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Migration of Antibody-Forming Cells and Antigen-Sensitive Precursors between Spleen, Thymus and Bone Marrow

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Summary. The appearance of 19S and 7S antibody-forming cells (producers) in mice following a single i.p. injection of sheep erythrocytes, and their recirculation between spleen, thymus and marrow, were studied by the direct and indirect Jerne plaque techniques. The appearance and distribution of antigen-sensitive precursor cells (memory cells) in the same tissues were determined by transferring aliquots of thymus, marrow and spleen cells to irradiated recipients, restimulating with antigen, and counting the plaque-forming cells (PFCs) in the recipient spleens. 2-ME sensitive and 2-ME resistant serum antibodies in donors and recipients were also titrated.

19S antibody production in mice reached a peak 4 days after antigen and declined rapidly thereafter. Direct PFCs were found in the spleen, but not in thymus or marrow. These cells probably do not recirculate, at least not within their lifespan as 19S antibody producers. Production of 7S antibody reached a peak 7 days after immunization. Indirect PFCs were most numerous in the spleen at this time and persisted in the body for several weeks. These cells probably recirculate since increasing numbers were found in the thymus and marrow with increasing intervals after immunization.

The appearance and distribution of antigen-sensitive precursor cells showed a pattern similar to that of the 7S producers, suggesting that they recirculate in a similar manner.

INTRODUCTION

The origin of the 'stem cell' and the nature of immunological memory are two problems of antibody production which have been of interest for some time. Previous studies have shown that the first cells to produce antibody following antigenic stimulation to an animal occur in spleen or regional lymph nodes, depending upon the route of antigen injection (McMaster, 1961). Although antibody production has been observed in other sites, e.g. peripheral blood, lung, liver, etc., responses are usually later and much lower in titre (Friedman, 1964).

Antibody production by thymus or marrow cells has been shown. In most of these experiments, donor animals had either been injected with antigen in adjuvant (Askonas and White, 1956) or with several doses of antigen (Stoner and Bond, 1963). In these cases, several weeks elapsed between primary exposure to antigen and evaluation of cells for antibody production. This was also the case when antigen was directly injected into the thymus (Marshall and White, 1961). The antibody-forming cells found in thymus

and marrow were considered to be indigenous to those organs. The recirculation of immunocompetent cells during the long period between immunization and cell evaluation was not considered in detail until the experiments of Gengozian, Makinodan and Shekarchi (1961). Here too, the greatest amount of antibody production by marrow cells occurred when there was a long interval between antigen stimulation of donor animals and cell transfer. Landy, Sanderson, Bernstein and Lerner (1965) reported different results; antibody-producing cells were found in the rabbit thymus only at 5 days after a single dose of antigen, at a time when thymus cellular destruction was maximal.

Preliminary findings (unpublished) from this laboratory indicated that cell suspensions from the thymus or marrow of previously immunized mice contained both antibody-producing cells and antigen-sensitive precursor cells, if a sufficient interval were allowed to elapse between antigenic stimulation and preparation of cells for Jerne plaque assay or transfer to irradiated syngeneic hosts. The present report represents a systematic attempt to define the kinetics of appearance of both cell types in these tissues. The results suggest that some antibody-producing cells (producers) and some antigen-sensitive precursor cells (precursors) move from the spleen to the thymus and marrow.

MATERIALS AND METHODS

The basic plan was to immunize donor mice with a single injection of sheep erythrocytes. Subsequently at varying intervals donor mice were sacrificed and antibody-forming cells were looked for in their spleen, marrow and thymus. Cell suspensions from these organs were then injected into irradiated recipient mice and antigen was given. Antibody-producing 'descendants' of these transferred cells were sought in recipient spleens.

TABLE 1

NUMBERS OF CELLS TRANSFERRED TO IRRADIATED RECIPIENTS

Donors	Thymus	Marrow	Spleen
42-day	36·1	10·0	1·0
28-day	35·7	10·0	1·0
14-day	21·5	10·0	1·0
7-day	45·7	9·8	1·0
4-day	58·2	10·0	2·6
Non-immune	52·0	9·9	1·1

Cells injected ($\times 10^6$).

Male LAF_1 mice obtained from the Jackson Laboratory were used in all experiments. Donors were from a single shipment, age 5–6 weeks, at the start of the experiment. Immune donors received a single intraperitoneal injection of 0·2 ml of a 10 per cent suspension of washed sheep erythrocytes (SRBC) at varying intervals prior to transfer of cells; days −42, −28, −14, −7 and −4. Normal donors (day 0) received no SRBC. At death, all donors were 12–13 weeks old. On day 0, the donors were killed, sera were obtained, and cell suspensions prepared in cold Saline F (Puck) from thymus, marrow and spleen (Claman, Chaperon and Triplett, 1966a) were counted. Aliquots of these suspensions were assayed for antibody-forming cells (see below). To test for precursors, other aliquots (Table 1) of these suspensions were injected i.v. into mice which had been

irradiated 1–3 hours previously (750 r in air, 250 kVp X-rays, 30 mA, 0·5 mm Cu, 1·0 mm Al). The dose of X-rays received by the mice was 855 r. Recipients were injected i.v. with SRBC (0·5 ml, 40 per cent) on day 0 and i.p. on day 4 (0·2 ml, 10 per cent). The i.v. injection also contained the transferred mouse cells. On day 8, the recipient mice were killed, bled for sera, and spleen cell suspensions were prepared for assay of plaque-forming cells.

For convenience, cells transferred will be described according to the cell type and the number of days between immunization of the donor and transfer of the cells, e.g. '4-day spleen' or '42-day thymus'.

Assay of plaque-forming cells (PFC) (producers)

Aliquots of the donor thymus, marrow and spleen suspensions and the recipient spleen suspensions were diluted in Earle's Solution and plated in duplicate according to the single cell haemolysin technique (Jerne, Nordin and Henry, 1963). After incubation for 1 hour at 37°, 2 ml of a 1:25 dilution of rabbit anti-mouse γ-globulin (Colorado Serum Company, Denver) were added to one of each pair of duplicate plates (Dresser and Wortis, 1965; Sterzl and Riha, 1965; Weiler, Melletz and Brenninger-Peck, 1965) and 2 ml of diluent (Earle's) were added to the other plate.

Following an additional hour of incubation, the plates were rinsed with Earle's solution and 2 ml of 20 per cent freshly reconstituted lyophilized guinea-pig serum were added to each plate. Development of plaques was permitted to proceed for 1 hour before they were counted. Plaques seen on plates covered with Earle's diluent were called 'direct' plaques. Plaques seen on plates covered with rabbit anti-mouse γ-globulin were called 'indirect' plaques.

Serum antibodies

The serum samples from each treatment group were pooled and titrated for both 2-mercaptoethanol (2-ME) sensitive and (2-ME) resistant antibodies. One volume of undiluted serum was mixed with 1 volume of 0·015 M 2-ME in 0·02 M phosphate-buffered saline, pH 7·0 (PBS) and incubated at 37° for 3 hours. Duplicate controls contained 1 volume of serum and 1 volume of PBS. $\log_2$ haemolysin and haemagglutinin titres were determined by means of a microtitre technique as previously described (Claman *et al.*, 1966b).

RESULTS

1. PLAQUE-FORMING CELLS (PFC) AND SERUM ANTIBODIES IN DONOR MICE (Figs. 1 and 2)

The number of direct PFC in the spleens of immune donors (Fig. 1) reached a maximum level by 4 days following immunization, fell off rapidly thereafter, and reached control levels by 42 days. 2-ME sensitive antibodies in the sera of these animals (Fig. 2) reached peak titre at approximately the same time as the direct PFCs and disappeared by 28 days. In contrast, direct PFCs in the thymus and marrow of immune donors never exceeded that of the non-immune controls.

In the duplicate plates, plaques were 'developed' by the addition of anti-mouse globulin. The number of these developed (indirect) plaques was greatest in those spleens plated 7 days after immunization of the donors and decreased rapidly thereafter, but

did not reach control levels by day 42. The numbers of indirect PFCs in donor thymus and marrow were still increasing 42 days after immunization. By this time the marrow activity equalled that of the spleen, while the thymus was only about one-third as active as marrow or spleen. 2-ME resistant serum antibodies reached a peak titre 7 days after immunization and this was maintained for at least 42 days.

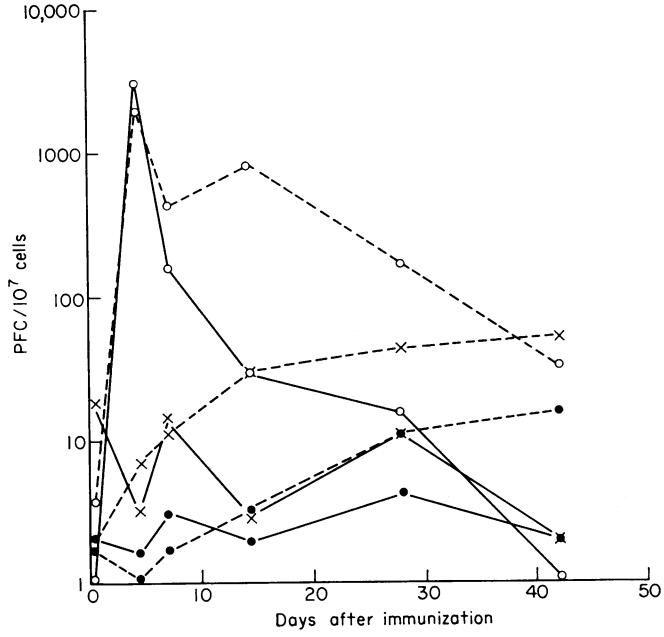

FIG. 1. Direct (—) and indirect (- - - -) plaque-forming cells in spleen (○), thymus (●) and marrow (×) of donor LAF_1 mice killed at varying intervals after a single i.p. injection of 0·2 ml of 10 per cent sheep erythrocytes.

2. PLAQUE-FORMING CELLS AND SERUM ANTIBODIES IN RECIPIENT MICE (Figs. 3 and 4)

The presence of antigen-sensitive precursor cells in spleen, thymus and marrow suspensions from the immune donor mice was evaluated by measuring the antibody response following transfer of these suspensions to irradiated syngeneic hosts, which were then stimulated with sheep RBC. The direct PFCs in recipient spleens (Fig. 3) were most numerous in those animals injected with 4-day spleen cells. The 2-ME sensitive serum antibody titres (Fig. 4) were also highest in those animals receiving 4-day spleen cells, and decreased thereafter as the interval between immunization of the donors and transfer of the cells was increased.

The patterns of direct PFC production in the spleens of animals receiving immune-donor thymus or marrow cells (Fig. 3) resembled the pattern of indirect PFC production in the donor suspensions. There were more PFC in spleens of mice receiving 4-day marrow than in controls and the number of PFC appeared to be approaching a plateau by day 42. PFC in spleens of mice receiving thymus cells also increased as the interval between immunization of donor and death was increased and appeared to reach a plateau at day 42. There were approximately ten times more PFC in spleens from recipients given

marrow cells than in spleens from recipients given thymus cells. The 2-ME sensitive serum antibody titres in these recipient animals were never high enough to establish the presence or absence of a similar trend.

The developed (indirect) PFC response curves of all groups of recipients, whether transfused with immune thymus, marrow or spleen cells, were strikingly similar (Fig. 3),

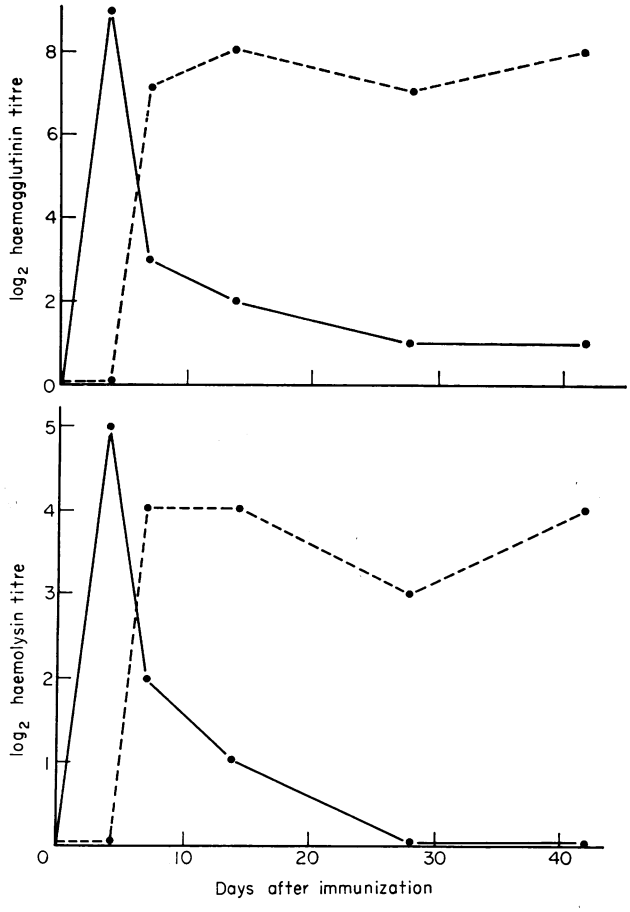

FIG. 2. Serum $\log_2$ haemolysin and haemagglutinin titres of donor LAF_1 mice killed at varying intervals after a single i.p. injection of 0·2 ml of 10 per cent sheep erythrocytes. 2-Mercaptoethanol sensitive (—) and resistant (- - -) titres are shown.

except for their differing degrees of magnitude. All responses still had not reached maximum levels when the interval between immunization of donors and transfer of cells was extended to 42 days. At this time, the developed PFC/10^7 injected cells exceeded 15,000 for spleen cells, 2000 for marrow cells and 140 for thymus cells. Serum 2-ME resistant antibody titres differed somewhat from the number of PFC in the spleens, but there was a general trend toward an increase in titre, particularly of agglutinins, as the interval between immunization and transfer of cells increased.

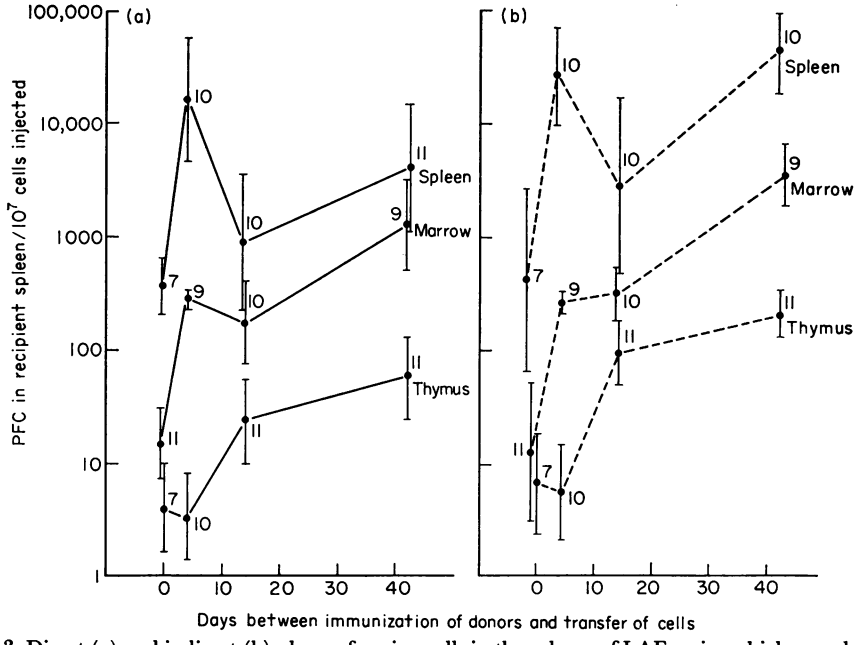

FIG. 3. Direct (a) and indirect (b) plaque-forming cells in the spleens of LAF_1 mice which were lethally irradiated, then injected with thymus, marrow or spleen cells from immune or non-immune donors. Sheep RBC were given (i.v. on day 0 and i.p. on day 4) and the mice were killed at day 8 following irradiation. Each point indicates the geometric mean ± the 95 per cent confidence limits. The numbers adjacent to the points represent the numbers of animals used.

DISCUSSION

PFC and serum antibodies in donor mice

The interrelations between direct and indirect PFC and 2-ME sensitive and 2-ME resistant antibody titres must be considered when interpreting the results of these experiments. The early appearance and rapid decline of 2-ME sensitive antibodies and the delayed but sustained rise of 2-ME resistant antibodies in the donors agrees with the results of other investigators (Uhr and Finkelstein, 1963; Sterzl and Riha, 1965; Wigzell, Möller and Andersson, 1966) and supports their designation of 19S and 7S, respectively. 2-ME sensitive 7S antibody in mice following a single i.p. injection of SRBC has, however, been reported by Adler (1965).

The probability that most of the direct PFC in the donors made 19S antibody is supported by the known great haemolytic efficiency of 19S antibodies (Humphrey and Dourmashkin, 1965) and by the parallel between 2-ME sensitive serum agglutinin titres and direct PFC in the spleen (cf. also Wigzell *et al.*, 1966). A similar correlation of indirect PFC with 7S antibody production has been made by Sterzl and Riha (1965) and Dresser and Wortis (1965). It was based on the finding that the peak level of indirect PFC occurred later than that of the direct PFC; the antiserum used to develop these plaques was primarily anti-γG; and although this antiserum developed plaques in spleen suspensions taken 9 days after immunization, it inhibited plaque formation in suspensions taken at 2 days (Wortis, Taylor and Dresser, 1966). We feel, therefore, that the direct PFC and 2-ME sensitive serum titres in the donors are predominantly indicators of 19S

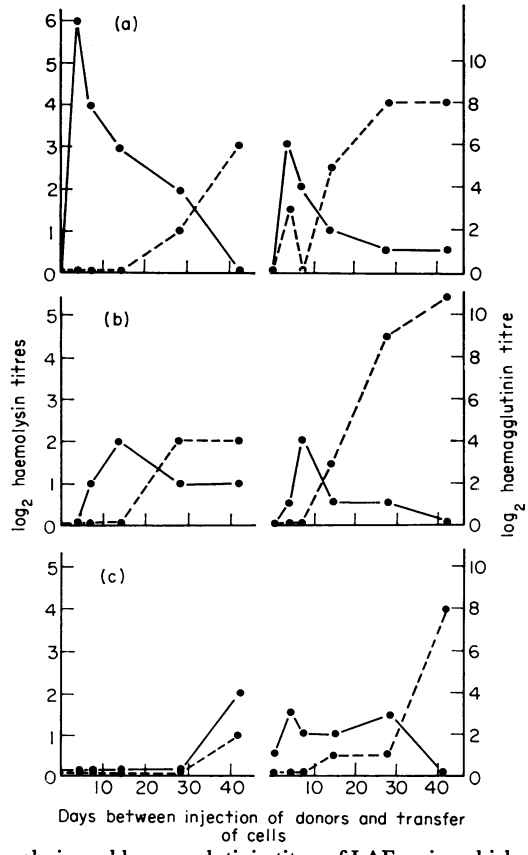

FIG. 4. Serum $\log_2$ haemolysin and haemagglutinin titres of LAF_1 mice which were lethally irradiated, then injected with: (a) spleen, (b) marrow or (c) thymus cells from immune or non-immune donors. Sheep RBC were injected (i.v. on day 0 and i.p. on day 4) and the mice were killed at day 8 following irradiation. 2-Mercaptoethanol sensitive (—) and resistant (- - - -) titres are shown.

antibody production, and that indirect PFC and 2-ME resistant titres probably represent 7S antibody.

In the donor mice, the number of direct PFCs in the spleens and 2-ME sensitive haemolysins and haemagglutinins in the sera reached a peak 4 days following a single i.v. injection of SRBC. Comparable direct PFC were never detectable in the thymus or bone marrow of these animals. It is known that in rats (and presumably in mice) the spleen is responsible for most of the antibody produced in response to intravenous SRBC (Rowley, 1950). It appears that most of the direct PFCs originate in the spleen but disappear rapidly thereafter. Significant recirculation of these cells to other lymphoid tissues was not demonstrable, at least not within their life-span as 19S antibody-producers.

The number of indirect PFC in the spleen and the 2-ME resistant serum antibody titres reached peak levels by 7 days following antigenic stimulation. Spleen activity declined thereafter, although 2-ME resistant serum antibodies remained at or near peak levels for the entire 42-day period. Indirect PFC in the thymus and marrow appeared at first in small numbers but continued to increase as spleen activity fell off until at 42 days the marrow was as active as the spleen (50 PFC/10^7 cells), and the thymus had about one-third the activity of the other two tissues. Harris and Ford (1964) have demonstrated

the recirculation of lymphoid cells, and Galton and Reed (1966) have shown that lymphoid cells enter the thymus. In addition, plaque-forming cells (Friedman, 1964) and stem cells (Santos and Owens, 1964) have been found among circulating leucocytes. We feel that the most likely interpretation of the data is that long-lived indirect PFC (7S producers) originate in the spleen, leave early in immunization and 'equilibrate' within the lymphoid tissues of the body, as exemplified by the thymus and marrow.

The late-appearing PFCs in the thymus and marrow may also have developed *in situ* (under the influence of antigen within these organs) from immunologically competent precursors, either indigenous to these organs or immigrants from some other source, e.g. the spleen. This is unlikely, however, since such precursors have not been found in marrow and thymus cells from unstimulated donor animals, when transferred to irradiated recipients and then stimulated with SRBC (Claman *et al.*, 1966a). Since precursors were found among spleen cells from unstimulated donors, one would expect also to find them in thymus and marrow if they circulated in significant numbers prior to immunization. In addition, since there is a partial blood–thymus barrier for at least some antigens (Clark, 1964), a delayed response might occur in the thymus, but the rapid clearance of circulating RBC in immunized animals (Hollingsworth, 1958) opposes this possibility. Also, the appearance of new PFC in the thymus and marrow while splenic PFC were disappearing is difficult to explain in the presence of decreasing concentrations of circulating SRBC antigen.

PFC and serum antibodies in recipient mice

In the recipients, the direct and indirect PFC and 2-ME sensitive and 2-ME resistant serum titres do not correlate, probably because of the multiple immunization procedure employed. Large numbers of direct PFC were observed in spleens of mice when no 2-ME sensitive serum antibodies were detectable. We suggest that developing such plates with anti-mouse globulin merely increased the haemolytic efficiency of 2-ME resistant antibody, resulting in an increased number of plaques of the same type.

The kinetics of PFC production in the spleens of recipient mice showed a striking similarity to indirect PFC population in the donor suspensions, with the exception of a secondary or 'late' rise in the spleens of those mice receiving 42-day immune-donor spleen cells. The only groups with high titres of 2-ME sensitive antibody were those receiving 4-day spleen cells (the serum antibody titres of the non-immune spleen recipients were negligible, probably because of the small number of cells transferred). Thus most, if not all, of the PFCs seen in the recipients of immune thymus, or marrow cells, in addition to the secondary rise in the recipients of spleen cells, were due to 2-ME resistant antibody. This would be in agreement with the results of Uhr and Finkelstein (1963), and indicates that the secondary response antibody is predominantly 7S.

The early appearance of precursor cells in the spleens of the donor mice and their later progressive appearance in the thymus and bone marrow suggest that they are recirculated. This hypothesis has already been proposed by Gengozian *et al.* (1961) to explain their success in conferring immune responsiveness on lethally irradiated mice by transferring marrow cells from immune donors. In addition, previous attempts to demonstrate the immune competence of transferred thymus cells have only been successful when donor animals were immunized (Stoner and Bond, 1963). These findings suggest that thymus and marrow cells from immune mice contain a substantial proportion of immigrant

cells, ostensibly coming from the spleen, which have reached these tissues during recirculation following antigenic stimulation.

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