rapid, and a titre of 15 was recorded on the third day of the response. A peak titre of 310 was achieved on day 6 compared to the first

peak on day 10 in the irradiated mice of only 4, and a maximum of only 13, even after 38 days. In the controls, following the peak, the titre fluctuated before falling to about 100 on day 12. Thereafter it declined more steadily, with a mean halving time of approximately 13 days.

Since the shapes of the curves for all three parameters are quite different in the control and irradiated groups, it is of limited value to draw conclusions based upon comparisons made at arbitrary times after immunization. (For instance, Kennedy et al (1965) obtained "survival curves" for the capacity of mice to produce 19S PFC by comparing the numbers on the 4th day, when the controls had reached the peak of their response, but the numbers were still increasing in the irradiated mice). Probably it is more useful to compare peak numbers of PFC, or peak titres, irrespective of whether or not they occur on the same day of the response. The times and levels of the different peaks are shown in table 2. Included in table 3 are the approximate minimum doubling times of the PFC and serum titres.

Table 2. **Effect of 500 rads on Peak Numbers of PFC and Haemolysin Titres**

Group	19S PFC		7S PFC		Serum Haemolysin	
	Day	Number	Day	Number	Day	Titre
Control	4-5	165,000	(i) 6 (ii) 10	400,000 390,000	6	310
Minus 1 day	(i) 12 (ii) 19	2,370 2,900	(i) 12 (ii) 24	1,760 4,900	(i) 10 (ii) 38	4 13
½ day	(i) 7 (ii) 16-22	920 3,800	16	17,300	16	116
1 day	(i) 6 (ii) 11	6,200 12,800	14	85,000	14	128
3 day	*16	5,100	19	31,000	(i) *8 (ii) *16	135 85
8 day	*19	24,000	*19	63,000	*14	170

* Secondary rise following radiation-induced depression

Table 3. Approximate Doubling Times (hours) of PFC and Serum Haemolysin

Group	19S PFC		7S PFC		Serum Haemolysin	
	†Day	Time	†Day	Time	†Day	Time
Control	1- 4	6.6	(i) 6 (ii) 7-10	- 25	3- 4 (maximum)	7.4
Minus 1 day	1-12	46	(i) 10-12 (ii) 16-24 10-24	21 132 88	8-24 (overall)	144
¼ day	(i) 1- 7 (ii) 10-16 1-16	32 41 57	11-16	35	7-24 (mean)	70
1 day	1- 6	20	6-14	42	5- 7	12.5
3 day	*7-16	55	8-16	32	3- 8 *12-16 *12-24	36 18 59
8 day	*12-19	26	*11-19	67	*9-12 8-19	19 constant

† Period during which doubling time estimated.
* Secondary rise following radiation-induced depression.

Alternatively, the overall degree of immunocompetence following irradiation may be determined by estimating the total production of PFC or antibody during the period of the experiment. To do this the relative areas under the curves in Fig. 9, 10 and 11 have been calculated, and are shown in table 4. The areas are equivalent to numbers of PFC x days, or titre x days, and are expressed as proportions of those under the control curves.

Table 4 Relative Areas under Curves in Fig. 9, 10 and 11

(Effect of 500 rads to whole mouse)

Time of Irradiation in relation to Immunization	19S PFC	7S PFC	Serum Titre
Control	1	1	1
-1 day	0.05	0.025	0.07
¼ day	*0.07	0.07	0.24
1 day	*0.13	0.26	0.95
3 days	*0.13	0.13	*0.46
8 days	*0.52	*0.31	*1.07

*Post-irradiation part of curve only.

The data in tables 2-4 confirm the conclusions drawn from the original figures. Irradiation prior to immunization had the

greatest overall effect, especially on the production of 7S PFC; irradiation 6 hours after immunization had less effect, and at 24 hours after, still less. The three groups in which the mice were irradiated either before, or 6-24 hours after immunization will now be compared, since different factors have to be considered in a discussion of the "3 day" and "8 day" groups.

There was only a slight difference between the "minus 1 day" and "¼ day" groups in their production of 19S PFC (Fig. 9), and the curves frequently overlapped. This was reflected by the similarity in the doubling times during the first 12 or 16 days (46 and 57 hours, respectively), and by the similar total areas under the curves (0.05 and 0.07 of the control area). The "1 day" group of mice produced a distinctly stronger response, the number of 19S PFC initially doubling in less than 20 hours, reaching a peak of 12,800 PFC per spleen, and with a relative area of 0.13.

When 7S PFC were considered (see Fig. 10) the relative differences were greater, with peak numbers of 4,900, 17,300 and 85,000 in the three groups, and relative total production of 0.025, 0.07 and 0.26 respectively, but again the "minus 1 day" group of mice was least active and the "1 day" group most active. This was also reflected by the earliest days upon which 7S PFC could be detected. AMS enhancement

occurred in the controls from day 5 onwards, in the "1 day" group from day 6, but not until days 10 and 11 (approximately) in the "minus 1 day" and "$\frac{1}{4}$ day" groups.

In the case of the levels of antibody in the sera (Fig.11), the relative differences were still greater, but still in the same sequence of activity. A high titre was never reached in the "minus 1 day" mice. While a titre of 116 was attained in the "$\frac{1}{4}$ day" group, compared to 128 in the "1 day" group, this occurred later and the response was much more erratic. Antibody became detectable in the sera of the "1 day" mice sooner than in the other two groups (by day 5 as opposed to days 7 and 8), and the titre also increased much more rapidly (doubling in only 12.5 hours at the time of greatest activity). While the maximum titre was never as high as in the controls, it remained at above-normal levels from day 14 onwards, so that the area under the serum curve of the "1 day" mice was 0.95 of that for the controls. This indicated only a slight depression of the overall response, despite the marked depression in the numbers of 19S PFC.

When the mice were irradiated on the 3rd day of the response 19S PFC had already reached an average of 25,000 per spleen, and haemolysin was readily detectable in the serum. Irradiation appeared to slow down the synthesis of haemolysin, increasing the doubling time of the titre from 7.4 to 36 hours over the period 3-8 days. There was then a somewhat erratic fall in the titre from 135 to 2 by day 12 (mean halving-time 16 hours), followed by a recovery to control levels by day 16 (doubling time 18 hours).

In contrast to this, the numbers of 19S PFC began to fall soon after irradiation, and were down to 9,500 24 hours afterwards. They continued to decline with a mean halving-time of 15.5 hours, and reached a minimum of 340 on day 7. After this time, however, the PFC again began to increase in number, though now at a much reduced rate, only doubling once every 55 hours, compared to the shortest doubling time of 6.6 hours in the controls. In fact this was similar to the initial doubling rates of the "minus 1 day" and "$\frac{1}{4}$ day" groups. Recovery continued until day 16, when the spleens contained a mean of 1500 19S PFC, as many as in the controls at this time. The number then began to fall at a similar rate to the decline in the control spleens.

7S PFC were still undetectable on day 3, and irradiation further delayed their appearance until day 8. The first peak seemed to be absent, and the main peak was both reduced from 390,000 to 31,000 and delayed from day 10 until day 19. Thereafter they declined at the same rate as the controls.

A somewhat similar pattern was followed by the "8 day" group of mice. Immediately after irradiation the number of 19S PFC began to fall at a much accelerated rate. The mean halving-time was only 12.3 hours, compared to over 120 hours in the controls. By day 11 the number had fallen to 370, and on day 12 it was only 320. Thereafter recovery occurred, as in the "3 day" group, except that the PFC doubled at a mean rate of once in 26 hours compared to once in 55 hours. The number also exceeded the control level on day 19 (24,000 compared to 5,000 PFC per spleen). However, this seemed to be temporary (or due to individual variation), as the number fell parallel with, and slightly below, the control level from day 24 onwards.

In the "8 day" group it was also possible to observe the numbers of 7S PFC from the time of irradiation. There was also an immediate and rapid decline, from 165,000 on day 8 to 8,700 on day 11, with a mean halving-time of 17 hours. This was followed by a recovery,

during which the PFC doubled in a mean time of 67 hours (compared to the time of 25 hours in the controls for the second 7S peak), to reach 63,000 on day 19, approximately the same as the control level. Thereafter both curves declined in parallel.

The serum titre seemed unaffected by the radiation, except that the 9-day titre was exceptionally low, and the general level seemed slightly below the control titre between days 8 and 12. This was reflected by the relative areas under the "8 day" and control curves in Fig. 11, which were not significantly different over the period 8-39 days after immunization. The falls in the numbers of PFC were shown by marked, although not extreme decreases in the corresponding relative areas in Fig. 9 and 10.

The relative areas in all three figures were less in the case of the "3 day" group of mice, and again this showed much more markedly when individual PFC had been estimated, as opposed to serum titres.

It was interesting to note that in this experiment, in the case of the 19S PFC, there was a kind of pattern in the effects of the radiation. If the "1 day" group of mice was excluded, the numbers of splenic PFC tended to follow common "pathways" when plotted graphically.

The two basic pathways were that of the controls, rising steeply to a sharp peak and then falling rapidly at first, but at a more gradual rate from day 7 onwards, and that of the "zero time" irradiated mice. This was represented by the "minus 1 day" and "$\frac{1}{4}$ day" groups, in which the PFC rose at a much slower rate and reached a plateau-like peak of about 3000 per spleen which lasted until the 24th day. At this time it merged with the descending control pathway, and thereafter the two pathways were not significantly different. It would appear from this limited data that the control pathway represents an upper "limit" to the response at all times. Irradiation close to the time of immunization abolishes the true "peak" of the response, since the maximum is only reached when the irradiated pathway merges with the descending control pathway.

Irradiation on the third day of the response causes the "3 day" mice to "switch" from the control to the irradiated pathway,

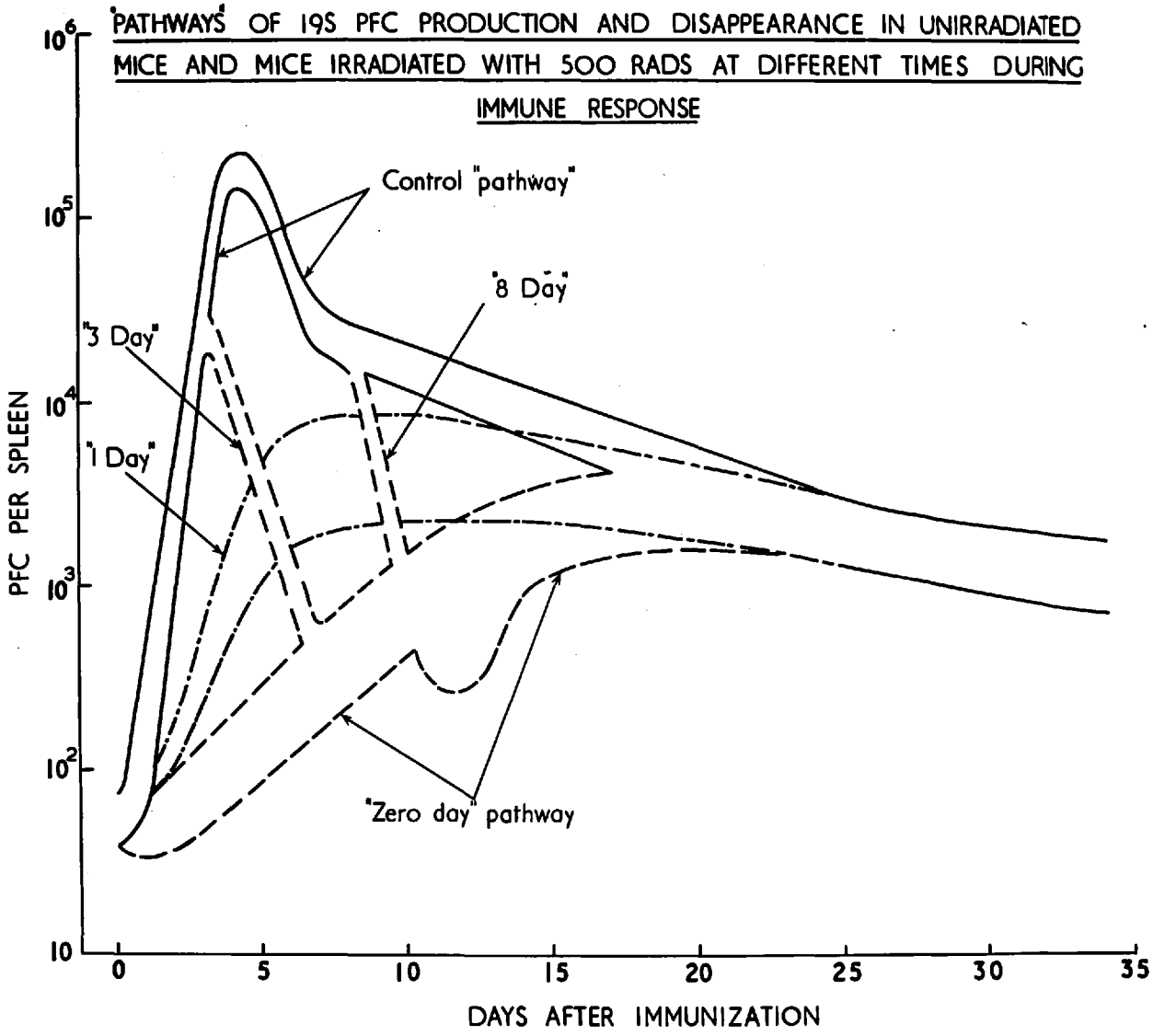

'PATHWAYS' OF 19S PFC PRODUCTION AND DISAPPEARANCE IN UNIRRADIATED MICE AND MICE IRRADIATED WITH 500 RADS AT DIFFERENT TIMES DURING IMMUNE RESPONSE
PFC PER SPLEEN
DAYS AFTER IMMUNIZATION
Control "pathway"
"8 Day"
"3 Day"
"1 Day"
"Zero day" pathway
10^6
10^5
10^4
10^3
10^2
10
0
5
10
15
20
25
30
35

FIGURE 12

which they followed closely from the 7th day onwards. Irradiation on day 8 had a similar "switching" effect, except that the initial fall was to a slightly lower level, and the recovery at a slightly faster rate than the irradiated pathway. However, these differences may not have been significant.

In the case of the "1 day" group, the numbers of PFC were generally above the "zero day pathway". Although the numbers fluctuated considerably days 6 and 14, there appeared to be a flattened peak, with the numbers declining somewhat before finally merging with the control pathway, again on day 24.

These pathways are represented diagrammatically in Fig. 12.

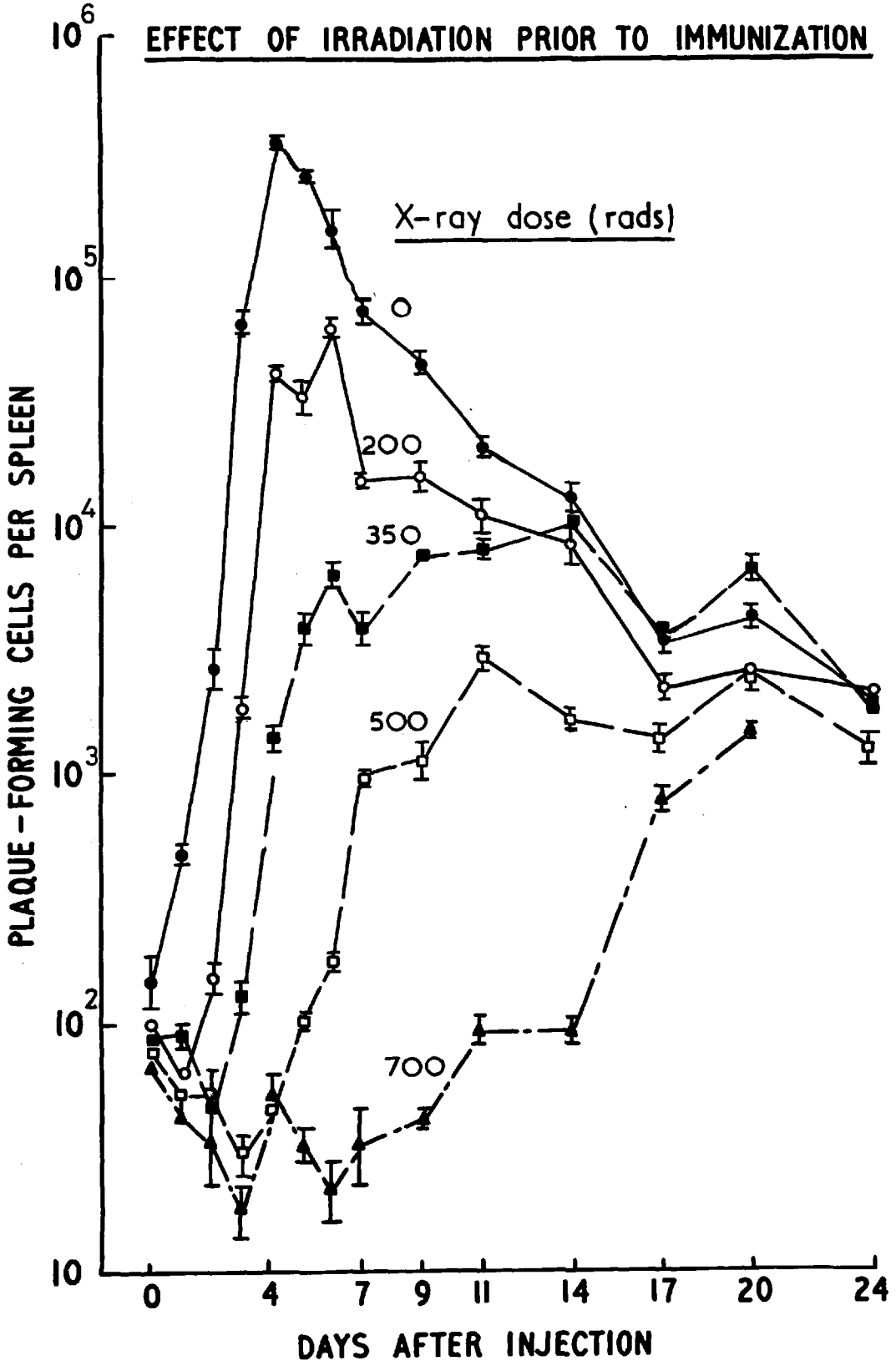
No similar pathways were visible for 7S PFC in Fig. 10 or serum titres in Fig. 11. Nevertheless, this observation may provide information indicative of the fundamental kinetics of 19S PFC production, as will be discussed in more detail in Part IV.

Section 6 : Dose-Effects of Radiation

(i) Irradiation before Immunization

As has already been shown, a dose of 500 rads exerts its greatest inhibitory effect when given before immunization. The subsequent response appears to commence at a lower background level and, following a delay, develops at a considerably slower rate than in unirradiated mice. The maximum number of PFC reached is also depressed and delayed, and generally below the control level on any given day, even several weeks after immunization. Three large-scale experiments were performed to investigate the development of PFC following a range of X-ray doses. The results were all comparable, but only the most comprehensive of the experiments will be described.

In this experiment the doses given were 0, 200, 350, 500 and 700 rads. 42 hours later the mice were immunized by a single injection of 10^9 SRC and groups were killed at intervals until the 24th day for the estimation of splenic 19S PFC and circulating haemolysin and agglutinin. The primary results are shown in Fig. 13 - 15, and further details in tables 5, 6 and 7 and Fig. 16 - 19.


EFFECT OF IRRADIATION PRIOR TO IMMUNIZATION
X-ray dose (rads)
0
200
350
500
700
PLAQUE-FORMING CELLS PER SPLEEN
10^6
10^5
10^4
10^3
10^2
10
0
4
7
9
11
14
17
20
24
DAYS AFTER INJECTION

Increasing doses of radiation reduced and retarded the response, but even a dose of 700 rads did not abolish it. 200 rads appeared to delay the production of PFC and synthesis of antibodies by approximately 24 hours, but this could equally well have been due to a reduction in the number of "immunocompetent units" able to respond to the antigen, requiring a longer period to reach observable levels. The peak number of PFC and peak titre of haemagglutinin were also reduced by approximately the equivalent of a 24-hour increment during the period of exponential increase, and the titre of haemolysin was also somewhat depressed. However the rates of increase did not seem to be affected significantly.

The doubling times for the PFC at the times of maximum increase were 5.2 hours in the controls, and 5.4 hours in the 200 rad-irradiated group, on days 2-3 and 3-4, respectively (see table 5). The mean doubling times for each group were 8.5 and 9.7 hours. Conversely, the peak number of PFC fell from 371,000 to about 66,000, or to 17.5% of the control number.

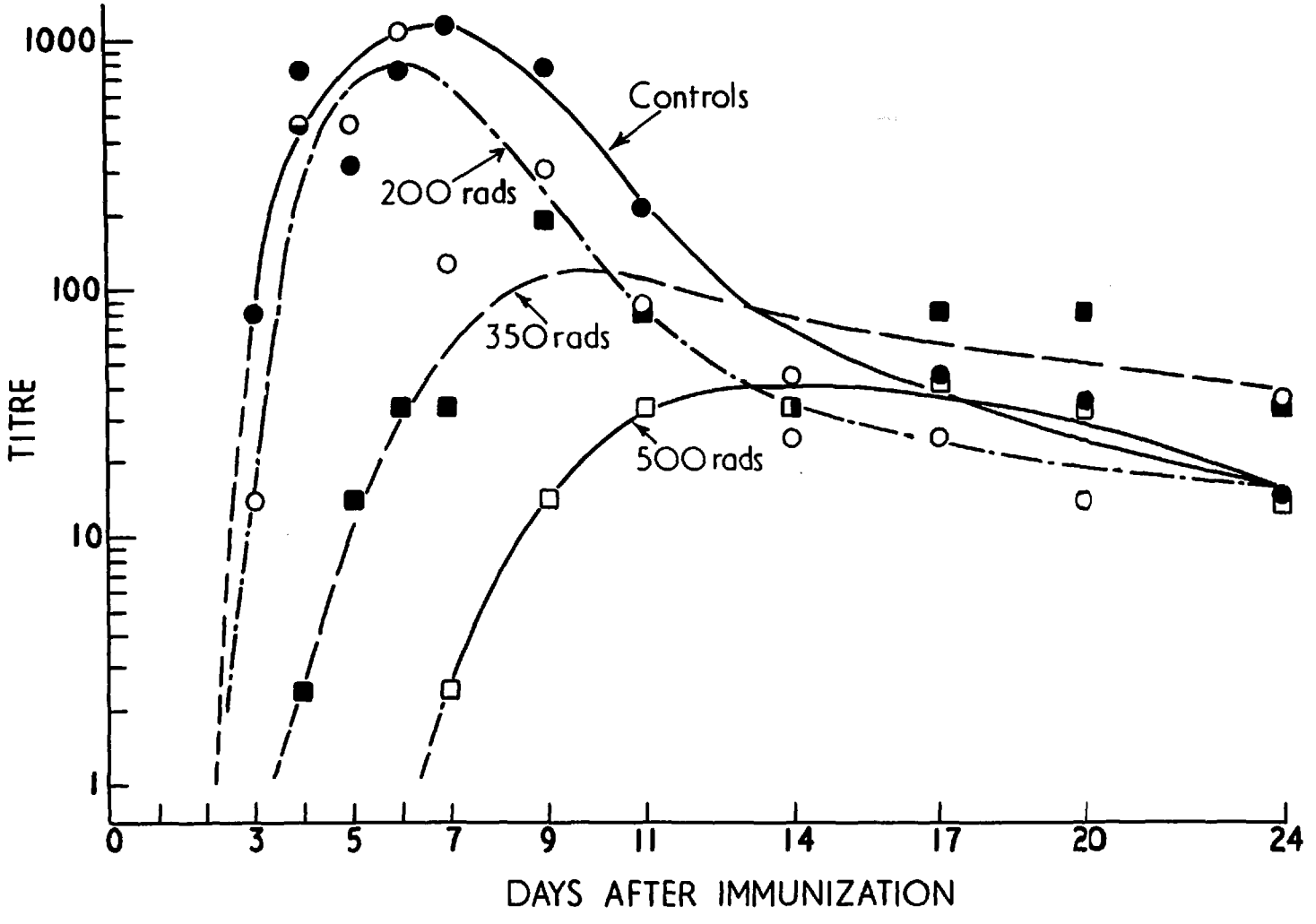

TITRES OF HAEMOLYSIN IN SERA OF IRRADIATED MICE
1000
100
10
1
TITRE
Controls
200 rads
350 rads
500 rads
0
3
5
7
9
11
14
17
20
24
DAYS AFTER IMMUNIZATION

FIGURE 14

Table 5 Production of 19S PFC by Irradiated Mice

Dose (rads)	Maximum Numbers of PFC (and Days reached)				*Doubling Times (hours)			
					Minimum		Average	
	Actual		*Estimated		Actual	Rel. Rate	Actual	Rel. Rate
0	371,000	(4)	371,000	(4)	5.2	1.0	8.5	1.0
200	65,900	(6)	54,000	(5)	5.4	0.95	9.7	0.875
350	10,500	(14)	8,750	(12)	7.0	0.74	11.1	0.77
500	3,010	(11)	2,700	(14)	9.6	0.54	23	0.37
700	1,540	(20)	1,700	(22)	23	0.225	43	0.20

*Estimated by inspection of graph (Fig. 13).

Increasing the X-ray dose to 350 rads further delayed and depressed the response. The peak number of PFC achieved was now only 10,500 (2.7%), and the maximum titres of haemolysin and haemagglutinin were only 10-12% of the control peak levels (see Table 6). The production of PFC and both types of antibody was now noticeably slower than in the controls, with the mean doubling time of the PFC increased by approximately 30%, and peak titres delayed 3 - 5 days.

TITRES OF HAEMAGGLUTININ IN SERA OF IRRADIATED MICE

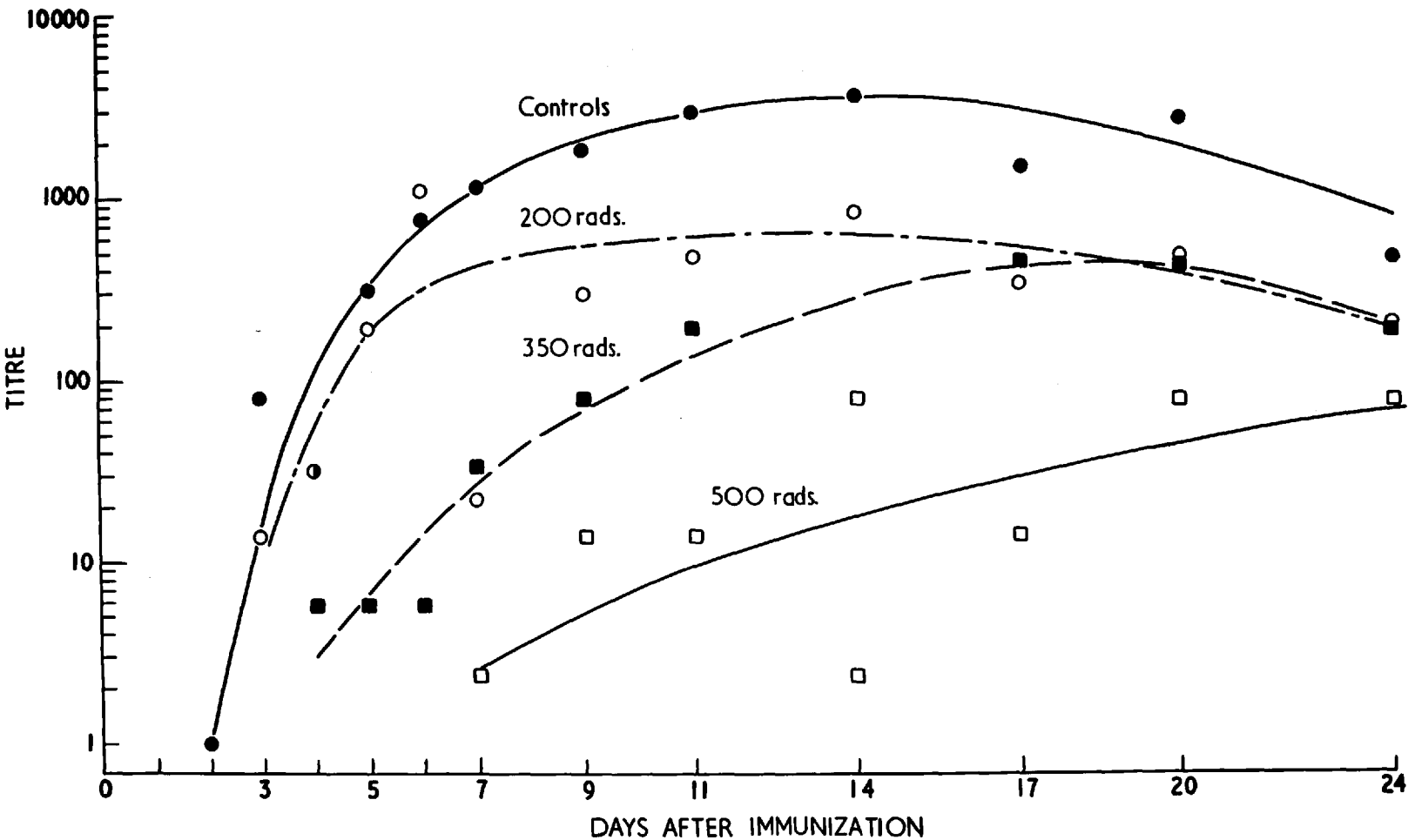


FIGURE 15

Table 6 Estimated Peak Numbers of PFC per spleen and Peak Titres of Serum Haemolysin and Haemagglutinin

Dose (rads)	*PFC/Spleen		Haemolysin		Haemagglutinin	
	Max. No. & day	% of Controls	Peak Titre & day	% of Controls	Peak Titre & day	% of Controls
0	371,000 (4)	100	1150 (7)	100	3500 (14)	100
200	54,000 (5)	14.4	820 (6)	71	650 (13)	18.5
350	8,750 (12)	2.33	120 (10)	10.4	440 (19)	12.5
500	2,700 (14)	0.72	40 (14)	3.5	70 (24)	2.0
700	1,540 (20)	0.41	-	-	-	-

*Values estimated from Fig. 13

Haemolysin " " " Fig. 14

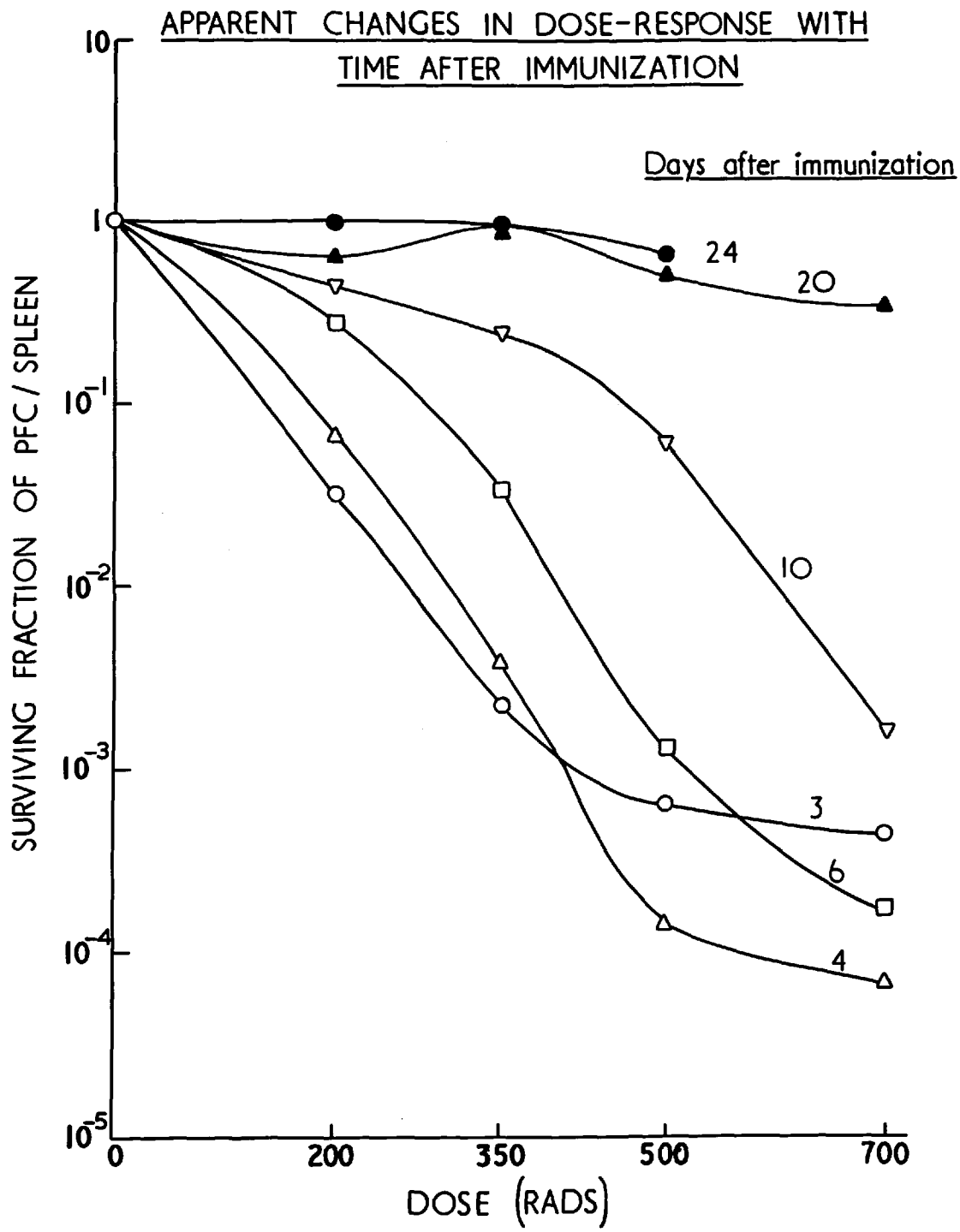
Haemagglutinin " " " Fig. 15

With 500 rads there was a distinct retardation of the response, besides further depression and a considerable lengthening of the "lag" phase before the numbers of PFC began to rise. Indeed, they appeared to fall progressively for the first 5 days after irradiation and only began to rise on the 4th day of the immune response. The maximum titre of agglutinin observed was not obtained until day 24, compared to day 14 in the controls, (see Fig. 15 and table 6) and was only 80,

compared to 3500. The peak in the haemolysin response, with an estimated titre of 40, occurred about day 14, compared to 1150 on day 7 in the controls, and the maximum number of PFC fluctuated between 1400 and 3000 from day 11 until day 20, compared to a sharp peak of 371,000 on day 4. The maximum doubling rate of the PFC was now slower than the average rate in the controls (doubling times 9.6 and 8.5 hours, respectively).

Unfortunately there was a 14% death rate among the mice which received 700 rads, and their response could only be followed for 20 days. Serum antibody titrations were not performed, but were presumably very low. The number of PFC remained below the unimmunised control level until the 17th day of the response, but by the 20th day it had reached 1540, i.e. about one-third of the day-20 control level. Therefore there was only a small further depression in the number of PFC attained, compared to the 500-rad group, but a considerable slowing: the mean doubling time between days 9 and 20 was approximately 43 hours, and the minimum (between days 14 and 17) was 23 hours, 4.5 times longer than in the controls.

As pointed out in Section 5 (see page 90) because of the changed timing and kinetics of the response to SRC following different doses of radiation it is difficult to compare the dose-effects quantitatively. A comparison of the numbers of PFC or levels of circulating antibody on any given day may yield misleading results.


APPARENT CHANGES IN DOSE-RESPONSE WITH TIME AFTER IMMUNIZATION
Days after immunization
24
20
10
3
6
4
SURVIVING FRACTION OF PFC / SPLEEN
10
1
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
0
200
350
500
700
DOSE (RADS)

The PFC begin to increase rapidly in number in the controls soon after immunization, whereas following the larger doses of radiation immunization has little effect. This results in a steep dose-response curve with a horizontal "tail" (see Fig. 16). Later, the controls are past the peak of their response, but in irradiated groups PFC are still increasing. Therefore the dose-response curve becomes progressively less steep, until by about 24 days after the antigenic stimulus the effect of irradiation is no longer apparent.

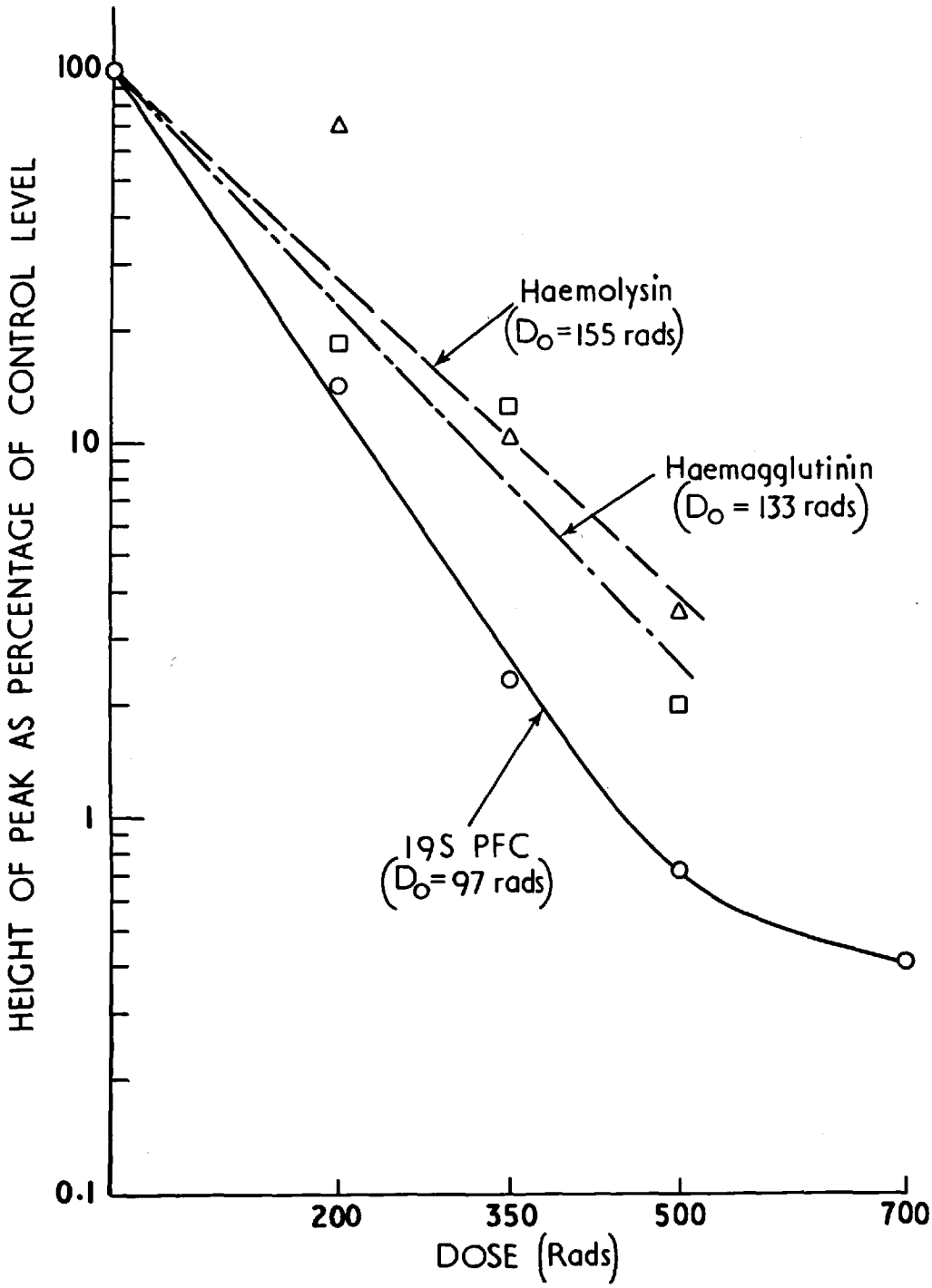
The curve obtained on the fourth day had a mean D_0 of about 60 rads (over the dose-range 0-500 rads), and was therefore steeper than the corresponding dose-response curve of Kennedy et al (1965), the D_0 of which was approximately 80 rads.

The time factor may be avoided by comparing the maximum numbers of PFC and peak titres of circulating antibody achieved in each group (see table 6 and Fig. 17).

In the control mice a peak number of PFC of 371,000 per spleen was attained about 4 days after immunization. Following 200 rads, this was reduced to 66,000 and delayed until the sixth day. However, the general trend of the curve would suggest that the activity

DOSE EFFECTS OF RADIATION ON IMMUNE RESPONSE:

Estimated peak numbers of PFC per spleen and peak Titres of serum Haemolysin and Haemagglutinin



of the individual mice killed on this day was above average, and probably a more representative figure would have been 54,000, achieved 5 days after immunisation. Similar best estimates for the peak numbers and their times are also given in table 6. The delay in the response was much more marked with doses of 350 rads or more, but as may be seen from Fig. 17, the number of PFC was depressed by a factor directly proportional to the dose of radiation until more than 500 rads has been given. Further increasing the dose to 700 rads seemed to have less effect and the curve tended to flatten out. However, it was approximately linear with doses of 0 - 500 rads, and had a mean D_o value of 97 rads. Thus it was less steep than the very steep dose-response curve obtained on the 4th day of the response (see Fig. 16), although the general shape was similar. No "shoulder" was apparent.

Since no attempt was made to estimate the (presumably) very small amounts of circulating antibody in the mice irradiated with 700 rads, only doses of up to 500 rads could be compared directly in their effects on peak antibody titres and numbers of PFC. The appearance and continued synthesis of both haemolysin and agglutinin were delayed, as were the PFC, and although the results were more variable it also appeared that there was a linear relationship between the dose of radiation and the depression of the peak titres, with no shoulders

in the curves. However the relative effects were less, since the curves were less steep, with D_o values of approximately 155 rads for lysin and 133 rads for agglutinin.

Another way of comparing the responses, which may be more useful for quantifying the overall level of immunity, is to calculate the areas under the curves in Fig. 13, 14 and 15 (in the way described on page 93 in Section 5) and compare those for the irradiated groups with their respective controls. This method of estimation is still affected by the times chosen, but to a lesser degree than the daily dose-effect curves. In the case of the 19S IFC, it is probably most accurate from the time of immunization (or from the time irradiation, if this occurs after the response has commenced) until about the 20th day, when all the curves tend to merge. After this time the most heavily irradiated group are favoured relative to the controls, since the differences are then slight compared to earlier in the response. In the case of haemagglutinin production a somewhat longer period might be more appropriate, since the response is lower and more extended. In table 7 the areas have been estimated for days 0 - 24. The results are also presented graphically in Fig. 18.

Table 7. Relative Areas under Curves in Fig. 13, 14 & 15

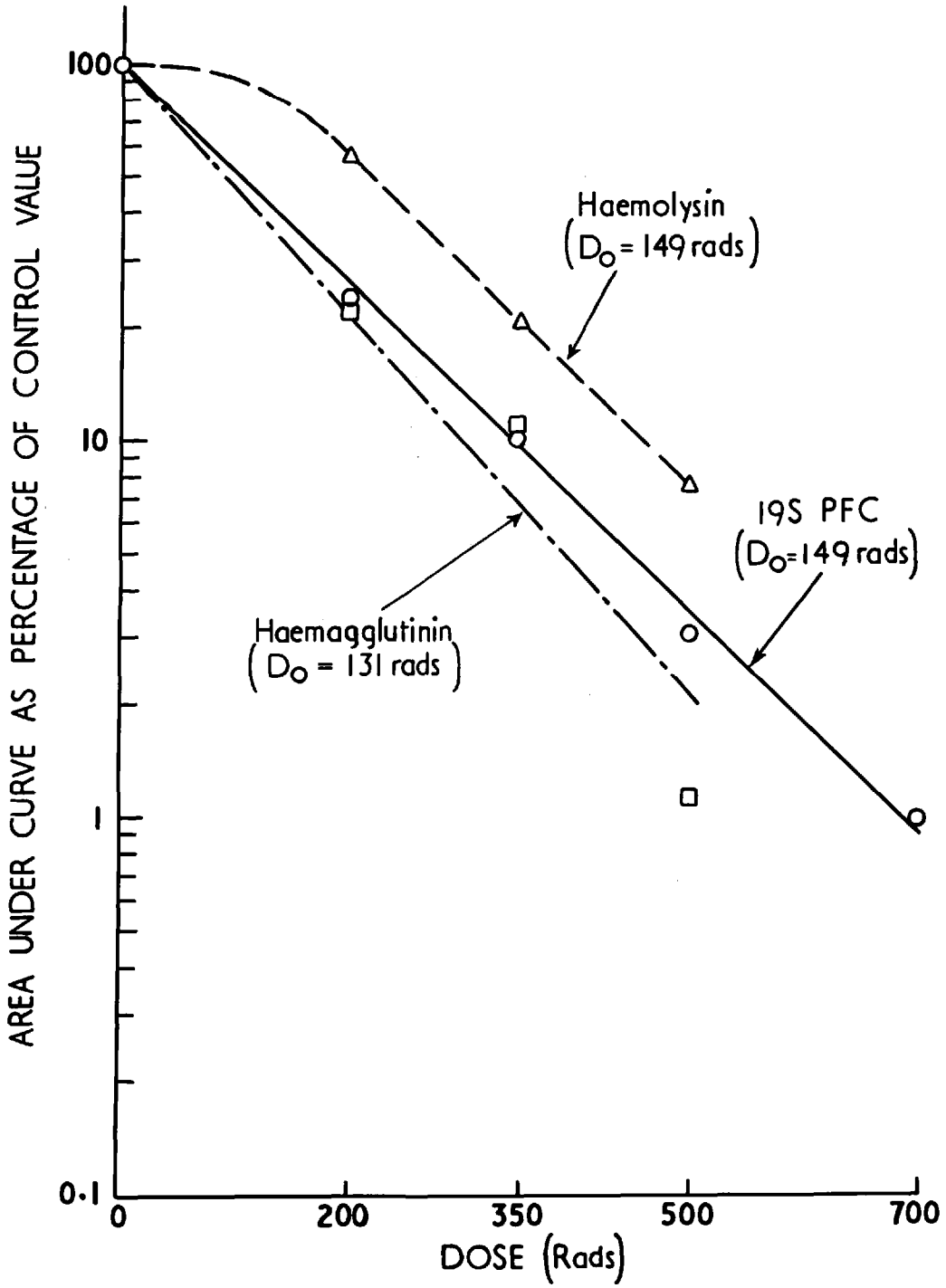
Dose (rads)	PFC/Spleen (Fig. 13)	Haemolysin (Fig. 14)	Haemagglutinin (Fig. 15)
0	1.00	1.00	1.00
200	0.237	0.566	0.216
350	0.102	0.206	0.108
500	0.0306	0.0756	0.0112
700	0.0099	-	-

In the case of the PFC, there was an exponential relationship between the dose and the response over the whole dose-range tested, with a mean D_o of 149 rads. The curve for haemagglutinin production was similar, with a D_o of 130 rads. However, as has been mentioned, possibly a slightly longer period of study would be more appropriate for a slow type of response such as this. If this had been possible, a less steep curve would probably have been obtained.

Unlike the other curves, that for the relative areas under the haemolysin titre curves appeared to have a "shoulder". This would suggest that the synthesis of this antibody was less affected by small doses of radiation than was the production of splenic PFC, but

DOSE EFFECTS OF RADIATION ON IMMUNE RESPONSE:

Relative total production of PFC, Haemolysin and Haemagglutinin during first 24 days of response.



the discrepancy may have been due to experimental error in the antibody titrations. Over the dose-range 200-500 rads the curves for total PFC and haemolysin production were approximately parallel, indicating similar effects of irradiation.

When considering the overall effect of radiation on the antibody response, especially in relation to immunity against post-irradiation infection, this may be the most useful indicator of sensitivity. It may not, however, reflect the initial radiation-induced damage, since homeostatic mechanisms may play a part as the response progresses.

A third possible method of comparing the effects of radiation, which is independent of the changed timing of the response, is to compare the rates of increase in the numbers of PFC between the end of the "lag" phase and the peak of the response. This could also be applied to the serum titres, except that there were fewer points in the upward parts of the curves. The doubling times for the PFC have been calculated and are presented in table 5. Both the fastest rates and the mean rates for the whole of the rise in their numbers are shown.

However, even in the case of PFC the accuracy with which their doubling times can be estimated is not very good. Moreover, interpretation of the results is uncertain without direct determination of their

true cell cycle times. PFC may be "recruited" from inactive precursors throughout the response (Perkins et al., 1969) and may die or become inactive again instead of giving rise to daughter PFC. This will be considered in more detail in the General Discussion (Part IV).

(ii) Irradiation after Immunisation

Having studied the effects of different doses of radiation on the development of the immune response, it was interesting to investigate the dose effects produced by irradiating mice already undergoing an active response. Mice in 9 groups were injected in succession. Precisely 47 hours after immunisation they were irradiated with the following doses:-

0, 50, 100, 200, 350, 500, 700, 1000 or 1500 rads.

Their responses were then followed for a further 30 days, or until all the mice in a group had died from the effects of the X-rays, as discussed in Section 3 (page 74). Adequate numbers survived until the 25th day of the response following doses up to and including 500 rads, but only until the 18th day following 700 rads. The supra-lethal doses of 1000 and 1500 rads caused deaths from the 4th day after irradiation, so that testing had to be terminated on day 9 of the response with 1000 rads, and on day 6 with 1500 rads.

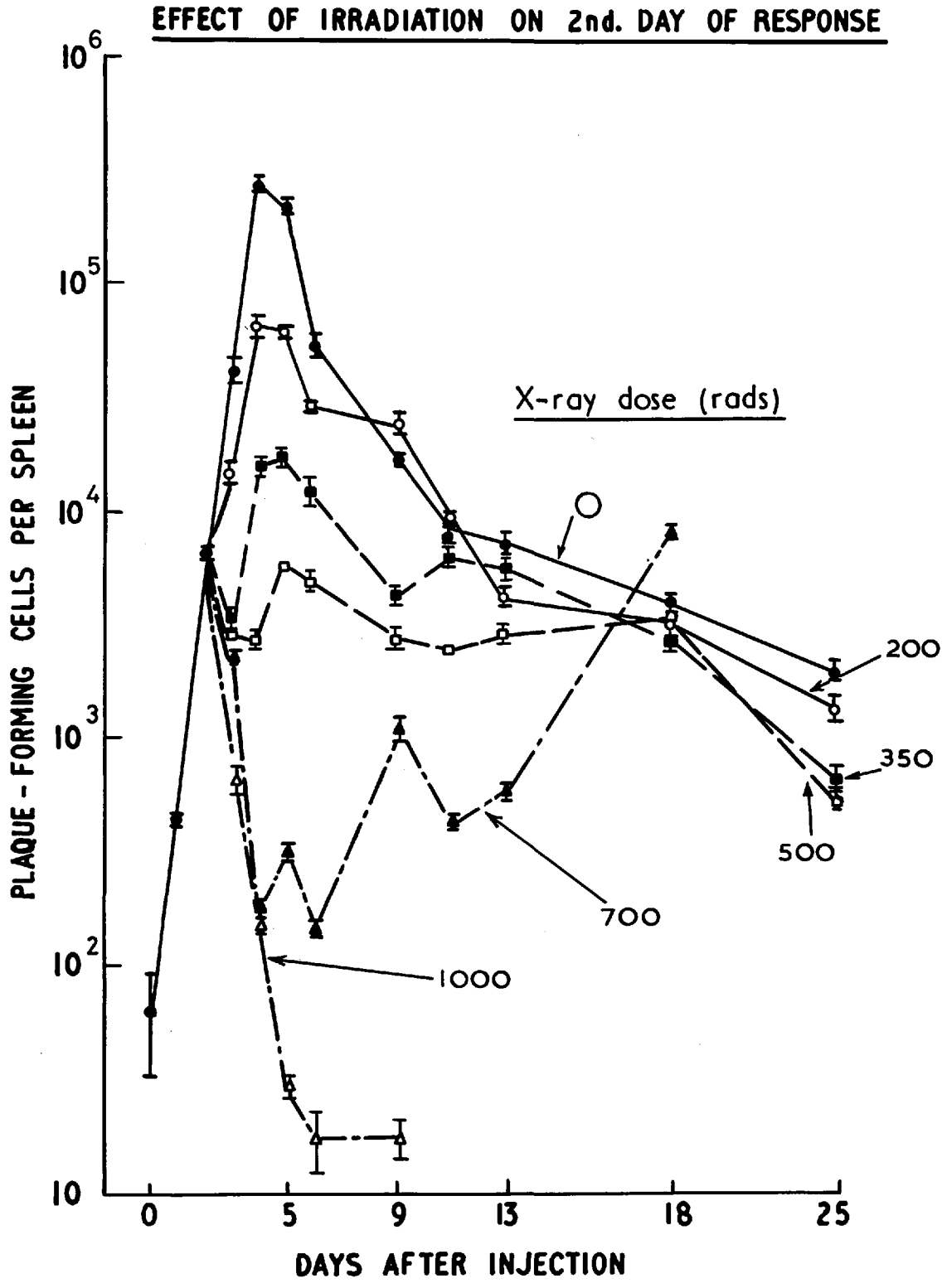

EFFECT OF IRRADIATION ON 2nd. DAY OF RESPONSE
PLAQUE-FORMING CELLS PER SPLEEN
10^6
10^5
10^4
10^3
10^2
10
X-ray dose (rads)
0
200
350
500
700
1000
0
5
9
13
18
25
DAYS AFTER INJECTION

Fig. 19 shows the numbers of 19S PFC per spleen in controls and in mice irradiated with 200-1000 rads. Curves representing those groups which received 50 and 100 rads, which overlapped the control curve, and 1500 rads, which was similar to that of the 1000-rad group, have been excluded for the sake of clarity. The ratio of PFC to total spleen cells for the first part of the response is shown in Fig. 20.

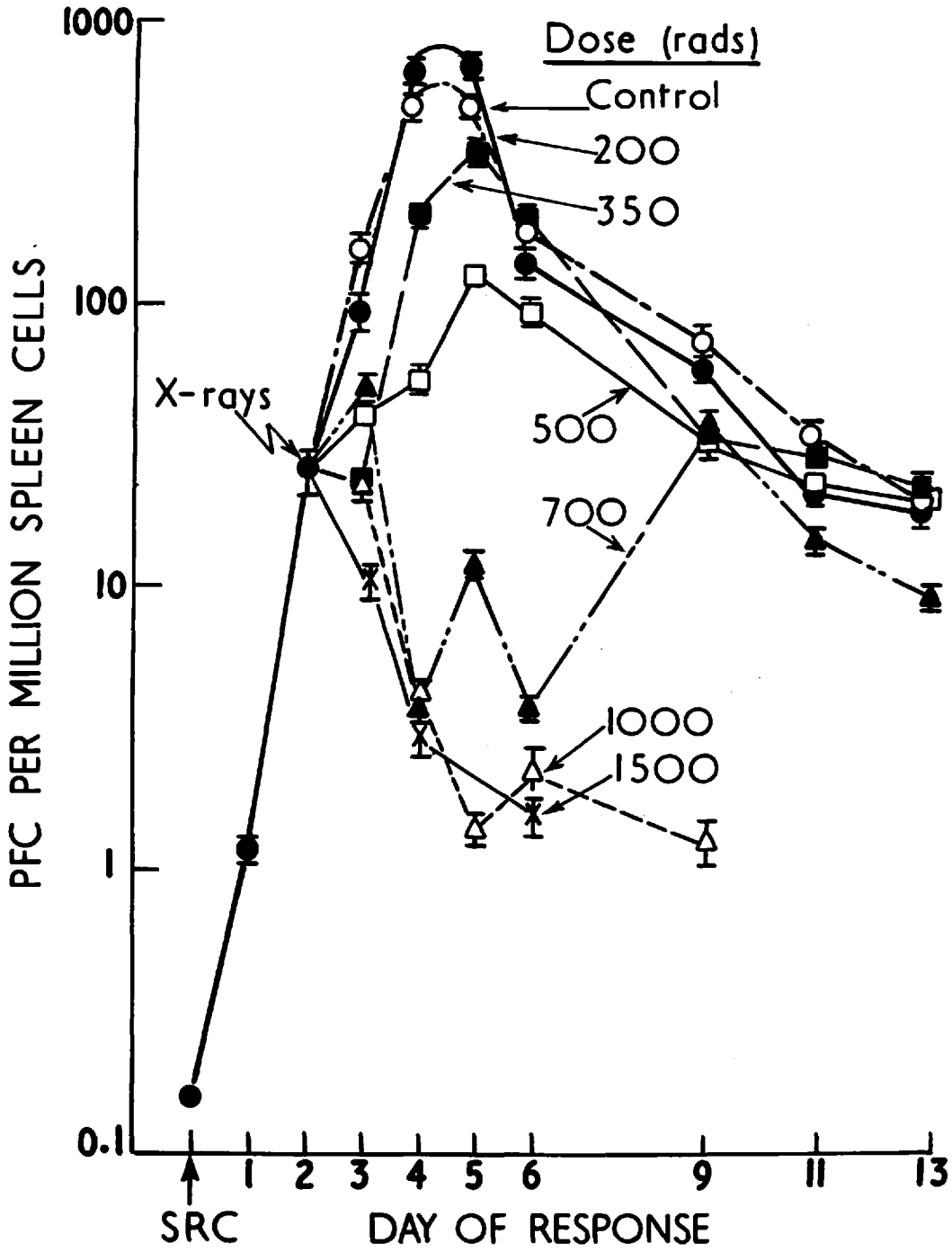
The development of 7S PFC is illustrated in Fig. 21. These were not detectable early in the response for technical reasons (see page 65), nor in those groups irradiated with 1000 or 1500 rads.

Haemolysin and haemagglutinin titres are shown in Fig. 22 and 23.

Further details of the results are summarised in tables 8 and 9, and Fig. 24 and 25. The effects of irradiation on total cell numbers in this experiment were discussed in Section 3, and illustrated in Fig. 5.

Fifty and 100 rads had little effect on either the spleen cell number, or the continuation of the immune response after the second day. However, 100 rads was sufficient to reduce the numbers of 7S PFC below the control values, and delay the second peak slightly. Conversely, there appeared to be some stimulation of the levels of circulating antibodies, particularly haemagglutinin (see Fig. 23), but there was no consistent pattern indicating a true effect. Increasing the dose to 200 rads did not alter the rise in antibody titres to any great extent.

EFFECT OF IRRADIATION DURING RESPONSE UPON PROPORTION OF 19S PLAQUE-FORMING CELLS



Following 200 rads the rate of the 19S PFC response was slowed, but although the peak number was reduced to 62,000 per spleen, about 4 times lower than in the controls, it was not delayed significantly, and the numbers began to fall after the 4th day. With this dose, the reduction in the rate of the response was matched by the overall cellular depletion of the spleens. Therefore the actual proportion of PFC was still almost the same as in the controls (see Fig. 20). 7S PFC were affected to about the same extent, but with these larger doses the first peak (on day 5 in the controls) was lost altogether.

350 rads further slowed the rate of the response; the peak of 19S PFC was reduced to less than 17,000 and slightly delayed until day 5. With this does, the effect on the PFC was greater than on the total cell number, and the actual proportion of PFC was slightly lower than in the controls on days 3 - 5 of the response. The single peak of 7S PFC was also significantly reduced and retarded, from almost 600,000 on day 9 in the controls to 175,000 on day 11.

The appearance of both types of antibody was also slowed, and somewhat reduced, although haemagglutinin titres were above the controls on days 9-13.

500 rads halted any further net increase in 19S PFC, the number remaining near or slightly below the level of 6500 already attained

EFFECT OF IRRADIATION ON SECOND DAY OF RESPONSE ON APPEARANCE OF 7S PLAQUE-FORMING CELLS

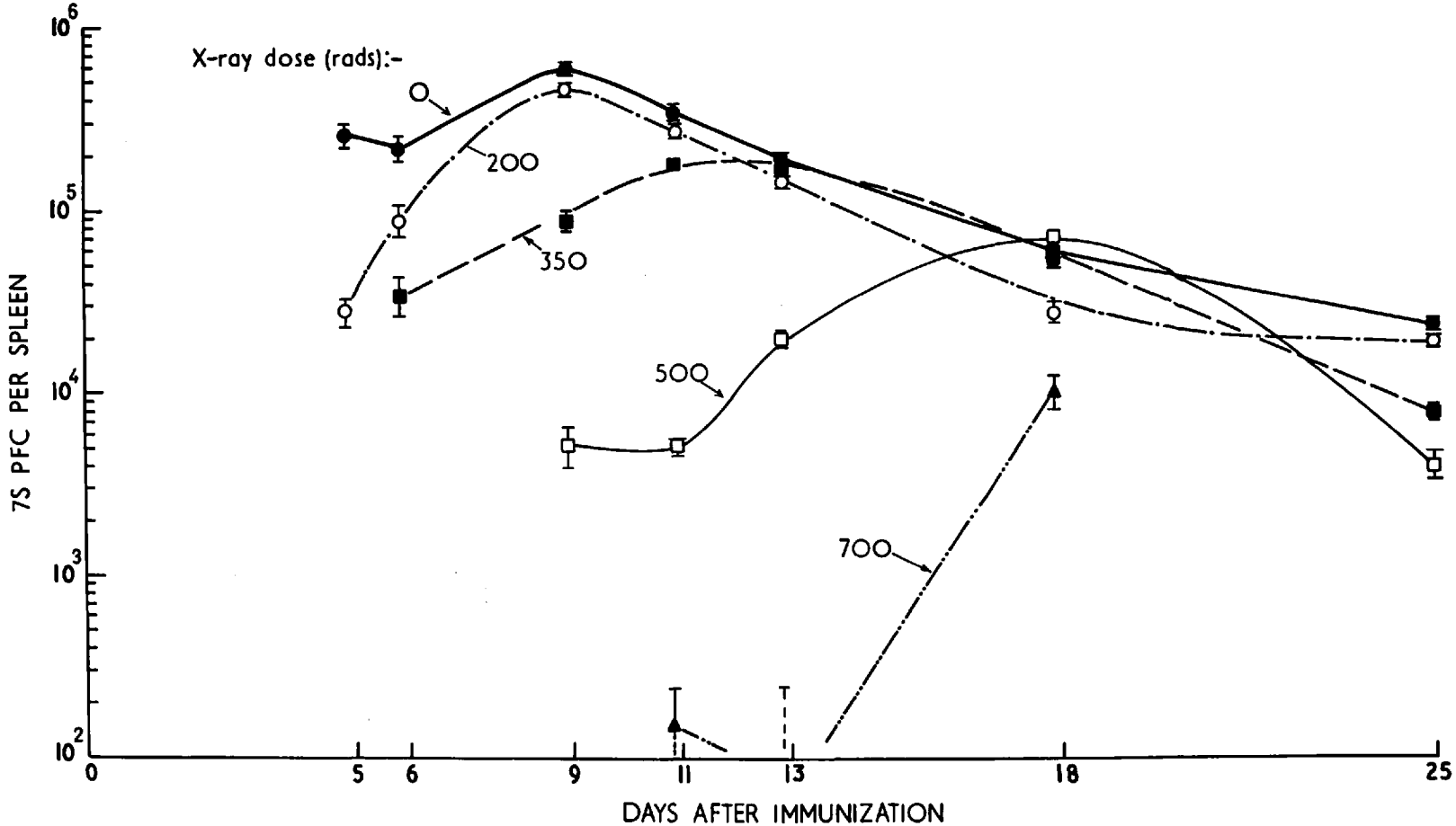


FIGURE 21

at the time of irradiation. However, due to the fall in total spleen cells, the proportion of PFC continued to rise at a reduced rate until the 5th day. The appearance of 7S PFC was delayed until day 9, and the peak number was only 71,000, which was not reached until the 18th day.

With both 350 and 500 rads the numbers of 7S PFC appeared to continue to rise until they reached the control level, which was by then declining. Therefore the curves merged on the graph. This was reminiscent of the "pathways" concept, as described in relation to the 19S PFC responses of irradiated mice (see page 99 and Fig. 12). Unfortunately too few mice survived 700 rads to allow the 7S PFC to be determined after the 18th day, so that a 7S peak was never reached.

While the number of 19S PFC remained fairly constant following 500 rads, both kinds of antibody continued to accumulate, but at subnormal rates. The peak titres were about 2½ times lower than in the corresponding controls, and in the case of haemolysin the peak was delayed by about 4 days, as was the first peak of agglutinin. The titres were shown to be very low on days 3 and 4, following 700-1500 rads, but were not determined after this time. Probably there was a slight increase during the first 24 hours after irradiation, but this was not maintained as the antibody-forming cells died.

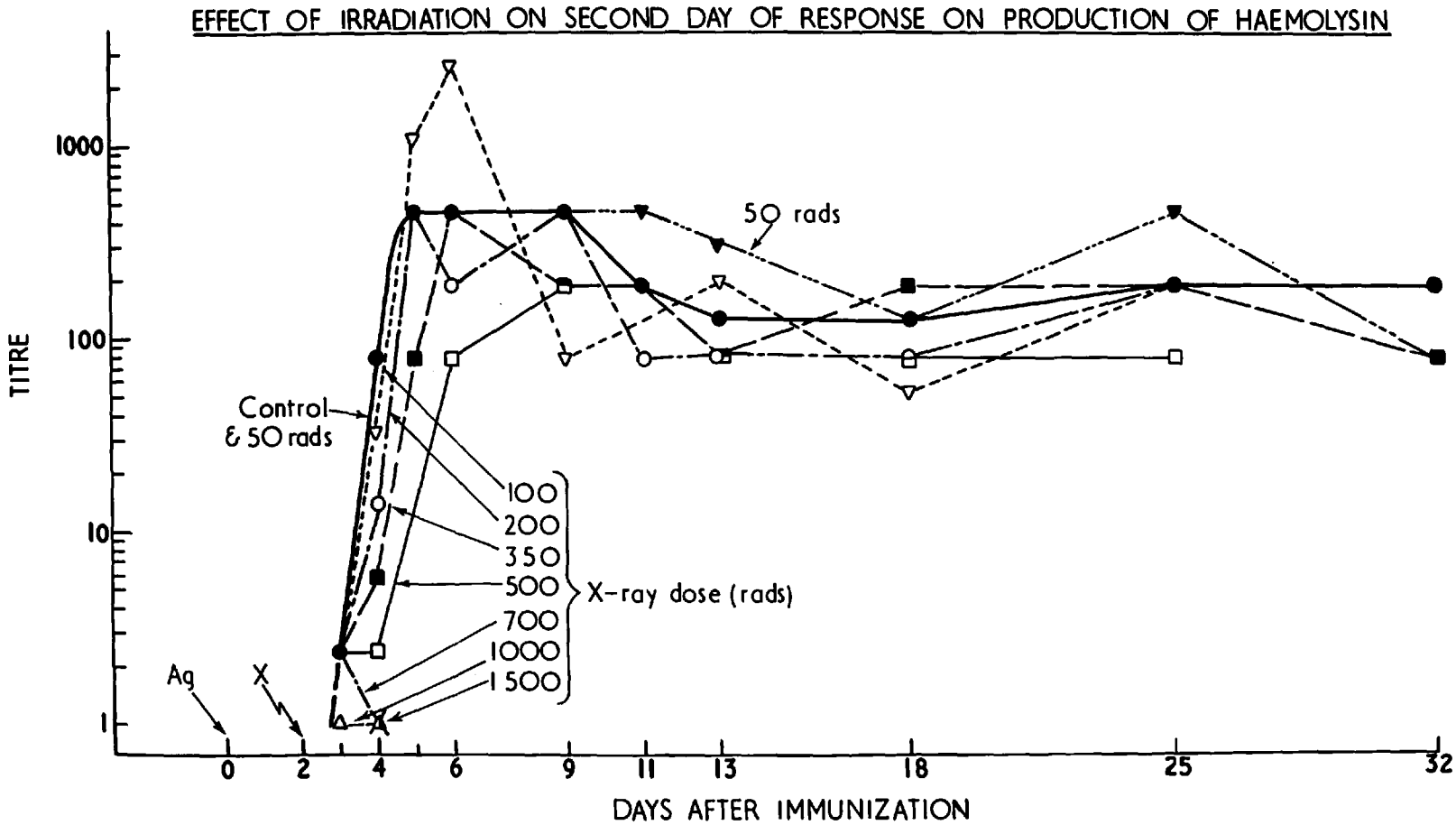

EFFECT OF IRRADIATION ON SECOND DAY OF RESPONSE ON PRODUCTION OF HAEMOLYSIN
TITRE
1000
100
10
1
50 rads
Control
& 50 rads
100
200
350
500
700
1000
1500
X-ray dose (rads)
Ag
X
0
2
4
6
9
11
13
18
25
32
DAYS AFTER IMMUNIZATION

FIGURE 22

With these large doses there was an immediate and sharp fall in the numbers of 19S PFC, which was considerably greater than would be expected from non-specific cellular depletion of the spleens. While the rate of decline of the PFC was related to the dose, there was not a great deal of difference between the three curves during the first 48 hours following irradiation. After this time, the fall was halted in the 700-rad group, and after a further 2 days the numbers of PFC began to rise again, although the total spleen cell number did not begin to increase until 9 days after irradiation. Moreover, the rate of increase of the PFC was slightly faster than that of spleen-repopulation. Although a definite peak could not be defined, because of the mice dying, by day 18 the number had surpassed that of the controls, and recovery seemed to be complete. 7S PFC had also nearly reached the control level by this time, despite being almost completely suppressed on previous days.

The decline in 19S PFC continued for 4 days with doses of 1000 and 1500 rads, in both cases reaching a level almost 40 times lower than on the 2nd day of the response, and almost 4 times below the normal background number of n-PFC. The number stabilized at this level in the 1000-rad group, but only one further test (on the 9th day of the response, 7 days after irradiation) was possible. There were no survivers in the 1500-rad group more than 4 days after irradiation. 7S PFC could not be detected so early in the response.

EFFECT OF IRRADIATION ON SECOND DAY OF RESPONSE ON PRODUCTION OF HAEMAGGLUTININ

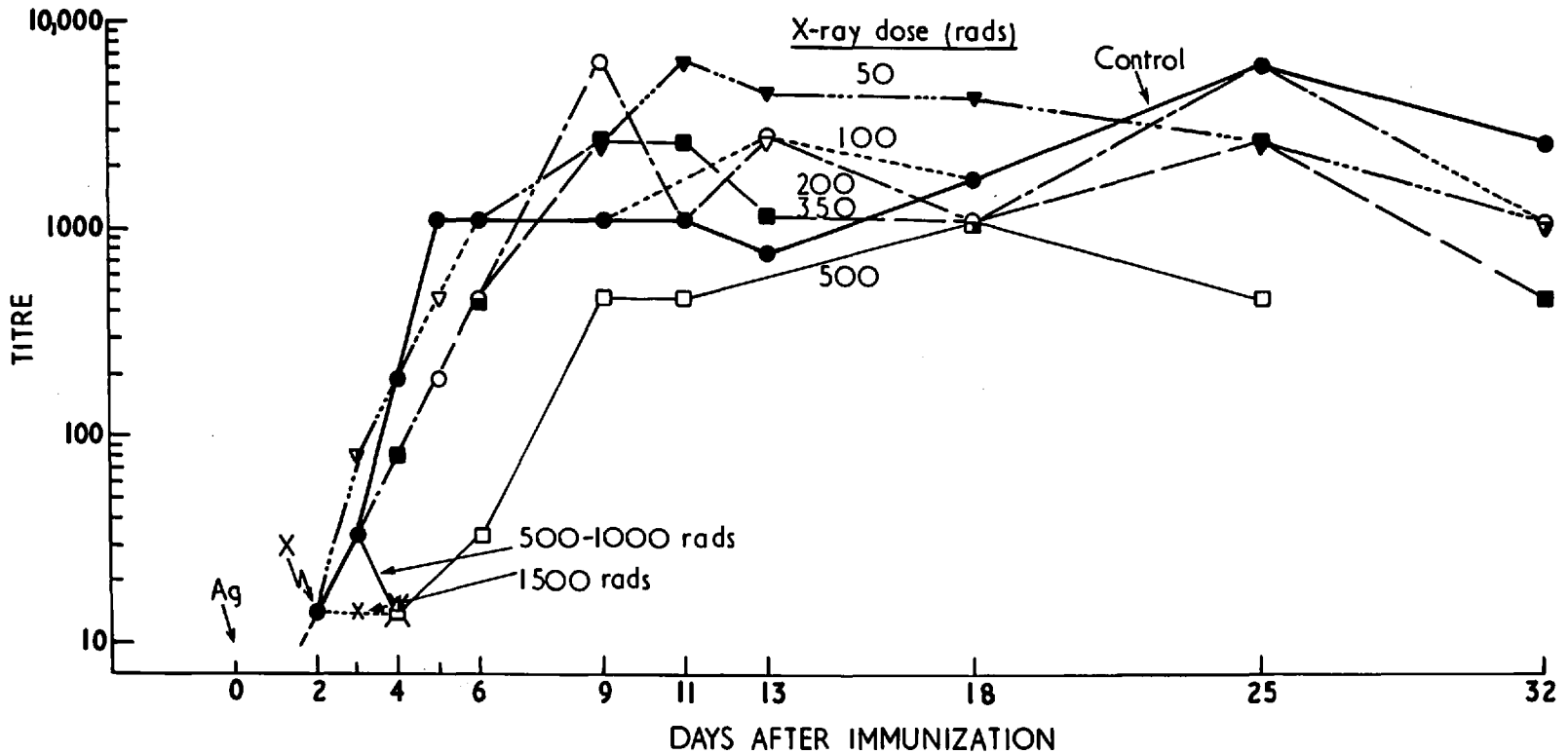


FIGURE 23

Since the response was already at an active stage at the time of irradiation, it was possible to observe simultaneous dose-effects during the first 48 hours afterwards, without the complications arising from changed timing effects which were obtained in mice irradiated prior to immunization. A set of dose-effect curves is shown in Fig. 24, which is based upon the estimated numbers of total spleen cells, 19S PFC, and haemolysin and haemagglutinin titres 48 hours after irradiation, expressed as proportions of the numbers or levels in the controls at this time.

There was a small "shoulder" in the curve for the 19S PFC, equivalent to 60 - 70 rads, but thereafter up to 700 rads it appeared to be exponential, with a D_0 of 92 rads, and an extrapolation number of approximately 2. Doses of 1000 and 1500 rads seemed relatively less effective and the curve became more horizontal. This might indicate a maximum rate of cell-loss being approached.

A similar result would be obtained by comparing the rates of increase or decrease of the PFC during the period 0 - 48 hours following irradiation. These are set out in table 8.

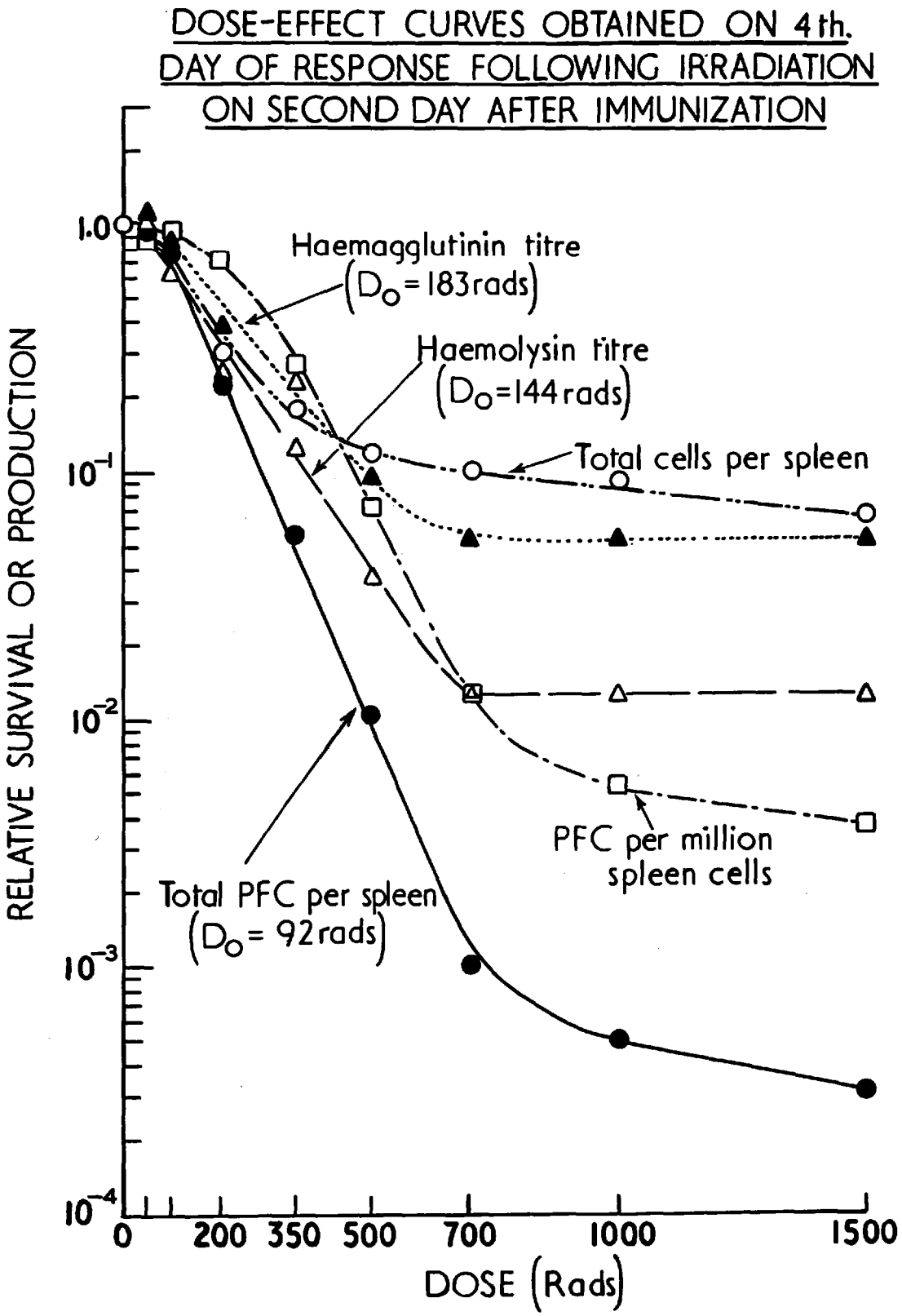

DOSE-EFFECT CURVES OBTAINED ON 4th. DAY OF RESPONSE FOLLOWING IRRADIATION ON SECOND DAY AFTER IMMUNIZATION
RELATIVE SURVIVAL OR PRODUCTION
1.0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
Haemagglutinin titre (D_o = 183 rads)
Haemolysin titre (D_o = 144 rads)
Total cells per spleen
PFC per million spleen cells
Total PFC per spleen (D_o = 92 rads)
0
200
350
500
700
1000
1500
DOSE (Rads)

Table 8 Mean Doubling and Halving Times of 19S PFC during first 48 hours following Irradiation on Second Day of Response

Dose (rads)	Doubling or Halving Time (hours)
0	+ 8.37
50	+ 8.64
100	+ 9.04
200	+14.5
350	+50.9
500	-37.0
700	-10.8
1000	- 8.70
1500	- 7.61

\+ = PFC increasing

\- = PFC decreasing

Doses up to 350 rads merely slowed the response during this period, but with 500 rads or more the slope actually became negative. Following 1500 rads the number of PFC halved every 7.6 hours, compared to a mean doubling time of 8.4 hours in the controls.

When the 4-day titres of haemolysin and haemagglutinin were considered, survival curves were obtained with small shoulders equivalent to about 50 rads, and exponential slopes between 50 and 500 rads with D_o values of approximately 144 and 183 rads, respectively. With doses above 500 rads the curves became shallower, and apparently horizontal with 700-1500 rads. Within experimental limits, there was good agreement between the survival curves for 19S PFC and haemolysin in the serum. Following the smaller doses, PFC continued to appear, although at a slowed rate, and new antibody was synthesized. However with over 700 rads, the response was virtually halted and antibody synthesis soon terminated. The levels detected therefore probably represented the remainder of the antibody produced by the initially-stimulated cells.

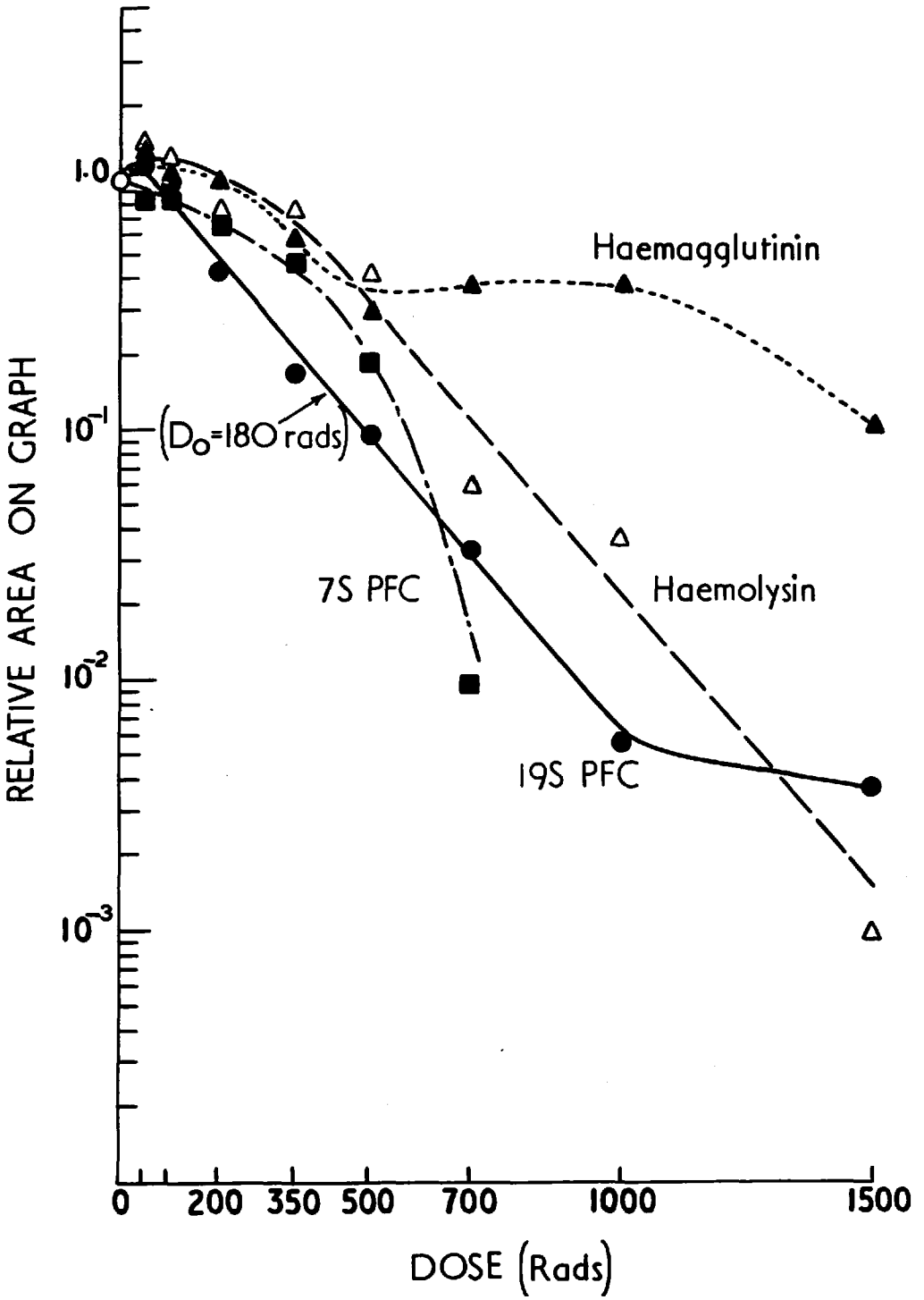
As in the case of mice irradiated before they were immunized, the initial depression was followed by a recovery in the numbers of PFC to near-control levels, so that the overall synthesis of antibody was much less affected than the initial inhibition of the response would suggest. The overall immune response was assessed by estimating the relative areas under the curves in Fig. 19, 21, 22 and 23 during the period 0 - 23 days after irradiation. The results are set out in table 9 and all included in Fig. 25.

Table 9 Effects of Irradiation on the 2nd Day on the Subsequent Development of the Immune Response, as Indicated by the Relative Areas Under the Curves in Fig. 19, 21, 22 & 23

Dose (rads)	19S PFC (Fig. 19)	7S PFC (Fig. 21)	Haemolysin (Fig. 22)	Haemagglutinin (Fig. 23)
0	1.00	1.00	1.00	1.00
50	1.16	0.817	1.33	1.22
100	0.983	0.835	1.22	0.988
200	0.431	0.662	0.743	0.976
350	0.171	0.461	0.755	0.581
500	0.0944	0.185	0.419	0.296
700	0.0337	0.0095	0.060	0.389
1000	0.0055	-	0.036	0.389
1500	0.0037	-	0.001	0.105

The response as indicated by the numbers of 19S PFC again showed a small shoulder (equivalent to 60-70 rads - see Fig. 25), unlike the corresponding "survival curve" in Fig. 18 for the immune response in pre-irradiated mice. However, since the latter curve had points corresponding only to doses of 0, 200, 350, 500 and 700 rads, it is possible that a similar shoulder would have been detected if low-dose groups had been included in the previous experiment (see page 102).

EFFECT OF IRRADIATION 2 DAYS AFTER IMMUNIZATION ON CONTINUATION OF RESPONSE



In any case, with doses in the range 100-1000 rads, the curve for 19S PFC in Fig. 25 was approximately exponential, with a D_o value of 180 rads, which was about 20% higher than that for 19S PFC in animals irradiated before immunization. This again seemed to confirm the greater degree of apparent radioresistance which develops as the immune response progresses. However, a strict comparison of these two curves may not be justified. Following 1500 rads the mice died before the number of PFC reached a minimum, and no significance can be attached to the shape of the curve in the high dose-range.

The curve for the production of 7S PFC was even more subject to bias with the large doses, since the 7S response was delayed, while the mice died sooner. (With the largest doses it could never be detected, due to their early mortality). Possibly with doses up to 500 rads 7S PFC were less affected than 19S PFC.

Haemolysin synthesis appeared to continue relatively unaffected by doses up to 350 rads, but thereafter it fell with increasing dose, approximately in parallel with effect on 19S PFC, allowing for the limitations of the data. On the other hand, haemagglutinin production, which was more readily detectable, appeared to continue for 24 hours after the larger doses of radiation before the titre began to decline, so that during the short period of survival the mean titre could not fall very much below the control value. In any case the titres during the first few days were too low for accurate determination.

Section 7: Effect of Split-Dose Irradiation

Preliminary experiments showed that there was a sparing effect if a total dose of 500 rads was given in two fractions of 250 rads separated by 1 hour or more. The following experiment was designed to confirm this sparing effect, and to show how the responses of mice which had received split-dose irradiation compared to those following various unfractionated doses.

Two doses of 250 rads were given to six groups of mice, separated by the following time intervals:- 1, 3, 4, 6, 9 and 12 hours. Single doses of 350, 450 and 500 rads were given to three more groups.

In order to diminish variations in the immediate effects of irradiation, the mice were left for a mean period of 46 hours before being injected with a standard dose of SRC. (In the case of split-dose irradiation, this period was calculated from the mean time of the two fractions).

Splenic plaque-forming cells were determined before immunization, and on days 3-20 of the response. The results are shown in Fig. 26 and 27.

In Fig. 26 the responses of the groups which received 250 plus 250 rads (split-doses) are shown. Also included is the basic curve for PFC in the mice irradiated with a single dose of 500 rads, which is also shown in more detail in Fig. 27, together with the results for the other single-dose groups (350 and 450 rads).

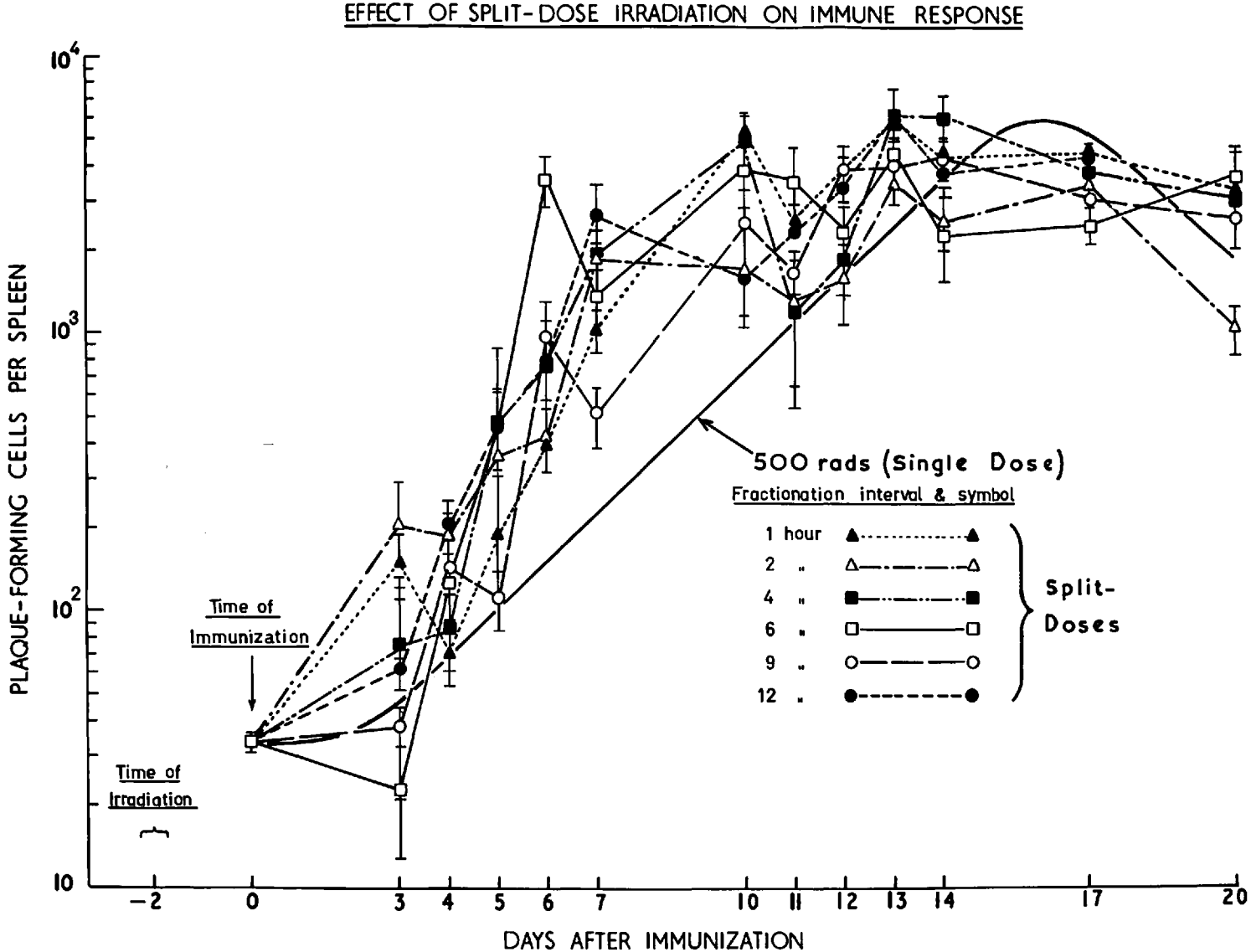

EFFECT OF SPLIT-DOSE IRRADIATION ON IMMUNE RESPONSE
PLAQUE-FORMING CELLS PER SPLEEN
10^4
10^3
10^2
10
Time of Immunization
Time of Irradiation
500 rads (Single Dose)
Fractionation interval & symbol
1 hour
2 "
4 "
6 "
9 "
12 "
Split-Doses
−2
0
3
4
5
6
7
10
11
12
13
14
17
20
DAYS AFTER IMMUNIZATION

FIGURE 26

Referring to Fig. 26, no consistent sequence of recovery between the six "split-dose" groups of mice was apparent. In general the differences were small and the curves frequently overlapped each other. However, with the exception of a few points, all were above the 500-rad single-dose curve between the 3rd and 14th days of the immune response, indicating that the effect of this dose in two fractions was less than for 500 rads as a "single shot" for all the fractionation intervals tested. The differences were most marked about the 7th day after immunization. Although the maximum numbers of PFC attained were about the same in all the groups irradiated with a total of 500 rads, they were reached sooner with the split-dose groups, especially in that with a fractionation interval of 6 hours.

It would seem, therefore, that a sparing effect occurs, and that this takes place within the first hour after irradiation.

Since there were no significant differences between the PFC-responses of the 6 split-dose groups, the combined means of the individual group means were calculated for each day, and have been included in Fig. 27 with the results for the unfractionated groups. The vertical lines indicate the standard errors of the

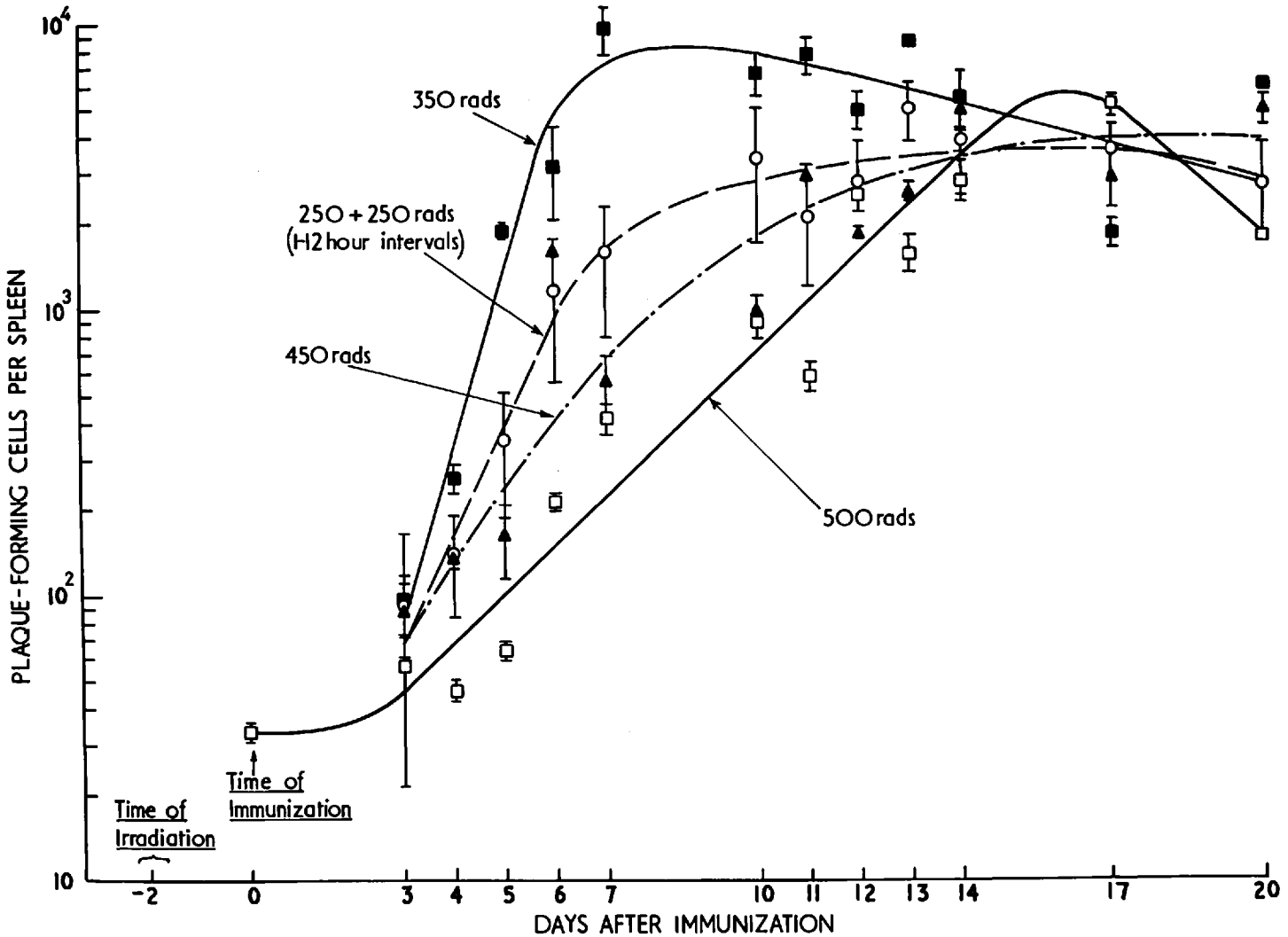

IMMUNE RESPONSES FOLLOWING SINGLE AND SPLIT DOSES OF X-RAYS
PLAQUE-FORMING CELLS PER SPLEEN
10^4
10^3
10^2
10
350 rads
250 + 250 rads
(H2 hour intervals)
450 rads
500 rads
Time of Irradiation
Time of Immunization
-2
0
3
4
5
6
7
10
11
12
13
14
17
20
DAYS AFTER IMMUNIZATION

FIGURE 27

combined means. (On all other points they indicate the 95% confidence limits, but these could not be calculated for the means of different groups with variances which were statistically significantly different).

A comparison of the pooled results for the split-dose groups and the "single shot" groups (see Fig. 27) shows that on the 7th day after immunization there were approximately 5 times as many splenic PFC in the split-dose groups as in the single-dose 500-rad group, twice as many as in the 450-rad group, but 4 times fewer than in the 350-rad group.

This indicates that fractionation of a total dose of 500 rads resulted in a response similar to that which would be expected if the mice were given an unfractionated dose of 400-450 rads. Thus the sparing effect was equivalent to a dose-reduction of approximately 75 rads.

There also appeared to be a slight sparing effect on the total spleen cell population, but the results were very variable. However, it was less than that on the PFC response, so that the actual proportion of PFC among other cell-types was generally higher in the split-dose irradiated groups than in the 500 rad (single dose) group.

Section 8: Recovery of Immunocompetence following Irradiation

In order to study the recovery of the ability to give a normal response to antigenic stimulus following irradiation, three main experiments were performed.

(i) Recovery of Immunocompetence as Indicated by 4-day Responses at Various Times following 500 rads

In the first experiment groups of mice from the same batch were irradiated with 500 rads at various times, and then all injected with SRC simultaneously. This allowed different lengths of time for the immune mechanism to regenerate. All the mice were tested 4 days after immunization at a time when the maximum differences between the responses of normal and irradiated mice are usually observed (see Section 5). The results are shown in tables 10, 11 and 12 and in Fig. 28.

The effects of irradiation on the "background" levels of n-PFC, which must be taken into account when estimating small immune responses, have been discussed in Section 4 and are illustrated in Fig. 7. To summarise: irradiation slightly depressed the number of n-PFC during the first 20 days following treatment, and then the number rose above the control background.

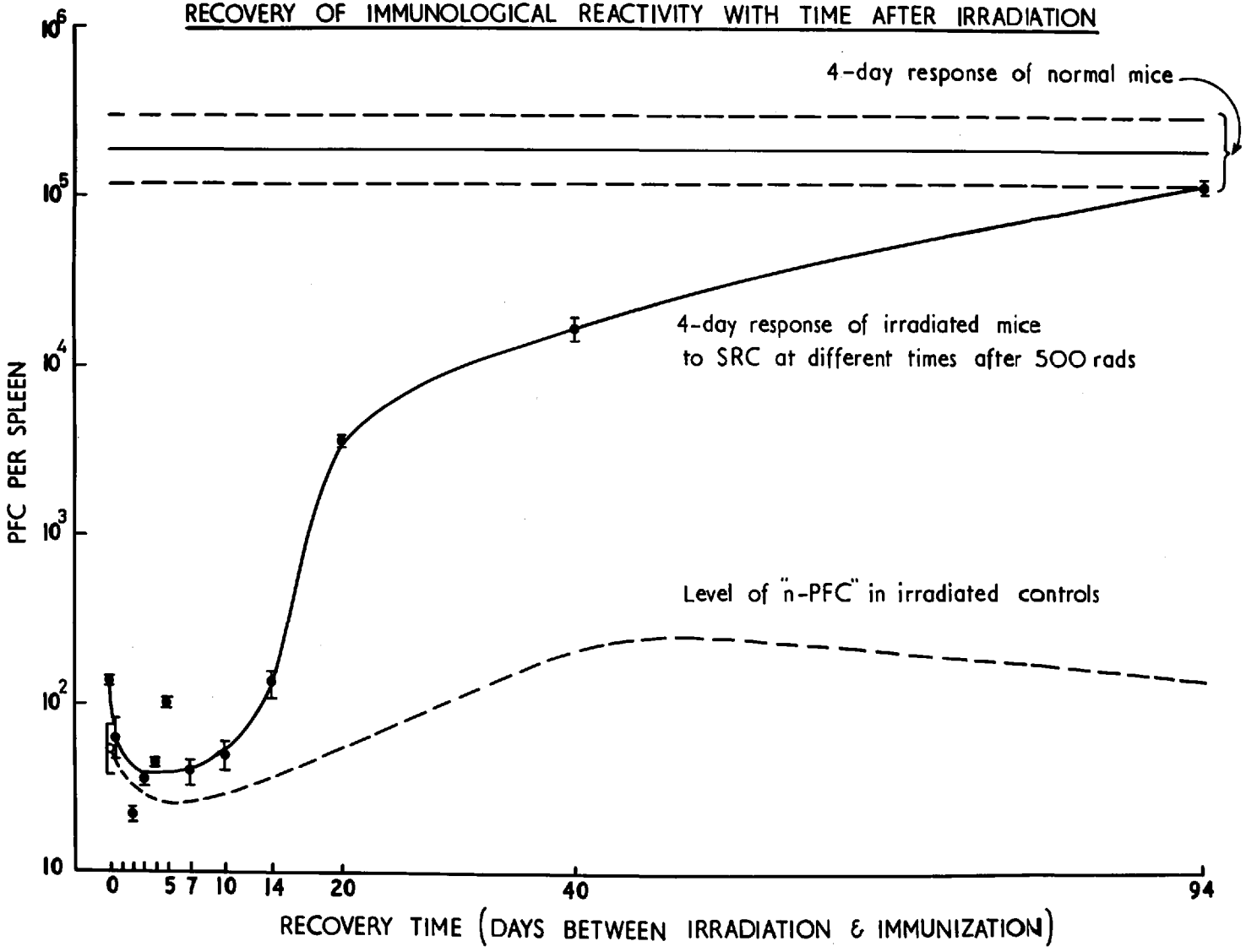

RECOVERY OF IMMUNOLOGICAL REACTIVITY WITH TIME AFTER IRRADIATION
4-day response of normal mice
4-day response of irradiated mice
to SRC at different times after 500 rads
Level of "n-PFC" in irradiated controls
PFC PER SPLEEN
10^6
10^5
10^4
10^3
10^2
10
0
5 7 10 14 20
40
94
RECOVERY TIME (DAYS BETWEEN IRRADIATION & IMMUNIZATION)

FIGURE 28

In table 10 (see overleaf) the immune response has been estimated as the increase in PFC in the immunized groups of mice, compared to the background level in corresponding unimmunized groups, which were irradiated at the same time. Four pairs of unirradiated control groups were also included in the experiment. The difference between the mean number of PFC in the immunized and unimmunized controls was taken as a 100% response, and the responses of the different irradiated groups are expressed as percentages in table 11.

Table 10. 4-day PFC Responses of Control and Previously Irradiated Groups of Mice

Time since Irradiated	PFC per Spleen Immunized	PFC per Spleen Unimmunized	Difference
5 minutes	135	-	-
3 hours	53	-	-
12½ "	64	14	50
24 "	70	21	49
2 days	22	43	-21
2 "	36	28	8
4 "	44	63	-19
5 "	100	19	81
7 "	40	27	13
10 "	50	38	12
14 "	135	63	70
20 "	3,642	42	3,600
40 "	16,703	287	16,416
94 "	117,164	127	117,037
Controls			
A	337,000	70	
B	151,000	40	
C	116,000	39	
D	205,000	73	
*Mean	186,000	53	186,000
±SE	(117,000-295,000)	(38-75)	

*Logarithmic mean ± standard error.
(This gave the best fit to the data; see also Dresser and Wortis, 1967).

Table 11 Percentage Responses at Different Times after Irradiation with 500 rads

Recovery Time (days)	Percentage Response
0-10	0
14	0.07
20	1.9
40	8.9
94	62
Controls	*100

*100% response = increase of 186,000 PFC/ spleen in 4 days following immunization

During the first 10 days after irradiation, the mice failed to give any significant response to SRC within the 4-day period. By the 14th day there was a possible slight response to about 3 times the background level. Thereafter the ability to respond increased steadily, as shown in table 11 and Fig. 28.

When 94 days elapsed between irradiation and the test immunization the response was 62% of the mean control response, and the number of PFC produced fell within one standard error of the control mean. Therefore the response was no longer statistically inferior, but all the other groups were significantly suppressed.

Basically similar results were obtained from antibody titrations of the sera. Haemolysin was determined by the 50% haemolysis microtitre method and by haemolysis on agar plates (Uyeki's method), and haemagglutinin was also determined. The results are presented in table 12.

Table 12 4-day Antibody Titres in Control and Irradiated Groups of Mice

Group	30% Haemolysis	Haemolysis-in-Gel	Haemagglutinin
Irradiated			
0-14 days	<1	<1	<1
20-day	2.4	14	14
40-day	80	190	190
94-day	>3000	1150	»3000
Controls			
A	83) log. mean	200) log. mean	83) log. mean
B	310) = 129	480) = 480	480) = 267
C	83)	1150)	480)
D	<4	3	14

It appeared that in Control group D partial denaturation of the serum may have occurred, resulting in exceptionally low titres. However, it is difficult to explain the extraordinarily high titres in the 94-day group of irradiated mice.

RECOVERY OF 19S PFC RESPONSE FOLLOWING IRRADIATION

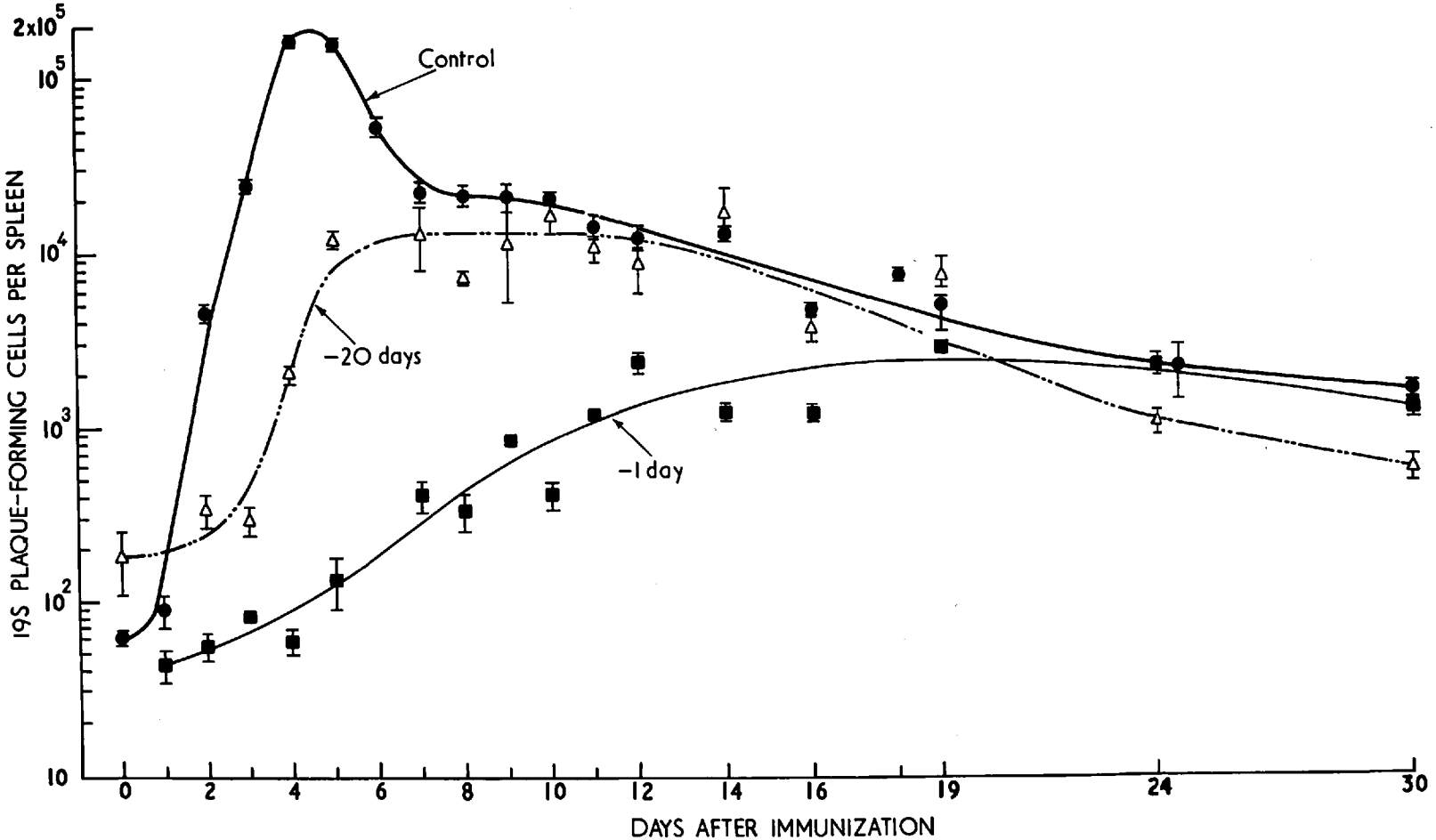


FIGURE 29

Nevertheless, moderately high titres were attained in the first three control groups, whereas no activity was detectable in any of the groups irradiated 14 days or less prior to immunisation. A low level of activity, both lytic and agglutinating, was present in the 20-day group, and this had risen considerably in the 40-day group, although it was still slightly less than in the controls.

Depletion and repopulation of the spleens following irradiation was similar to that shown in Fig. 6 (page 77). The total cell number had already recovered to 64% of the control level within 14 days, and seemed to stabilise at this level. Nevertheless, it was between the 14th and 20th days that the greatest recovery in the ability to produce PFC appeared to occur, and no recovery at all was observed before the 10th day. Even after 3 months recovery was still not complete. Production of both haemolytic and agglutinating antibodies followed a similar recovery pattern, as far as could be ascertained from the results. Thus the recovery of immunological responsiveness seemed unrelated to the general cellular repopulation of the spleen.

(ii) Comparison of Responses of Normal Mice and Mice Irradiated with 500 rads 1 or 20 days before Immunisation

Since the kinetics of the immune response have been shown to be altered by irradiation before immunisation, the recovery of immunocompetence was studied in more detail by comparing the response over a 40-day period. This was performed as part of the experiment described

RECOVERY OF 7S PFC RESPONSE FOLLOWING IRRADIATION

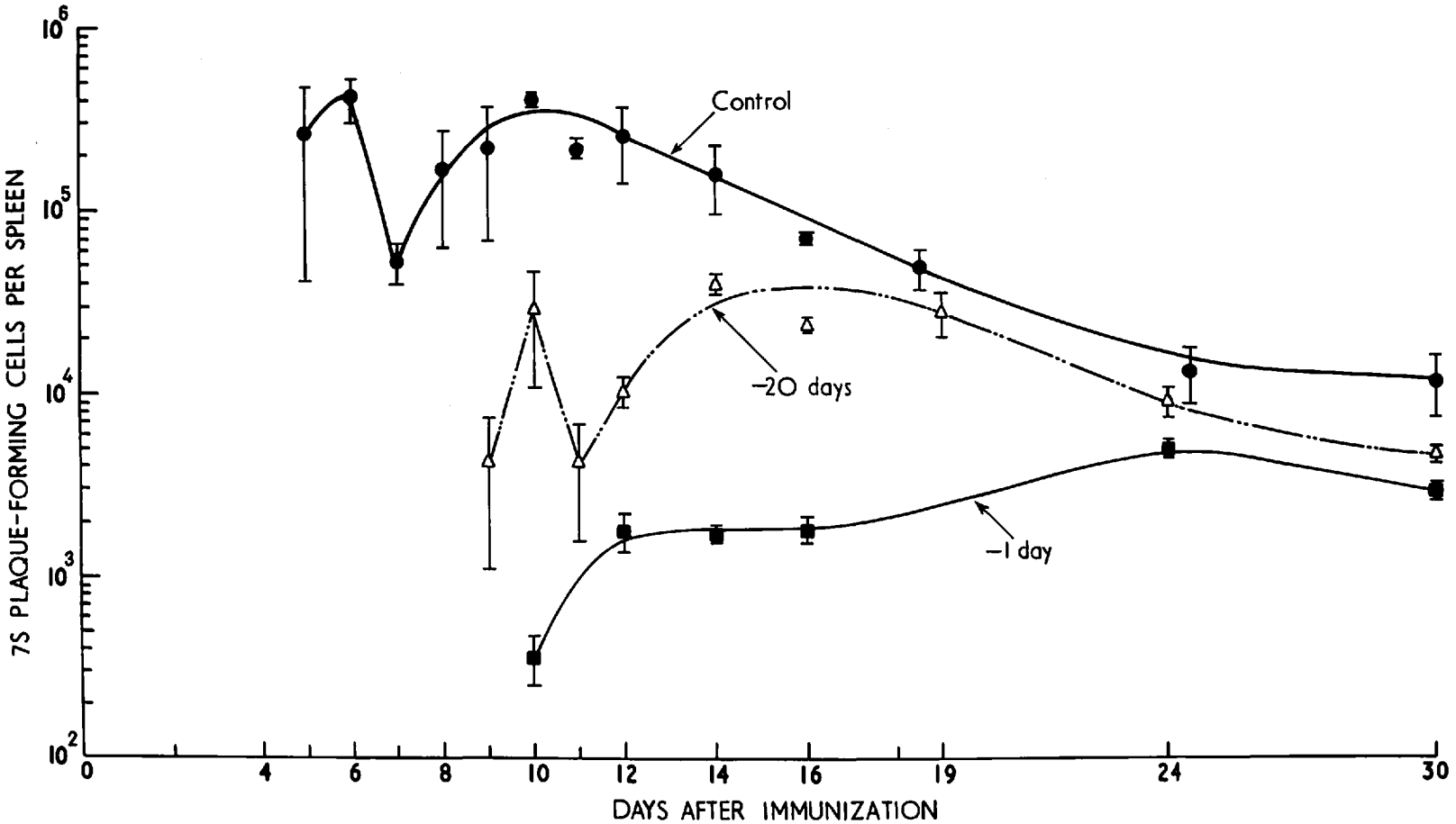


FIGURE 39

in Section 5. The control and "minus 1 day" groups were as already described, but a "minus 20 days" group was also included.

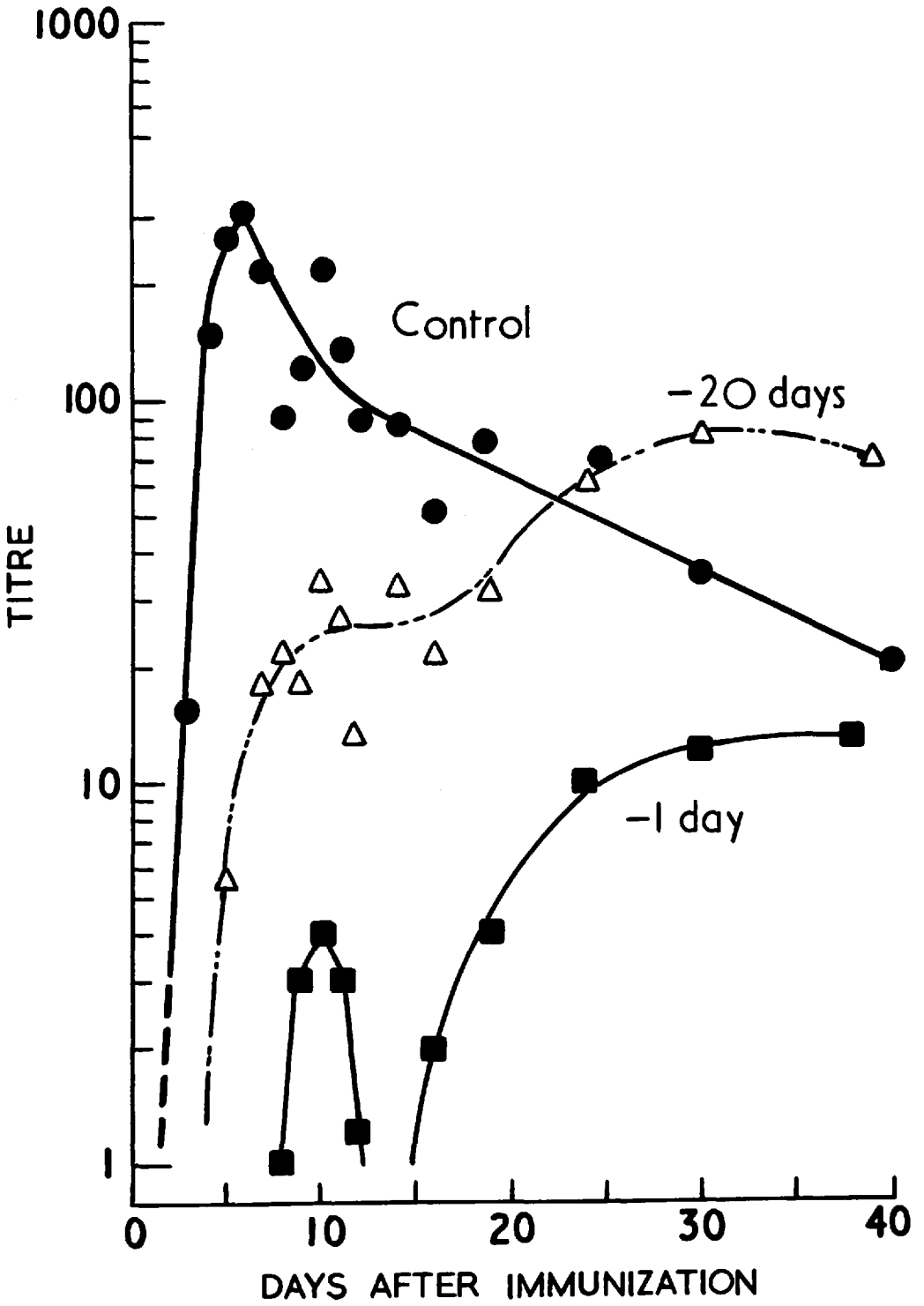
The two irradiated groups received 500 rads 1 or 20 days prior to immunization, and their responses were compared to that of the controls. 19S PFC, 7S PFC and serum haemolysin titres were estimated at frequent intervals, and the results are shown in Fig. 29, 30 and 31, respectively, which correspond to Fig. 9, 10 and 11 in Section 5. Peak numbers of PFC and titres of haemolysin are summarized in table 13, and the relative areas under the curves were also calculated and combined in table 14.

Table 13 Peak Numbers of PFC and Haemolysin Titres in Control and Irradiated Groups of Mice

[a]Recovery Time	19S PFC		7S PFC				Haemolysin Titre			
			1st peak		2nd peak		1st peak		2nd peak	
	day	number	day	number	day	number	day	titre	day	titre
1	19	2,900	12	1,760	24	4,900	10	4	38	13
20	9	13,200	10	28,000	15	37,000	10	30	30	82
Control	4½	190,000	6	406,000	10	388,000	6	300	-	-

[a]Days between irradiation and immunization.

RECOVERY OF HAEMOLYSIN RESPONSE FOLLOWING IRRADIATION



Although the response of the "minus 20 days" group was still considerably weaker than that of the controls, whichever parameter was considered, it showed considerable recovery when compared to the "minus 1 day" group. The various peaks were generally higher, and occurred earlier (see table 13), while the curves were higher throughout the period of observation (Fig. 29-31). The rate of production of 19S PFC, although lagging approximately 48 hours behind the controls, was almost as rapid between days 3 and 5, whereas there was a long slow rise in the "minus 1 day" group.

While the differences between the numbers of 19S PFC were greatest between days 4 and 5 (see Fig. 29), the three curves did not finally merge until about the 19th day. Therefore it was probably useful to compare the areas under the curves in this figure in order to estimate the degree of overall recovery of the immune mechanism. This method was perhaps still more useful for comparing the other two parameters, with their more complex curves.

Table 14 Areas under Curves of Irradiated Groups of Mice in Fig. 29, 30 & 31, as Proportions of Control Values

Group	19S PFC (Fig.29)	7S PFC (Fig.30)	Haemolysin Titre (Fig.31)
(i) Minus 1 day	0.05	0.025	0.07
(ii) Minus 20 days	0.32	0.13	0.54
Relative Recovery (ii/i)	6.4	5.2	7.7

The overall response, as indicated in table 14, showed good agreement between 19S and 7S PFC, and haemolysin titres as indicators of the recovery of the capacity to respond to SRC. This appeared to have increased approximately 6 times between days 1 and 20 following irradiation.

(iii) Recovery of Immunocompetence as Indicated by Responses of Spleen Cells Transferred to Lethally Irradiated Recipients

A second type of experiment was also performed. This was designed to avoid the possibility that the ability to respond to the antigenic stimulus was influenced by physiological, as opposed to cellular, factors. Lethally (850 rad") irradiated recipient mice were therefore used as *in vivo* "culture chambers" for transferred donor spleen cells.

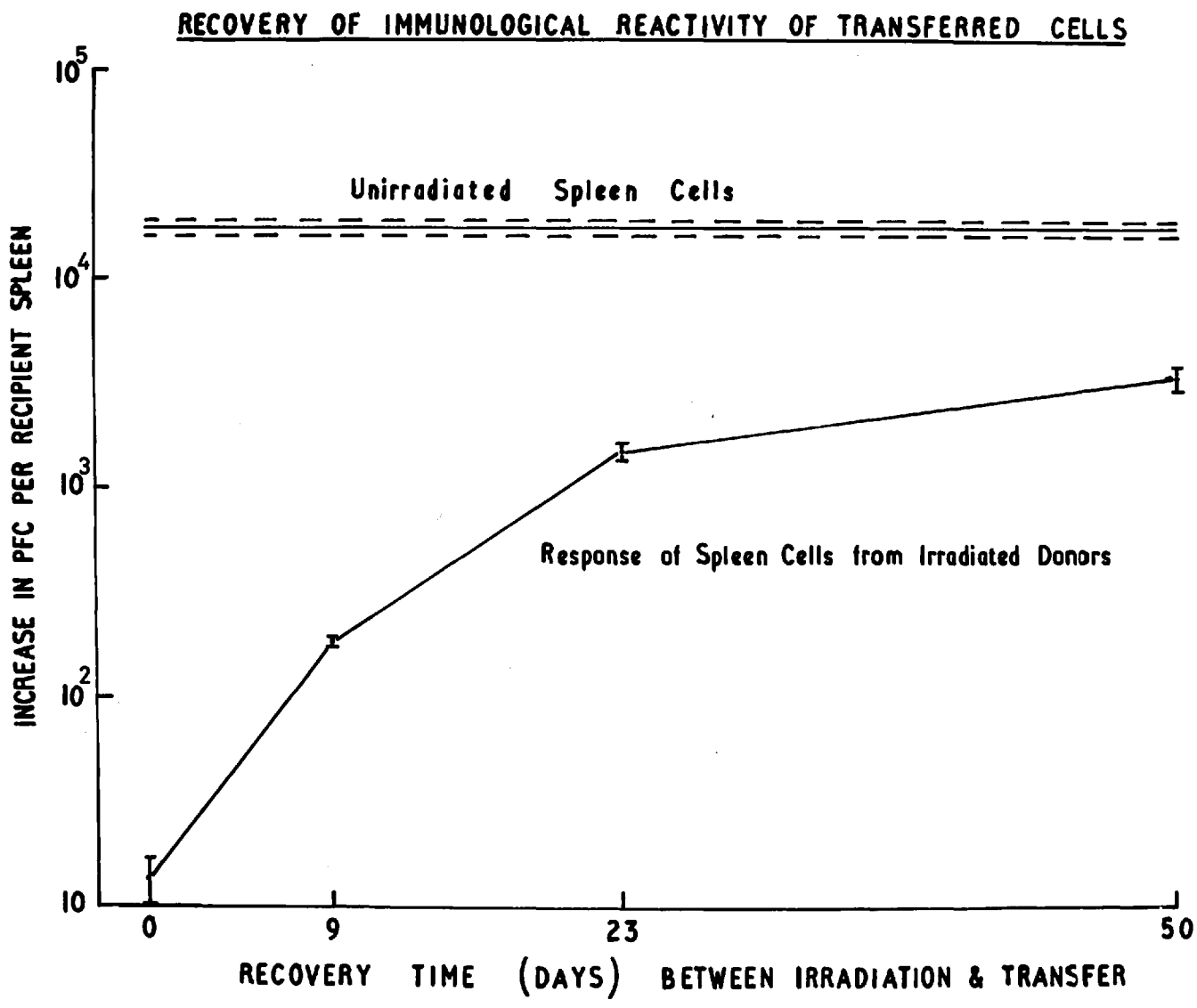

RECOVERY OF IMMUNOLOGICAL REACTIVITY OF TRANSFERRED CELLS
INCREASE IN PFC PER RECIPIENT SPLEEN
10^5
10^4
10^3
10^2
10
Unirradiated Spleen Cells
Response of Spleen Cells from Irradiated Donors
0
9
23
50
RECOVERY TIME (DAYS) BETWEEN IRRADIATION & TRANSFER

FIGURE 32

The spleen cells were obtained from donor mice which had been irradiated with 500 rads 1 hour, 9 days, 23 days or 50 days previously*, or from unirradiated controls. Each recipient was injected intravenously with 50 x 10^6 donor cells from one of the 5 pools. Therefore the only difference was that the cells were from irradiated animals, which had had different lengths of time to recover from the effects of the radiation, or from normal mice. The various donor cells were all injected into similar physiological environments.

Some of the recipients were then injected, about an hour later, with SRC, and all were killed and tested on the 7th day after transfer. One group received no spleen cells, but was injected with the antigen, in order to correct for the response, if any, of the hosts themselves.

The results are shown in tables 15 and 16 and Fig. 32, and were again very clear-cut.

Recipients of unirradiated spleen cells developed 17,360 PFC in their own spleens following the injection of SRC (see

*Cells were also to be transferred from a group of donors irradiated 2 days previously, but this failed for technical reasons.

Table 15 7-Day PFC Responses and Spleen Cell Numbers of Recipients of Spleen Cells from Previously Irradiated Donors

Time since donors Irradiated	PFC per Recipient Spleen & 95% limits	Total Cells per Recipient Spleen ($\times 10^6$) ± 2 x standard error
No donor cells	14.4 (12.0-16.8)	22.3 (20.5-24.1)
1 hour	28 (24-31)	33.7 (31.3-36.1)
9 days	200 (189-210)	53.8 (49.5-58.0)
23 "	1510 (1370-1650)	54.1 (49.9-58.2)
50 "	3410 (2970-3840)	76.2 (69.9-82.6)
Unirradiated	17,360 (15,900-18,830)	69.1 (64.1-74.0)

Table 16 Serum Antibody Levels on the 7th Day after the Transfer of Normal and Irradiated Spleen Cells

Group	50% Haemolysis	Haemolysis-in-Gel	Haemagglutinin
0 & 9-day	<1	<1	<1
23-day	<1	5.8	<1
50-day	5.8	33	33
Unirradiated	33	80	80

table 15). There was also moderate repopulation of the spleens, as compared to the mice irradiated and injected with SRC, but no donor spleen cells. The latter group also had very few PFC, indicating that they themselves were unable to respond significantly to the antigenic stimulus.

The injection of donor spleen cells from mice irradiated shortly before sacrifice allowed some cellular repopulation of the recipients' spleens, but probably the increase in PFC was insignificant. No antibody was detectable in the serum (table 16).

Cells from mice irradiated 9 days previously produced repopulation almost within the control range, but the PFC response was still very slight, and below the threshold necessary for antibody detection. Increasing lengths of time for recovery allowed progressively stronger responses, both in the production of PFC and the appearance of circulating antibodies.

Nevertheless, even a 30-day recovery period for the donor mice was insufficient to allow their spleens to return to the control level, when testing their ability to reconstitute recipient mice immunologically. Thus, again, the activity of repopulating stem cells appeared to precede the production of fresh immunocompetent cells

by a considerable margin of time. Moreover, by transferring a constant number of cells, it is fairly certain that there was both an overall fall in the number of immunocompetent spleen cells, and a decline relative to other cell types.

While the experiments in parts (i) and (iii) of this section showed good general agreement on the repopulation of the spleens of irradiated mice with antigen-reactive cells, there was a slight difference which may have been due to technical reasons. Whereas whole mice immunized 10 (or perhaps even 14 days) after irradiation with 500 rads produced no detectable response to SRC (see table 10), recipients of cells obtained from similarly treated donors 9 days after irradiation showed a small PFC response (table 15). This may have been due to one or both of two factors:-

Firstly, the spleens were still somewhat depleted on the 10th day, whereas the 50×10^6 transferred cells was equivalent to approximately 20% of the entire spleen cell population of un-irradiated mice, and may therefore have contained a larger number of immunocompetent cells than the irradiated spleens <u>in situ</u>. Secondly, and more probably, the longer period between antigenic stimulation and PFC assay (7 days, as opposed to 4) in the transfer

experiment may have allowed the response of the surviving cells to develop further.

In any case the response was very minor, and recovery was only marked after 23 days.

Section 9: Gross Distribution of Antibody-Forming Cells in Relation to Autochthonous Spleen Nodules

Four experiments were performed in which an attempt was made to separate the nodules which appear during the self-regeneration of spleens following large doses of radiation (Brecher, Endicott, Gump and Brawner, 1948; Celada and Wigzell, 1966). It was assumed that in these nodules there would be a relatively high proportion of cells derived from surviving stem-cells, which were stimulated to proliferate in the depleted lymphoid tissues, since Becker, McCulloch and Till (1963) have demonstrated that similar nodules arising in lethally irradiated mice injected with cytologically distinct bone marrow cells are probably colonies, each derived from a single stem cell.

Complete separation of the nodular tissues from the non-proliferating parts of the spleens was not possible, but careful dissection resulted in the preparation of two suspensions, one relatively rich in nodular cells, the other in "non-nodular" cells. The ratio of PFC to other cells in each pool was then estimated by plating and incubating samples.

By immunizing the mice at different times after irradiation it was hoped to show whether antigen-sensitive

precursors were as frequent among developing nodular cells as among the remainder of the splenic tissues. However, the results of such a test would be difficult to interpret if there were considerable cell-migration between the different regions of the spleen.

The X-ray dose used was 500 rads (whole-body), since the majority of mice which received this had several spleen nodules 9-12 days after irradiation. After this time the nodules became so large that confluence made them difficult to distinguish. In all the experiments pooled cell suspensions were used. Individual nodules were not tested separately.

In each experiment unimmunized controls were included, while other groups of mice were injected with SRC either within 3 hours of irradiation (day 0) or 2 or more days later. N-PFC, and/or PFC arising from direct antigenic stimulus, were assayed in the different spleen regions, and an increase in their numbers relative to other cells following immunisation was taken as an indication of the presence of immunocompetent cells. For instance, if the relative increase were greater among nodular cells, this would indicate that the nodules were richer in immunocompetent

cells than the other regions of the spleen. The main findings are summarized in table 17 which shows the results of the 4th and most comprehensive experiment of the series.

Table 17 PFC per Million Spleen Cells in Nodules and Inter-Nodular Tissues of Mice 11 Days after Irradiation

Group	a	b	c	d	e	f	g	h
Days Injected	Control (none)	0	2	7	0/8	2/8	7/8	8
Internodular Tissues (i)	0.80 ±0.08	31.5 ±3.1	25.3 ±2.2	2.52 ±0.24	approx. 222	102 ± 8	3.04 ±0.30	0.85 ±0.11
Nodular Tissues (ii)	1.68 ±0.37	27.2 ±4.9	10.4 ±1.0	3.43 ±0.33	approx. 278	65.6 ±5.7	2.18 ±0.26	1.00 ±0.16
Ratio (i/ii)	0.47 (0.23-0.72)	1.16 (0.95-1.36)	2.44 (2.31-2.56)	0.73 (0.60-0.87)	0.80	1.55 (1.43-1.67)	1.40 (1.24-1.55)	0.85 (0.65-1.05)

It was found in the first three experiments that in unimmunized mice there was a higher proportion of n-PFC among the nodular tissues than in the non-nodular regions of the regenerating spleens. The mean of these was 1.06 PFC per 10^6 in the internodular regions to 1.45 PFC

per 10^6 in the nodular tissues. This gave a ratio of 0.73, indicating that the internodular tissues were relatively poorer in n-PFC than were the nodules. In the 4th experiment the ratio was 0.47 (see group a in table 17).

In groups immunized with a single injection of SRC late in the experiment (d and h), the response had only just begun by the 11th day and the distribution of i-PFC was quite similar to that of n-PFC in group a. Possibly there was a slightly better response in the internodular tissues than in the nodules, which resulted in ratios of 0.73 and 0.85, respectively.

When the first injection of antigen was given on day 2, either alone (group c) or followed by a second injection on day 8 (group f), there was a much better-developed response, with a clear preponderance of i-PFC in the internodular regions of the spleens, especially when compared to the controls (ratios = 2.44 and 1.55).

While the largest responses were observed in groups b and e, in which the mice were injected shortly after irradiation, with a booster injection on day 8 in group e, the distribution of i-PFC was intermediate between those of groups c and f and of the n-PFC in the controls. The same was true in group g, which received two injections late in the response.

Thus it would appear that if the mice are immunized about the time of nodule initiation (2-3 days after irradiation), the development of PFC is greater in the internodular tissues than in the nodules. Immunization earlier or later in relation to nodule development seems to result in a higher relative proportion of PFC in the nodules.

This may be because PFC stimulated by an early antigenic challenge either become numerous in the peripheral regions of the spleen, and so tend to be removed together with the nodules, or possibly they have more time to migrate into, or become engulfed by the developing nodules. These possibilities could therefore be classified as "contamination" of the nodular tissues. Alternatively, some nodules may have actually been PFC-clones. So far individual nodules have not been analysed, but the findings of Celada and Wigzell (1966) would not support the latter possibility.

The low ratio of PFC in the internodular tissues in mice immunized later during the development of the nodules is probably due to the immune response not having developed, with the n-PFC in the nodules still biasing the results.

In conclusion, it would appear that spleen nodules, whether because of physiological "immaturity" or because they are poor in

SRC-responsive cells, do not provide such a suitable environment for the development of PFC as does the "mature" tissue of the remainder of the spleen. Nevertheless n-PFC are relatively numerous in the nodules of unimmunized irradiated mice. Once PFC development has been initiated, a second injection of antigen does not change the relative distribution greatly, but stimulates a larger response. These findings are in general agreement with those of Celada and Wigzell (1966).

Section 10: Effect of Irradiation in vitro on the Immunocompetence of Spleen Cells

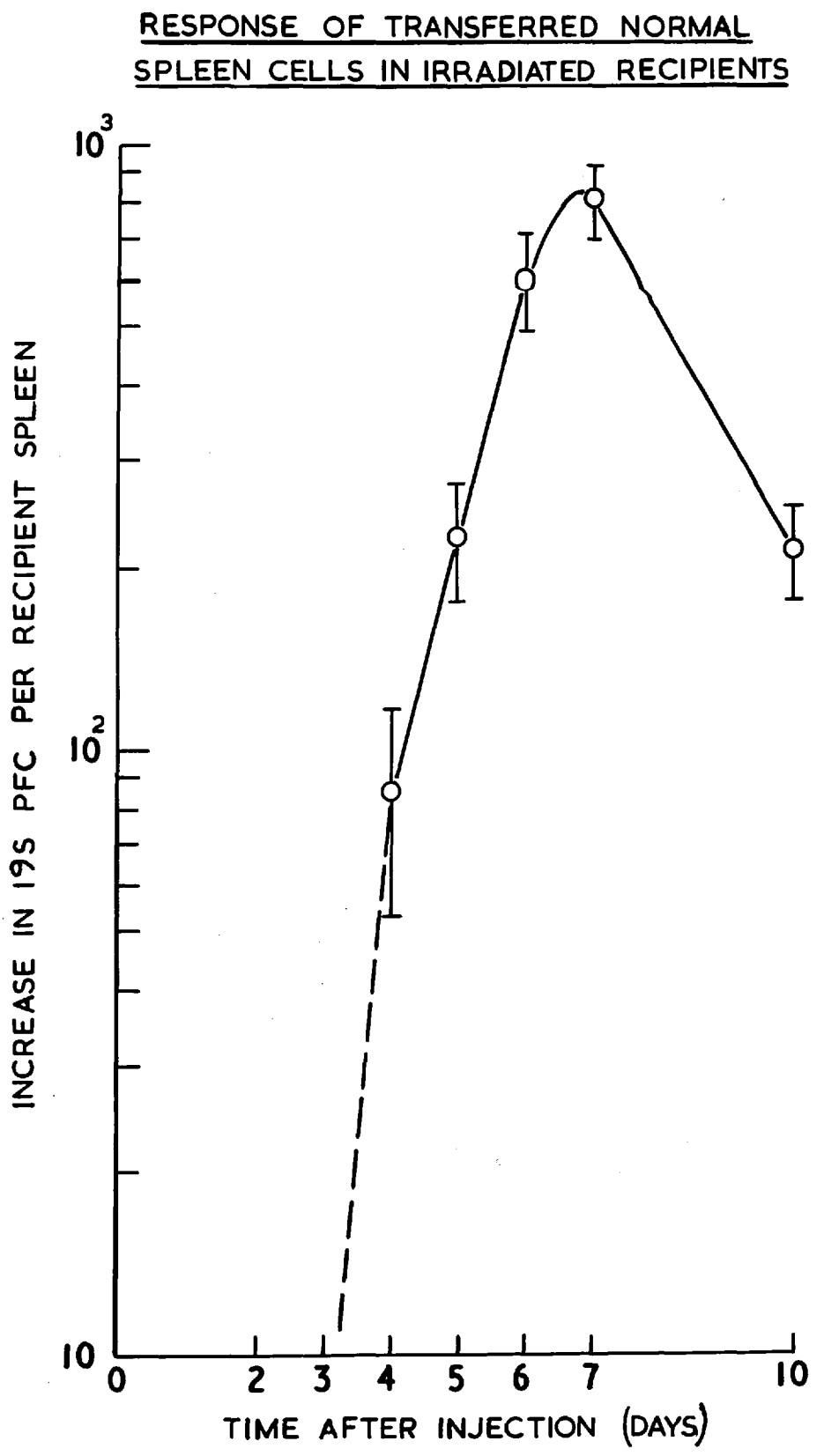
A series of transfer experiments was performed in which the responses of normal and irradiated spleen cells were investigated when "cultured" in lethally irradiated hosts. This allowed the manipulation of the number of competent cells, as well as the dose of radiation they received.

(i) Response of Normal Spleen Cells to Antigenic Stimulation in Lethally Irradiated Hosts

In the first experiment 0.4 ml. volumes of phosphate-buffered saline (PBS) containing 8×10^6 spleen cells from normal, unimmunized, 10-week-old donor mice were injected into similar recipients $2\frac{1}{2}$-$4\frac{1}{2}$ hours following 850 rads whole-body irradiation. The recipients were then injected with SRC about 2 hours later. Groups were killed and the numbers of PFC in their spleens determined on days 3, 4, 5, 6, 7 and 10 after immunization. Various controls were also included: no spleen cells, no SRC; SRC, no spleen cells; spleen cells, no SRC; or untreated (neither irradiated nor immunized). The results are shown in table 18 and Fig. 33.

Untreated mice had a mean of 121 n-PFC per spleen, which was reduced to 31 within 3 days following 850 rads, and remained suppressed. These irradiated, but otherwise untreated mice completely failed to respond to SRC within the 10-day period. The number of n-PFC increased to 72 when normal spleen cells, but not SRC, were injected.

FIGURE 33


RESPONSE OF TRANSFERRED NORMAL SPLEEN CELLS IN IRRADIATED RECIPIENTS
INCREASE IN 19S PFC PER RECIPIENT SPLEEN
10³
10²
10
0
2
3
4
5
6
7
10
TIME AFTER INJECTION (DAYS)

Mice which received spleen cells began to respond to the SRC after 3 days (see Fig. 33). On day 3 the number of PFC per spleen was slightly,

Table 18. PFC per Spleen in Lethally Irradiated Recipients

Irradiation	SRC	Spleen Cells	Day	PFC per Spleen & 95% limits
-	-	-	0	120 (100-141)
			0	122 (91-153)
+	-	-	3	31 (28-33)
			7	23 (19-27)
+	+	+	3	38 (27-49)
			6	19 (15-22)
			10	25 (19-30)
+	-	+	7	72 (51-92)
+	+	+	3	52 (40-64)
			4	157 (131-183)
			5	296 (263-329)
			6	674 (641-708)
			7	886 (820-952)
			10	287 (240-334)

but not significantly, below the unimmunized background. By day 4 this had risen significantly to 157, and continued to rise, approximately exponentially, with a minimum doubling time of 23

hours until it reached a peak of 886 on day 7. By day 10 the response had diminished, and only 287 PFC per spleen were still detectable.

Levels of antibody in the serum were very low in all groups, and never sufficient to produce 50% haemolysis. However, haemolysin was detectable by Uyeki's method on days 6 and 7, with titres of approximately 1 and 2.4 respectively. An agglutinin titre of 14 was also detected on day 7, though this was insignificant on the other days.

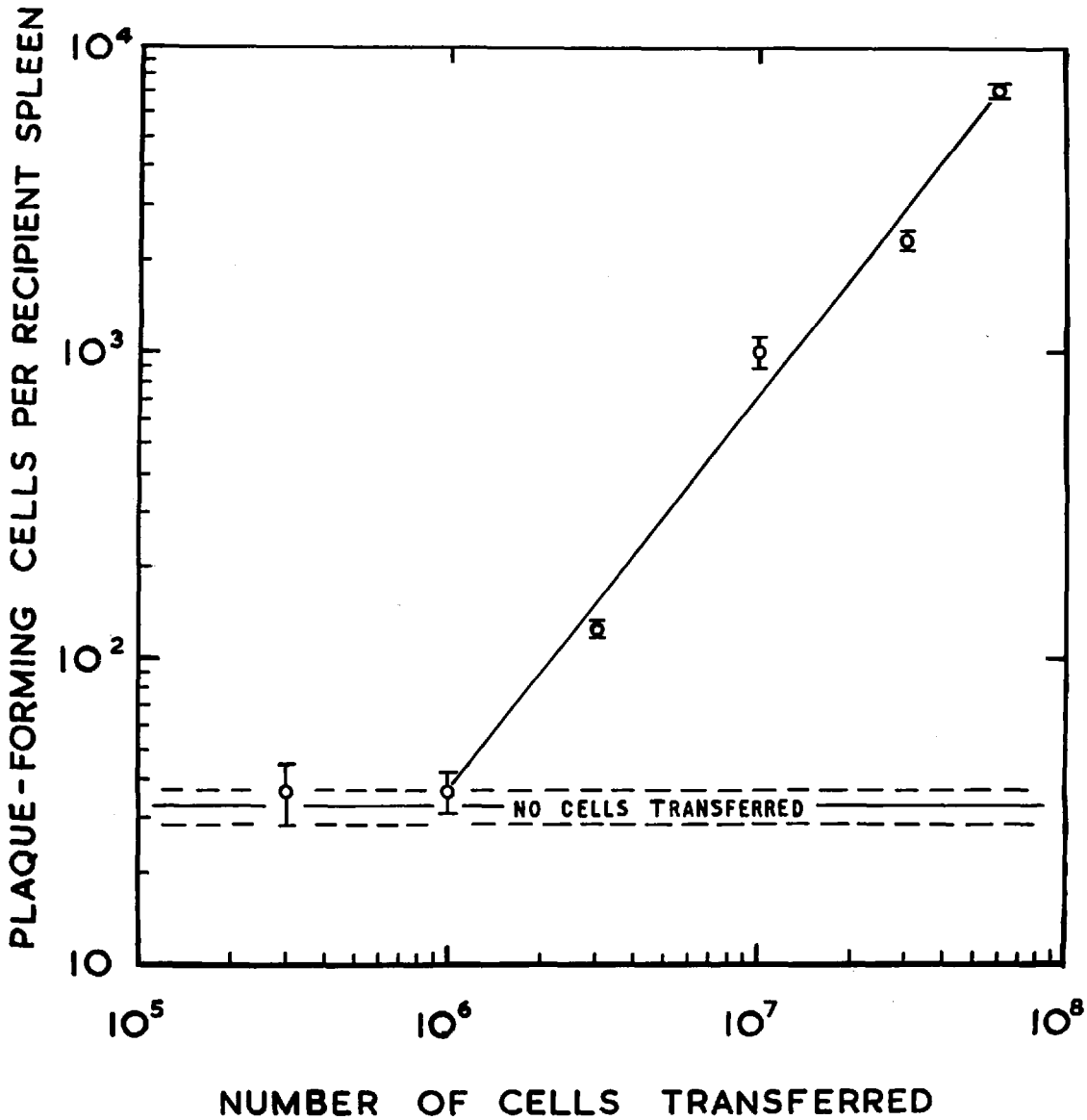
(ii) Relation between Cell Number and Response of Transferred Spleen Cells

In a similar experiment the number of normal spleen cells transferred to irradiated recipients was varied from 0 to 60 x 10^6 per mouse. All the recipients were immunized and their spleens assayed 5 days later. The results are shown in Fig. 34.

Up to 1 million transferred cells had no significant effect on the response of the hosts to SRC. However, increasing the number of transferred cells from 10^6 to 60 x 10^6 produced increasing responses, which when plotted on a two-way logarithmic scale, gave a straight-line relationship between the number of cells injected and the number of PFC obtained. The slope (β), according to the equation

$$y = k. x^{\beta}$$

RESPONSE OF TRANSFERRED SPLEEN CELLS IN IRRADIATED HOSTS



where y = number of PFC, x = number of spleen cells and k = constant, was approximately 1.3, indicating that the response became more efficient as the number of transferred cells was increased.

The most likely explanation for this is that more than one immunocompetent cell-type may be involved in the response to SRC, and that the probability of a synergistic event occurring between them increases in proportion to the product of their individual numbers. Groves, Lever & Makinodan (1969, 1970) have proposed a more complex stochastic model to explain this, which will be discussed in Part IV (see page 20). If two kinds of cell are involved, under ideal conditions this should result in a slope where $\beta = 2$, but various factors are likely to reduce this, e.g. one cell-type may become limiting during the response if the other type is in excess, or divides more rapidly. Such a co-operative mechanism has considerable experimental support, the cells involved being thymus- and bone-marrow-derived lymphocytes (see page 21 in the Introduction).

It is therefore necessary to think of the response originating from "antigen-sensitive units" (ASU) (Shearer, Cudkowicz, Connell and Priore, 1968) which when stimulated give rise to PFC progeny. An ASU may be created when one or more of each cell-type come into contact with each other in the presence of antibody.

From the intercept of the curve with the "background" level of PFC observed when no donor cells were injected, it would appear that the probability of an ASU occurring, or being formed from sensitive precursors among the spleen cell population, is not significantly above zero when 10^6 cells or less are transferred. Since each ASU probably gives rise to many PFC (Playfair, Papermaster and Cole, 1965, estimated this as 200-1000 PFC per precursor), the type of experiment described above does not indicate the original number of ASU in the spleen cell inoculum. However it may be concluded that the probability of one or more ASU occurring increases as the cell number is raised above 10^6. If it is assumed that each ASU produces a mean of 200 PFC by day 5, this would indicate that on average approximately 4×10^6 spleen cells include one detectable ASU (see Fig. 34). Similarly, about 10 ASU are detectable among 22×10^6 cells.

Since only a small proportion of donor cells reach the host's spleen (Playfair et al., 1965, estimated this to be 4% in the case of ASU to SRC), the true proportion of ASU among other cells should be increased by a factor of approximately 25, i.e. to one in 160,000. The corresponding figure obtained by Playfair et al was one per 20,000 donor cells. Shearer et al (1968) obtained an intermediate figure.

Serum antibody titres are shown in table 19.

Table 19. 5-day Antibody Titres in Irradiated Recipients

Number of cells transferred	50% haemolysis	Haemolysis-in-gel	Agglutination
3×10^6	<1	<1	<1
10×10^6	<1	1	2.4
30×10^6	2.4	2.4	1
60×10^6	14	190	80

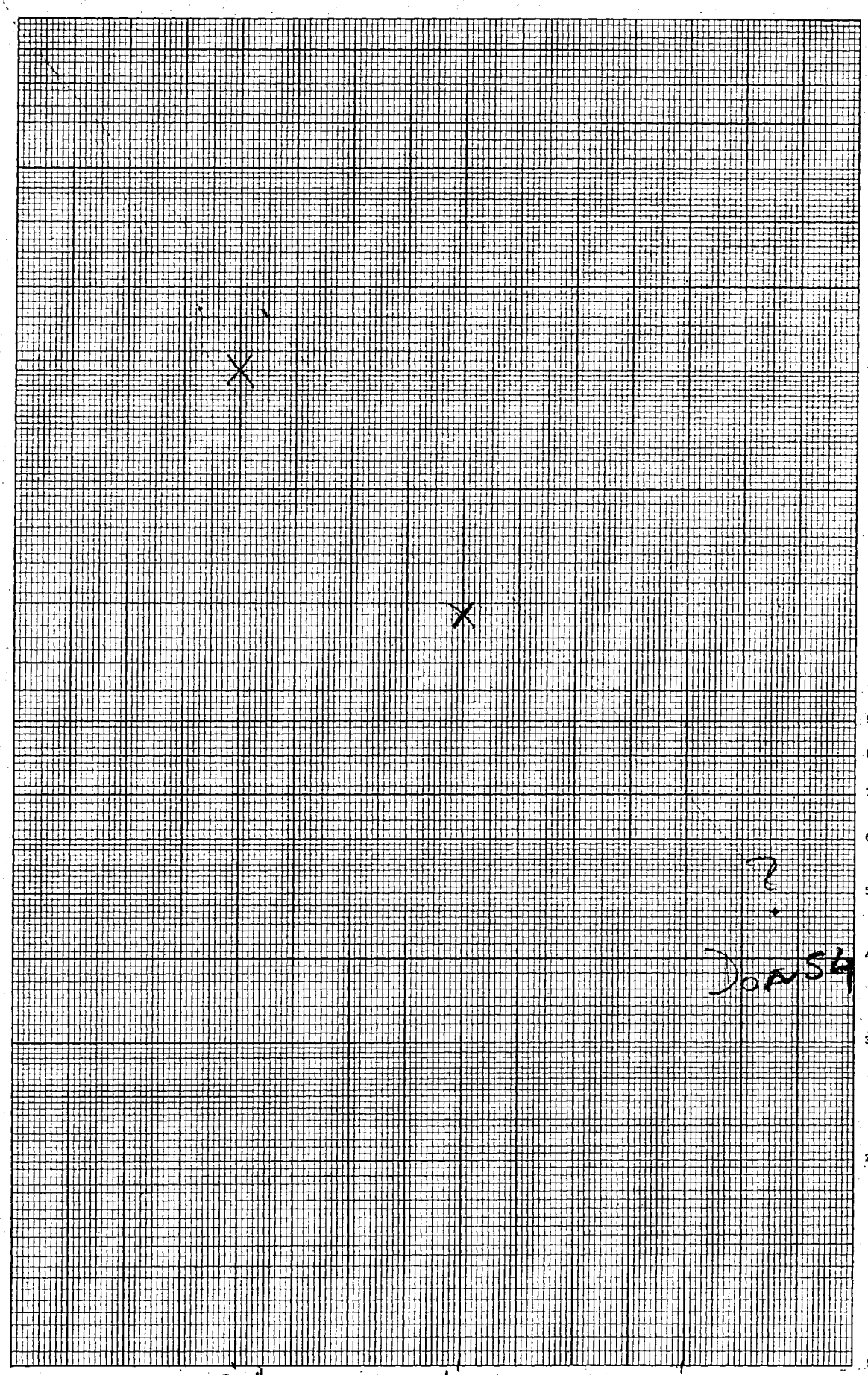
Increasing doses above 3×10^6 transferred spleen cells resulted in increasing antibody titres, but the results were not as consistent as those for the PFC.

(iii) Effect of Irradiation on Response of Transferred Spleen Cells

In the next experiment an attempt was made to estimate the radiosensitivity of the precursors of PFC by determining the numbers of PFC appearing in the spleens of irradiated recipients injected with a fixed number of donor spleen cells. A uniform suspension of normal spleen cells in ice-cold PBS was divided into aliquots which were X-irradiated *in vitro* with doses of 0, 50, 100 or 200 rads at a dose-rate of 40 rads per minute. Each recipient was then injected with 22.3×10^6 cells from the appropriate suspension

Fig 35

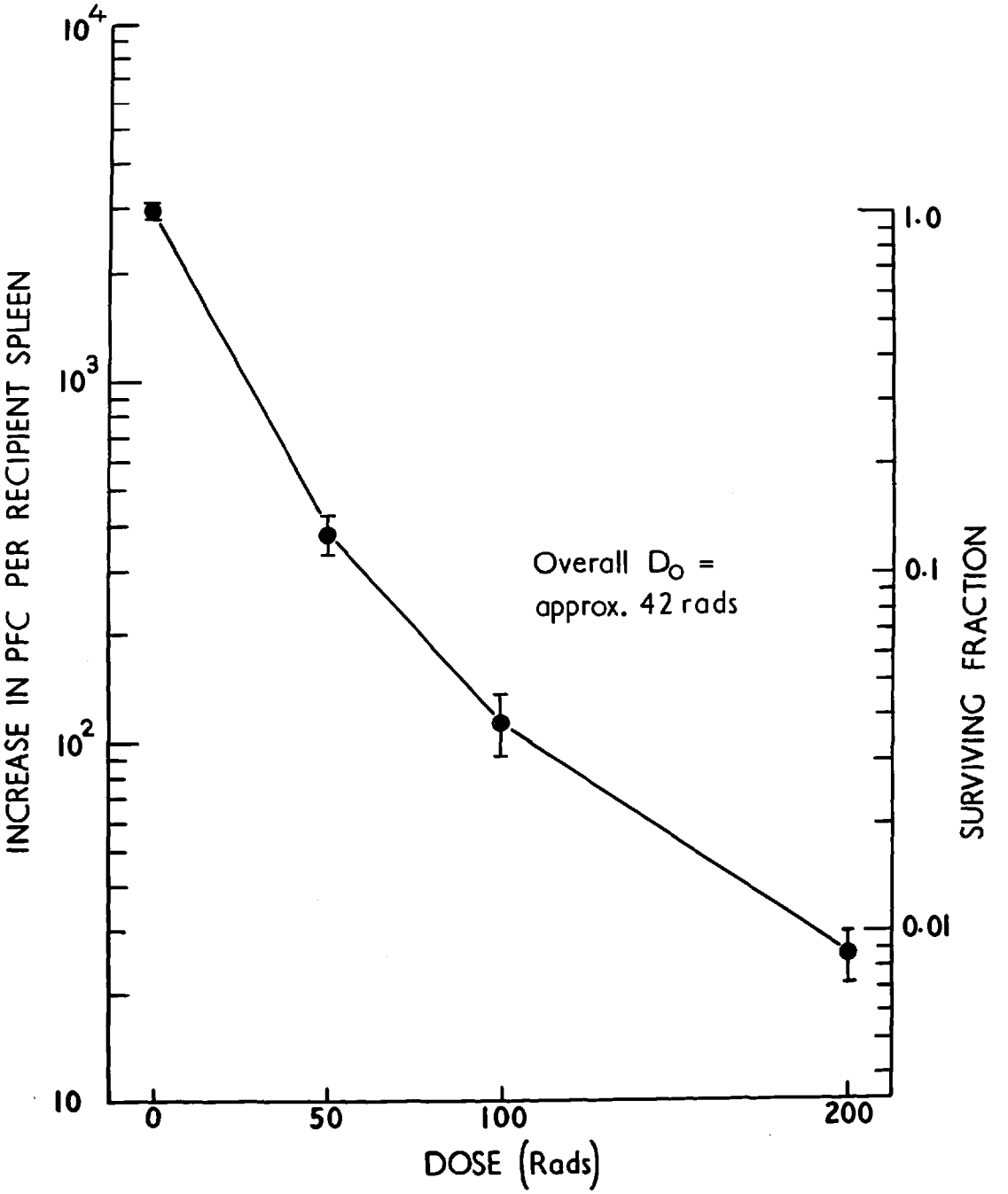
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EFFECT OF IRRADIATION *in vitro* ON RESPONSE OF TRANSFERRED SPLEEN CELLS



(8 recipients per group). Shortly afterwards they were immunized and their responses tested on the sixth day. The results are shown in Fig. 35.

Control mice, which were irradiated and immunized, but received no spleen cells, had a mean of 16 (11-20) PFC per spleen. This number was subtracted from the counts in the experimental groups to give the response due to the transferred cells. Unirradiated cells produced a response of 2990 PFC per spleen, which was severely reduced by the *in vitro* irradiation. The response following 200 rads was only just significant, at 25 PFC per spleen.

As can be seen from the graph, the radiation dose-response for the production of PFC was not exponential. However, Makinodan et al (1962), who determined levels of circulating antibody in a similar type of experiment, obtained an exponential dose-response, suggesting that the curve shown in Fig. 35 may have been subject to experimental error. It may therefore be justified to conclude from it that the overall D_o for this response was in the order of 42 rads, although this is only an approximation.

The D_o obtained by Makinodan et al was approximately 70 rads, but as shown in Section 6 (i), the immune response *in vivo*, as indicated by the appearance of PFC, seems more radiosensitive than

when estimated from the levels of circulating antibody, especially when peak levels in the response are compared (see table 6). Unfortunately the levels of serum antibody were too low for accurate determination in this experiment, so a direct comparison could not be made.

The closest comparison between the above experiment and the results obtained in Section 6 (i) is probably obtainable by considering the 4-day curve in Fig. 16 and the curve in Fig. 35, since these both represent radiation dose-response curves obtained at a single time shortly before the normal peak of the immune response in each system. The D_o values obtained were approximately 60 and 42 rads, respectively, indicating that the precursors of PFC irradiated *in vitro* seem more sensitive than those irradiated in the intact animal. Such an apparent increase in the radiosensitivity may be due to the different test system, lack of repair in the transfer situation, or an actual enhancement of the immediate damaging effects of radiation on the cells due to the conditions *in vitro*. Speculation must, however, be restricted by the limitations of the data so far available, especially with regard to the transfer experiment.

It is probable that data from transfer experiments, in which only the total number of PFC produced is determined, should in any case be transformed in order to obtain a better estimate of the radiosensitivity of their antigen-sensitive precursors (antigen-sensitive units; ASU). It was shown in Fig. 34 that increasing the number of transferred spleen cells, and therefore presumably the numbers of cells potentially able to form ASU, resulted in a relatively greater increase in the number of PFC produced. Conversely, reducing the number appeared either to reduce the number of PFC produced by each ASU, or to reduce the probability of ASU being formed.

If by killing or preventing the proliferation of immunocompetent cells radiation effectively reduces the number of cells transferred, it may also result indirectly in a steeper fall in the production of PFC than in the numbers of surviving immunocompetent cells, due to a "dilution effect". Alternatively, it might reduce the number of ASU directly in proportion to the number of surviving immunocompetent cells, but the activity of individual ASU may be reduced, resulting in fewer PFC per ASU.

The work of Playfair et al (1965) indicates that each ASU gives rise to many PFC, which would require at least 6-7 divisions of a single precursor cell. Therefore in order to produce a significant number of PFC the immunocompetent cell or cells of an ASU must be able to pass through several proliferative cycles. If radiation-injury caused them to die within the first few divisions, the number of PFC produced would be greatly reduced. This would suggest that the number of PFC arising from damaged ASU would be very small compared to those produced by uninjured ASU, due to differential survival at each mitosis.

Therefore, taking into account the apparent fall in the efficiency of the response with reduced cell numbers, even in the absence of irradiation, it would seem more likely that the main cause of inhibition of PFC-production is the killing of immunocompetent cells combined with a "dilution effect" which further reduces the chances of viable survivors coming together to form potentially active ASU.

The numbers of PFC, particularly several cell cycles after antigenic stimulation, may be approximately proportional to the numbers of viable ASU, but the numbers of ASU may be less than proportional to the numbers of immunocompetent cells, whether this is due to simple dilution of all spleen cells transferred, or to irradiation-induced cell death. If the same relationship between the numbers of PFC and

the numbers of spleen cells indicated in Fig. 34 applies to PFC and ASU-constituent cells, it may be necessary to increase the apparent D_o obtained from Fig. 35 by a factor of 1.3 (see equation on page 168). i.e. to approximately 55 rads, to obtain a closer measure of the true radiosensitivity of immunocompetent cells. Possibly a similar correction should be made when determining the radiosensitivity of the individual cells in vivo, but this system is hardly amenable to direct experimental manipulation.

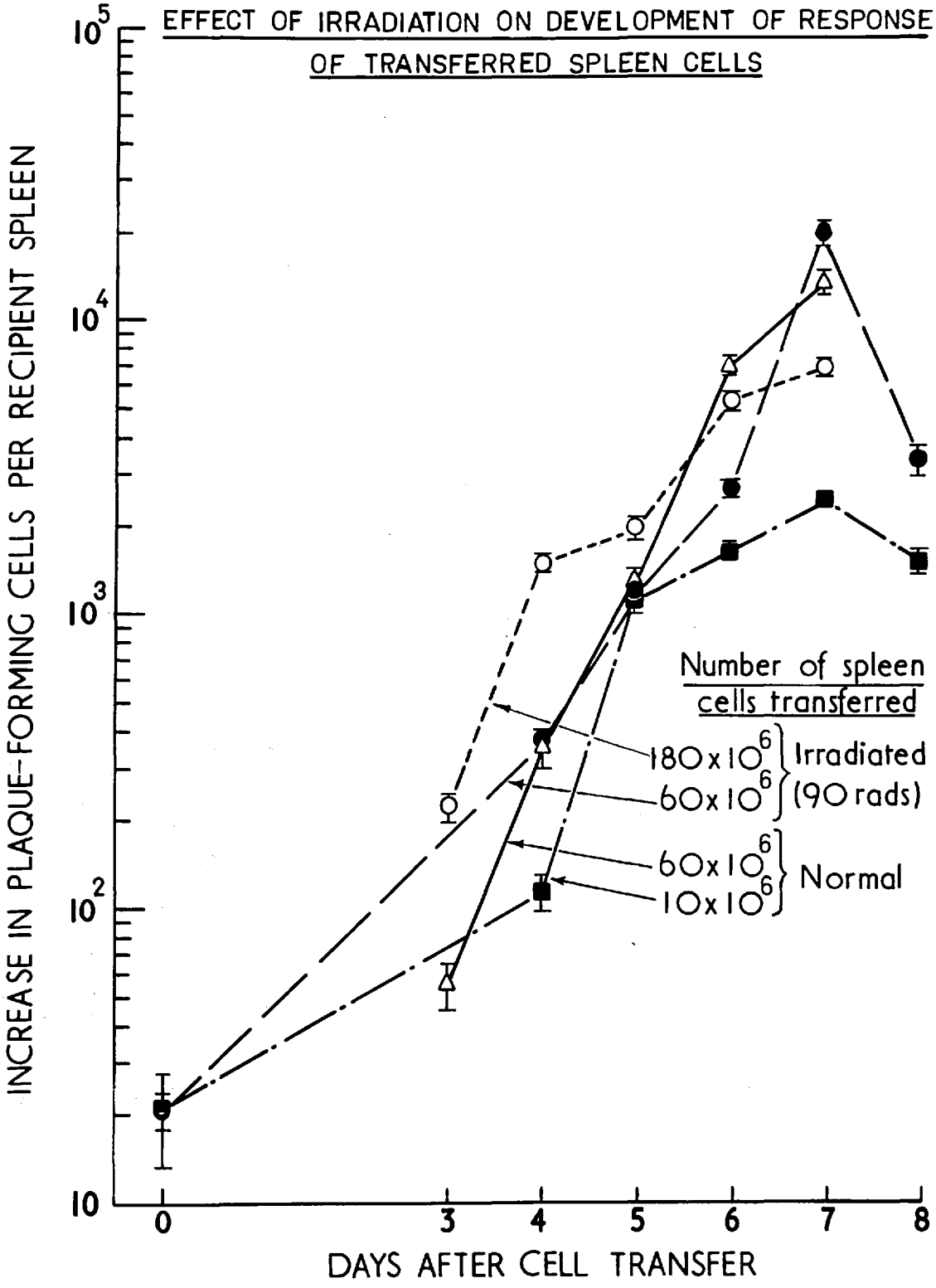
(iv) Effect of Variations in Cell Number and Radiation Dose on Response of Transferred Spleen Cells

The three previous experiments were all performed in preparation for the one described below, in which an attempt was made to study the interaction of cell number and irradiation on the development of an immune response by cells injected into the relatively homogeneous environment of lethally irradiated host mice. In subsection (i) it was shown that the development of PFC in the recipients was dependent upon the injection of unirradiated spleen cells, as well as antigen. It was also shown, for a single cell number transferred, that the response followed a somewhat different time-course from that in intact mice and was considerably slower. Subsection (ii) showed that the response at a given time was highly dependent upon the number of cells transferred. Subsection (iii) showed that increasing doses of radiation appeared to have the opposite effect to increasing the number of transferred cells.

If it is assumed that radiation acts by reducing the number of surviving immunocompetent cells, then increasing the dose of cells transferred should counteract this, and a suitable balance should result in similar responses by a given number of unirradiated cells and by a larger number of irradiated cells, both populations including the same number of viable immunocompetent cells. However other factors may also operate and there may be certain differences in the response, particularly in its rate of development.

In this experiment a single radiation dose of 90 rads was given to normal spleen cells suspended in PBS. The response of 10×10^6 normal cells was compared to that of 60×10^6 irradiated cells from days 4 to 8 inclusive. The responses of 60×10^6 normal and 180×10^6 irradiated cells were also compared (from day 3 to day 7). All four responses could be compared during the overlapping p riod from day 4 to day 7. (It had been intended to transfer 360×10^6 irradiated cells into the fourth group of recipients, giving a 36-fold overall cell dose-range, but this number was too great to be injected.)

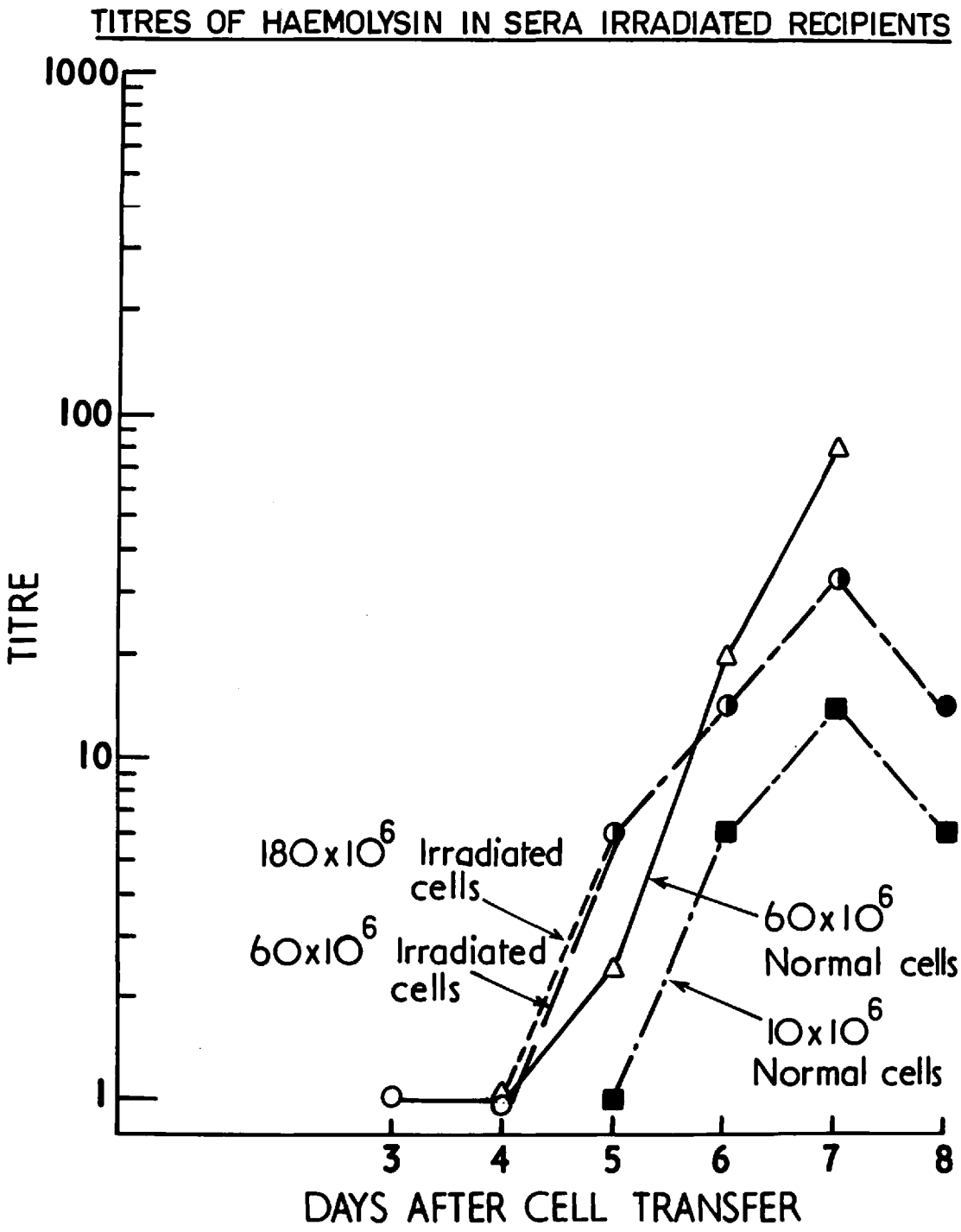
If the D_o for immunocompetent cells is taken as approximately 55 rads (see Section (iii)), the survival following 90 rads should be


EFFECT OF IRRADIATION ON DEVELOPMENT OF RESPONSE OF TRANSFERRED SPLEEN CELLS
INCREASE IN PLAQUE-FORMING CELLS PER RECIPIENT SPLEEN
10^5
10^4
10^3
10^2
10
0
3
4
5
6
7
8
DAYS AFTER CELL TRANSFER
Number of spleen cells transferred
180 x 10^6
60 x 10^6
Irradiated (90 rads)
60 x 10^6
10 x 10^6
Normal

in the order of 4%, i.e. about a 25-fold decrease in their number. Therefore it was hoped both to over and to under-compensate for this reduction, but in the event it may have been marginally under-compensated for. The higher number (60×10^6) of unirradiated cells was also included so that a direct comparison could be made between the responses of equal numbers of normal and irradiated spleen cells, with no compensation for the effects of irradiation. The results of the PFC estimations are combined in Fig. 36, while the serum titres are shown in Fig. 37 and 38.

Fairly consistent results were obtained by all methods of determination, except the 50% haemolysis-in-suspension method. This seemed insufficiently sensitive and too variable, and was ignored. Fortunately, however, the haemolysis-in-gel method of Uyeki was very satisfactory and gave clear-cut answers. As far as the results of these two methods could be compared, they appeared to be compatible with each other.

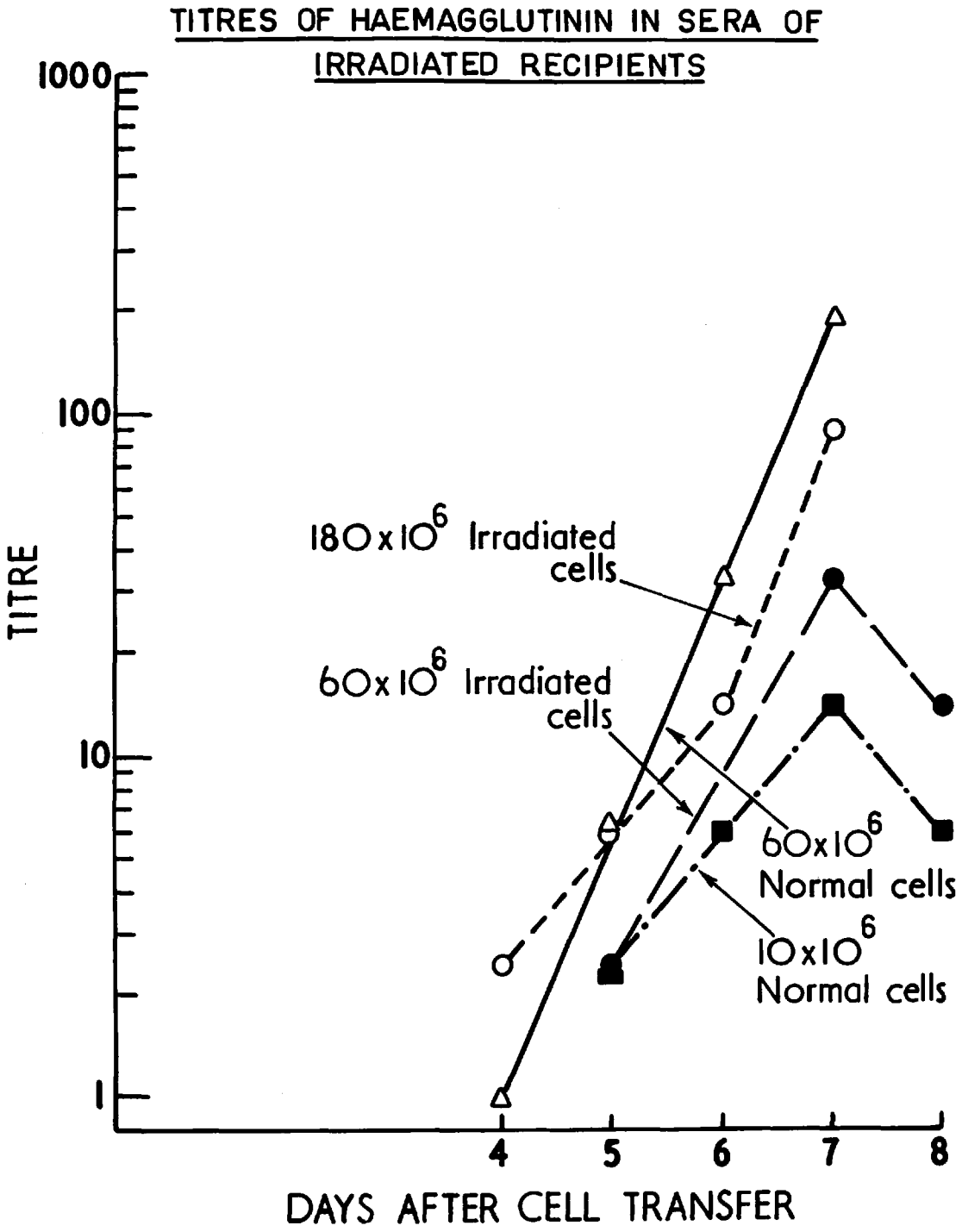
Both the numbers of splenic PFC and the titres of haemolysin and agglutinin increased approximately exponentially in all four groups of mice. In those which were tested on the 8th day after transfer (those which received 60 million irradiated or 10 million unirradiated spleen cells), the response seemed to have passed its peak.


TITRES OF HAEMOLYSIN IN SERA IRRADIATED RECIPIENTS
1000
100
10
1
TITRE
180x10^6 Irradiated cells
60x10^6 Irradiated cells
60x10^6 Normal cells
10x10^6 Normal cells
3
4
5
6
7
8
DAYS AFTER CELL TRANSFER

The numbers of PFC in the groups which received 60 million normal or 60 million irradiated cells were quite similar on days 4 - 7, and both had mean doubling times of approximately 13.2 hours during this period (from day 3 in the former group). Both types of circulating antibody accumulated in the serum slightly faster in the group receiving unirradiated cells, but the difference was only marked for the synthesis of haemagglutinin.

10 million normal cells gave a poorer response than 60 million normal cells, whichever parameter was considered. The doubling times of the PFC seemed to be rather variable, but on average were slightly longer (16.3 hours) in the 10-million-cell group, so that the difference between the two groups increased from 3 times on day 4 to 6 times on day 7. There was a similar divergence in the haemolysin and agglutinin titres, with differences on the 7th day of 6 and 14 times, respectively.

With 180 x 10^6 irradiated cells, the response seemed to start at a higher level, but to develop at a somewhat slower rate than with 60 million unirradiated cells. The mean doubling time of the PFC was 19.4 hours between days 3 and 7 (compared to 13.2 hours with normal cells), and by the 7th day both groups which had received 60 million


TITRES OF HAEMAGGLUTININ IN SERA OF IRRADIATED RECIPIENTS
1000
100
10
1
TITRE
180 x 10^6 Irradiated cells
60 x 10^6 Irradiated cells
60 x 10^6 Normal cells
10 x 10^6 Normal cells
4
5
6
7
8
DAYS AFTER CELL TRANSFER

cells had larger numbers of splenic PFC. In the case of the haemolysin and agglutinin titres, there were similar differences in the rates of accumulation of circulating antibody, with doubling times of 14.3 compared to 11.4 hours, and 14.3 compared to 9.5 hours, respectively. However, during the 4 - 7 day period both types of antibody appeared to increase faster than the numbers of PFC would suggest. Again, by the 6th and 7th days the highest titres were obtained in the 60-million unirradiated-cells group.

While the numbers of PFC increased faster in the group receiving 60 million irradiated spleen cells than in that receiving 180 million irradiated cells (with a doubling time of 13.2 compared to 19.4 hours), there was little difference in the rates of accumulation of circulating antibodies. Haemagglutinin titres were consistently lower with the lower number of cells, but the titres were almost identical when haemolysin levels were considered.

Comparing unirradiated cells injected into irradiated recipients, the haemolysin response, and the numbers of PFC, seemed directly related to the number of cells transferred by the 7th day of the response. Earlier in the response the difference was less marked, indicating a somewhat faster rate of response with a larger cell number.

However, with 60 million or 180 million irradiated cells there was little difference in the responses, except that it appeared to start slightly sooner with the larger cell number, but to proceed at a slightly slower rate.

An equal number of normal cells gave about the same response as irradiated cells when the number of PFC were compared, but a faster response when the circulating antibody was measured. It appeared that in this experiment a dose of 90 rads only had a small effect on the response to SRC, equivalent to an approximately 3-fold reduction in the transferred cell number.

This was an unexpectedly small effect. The donor cells were irradiated under slightly different conditions: the cell suspension was placed in flat plastic Petri dishes, instead of plastic tubes, as in subsection (iii), and the dose rate was 205 rads per minute, compared to 40 rads per minute, due to a shorter target distance. Otherwise the conditions and treatment were the same. The cells were suspended in PBS and kept cold throughout the preparative procedure, and were only in the Petri dishes for a few minutes. Anoxia would seem less likely in this experiment than in the previous one. Another difference was that the recipient mice were injected with SRC before, instead of after, the spleen cell transfer. However, in a preliminary experiment this had no effect on the responses of normal cells. It may possibly have

altered the dose-responses, both to irradiation and cell-number variation, although this seems unlikely. It did, however, facilitate the experimental operations somewhat.

The results appeared rather irregular from the graphs, and this may have biased their interpretation to some extent. Possibly the results for the PFC numbers were most representative on the 6th day of the response. The relative numbers at this time were as follows:-

Number of spleen cells				Relative No. PFC
10 million		normal	:	1.0
60	"	irradiated	:	1.7
60	"	normal	:	3.3
180	"	irradiated	:	4.4

Even so, they were not consistent with the results in subsections (ii) and (iii). Increasing the number of normal cells from 10 to 10^6 should have resulted in an increase in the numbers of PFC of at least 6 times by the 5th day, and perhaps as much as 10 times ($6^{1.3}$). Moreover, even increasing the number of irradiated cells to 180×10^6 should, according to the results in subsection (iii), have at best been only sufficient to compensate for the effects of irradiation, compared to the response by 10×10^6 normal cells, whereas it resulted in a markedly stronger response. Therefore it appeared that neither cell-dose variation, nor irradiation, had as much effect as expected, but the effect of cell number was the more important.

All that can be concluded is that the immune response is greater with higher numbers of transferred cells, and the effect of an increase in the cell number is partially offset by irradiation with a dose of 90 rads. It is difficult to decide whether the inconsistencies in the results are due to experimental error, to minor differences in technique, or to the use of non-syngeneic mice. The latter possible cause of variation may be particularly marked in the transfer-type of experiment, although it may not affect the experiments with intact mice.

Part IV

DISCUSSION

(i) General Observations

The introduction by Jerne of the haemolytic plaque technique made possible a closer study of the effects of irradiation on the immune response. In particular, it permitted observations on the immediate effects, even after antibody production had reached significant levels. Previous methods, such as antibody titration, were limited by the relatively long half-life of antibody molecules, compared to antibody-forming cells. The fluorescent antibody technique of Coons (Coons, Leduc & Connolly, 1955) was also limited by the possible superficial uptake of antibody onto inactive cells which might have persisted after active synthesis had ceased. With plaque-forming cells antibody is only released when conditions are suitable for active metabolism.

Berenbaum's study (1966a) was the first to show the true radiosensitivity of antibody-formation in animals one or more days after the injection of erythrocytes. He showed that PFC began to decline in number, compared to the controls, almost immediately after irradiation, whether the mice were irradiated on the first, third, sixth or twentieth day of the response. Moreover, the number always fell to slightly below the background level following a dose of 450r. Although this was followed by a slow recovery, it indicated a much greater radiosensitivity in immunized mice than had formerly been supposed.

The results in Sections 5 and 6 confirmed and extended this observation. However, they also agreed better with the earlier findings of Taliaferro, Taliaferro & Janssen (1952a, b) that the response appeared to be less radiosensitive if the animals were irradiated after immunization than before. Nevertheless, there still seemed to be a significant species difference between the response of rabbits and that of mice. It was never possible to achieve with mice any degree of enhanced antibody production as obtained in irradiated rabbits (e.g. Taliaferro et al (1952b); Dixon and McConahey (1963)).

When the effects of different doses of radiation on the subsequent immune response were considered (see Part III, Section 6 (i)), three main factors were apparent. 1) The maximum number of PFC which appeared was progressively reduced by increasing doses. 2) The time before the number rose significantly above the background was also progressively lengthened. 3) Even after this lag, the rate of production of PFC was slowed with the larger doses.

(ii) Possible Causes of Radiation Effects

These observations impose certain restrictions. Since the maximum number of PFC is not the same, and in fact differs by a factor of about 100 between controls and mice irradiated with 500 rads, it would appear that the 19S PFC response is limited either by the

availability of the antigen or is intrinsically limited by the effects of the irradiation. It is unlikely that it is terminated by the build-up of antibody: even if 7S antibody is the main agent of "feed-back" repression (Möller and Wigzell, 1965; Wigzell, 1966a, b; Möller, 1968), it is probably relatively ineffective in heavily irradiated animals, since 7S PFC seem equally suppressed by irradiation.

The "processing" of antigen by macrophages had been suggested as a possible radiosensitive step in the response to certain antigens (Gallily and Feldman, 1967; Pribnow and Silverman, 1967). However, there is little evidence that the function of macrophages in the response to strong antigens, such as SRC, is so radiosensitive that doses of 500 rads or less would have any great effect. As has already been described in Section (ii) of the Introduction, the uptake of antigens by phagocytic cells is not directly affected by sublethal doses, and irradiated macrophages are still able to transfer a specific immunological stimulus to unimmunized recipient animals (Unanue and Askonas, 1968) or tissue cultures (Roseman, 1969). The converse is also unlikely; namely that radiation stimulates phagocytes to take up and destroy increased quantities of antigen, since transferred spleen cells respond as strongly to SRC injected 2 days previously into lethally irradiated mice as to SRC injected immediately after transfer (unpublished observations).

Therefore it appears most likely that the response is mainly determined by the number of viable cells which actually respond to the antigen. If their numbers are reduced by irradiation, this would explain the apparent lag in the response; until this time the number of survivors would be too low to provide an appreciable increase in the numbers of PFC, since there are already in the order of 100 n-PFC per spleen present before immunization, which are not greatly affected by irradiation (see Part III, Section 4).

A reduction in the numbers of cells initially able to respond to the antigen would also account for the reduction in the peak numbers of PFC attained if the number of divisions during the proliferative response were constant. However, direct evidence is not yet available to confirm this, although it appears that even with multiple antigenic stimulation 7S PFC will not proliferate indefinitely. Möller (1968) serially passaged spleen cells from SRC-immunized mice through lethally irradiated recipients, which were also injected with SRC. The PFC ceased dividing after an estimated 38-40 divisions.

The best estimate for the D_o of a dose-response curve in which the peak numbers of 19S PFC were plotted against the dose of radiation (see Fig. 17) was 97 rads. If the immune response were a simple process this might reflect the radiosensitivity of the precursors of PFC.

However, the change in the rate of production of PFC requires a more complex explanation. In other simpler radiobiological situations in which proliferating cells suffer such an initial depletion in their numbers, the survivors usually multiply at a normal or near-normal rate following a dose-dependent lag before mitosis recommences (Alper, 1957). This results in a growth curve approximately parallel to that of unirradiated controls, but lagging behind them by a factor related to the surviving fraction and the mitotic delay.

Although approximately parallel "growth curves" for 19S PFC were obtained following doses up to about 350 rads (see Fig. 13), with higher doses their development slowed. This was observed consistently in several experiments in which doses of 500 rads were given (see also Fig. 9, 27 and 29, and compare with Fig. 2).

Three different kinds of explanation for this divergence in the experimental curves will be considered: that the survivors were in an unfavourable environment, that mainly cells with long cycle times survived irradiation, or that the overall numbers of PFC observed depend upon a complex balance between repeated "recruitment" events and proliferation on one hand, and cell-loss on the other, all of which may be affected by irradiation.

The first of these possibilities is unlikely to be of importance as transferred spleen cells give moderately good responses to SRC

when injected into heavily irradiated recipients. Indeed, with small numbers (8×10^6), the response in mice given 850 rads was as fast as that of mice irradiated with 500 rads, which were not reconstituted. With 60×10^6 cells the response was almost as fast as in mice given only 350 rads.

The second possibility also seems unlikely. If there are PFC-precursors which are both less radiosensitive and slower to divide than the majority of precursor cells, this would be expected to show as an upward inflexion in the dose-response curves with radiation doses sufficient to reduce the number of fast-dividing, sensitive precursors below the level of surviving more resistant cells. Although "levelling-out" of dose-response curves was observed in some cases (see Fig. 16, 17, 24 and 25), this appeared to be due to different causes, e.g. the presence of n-PFC which made it impossible to determine very low levels of PFC production, or may have been insignificant because too few experimental determinations were possible. When the overall production of PFC following doses of radiation up to 700 rads was considered (see Fig. 18), the dose-effect appeared to be exponential over the complete range tested. The same seemed true in Fig. 25, in the case of irradiation given on the second day of the response, at least with doses up to 1000 rads. (The effect of 1500 rads could not be followed long enough for a significant comparison to be made).

It would seem, therefore, that the overall production of PFC was limited by a single class of radiosensitive precursors, and a more complex explanation for the observed slowing of the response must be considered.

(iii) Stochastic Models for Immune Response

Various stochastic models have been proposed to explain the observed rates of increase in the numbers of PFC following irradiation, and also the relation between numbers of spleen cells and PFC produced in different culture systems.

Those of Berenbaum (1966a, b), which he proposed to explain the effects of *in vivo* irradiation and the effects of a mitotic inhibitor, were, on his own admission, probably oversimplified. Another model suggested by Dutton and Mishell (1967a), although still assuming a single immunocompetent cell-type to be involved, represented an advance in that it was suggested that the precursors of PFC were also proliferating in response to the antigenic stimulus. "Recruitment" of active PFC from these precursors was assumed to occur throughout the period of exponential increase in their numbers, resulting in a more rapid rate of increase than cell division alone would account for. Moreover, since the precursors were increasing exponentially, so the rate of new recruitments would increase until it finally overtook the number of precursors available.

However, neither of these models took into account the probable synergistic roles of thymus-derived and bone marrow-derived lymphocytes suggested by the classic experiments of Claman, Chaperon and Triplett (1966a, b). Indeed, *in vitro* at least, it seems likely that a third cell-type, the macrophage, is also necessary in the response to SRC (Mosier and Coppleson, 1968). These are probably also necessary *in vivo*, at least in order to obtain a maximal response, but the effect of varying their number cannot be studied in the transfer-type of experiment, owing to the persisting activity of donor-derived macrophages (Roseman, 1969).

The macrophages, which may be separated *in vitro* by their adherence to plastic, are very efficient at taking up SRC and transferring the immunogenic stimulus to the non-adhering lymphoid cells (Mosier and Coppleson, 1968). This function is not significantly affected by previous irradiations of the macrophage donors with doses of up to 1000 r., but the lymphoid cells are highly radiosensitive (Roseman, 1969). Both types of lymphoid cell appear to divide in response to the antigenic stimulus (Davies et al., 1967; Claflin and Smithies, 1967; Tannenberg and Malaviya, 1968), but while the proliferative response is much stronger among thymus-derived cells, only the bone marrow-derived cells become antibody-synthesizing cells (PFC) (Davies et al., 1967; Nossal et al., 1968).

It appears that the thymus-derived cells have an auxilliary function, but the nature of this is still unknown. Probably their presence enables the activated marrow-derived cells to transform into active PFC, or raises the probability of this occurring, and they may also accelerate proliferation of PFC or increase the rate of antibody synthesis. Direct cellular contact is almost certainly necessary for the synergistic process. Mosier (1969) demonstrated the importance of the formation and maintenance of clusters of spleen cells for the immune response to SRC *in vitro*. Most PFC were found to be present in the clusters, and each cluster usually contained several PFC, but all of the same specificity. In cultures containing a second type of red cell, PFC of the second specificity always occurred in separate clusters.

Antigen-bearing macrophages may act as nuclei in the formation of clusters. This may be the explanation of the small lymphocytes observed clustering around sensitized macrophages in rabbit spleen cell tissue cultures by Harris (1965).

By analysing the results from *in vitro* spleen cell cultures mathematically, Mosier and Coppleson (1968) concluded that three cell-types were involved in the immune response to SRC. This was possible because one type of cell (mainly macrophages) could be largely separated from the remainder of the spleen cell population,

which included the two other kinds (lymphoid cells), by their adherence to plastic. The numbers of adhering and non-adhering, or total spleen cells, could therefore be varied with respect to each other. PFC were produced in successfully-immunised cultures, and their numbers were plotted logarithmically against the variable class of cells (also plotted logarithmically). (For an example of this type of dose-effect curve see Fig. 34 in Part III, Section 10 (ii).)

When the adherent cells were varied in number while non-adhering cells were in excess, the slope on the resulting graph had a slope of approximately 1. This proved the necessity for the cell-type in the former population. However, when the number of non-adhering cells was varied the slope was increased to about 1.9. This was interpreted as being due to the interaction of two more cell-types in the non-adherent population. As their individual numbers were increased (in direct proportion to the total cell number), so the probability of interactions increased approximately in proportion to the <u>product</u> of the individual numbers. Finally, when the total (un-separated) cell population was increased in concentration, the resulting dose-effect curve had a slope of about 2.6. Thus the response seemed to increase almost in proportion to the product of all three cell-types. Other explanations were possible, but Mosier and

Coppleson concluded that this was the most likely.

Makinodan and collaborators (Groves, Lever & Makinodan, 1969, 1970), using a different culture system, came to a different conclusion. Spleen cells from unimmunized mice were placed in diffusion chambers, as described by Nettesheim, Makinodan and Chadwick, (1966), together with SRC, and these were implanted intraperitoneally in irradiated recipient mice. PFC were determined at the peak of the response. This method had the advantage that the spleen cell concentration could be increased considerably above that required for an optimal response in the in vitro cultures of Mosier and Coppleson (1968), who used the method developed by Mishell and Dutton (1966).

Increasing the cell number up to 12 x 10^6 per chamber, which was equivalent to 60 x 10^6 per ml., resulted in a dose-response curve with a slope of 2.75, similar to that obtained by Mosier and Coppleson with unseparated spleen cell suspensions. However, as the number was further increased, the number of PFC increased only linearly, i.e. the slope changed abruptly to approximately 1. Such a cell concentration could not be studied by Mosier and Coppleson (1968), who were limited to about 20 x 10^6 cells per ml.

It therefore appeared that one cell-type became limiting with the larger starting numbers. Conversely, as the cell number was reduced, so the proportion of non-responding cultures increased. To reconcile these findings, Groves et al (1970) proposed a more complex stochastic model.

They suggested that the limiting cell-type was one of the lymphoid cells, which was termed the "antigen-reactive cell" (ARC), while the second type consisted of PFC-precursor cells (PC). These corresponded to thymus-derived and bone marrow-derived lymphocytes, respectively. However, the interaction between them was not simple; it was postulated that one to several PC could be stimulated by each antigen-activated ARC, but that this number was limited. The form of this stimulation was unknown, but the result was presumed to be the transformation (or "recruitment") of PC into active PFC, and may well have involved a mitotic stimulus (Davies et al, 1967). PFC then increased in number by proliferation. In the whole spleen this probably results in the appearance of haemolytic "foci", which are rich in PFC (Kennedy, Siminovitch, Till & McCulloch, 1965; Playfair, Papermaster & Cole, 1965; Berenbaum and Stringer, 1970).

As the number of PC and ARC was increased, so the probability of interaction would increase approximately in proportion to their product, until all available sites on each ARC would be filled during the short period in which they were postulated to be active. Thereafter, as the cell concentration was further increased, the number of PFC could only increase in direct proportion to the number of ARC, assuming each PC to give rise to a constant number of daughter PFC, irrespective of cell concentration.

Groves et al (1970) did not discuss the role of macrophages, but the work of others (Mosier and Coppleson, 1968; Roseman, 1969) would suggest that with cell numbers at which ARC are not the limiting cell-type, increasing the concentration of antigen-sensitised macrophages will increase the chances of PC and ARC interacting. Again the mechanism is unknown. Prior interaction between ARC and macrophages may be necessary for their initial activation, or it may increase or lengthen this.

When considering the results in Section 10 (i), it is unlikely that ARC would be in the "saturated" condition which makes them the limiting cell-type, since a total of 60×10^6 spleen cells were dispersed throughout the body of an entire mouse, and would therefore be well below the threshold concentration for this. The cell-types may therefore be thought of as being effectively independent, except that macrophages from the recipient mouse would be in considerable excess.

Groves et al (1970) estimated the frequency of ARC to sheep erythrocytes to be approximately 1 per 140,000 spleen cells, or about 1000 per whole spleen. Owing to the culturing efficiency (6.5%), this was reduced to 1 per 2.2×10^6 cells, the value estimated experimentally. It was postulated that if foci of PFC among spleen tissues were analysed, each would require a minimum of one active ARC. Probably

one would be sufficient, since Playfair et al (1965) observed an average of 1 focus of haemolytic activity to be produced by transferring 5×10^5 normal spleen cells into irradiated hosts. Again correcting for cell-loss, the frequency of ARC was estimated as 1 per 20,000 cells, about 7 times as high as the estimate of Groves et al (1970). Applying similar corrections, the estimate of Shearer et al (1968) was 1 in 40,000, and that in Section 10 (ii) was 1 in 160,000. All these estimates are within the range expected using different strains of mice and different culturing systems.

Further experiments by Makinodan's group (Perkins, Sado & Makindan, 1969; Sado, Perkins & Makinodan, 1970) have recently shed more light on the events following antigenic stimulation. In the primary response to SRC *in vivo* antigen-induced proliferation began after a lag of 12 hours, and all responsive cells appeared to be incorporating labelled thymidine within 18 hours. In the secondary response of dispersed spleen cells in diffusion chambers proliferation commenced within 10-30 minutes. There was a further lag in both cases before the numbers of PFC begin to rise. It appeared that the first recruitment events occurred very early, with new PFC becoming active 1-2 hours after the first mitosis.

Proliferation and further transformation of PC occurred in a non-random manner, resulting in a "staircase"-like increase in the numbers of both 7S and 19S PFC. It appeared that PFC divided approximately synchronously, each giving rise to two daughter PFC, while at the same time new PFC were recruited. In the primary response the height of each "step" was equivalent to a 2.5- to sixfold increase, while in the secondary response recruitment was still greater, giving increases of 10- to 40-fold.

Despite the staircase-type of response, the overall rate of PFC production was approximately exponential. Therefore it is probable that ARC and perhaps also PC were caused to divide by the initial antigenic stimulus and also increased exponentially. This allowed the number of possible recruitment events to increase correspondingly as the response progressed. Due to repeated recruitment the observed doubling times of the PFC were shorter than the mean generation time, as indicated by the period between successive "steps". Estimates of each were as follows:-

	Doubling time (hours)	Approx. cell cycle time (hours)
Primary Response		
19S PFC	6	8.5
Secondary Response		
19S PFC	7	8-16
7S PFC	4	8-14

In the secondary response it is likely that the majority of PC were "memory cells" produced in large numbers during primary immunisation. These would account for the massive recruitment and very rapid increase in 7S PFC. 19S PFC may have been recruited to a greater extent from previously unstimulated precursors.

It was on the question of proliferation and continuing recruitment of precursor cells that the models of Groves et al (1970) and Perkins et al (1969) seemed somewhat uncorrelated, despite the co-authorship of Makinodan. This may have been due to insufficient detail having been published rather than to any disagreement between the hypotheses. However, an alternative explanation may not only make their experimental findings fully compatible, but also correlate more easily with the observations in this thesis:

(iv) New Synergistic Model

1. Following the initial contact with antigen, thymus-derived ARC are stimulated to divide (Davies et al, 1967). It is not clear whether or not the bone marrow-derived precursors of PFC (PC) are also stimulated to divide independently, but this is possible, since in one of their groups of mice Davies et al (1967) detected cells of bone marrow origin (bearing the T6 chromosome "marker") in mitosis. However, division of unassociated PC is not a prerequisite for the operation of this model.

2. Activated ARC may interact with PC at any time during the period of antigenic stimulation, although this may be restricted to certain periods during each mitotic cycle. The mechanism of this interaction is uncertain, but possibly each ARC bears a receptor site on its membrance to which antigen may bind. PC may have similar sites. Therefore it may be possible for the antigen particles to form a "bridge" holding the two kinds of cell together. The presence of excess antibody, by combining with the antigen, may prevent such bridge formation (see below). However, once it has occurred, the antigen will be protected. At the same time this will prevent further uptake of antigen, and the magnitude (and perhaps the duration) of the immunogenic stimulus will therefore be limited unless the association is disrupted. When the antigen becomes exhausted proliferation will terminate. ARC associated with PC are termed ARC* (Groves et al, 1970). ARC-associated PC will also divide, probably synchronously, while the stimulus persists, resulting in the formation of a haemolytic focus.

3. It is only necessary to postulate a 1 to 1 ratio of PC and ARC in each newly formed ARC.* However, due to cell division the numbers of each will increase exponentially during the subsequent proliferative phase. Finally the ARC will cease dividing, and this will cause PC to terminate DNA synthesis, and either die or transform, perhaps into "memory cells".

4. Formation of ARC^{M} will occur independently of each other. ARC not interacting with PC during the first cell cycle may divide and each daughter cell may form an ARC^{M}, or divide again without interacting with PC. Therefore in the absence of PC the number of ARC may increase exponentially for an unknown length of time. However, once an ARC has become an ARC^{M} it will be unable to interact with more PC unless dissociated before the antigenic stimulus is exhausted.

5. Throughout the proliferative phase recruitment of PC into antibody-secreting PFC will occur, probably in synchrony with cell division, but only a fraction of the PC in any focus will be recruited each cell cycle. Therefore the foci will grow into clusters of ARC, PC and PFC, all proliferating. This limited recruitment will allow the numbers of precursor cells to increase approximately exponentially, so that the rate of recruitment also rises exponentially. It would also result in a staircase pattern of PFC development.

Where this differs radically from the model of Groves et al (1970) is in the constant ratio between PC and ARC at all initial cell densities. How this could be consistent with their observations will be described below.

It is possible that PC in a focus stimulated to become PFC early in the response may cease proliferating after a few divisions

(Perkins et al, 1969). If they continued antibody synthesis for a while, they would appear as unlabelled PFC, such as were observed, particularly later in the logarithmic phase of the response, even following several injections of radioactively labelled thymidine (Perkins et al, 1969). However such cell-loss would be offset by new recruitments. Therefore the "steps" observed may be the net product of cell division, loss and recruitment of PFC.

(v) Effects of Dissociation and Irradiation on the Continuation of Antibody Synthesis

Dissociating the cells of a haemolytic focus may be equivalent to dissociating the cell clusters which form during the immune response *in vitro* (Mosier, 1969). If this was done at an early stage in the response it was prevented from developing. However, if the clusters were allowed to reform, the response continued. Both reformation and the development of PFC were inhibited by specific antiserum, although normal serum or antiserum of a different specificity had no effect. Antiserum had little effect on intact clusters (Mosier, 1969; Lang et al, 1969), nor was it effective in allowing the response in already immunized intact mice for a period of at least 40 hours (Möller and Wigzell, 1965). It is likely, therefore, that antigen is essential in the interaction between thymus- and bone marrow-derived lymphoid cells, since antiserum would specifically block its binding with antibody-like sites on the lymphocyte membranes.

As already suggested above, the ARC may actually cause their associated PFC to become inactive. This would explain the ability of dissociated PFC, taken from immunized mice, to continue secreting antibody for long periods of time (Makinodan et al, 1966; Sado, 1969 - see also Section (iv) of Introduction). Moreover, their activity was unaffected by irradiation with doses of 5000 or 10,000 r. if cultured in diffusion chambers. However no new PFC were produced, and the original ones had a mean half-life of about 5 days. If, by separating PFC from ARC, the mitogenic stimulus were also cut off, (probably the handling involved during the irradiation procedure would cause some disruption among the cultured cells), PFC would no longer be induced to divide, either until they became terminal cells, or else to die from the effects of the radiation in the case of the injured cells. The actual process of antibody synthesis is probably unaffected significantly by such doses (see Part III, Section 2).

Conversely, in the intact animal the cellular associations are probably not disrupted and the potentially lethal mitogenic stimulus may still be received by the active PFC. This would account for their very rapid disappearance <u>in vivo</u> (see Sections 5 and 6 (ii)). A dose of 350 rads was sufficient to arrest the response, while larger doses caused the PFC to cease functioning considerably more

rapidly than during the normal decline in their numbers following the peak of the response (see Fig. 19). Emigration from the spleens may have contributed to this fall, but this seems unlikely.

(vi) Effects of Variation in Cell Number and Doses of Radiation

Despite the apparent multiple recruitment of PFC indicated by the results of Perkins et al (1969), the stochastic model of Groves et al (1970) seemed to indicate that PC and ARC combined at an early phase during the response, and that the mean number of PC per ARC* was determined by their relative concentrations from 1 up to a maximum limit. Therefore a few days later, at the peak of the response, the number of PFC per focus was proportional to the number of PC per ARC*. If ARC (and possibly PC) were proliferating, and did not interact at first their concentrations would change and this could affect the numbers of PC eventually combining with the ARC.

In the alternative model all ARC* would be similar, if not identical, in the relative numbers of ARC and PC. However, if the initial numbers were very low, e.g. with less than 10^6 cells cultured per diffusion chamber, the number of ARC to a single antigen may be 0 or 1, and PC also rather infrequent. Therefore no interactions may occur for some time until several ARC are present. ARC* formed at

this time might still proliferate for the usual period, but at the time of assay the foci would still be small, or quite possibly undetectable (since Berenbaum and Stringer, 1970, have shown how difficult it is to determine the numbers of small haemolytic foci with accuracy). Indeed, no interactions may occur during the period of antigenic excitation and the culture may give a negative result, even though ARC are present.

Averaging the results of groups of similar cultures, each responding at a different time, or failing to respond, would result in an overall "peak" in the response when the majority of responding cultures reached their individual peaks. However, since they may have started to respond at different times, at this time only some will contain foci with the maximum number of PFC (after which the number declines), and the mean number will appear sub-optimal.

In the case of diffusion chamber cultures with many cells (in excess of 12×10^6), or perhaps normal intact mice, the probability of the ARC present initially interacting with PC may be sufficiently high for all of them to become ARC* within the first one or two cell cycles. Therefore they will in effect be synchronized, and all cultures will begin responding about the same time. All will then reach their peaks approximately simultaneously and will appear at this

time to have the maximum possible number of PFC per focus. The number of foci will also be directly proportional to the numbers of cells initially present, no increase in the numbers of ARC having taken place, and ARC will be the limiting cell-type.

With fewer cells initially the probability of an ARC interacting with a PC in time to develop into a detectable focus, or to produce large numbers of PFC will depend upon the product of their individual concentrations. Again, in this situation the presence of antigen-sensitized macrophages may further increase the probability of such interaction, and so the concentration of the latter may be important.

In short, the model of Groves et al (1970) postulates a reduction in the number of PC per ARC^{*}, whereas the above described version of it postulates a delay in ARC^{*} formation, limiting the number of PFC produced within the period of investigation.

Where the revised model seems superior to the original is in its prediction of the slowing and delaying of the overall appearance of PFC. For the purposes of discussion, the effect of irradiation will be equated with disappearance of precursor cells (ARC and PC) and will be equivalent to a reduction in the cell number. However, it is unlikely that the numbers of activity of macrophages are affected in the short-term by irradiation.

Considering first a small reduction in the optimal number of cells per culture, or the spleen of a mouse irradiated with a small dose of radiation (e.g. 100 rads), the numbers of ARC and PC will be reduced correspondingly. However, it will remain probable that ARC^{M} formation will still occur early in the response and the responses of individual foci of PFC will be fairly synchronous, reaching their peaks about the same time as in controls (i.e. cultures with an optimum cell number or unirradiated spleens). The height of the combined peak of all foci per spleen or culture will therefore be directly proportional to the number of ARC present initially.

If the numbers of PFC are followed throughout the exponential period they will be seen to rise above the background level first in the controls, and shortly afterwards in the reduced cell group, due to the smaller numbers initially recruited. Thereafter the curves will rise in parallel, with the controls always above the second group by a factor approximately equal to the initial reduction in ARC. The peaks will occur about the same time.

With a greater reduction in the cell number (or a dose of perhaps 200 rads), some ARC may at first fail to come into contact with PC of the corresponding specificity and the development of their foci will be delayed slightly. However the number of ARC^{M} will be slightly

increased due to free division of such ARC before interaction. Thereafter PFC will be recruited in all surviving ARC* foci at similar rates, but some may lag one or two cell cycles behind the others.

While the reduction in the total number of PFC produced may be slightly less than the initial reduction in the numbers of ARC, due to the increased number of ARC*, the individual foci will not attain their peak PFC numbers simultaneously and the overall peak number will still be approximately proportional to the number of ARC initially present. Overall, the development of PFC will still parallel the controls, but the lag will be greater, and the peak, by being broadened, will be slightly delayed.

By using the data at present available, it may be possible to quantitate the effects of reduced cell numbers, or irradiation approximately. Groves et al (1970) estimated the number of ARC per normal mouse spleen to be in the order of 1000. Several other authors estimated similar figures (Kennedy et al, 1965; Playfair et al, 1965), while others obtained closely compatible results (Shearer et al, 1969; see also Part III, Section 10 (b)). Assuming the D_o of ARC to be most closely reflected by the relative heights of PFC peaks attained following subsequent immunization, a value of 100 rads should be a fair approximation (see Fig. 17).

Therefore a reduction to 3% of the spleen cell number of a normal mouse would be approximately equivalent to the effect of 350 rads, and would result in a reduction from about 1000 to 30 in the number of ARC per spleen. If PC were equally affected the chances of interaction within the first cell cycle would be approximately 1000 times lower than in a normal spleen. This would suggest considerable delay before all ARC became activated and possibly some would pass through the reactive phase without becoming ARC^{M}.

Nevertheless, probably the majority would become active in the first few cycles and again the numbers of PFC would rise at the normal rate thereafter, again below and behind the controls. The peak number would be expected to be about 0.03 of that in controls, but still further flattened and the mean time delayed.

A dose of 500 rads (or an equivalent 140-fold reduction in the cell number, using the above D_o to estimate the relationship) might result in very wide variations developing between mice which were originally identical, since the mean number of ARC would be in the order of only 7. Assuming the number of ARC per spleen to follow a Poisson distribution (see Part II, page 57), the standard error of 7 would be approximately 2.5, so mice might well have between 4 and 10 ARC, reflecting normal statistical variation. Some might now fail to respond at all for several cycles, while individual

ARC^{M} may begin proliferating sooner in other animals (or cultures). The overall effect would be asynchrony of the response, both within individual spleens and groups of mice. Within individual foci the numbers of PFC will still increase at a normal rate, but some may pass their peaks before others have reached significant numbers. The probability of an ARC^{M} forming will increase with time as the precursors divide so the response in an individual will also tend to continue building up until unrecruited ARC cease to be reactive (or the antigen is destroyed or neutralized with antibody).

Therefore the total number of PFC per individual may increase erratically, but if a group of spleens are pooled, this may result in a smoother curve with a slope considerably less than in the controls. Due to the larger number of ARC^{M} appearing later in the response, the peak may be extended long enough to meet the downward curve of the controls, when the numbers of PFC are plotted graphically.

Due to the combined effects of reducing and delaying the response, a comparison of the numbers of PFC present in all groups at the time of the control peak will show very wide divergence, whereas if the peak numbers of each group are compared, irrespective of the time they are reached, the differences will be much less. What was lost due to delaying the response may be partly made up by the proliferation of ARC before activating PC. Also due to the latter

cause, which may act as an automatic homeostatic regulatory mechanism, the overall production of PFC will be considerably less depressed than the initial reduction in ARC might suggest. The two methods of assay might well give D_o values of 60 and 100 rads, respectively (see Part III, Section 6 (i)).

With lethal doses of radiation, or very few cells in culture, some individuals may fail to respond to the antigenic stimulation due to total loss of ARC. If these have a D_o of 100 rads, 700 rads would reduce the mean number of survivors per spleen to less than 1, while following 850 rads only about 2 mice in 10 should respond at all.

Furthermore, even if a single surviving ARC is present, few, if any, of its daughters may ever interact with PC. Although Groves et al (1970) postulated the latter to exceed ARC in number by a factor of about 5 in normal spleens, very few would be expected to survive 850 rads, particularly if the survivors do not proliferate. Hence even injecting bone marrow cells will fail to restore immunological responsiveness so long as ARC are absent. Similarly, injecting thymus cells will also fail to elicit a response, due to the absence of bone marrow-derived PC. The response observed following 700 rads (see Part III, Section 6 (i) and Fig. 13) was so much delayed that it may have been initiated by new ARC produced by the thymus. This may have represented the beginning of recovery of immune capacity, rather than a response

by the cells present at the time of injection.

(vii) Recovery of Immune Response

Considering the recovery of the immune system following irradiation (see, also, Part III, Section 8), it will be expected to be limited mainly by the "processing" of thymus-derived lymphocytes before they can become ARC. This view is shared by Gregory and Lajtha (1970). Tyan, Cole & Nowell (1966) estimated cells responding to an allograft required a period of about 30 days in the environment of the thymus to acquire immunocompetence, and this may well be similar to that required in the development of cells reactive to erythrocytes.

ARC are themselves derived from cells originating in the bone marrow (McGregor, 1968; Doria and Agarossi, 1969) (or the liver in the foetus - Umiel, Globerson & Auerbach, 1968), so in heavily irradiated animals the number of stem cells will be much reduced for a number of days, further delaying the appearance of ARC precursors, and the supply of these may remain subnormal for several weeks. Therefore it is not surprising that immunocompetence does not begin to recover for at least 10 days and is still less than in controls 3 months after irradiation with 500 rads (see Part III, Section 8).

By injecting bone marrow suspensions into mice irradiated with 925r., Shearer and Cudkowicz (1969) induced recovery of the immune system considerably sooner than in unrestored mice. With 10^8 cells, 30% of recipients had recovered fully after approximately 30 days. In

a similar experiment, Gregory and Lajtha (1970) showed that the injection of thymus cells as well as bone marrow cells resulted in immediate restoration of the response, but their relative effectivenss fell with time, presumably due to the regeneration of thymus-derived cells in the recipients.

As was shown in Section 8 (iii), immunological unresponsiveness was due to the lack of immunocompetent cells, and not to other factors.

(viii) Spleen Nodules

In Part III, Section 9, the immunocompetence of spleen nodules was assessed very crudely, and shown to be slightly subnormal, with respect to the remainder of the spleen. Probably this was due to the cells arising in the nodules being of direct bone marrow origin, and therefore lacking in ARC, although PC may have been produced in them. Since thymus-derived lymphocytes move freely in the circulation (for instance McGregor, McCullagh & Gowans, 1967; Miller & Mitchell, 1968), activated ARC may enter the nodules and initiate the development of foci of PFC. However, ARC may already be present in the older parts of the spleen, or perhaps these may have a better blood supply. Therefore PFC development may be initiated earlier in these regions. This would result in the higher concentrations of PFC observed in the intranodular tissues about the eleventh day.

Celada and Wigzell (1966) obtained evidence that clones of PFC arose randomly among nodules and increased in size and number with time. This would be consistent with the entry of surviving migratory ARC from other tissues, but it could also be due to PC also entering via the circulation, and not being derived from the original stem cells of the nodules.

(ix) Sparing Effect

It was shown in Part III, Section 7, that a small sparing effect occurred if a total dose of 500 rads was given as two fractions one or more hours apart, when the ability to respond to a subsequent injection of SRC was tested. The effect appeared approximately equivalent to reducing the total dose to about 425 rads.

Such sparing effects are more usually associated with the production of sublethal damage within cells which, if allowed to repair, results in reduced cell-death. In biological systems in which an accumulation of sublethal damage occurs before the first lethal events, the survival curve obtained following a single dose of radiation shows a "shoulder", equivalent to the threshold of effectiveness, when the number of survivors is plotted logarithmically against the dose (Elkind & Sutton, 1960). Although the data in the studies reported here are rather variable for radiobiological analysis, there does not appear to be such a shoulder in the

corresponding unfractionated dose response curves (see Fig. 16, 17 and 18), at least when PFC are considered, except for reasons unconnected with this. The results of Kennedy et al (1965) were comparable to those shown in Fig. 16, but again showed no shoulder when plotted as dose-response curves. A shoulder of approximately the expected width was, however, obtained when mice were irradiated during the immune response (see Fig. 24 and 25), but this was not a strictly comparable situation.

Two different kinds of explanation will be suggested to reconcile the occurrence of a sparing effect with the absence of a shoulder.

Firstly, the effect of the first fraction of radiation may cause the release of a non-specific stimulatory factor, e.g. lymphopoietin, which may cause the undamaged cells to enter a more resistant part of the cell cycle. Perhaps this produces rise in the levels of enzymes associated with DNA synthesis which may non-specifically repair part of the damage cuased by the first dose of radiation.

Alternatively, PC may normally be in a state in which sublethal injury is repaired, but the "survival curve" for the production of PFC is exponential because ARC are not able to do this. It has already

been suggested that in the normal spleen, or in animals exposed only to small doses of radiation (when a "shoulder effect" would be most marked), the response is directly limited by the number of thymus-derived ARC. Therefore increased recovery of the bone marrow-derived PC will have little effect, except possibly for a marginal speeding of the commencement of the response.

Following 500 rads, however, the survival of PC will be important, but the effect (according to the model proposed earlier) will be to shorten the lag period before ARC^{M}-forming interactions take place. The height of the PFC peak will not be significantly affected, although it would occur rather earlier. This would agree quite well with the observations illustrated in Fig. 26.

To summarize, the repair of sublethal damage by PC may normally be obscured by the lack of repair in ARC. However, this may not apply to the effects of radiation during the exponential phase of the PFC response, since both ARC and PC may then be proliferating and equally able to repair sublethal damage.

(x) Continuation of Response after Initial Peak

So far little attention has been given to the kinetics of the response following the initial peak in the numbers of 19S PFC, nor has this been considered in the stochastic models of others. However, it would seem probable from the similar effects of radiation on the numbers

of PFC throughout the response (Berenbaum, 1966a, and Part III, Section 5) that the basic proliferative process is similar.

Some ARC may escape activation at first, while new cells may be produced continuously in normal mice. It is not inconceivable that the precursors of ARC and PC are already antigen-specific, and may also be stimulated by the antigen to produce new immunocompetent cells. Therefore while the concentration of antigen exceeds that of antibody, new foci of PFC may be initiated continuously. Since this will probably only occur at a slow rate, the overall height of the response will not be very high. (If 1000 ARC stimulated simultaneously produce a peak of 2×10^5 PFC, each focus would on average contain only 200 PFC before ceasing to function).

Alternatively a proportion of ARC may respond more slowly and/or for a longer period, and so continue theresponse beyond the time when the majority become inactive. Indeed this may be the case in the production of 7S PFC. Shearer and Cudkowicz (1969) and Shearer et al (1969) suggested on the basis of transferring limited numbers of spleen cells to lethally irradiated recipients that 19S PFC, 7S PFC and haem-agglutinin-producing cells arose from independent antigen-sensitive units (ASU; equivalent to ARC* in the nomenclature of Groves et al, 1970). These occurred at different frequencies, those producing 19S PFC being approximately twice as numerous as the other types. It would not,

therefore, be surprising if they multiplied at different rates. Moreover, there may well be at least two types of 7S PFC ASU, each producing a separate peak. These would correspond to the peaks observed on days 6 and 10, as reported in Part III, Section 1 (see Fig. 2).

Cells producing agglutinins ("rosette-forming cells") were not assayed in these studies, but there was considerable similarity between the results of the haemolysin and haemagglutinin titrations, so a similar cellular basis may underly their production.

Recently, Playfair (1970) has presented evidence suggestive of a direct response by cells present in the bone marrow of mice to SRC, which resulted in the appearance of direct PFC in the spleens of lethally irradiated recipients. Such a thymus-independent response was predicted by Tyan et al (1969). The number of PFC produced was low, but increased in direct proportion to the number of bone marrow cells transferred. They also appeared less susceptible to the tolerogenic effect of cyclophosphamide, and were postulated to belong to a second class of PC. Indirect evidence suggested these might also enter the spleen, but probably in small numbers than PC of the type which must interact with ARC.

Therefore the numbers of splenic PFC observed in intact mice may be derived mainly from synergistically produced PC (PC_2), but also from

directly produced PC (PC_1). While these may have different radiosensitivities, the difference is unlikely to be great and so the responses may be similarly depressed by irradiation. However, since no interaction with ARC may be involved, there should be little delaying effect on the PC_1, except possibly that produced by sublethal damage which is repaired before proliferation commences. No radiobiological data is available as yet, so further speculation on the implications of such a second population is not advisable.

There appeared, on the whole, to be quite good agreement between the results for 19S and 7S PFC, when these were both studied (see Part III, Section 5). The appearance and peak numbers of 7S PFC were delayed, and their general levels depressed by irradiation (Fig. 9 and 10, and Table 4). When mice were irradiated on the 8th day of the response, both 19S and 7S PFC disappeared rapidly from the spleens. Although 19S PFC fell more rapidly, this may have been because they were already declining, whereas 7S PFC were approaching their second peak and the survivors may have been more active. Alternatively, this may have reflected a faster rate of turnover among 19S cells. Nevertheless, the difference in their halving times was less than 5 hours.

(xi) Estimation of Cell Cycle Time

As suggested in Section 6, the rate of disappearance of 19S PFC (see Fig. 19) following the largest doses (1000 and 1500 rads) may be closely correlated with the cell cycle time during their production. PFC are likely to remain active until caused to divide, when damage insufficient to prevent the synthesis of antibody may be sufficient to cause death during, or shortly after, mitosis. Therefore this would indicate that approximately 50% of PFC reach their next mitosis within 7.6 hours, and that this may be equivalent to the mean length of interphase. Allowing 2.4 hours for mitosis, the estimated cell cycle time of 19S PFC would be about 10 hours. If mitosis takes longer, this estimate should be increased, but if it is shorter, or if only a brief phase in mitosis is sensitive to these doses of radiation, it should be decreased. Perkins et al (1969) estimated the mean PFC cycle time in the primary response to be about 8.5 hours, if the "shelf" times during the "staircase" rise in their numbers were equivalent to this. The results were therefore quite compatible.

Tannenberg and Malaviya (1968), using the double-labelling technique of Wimber and Quastler (1963), estimated the mean cycle time of 19S PFC to be 13 hours. However, as pointed out by Perkins et al (1969), the assumptions they made were suitable for the

analysis of randomly dividing cells, whereas PFC appear to increase non-randomly. Therefore their conclusions may have been somewhat biased.

(xii) Apparent Changes in Radiosensitivity

It was indicated in Part III, Section 5, table 4, that the overall production of PFC was least affected by a dose of 500 rads when given 24 hours after immunization (not counting irradiation on day 8, after the 19S PFC peak had been passed). This was noted in all the parameters determined, particularly in the production of circulating haemolysin. Berenbaum (1966a) took the view that the radiosensitivity of the immunocompetent cells was unlikely to vary greatly at different times during the response, but that such an apparent fall in the radiosensitivity of the response reflected an increase in their numbers. No evidence is available to contradict this, except that the antibody-releasing capacity of PFC is not affected significantly by sublethal doses of radiation. However, since these are also proliferating cells (Claflin & Smithies, 1967) they remain highly sensitive when stimulated to divide.

The stochastic model proposed in this Discussion would also predict that the numbers of immunocompetent cells (ARC and PC, and possibly also PFC) would have increased in number 24 hours after immunization. Therefore the number of survivors would presumably increase proportionately, so enabling the response to continue more rapidly than in unstimulated

animals. As has already been suggested, a dose of 500 rads would reduce the numbers of surviving cells to 0.7%, assuming a D_o of 100 rads. In the case of ARC a mean survival of 7 per spleen would therefore be expected without antigenic stimulation. It would appear possible from Fig. 9 that dissociation of ARC-PC clusters may have occurred, or more probably that dead partner cells may have been shed, leaving free ARC and PC. The resulting "growth curve" of 19S PFC did not appear dissimilar to that obtained following a dose of 350 rads given before immunization (see Fig. 13). This would indicate the survival of approximately 30 ARC per spleen, which would be expected if 2 doublings had already occurred in the original number since immunization. The peak was reached on the 6th day after immunization in each curve, following a slower than normal rate of increase, and was followed by an extended irregular plateau. According to the model, dissociated surviving ARC and PC may have interacted a second time, possibly following some proliferation, and would form unsynchronized foci reaching individual peaks at various intervals.

Irradiation 6 hours after immunization was less effective than irradiation 24 hours <u>before</u> injecting the antigen. Since proliferation did not appear to begin for 12 hours (Perkins et al, 1969), it seems unlikely that there was any significant increase in the numbers of immunocompetent cells, although some surviving PC may already have received a mitogenic stimulus from ARC which were to receive a lethal

dose of radiation. These might then double, providing a small compensatory effect to that of irradiation. It is also possible that metabolic changes following contact with antigen, particularly within ARC, may increase their ability to survive or repair radiation injury. In any case, the response was much less marked than in mice irradiated a further 18 hours later.

When 500 rads was given on the third day of the response the numbers of ARC and PC were probably very large, as were those of PFC. However the response not only declined very rapidly, but was slow to recover. Therefore it would appear that cells already in active haemolytic foci may not dissociate, even though perhaps 99% are killed. The antigenic stimulation may then run its course among the survivors, leaving them inactive.

However, as suggested previously, the presence of antigen may stimulate the production of fresh immunocompetent cells of the corresponding specificity. It may therefore be that the survivors from among these new cells produce the apparent recovery seen after the 7th day. By killing the majority of the initially-stimulated PFC, it may be possible that some of the cells which normally produce the continuation of the response are being detected. Their numbers will be very low, due to irradiation, and therefore the response will appear

slow. Nevertheless it continues to rise until it equals the number of PFC produced by the controls late in the response. Because there are few survivors, the response may by chance resemble that of the initial response of mice irradiated prior to immunization, and follow the same "pathway" (see Section 5).

The same may apply when the mice are irradiated at other times during the response, e.g. on the 8th day.

(xiii) Discrepancy between 19S PFC Number and Haemolysin Titre

In the experiment described in Part III, Section 5, it was noted that the titre of haemolysin detected in the group of mice irradiated 24 hours after immunization (the "day 1" group) remained at a higher level than in the controls from about the 14th day of the response onwards. Although it was slower to develop than in the control group, this persistence, instead of the normal decline, resulted in an overall haemolytic activity 95% of the control level during a 39-day period (as estimated by comparing the areas on the graph; see table 4). The corresponding production of 19S PFC was only 13% of the control level during the same period.

More recent investigations (not described) suggest that it is improbable that extra-splenic sites of haemolysin synthesis account for this apparent discrepancy, and in any case it would be difficult to explain why these were not as severely affected by irradiation as

splenic PFC. Since the volume of blood obtained per mouse appeared to be slightly less from the irradiated mice than from normal animals (although this was not quantitated accurately), the total amount of antibody may have been non-specifically "concentrated" in the smaller blood volume. This would be detected as a higher titre.

Another possible explanation is that some degree of selection takes place as the response proceeds, which progressively increases the affinity of the antibody produced. This process might be more marked in the irradiated mice. If it were the case, the same number of PFC might produce an antiserum of higher titre. However, it is difficult to understand why such a selective process should be more effective in irradiated than in normal size mice, particularly as the number of antigen-reactive cells is probably drastically reduced by the radiation. The only suggestion which can be offered at present is that antigen-reactive precursors are preferentially stimulated to produce fresh immunocompetent cells which play a more important part in the later development of the immune response. Selection may operate at this level, causing PC of higher efficiency to become available. In unirradiated mice, due to the rapid neutralization of antigen by the antiserum, the precursor cells may be less strongly stimulated.

Assuming this to be correct, it would follow that only those precursors which interact most strongly with SRC would be stimulated to divide. Therefore when irradiated at 24 hours the proportion of high-affinity precursors would be elevated, and would be reflected by an increased number of survivors. Irradiation before immunization would result in fewer total surviving precursor cells, and no selection. Irradiation a day after immunization results in a stronger PFC response and the antibody produced by these may have above-normal affinity.

(xiv) Enhanced Antibody Synthesis in Irradiated Animals

Related to the increase in the response if irradiation is delayed until a suitable time after immunization is the question of enhancement (see also Introduction, Sections (i) and (iv)). Such enhancement has been obtained by several groups studying rabbits (e.g. Taliaferro et al, 1952; Dixon & McConahey, 1963), but has not been reported in mice. It was never observed in these studies, and species differences seem the most likely explanation. However it may be worthy of a few comments.

Such enhanced output of antibodies appeared correlated with hyper-active plasma cell proliferation within the depleted lymphoid tissues (Dixon & McConahey, 1963). Perhaps PC, once stimulated to divide by activated ARC in rabbits, may have the ability to continue

proliferating spontaneously until caused to stop. Irradiation may kill many of both types of cell, but some PC may survive while their regulating ARC die or are dissociated from them. Therefore these PC (or PFC, as they may have become) increase until their numbers more than compensate for the initial loss. No further recruitment of unstimulated cells would be expected, and this would agree with the observation that the rate of increase in the antibody levels was slower than normal. The response may only terminate due to spontaneous degeneration of the PFC. In the mouse, on the other hand, PFC may require a continuous mitogenic stimulus from ARC to proliferate, although they may be able to continue antibody synthesis unaided.

(xv) Natural Plaque-Forming Cells

The nature of "natural plaque-forming cells" remains unresolved. Kennedy et al (1965) pointed out that these were too few in number to account for all the precursors of PFC, which they estimated to be approximately 5000 per spleen. Moreover it was shown in Part III, Section 4, that their number changed only slightly following irradiation, whereas in Sections 5 and 6 it was shown that the immune response varied widely with the time and dose of radiation.

Nordin (1968) detected n-PFC in the spleens of unimmunized germ-free

mice. Therefore it is probable that they arise spontaneously, and not in direct response to antigenic stimulation, even to antigens with common antigenic determinants to those of SRC. They were shown by Wigzell (1966) to be unaffected by specific 7S anti-erythrocytic serum. Following 500 rads of X-rays their number declined slowly, before recovering, indicating that n-PFC belong to a slowly turning-over population of cells (see Fig. 7).

It was noted in Section 9 that n-PFC were relatively frequent among spleen nodules in irradiated mice. Therefore they probably arise rapidly, but seem unable to respond to SRC. Possibly they represent PC which have already passed the immunocompetent phase and can no longer interact with ARC. They may therefore be terminal bone marrow-derived cells which are continually replaced by fresh PC. It would be desirable to have more information concerning their significance and activity.

Part V

CONCLUSIONS;

NEW ADVANCES

AND

FUTURE STUDIES SUGGESTED

V. CONCLUSIONS: NEW ADVANCES AND FUTURE STUDIES SUGGESTED

It was concluded that the principal cause of the inhibitory effects of ionizing radiation on the immune response is due to the death of immunocompetent lymphoid cells. Although the precursors of macrophages may be killed by radiation (Virolainen, 1968), mature macrophages apparently continue to function normally following sublethal doses (Muramatsu et al, 1965). Due to their loss during the normal turnover of these cells, the phagocytic capacity of the animal may decline temporarily, but there was no direct evidence from these studies that this had any significant effect on the response to SRC.

The lymphoid cells which take part in this response are probably of two main classes, thymus-derived and bone marrow-derived (Mitchell and Miller, 1968 a,b), which co-operate with each other. However, a direct response by bone marrow-derived cells to the antigenic stimulus may contribute to a lesser degree (Playfair, 1970). Both cell types undergo mitosis before marrow-derived precursors are "recruited" to become PFC (Davies et al, 1967; Nossal et al, 1968), and it is probably before or during this antigen-induced proliferation that the damage caused by irradiation exerts

its lethal effects. PFC already recruited may continue antibody synthesis for several days despite lethal doses of radiation (Sado, 1969), provided the mitogenic stimulus is cut off. The effect of this cell-killing may be similar to a reduction in the concentration of lymphoid cells in tissue cultures.

In these studies it was observed that the immune response was reduced, delayed and slowed by irradiation at various times before and after immunization. A stochastic model based on that of Groves et al (1970) has been proposed to explain this in terms of changes in the numbers of viable cells. While this model is still highly speculative, it appears to be compatible both with the major findings reported in the recent literature and with most of the additional observations here. To this extent, this thesis represents an attempt to integrate the findings of many other authors and to provide a basis for future investigation.

New findings included the following:-

1. An approximate value for the Oxygen Enhancement Ratio of mature PFC irradiated <u>in vitro</u> was obtained, but their comparatively low radiosensitivity was already known.

2. Berenbaum (1966a) had already shown that the immune response could be inhibited temporarily by irradiation at any time following the injection of SRC. However, the experiment described in Part III, Section 5, was more extensive than his and included results for 7S PFC and haemolysin titres, as well as 19S PFC. Similarities in the numbers of 19S PFC in the different groups of irradiated mice were noted.

3. When a direct comparison was made between the numbers of 19S PFC and the levels of haemolysin (as far as this was possible), both similarities and discrepancies were noted. A tentative explanation for the differences was proposed.

4. Effects of different doses of radiation prior to immunization were described, and the difficulties involved in obtaining useful dose-response curves were discussed. The D_o for the survival of the immunocompetent cells involved in the response to SRC was estimated to be approximately 97 rads, although the "survival curve" obtained 4 days after immunization indicated a D_o of only about 60 rads. The overall radiosensitivity of the immune response

was estimated from an integrated dose-effect curve, the D_o of which was in the order of 150 rads. The possible reasons for this wide range of results was discussed in terms of the changed rate of the immune response and the theoretical model.

5. Dose-response curves were also obtained for the first time when mice were irradiated two days after immunization. That for 19S PFC also indicated a D_o value of between 90 and 100 rads for their precursors (or their associated thymus-derived cells). From the maximum rates of disappearance of 19S PFC, their mean cell cycle time was estimated to be in the order of 10 hours.
6. A sparing effect was shown for the first time in the immune mechanism by the use of fractionated irradiation.
7. The recovery of immunocompetence following irradiation has been studied by many previous investigators, but an experiment in these studies provided additional information on the kinetics of the response in partially recovered mice. The use of the spleen cell transfer system showed that recovery was accompanied by the re-appearance of immunocompetent cells.
8. Information additional to that of Celada and Wigzell (1966) on the appearance of PFC in spleen nodules of

irradiated mice was presented in Part III, Section 9, but the conclusions were similar.

9. Results obtained in Section 10 using the spleen cell transfer system were somewhat disappointing and should be repeated. An approximate estimate of the sensitivity of the precursors of PFC to irradiation in vitro was obtained, but a degree of uncertainty was introduced by the need to use a correction factor to compensate for the non-linear relationship between the number of cells transferred and the number of PFC produced. The best estimate for their D_o was 55 rads. No clear effect of varying the number of cells or the dose of radiation on the rate of appearance of PFC was obtained, although the response seemed more rapid with a larger number of transferred spleen cells.

Several lines of investigation in this thesis are far from complete and further study might be profitable.

Experiments are already in progress to determine the radiosensitivity of mature PFC irradiated in vitro with greater accuracy. Others are designed to determine the relative importance of PFC in extra-splenic sites in normal and irradiated mice.

It would also be of considerable interest to study the effects of radiation on transferred thymus and bone marrow cells in experiments similar to those of Claman et al (1966 a, b).

More complex, perhaps, would be an investigation of the cell cycle times of the precursors of PFC in normal and irradiated mice, to determine whether this is affected by irradiation, or whether the apparent slowing of the response is due to delayed recruitment of PFC.

Also the factors which limit the peak numbers of PFC require investigation, and a comparative study of mice and rabbits might be of particular interest. Related to this, an examination of the haemolytic efficiency of the antibody produced by PFC at different stages in the response of normal and irradiated mice might provide a direct explanation for the apparent discrepancy between the numbers of PFC and the titre of antibody reported in Part III, Section 5.

The precise role of macrophages in the response to SRC is still unknown. Possibly tissue culture experiments would be most fruitful in determining this.

Use of the cell transfer system would allow a comparative study of the effects of ionizing and ultra-violet radiation on immunocompetent cells, which has not yet been done. Similarly, it would also facilitate a study of the effect of anoxia on their sensitivity.

As knowledge of the mechanism of the immune response increases, so will the value of experiments using it in order to elucidate the effects of radiation on biological systems in general.

R E F E R E N C E S

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A D D E N D U M

" P L A Q U E S "

C O M P U T E R P R O G R A M

F O R C A L C U L A T I O N O F

R E S U L T S

C O M P U T E R P R O G R A M

```
PLAQUES;
"BEGIN""REAL"TOTAL,SS,VAR,SE,VARC,SEC,PFC,VP,VC,U,L,UT,LT,T,
RAT,CELL,X,A;
"INTEGER"J,I,N,MICE,NUM1,NUM2,K,C,CDASH;
"READ"NUM1;
"FOR"J:=1"STEP"1"UNTIL"NUM1"DO"
"BEGIN""READ"NUM2,N,T;
"BEGIN""REAL""ARRAY"Y[1:N];
"FOR"K:=1"STEP"1"UNTIL"NUM2"DO"
"BEGIN""READ"A,VP,VC,C,CDASH,MICE;
TOTAL:=SS:=0.0;
"FOR"I:=1"STEP"1"UNTIL"N"DO"
"BEGIN""READ"Y[I];
TOTAL:=TOTAL+Y[I];
SS:=SS+Y[I]*Y[I];
"END";
X:=C/CDASH;
SAMELINE;
"PRINT""'L7'',ALIGNED(3,2),A,
''L'VP=',ALIGNED(5,2),VP,
''L'VC=',VC,
''L'',DIGITS(4),C,' CELLS COUNTED IN VOL. ',
```

```
ALIGNED(2,2),X,
''L`MICE=`,DIGITS(2),MICE,''L2``;
"FOR"I:=1"STEP"1"UNTIL"N"DO"
"PRINT"ALIGNED(4,0),Y[I],''S1``;
VAR:=(SS-TOTAL*TOTAL/N)/(N-1);
SE:=SQRT(VAR);
TOTAL:=TOTAL/N;
"PRINT"''L2`MEAN=`,ALIGNED(5,2),TOTAL,
''L`VAR =`,VAR,
''L`S.D.=`,SE;
PFC:=VP*TOTAL/MICE;
VAR:=VP*VP/MICE/MICE*VAR*(1/N-1/VP);
SE:=SQRT(VAR);
L:=PFC-2*SE;
U:=PFC+2*SE;
LT:=PFC-T*SE;
UT:=PFC+T*SE;
"PRINT"''L2`PFC/SPLEEN=`,ALIGNED(7,2),PFC,
''L`VAR =`,VAR,
''L`S.E.=`,SE,
''L`PFC/SPLEEN +,- 2S.E. `,L,''S2``,U,
''L`95% C.I. PFC/SPLEEN `,LT,''S2``,UT;
```

```
CELL:=CDASH*VC*10^4/MICE;
VARC:=CELL*CELL/C;
SEC:=SQRT(VARC);
L:=CELL-2*SEC;
U:=CELL+2*SEC;
"PRINT""L2`CELLS/SPLEEN=`,SCALED(4),CELL,
""L`VAR =`,VARC,
""L`S.D.=`,SEC,
""L`CELLS/SPLEEN +,- 2S.E. `,L,""S2``,U;
RAT:=PFC/CELL*10^6;
VAR:=RAT*RAT*(VAR/PFC/PFC+1/C);
SE:=SQRT(VAR);
L:=RAT-2*SE;
U:=RAT+2*SE;
"PRINT""L2`PFC/10^6 CELLS=`,FREEPOINT(4),RAT,
""L`VAR =`,FREEPOINT(5),VAR,
""L`S.E.=`,FREEPOINT(4),SE,
""L`PFC10^6 CELLS +,- 2S.E. `,L,""S2``,U;
"END";
"END";
"END"
"END";
```