Immunology, 1974, **27**, 167.

Immune Competence of Hereditarily Asplenic Mice

B. B. Lozzio and Liliana B. Wargon*

The University of Tennessee, Memorial Research Center and Hospital, Knoxville, Tennessee, U.S.A.

(*Received* 10*th October* 1973; *accepted for publication* 3*rd December* 1973)

Summary. Antibody formation and cellular immunity were studied in congenitally asplenic mice challenged with sheep erythrocytes (SE) and transplants of skin and spleen. Asplenic mice had a significantly lower antibody production than normal animals, albeit higher than splenectomized littermates in the primary and secondary immune responses. The serum levels of immunoglobulin (Ig) M of asplenic mice were diminished after a primary immunization and normal in the secondary response. Although the serum concentration of IgG2 was normal after a single antigenic stimulation, there was a reduction of IgG2 serum levels during the secondary response. The serum concentration of IgG1 was significantly higher in asplenic than normal mice. The number of 19S PFC was markedly reduced throughout the lymphopoietic system of asplenic mice which had a large number of 19S and 7S PFC in the blood stream, when compared to normal littermates, after a single antigen injection. During the secondary immune response there was a great improvement of the number of PFC in lymph nodes of normal and splenectomized mice but not in that of asplenic mice. The number of 7S PFC in other lymphoid tissues of asplenic mice was markedly diminished. Hereditarily asplenic mice had normal cellular immunity as indicated by normal rejection times of spleen and skin allografts. The results are consistent with the concept of decreased antibody production associated with asplenia and demonstrated the important function of the spleen during embryogenesis to achieve normal humoral immunity in adult life.

INTRODUCTION

The function of the spleen in lymphopoiesis (Fichtelius and Back, 1970) and antibody formation of adult mammals has been extensively studied and is now well established (Arvy, 1965; Mellbye, 1970; Rowley, 1950; Taliaferro, 1956; Taliaferro and Taliaferro, 1950, 1951). The spleen is a major site of cellular reactions leading to the synthesis of antibodies in rodents (Mellbye, 1970; Rowley, 1950; Taliaferro, 1956; Taliaferro and Taliaferro, 1950, 1951). The vast majority (>90 per cent) of serum antibodies during the 1st week after injection of non-primed and primed spleen cells are synthesized by immunocompetent cells which colonize the spleen (Bosma, Perkins and Makinodan, 1968). Further, the spleen accounts for 98–99 per cent of the total antibody produced after an intravenous or intraperitoneal antigenic stimulation (Perkins, 1970).

* Pre-doctoral Research Trainee from the Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Argentina.

Correspondence: Dr Bismarck B. Lozzio, The University of Tennessee, Memorial Research Center and Hospital 1924 Alcoa Highway, Knoxville, Tennessee 37920, U.S.A.

A classical method for studying the function of an organ is to remove it and follow the alterations developing thereafter. Although well defined immunological (Rowley, 1950; Taliaferro and Taliaferro, 1950) and haematological (Crosby, 1963; Dameshek, 1955) changes develop in animals after splenectomy in adult and neonatal life (Bucsi, Borek and Battisto, 1972; Haupfeldova-Dolejskova, Nouza, Matousek and Hauptfeld, 1969) these experiments do not define the contribution of the spleen to the development and maturation of the immunological system during embryogenesis. For that reason, considerable interest has recently arisen in the study of the haematological (Lozzio, 1972) and immunological (Battisto, Cantor, Borek, Goldstein and Cabrerra, 1969; Meier and Hoag, 1962) modifications that may be present in congenitally asplenic mice.

In the present study we wished to demonstrate the pattern of immunoglobulin production during the primary and secondary immune responses of congenital asplenic and splenectomized littermates challenged with sheep erythrocytes. Emphasis was given to the sequential pattern of IgM and IgG production after a single antigenic stimulation and that of IgG during the secondary immune response, because it appears that spleen cells are essential for IgM synthesis (Haupfeldova-Dolejskova *et al.*, 1969; Meier and Hoag, 1962; Nakov, Sejkorova and Nouza, 1969). Several additional features of the immune response such as serum levels of 19S and 7S antibodies and the number of antibody-forming cells throughout the lymphopoietic system, were also studied to establish conclusively the immunological capability of congenitally asplenic animals. Cellular immunity was also studied in adult asplenic mice recipients of spleen or skin allografts.

MATERIALS AND METHODS

Animals

Congenitally asplenic mice, heterozygous (Dh/+) for dominant hemimelia (Dh) and normal (+/+) mice with spleen hereafter described as 'asplenic' and 'normal' respectively, were bred at our Institution as previously reported (Lozzio, 1972). Mice of both sexes were 2–3 months old and were in the fifth and sixth generation of inbreeding at the time of these experiments. Splenectomy was performed in 1-month-old mice under methoxyflurane anaesthesia (Metofane, Pitman Moore, Fort Washington, Pennsylvania) Sham-splenectomy was also made by exteriorizing, then returning, the spleen to the peritoneal cavity followed by gentle manipulation of adjacent viscera. Mice were given oxytetracycline in the drinking water for 1 week after surgery to prevent post-operative infection. Splenectomized and sham-splenectomized mice were used 2 months after surgery because splenectomized mice remained anaemic for at least 45 days after surgery (Lozzio, 1972).

Preparation of sheep cells for immunization and plating

Sheep erythrocytes (SE) were washed three times with saline and a 10 per cent suspension (v/v) containing 20×10^9 SE/ml of saline was prepared. Mice were injected i.v. with 0·2 ml and killed at 3-day intervals for 12 days. To study the secondary immune response mice were given a second injection (0·2 ml) of a 10 per cent SE suspension 30 days later and were killed 5 days after the second injection of SE. For plating, SE were aged for 15–20 days, washed as indicated above and made up to 10 per cent in Eagle's MEM medium for use in the plaque-forming cells (PFC) assay.

Preparation of cell suspension for plating

Mice immunized with SE were killed by exsanguination and the serum collected was stored at −20° for antibody titration. Each spleen (when present) and all visible para-aortic and iliolumbar lymph nodes were removed and collected in plastic Petri dishes containing 1 ml of cold Eagle's MEM with Hanks's salts medium. The spleen and lymph nodes were minced with scissors and teased over ice until no macroscopic clumps were visible. Each cell suspension, free of connective tissue, was aspirated with a syringe and the dish rinsed once with 1 ml of chilled medium. Each homogenous cell suspension was kept in an ice bath until used; in general, no more than 2 hours. The marrow from both femurs was flushed out and clumps gently dispersed with the aid of a Pasteur pipette and a Vortex mixer until a homogenous cell suspension was obtained in 2 ml of the medium. Mice, in which peripheral blood cells were to be collected, were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.) and exsanguinated by puncturing the inferior cava vein. The blood was collected into a heparinized syringe, transferred to a macrohaematocrit tube, and spun down at 1300 ***g*** for 10 minutes at 4°. The buffy coat was picked up with a Pasteur pipette and delivered into another tube containing 2 ml of chilled Eagle's medium. The leucocytes containing a small number of erythrocytes were then ready for plating. Aliquots (0·1 ml) of each spleen cell suspension were diluted 1:200, whereas those from lymph nodes, marrow and leucocytes were diluted 1:20 in Turk's solution. Cells were counted in a haemocytometer.

Determination of cell viability

Cell viability was determined by the dye exclusion test using a 0·2 per cent stock solution of Trypan Blue in distilled water. The number of antibody-forming cells given throughout this publication represents the number of plaques per million (ppm) viable cells.

Plaque-forming cells assay

Direct (19S) PFC were detected according to the method of Jerne, Nordin and Henry (1963). Half a millilitre of the appropriate cell suspension was added to each plate. Indirect (7S) antibody-forming cells were developed using a goat-antiserum to mouse 7S γ-globulin (Hyland, Incorporated, Costa Mesa, California) diluted 100-fold, a concentration previously found to give optimal development of indirect PFC following the method of Dresser and Wortis (1965). Plaques were scored under magnification × 7.

Titration of serum antibodies

Sera were titrated from haemagglutinin and haemolysin by standard two-fold dilution method as previously described (Lozzio and Comas, 1969). Titres were expressed as $\log_2$ of the reciprocal of the highest dilution showing macroscopic haemagglutination or complete haemolysis (100 per cent).

Treatment of sera with 2-mercaptoethanol (ME)

Equal amounts (0·2 ml) of undiluted serum and of 0·2 M solution of ME were mixed and incubated at 37° for 30 minutes. Control tubes contained either ME alone or another sample with equal amounts of serum and saline. The antibody titre remaining after treatment with ME was considered to be ME-resistant antibodies of 7S type.

Quantitative determination of serum immunoglobulins (Ig)

All details of the method used were identical to the Mancini, Carbonara and Heremans (1965) radial diffusion technique. An equal volume of a mouse anti-SE serum adjusted to 300 μg of protein per millilitre and warmed to 55°, was mixed with the agar, and the mixture poured into the plastic mold. The final concentration of 150 μg of protein per millilitre of mouse serum was found to give the best results. Serum from non-immunized mice were used in control plates. Two millilitres of goat antiserum to mouse gamma-globulin-M, gamma-1 (7Sγ2) and gamma-2(7Sγ2) (Melpar Biological Products Laboratory, Springfield, Virginia) were added to different wells of agar plates, which were incubated at 37° for 10 days. Alternatively, goat antiserum to mouse IgG and IgM was incorporated into the agar, whereas the mouse anti-SE serum was added to each well. The incorporation of mouse serum into the agar gave the best results. The surface of radial precipitates was determined with the aid of a viewer (Miles Laboratory, Incorporated, Kankakee, Illinois). From these data the proportional amount of each Ig was calculated.

Skin and spleen allografts

Donors and recipients of skin or spleen transplants were anaesthetized with sodium pentobarbital as indicated earlier. A full-thickness skin graft of approximately 1 cm in diameter was prepared from mice of the Dh (isograft) and BALB/c (homograft) strains. Grafted skin was firmly secured with three to five silk stitches on the back of the recipient. Wounds were topically treated for 2 consecutive days with a terramycin solution containing 125 mg/ml of the diluent.

Transplants of a whole spleen, either intact or cut in two pieces, were also made by implanting it into the peritoneal cavity of adult asplenic and splenectomized mice. The fate of the grafted spleen was determined using leucocyte counts as an index of the initiation of the rejection time. The rejection of the spleen produced marked leucocytosis. Leucocytes were electronically counted prior to and every 2 days after a spleen implantation. An increase of two to three times the leucocyte number was regarded as the first sign of spleen rejection. Then mice were killed and spleens macroscopically examined.

RESULTS

PRIMARY IMMUNE RESPONSE

None of the normal (non-immunized) control mice exhibited detectable haemagglutination titres when tested against SE. Background plaque counts were obtained on spleen, lymph nodes, bone marrow and peripheral blood cells. Counts from ten normal and ten sham-splenectomized mice of up to 1·5 ppm cells are considered normal background.

Immunization of normal and sham-operated mice with a single injection of SE resulted in a similar and rapid increase of serum agglutinin titres. Asplenic animals had approximately 25 per cent of the serum antibody titres found in mice with spleen (Fig. 1). The agglutinin titre of mice splenectomized 2 months prior to immunization was around 5 per cent of that found in mice with spleen and 25 per cent of that observed in mice with agenesis of the spleen. The pattern of haemolysin titres was completely similar to that of agglutinin titres even though the rise and fall of serum haemolytic antibodies took place faster than agglutinating antibodies. The highest haemolysin titre (100 per cent haemolysis) was found 6 days after immunization. Normal mice had a mean $\log_2$ titre of 8 (100 per

cent), asplenic mice 6 (25 per cent) and splenectomized mice 4 (6·3 per cent). A titre of 8·6 was found in seven sham-splenectomized mice.

Serum antibodies were predominantly 2-ME resistant throughout a period of 12 days (Fig. 1). The analysis of circulating antibodies by the 2-ME method indicates that asplenic and splenectomized mice have an insufficient synthesis of IgM early in the primary

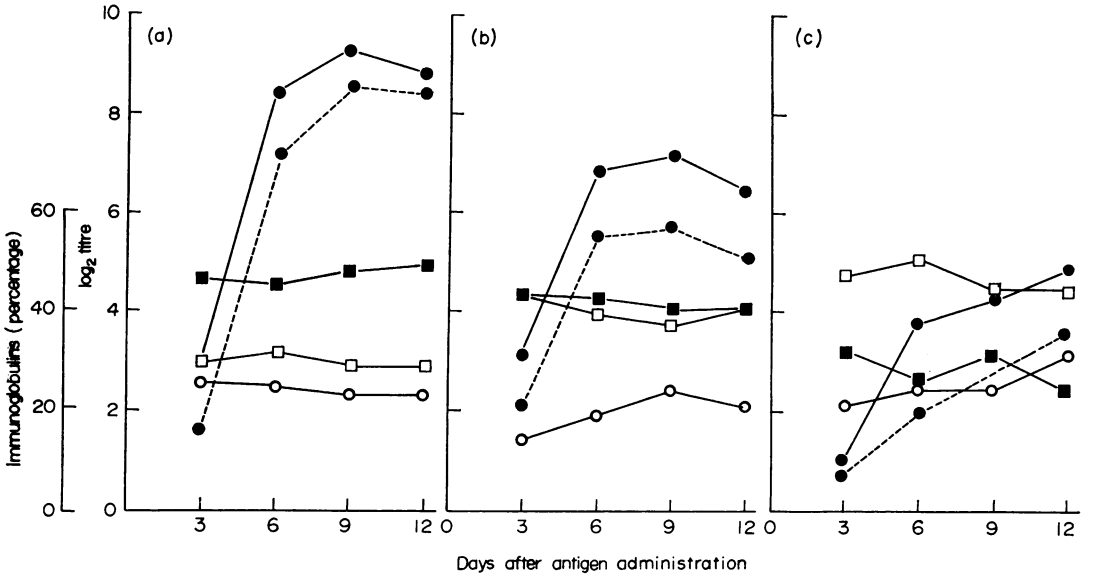

FIG. 1. (●—●) Total serum agglutinin titres and (● - - - ●) 2-mercaptoethanol-resistant antibodies of 7S type in (a) normal, (b) asplenic and (c) splenectomized mice immunized with a single injection of sheep erythrocytes (ten to fifteen mice in each point). Other titres of sham-splenectomized mice (five in each point) were nearly identical to those of normal littermates and are not represented. The proportional concentration of immunoglobulins (Ig) is also given as indicated: (□) IgG1; (■) IgG2; (○) IgM.

TABLE 1

DIRECT (19S) AND INDIRECT (7S) PLAQUE-FORMING CELLS (PFC) IN THE SPLEEN OF NORMAL MICE

Days after immunization	Number of PFC			
	19S/10^6 cells	19S/spleen	7S/10^6 cells	7S/spleen
3	89·5±30·0	15,953±8824	6·0±0·5	1027±269
6	84·7±14·1	14,533±5282	5·8±1·7	947±471
9	14·5± 3·1	2,053± 695	6·0±0·2	787± 80
12	12·2± 1·5	2,133± 550	15·4±4·2	2578±779

Values are the mean±1 s.e. of ten to eighteen mice.

immune response, and that the proportional amount of IgM in subsequent periods of observation was greater in asplenic than in normal mice. The delayed increase of IgM levels was also demonstrated in splenectomized animals which had a steady rise of IgM in the 3–12-day period after immunization.

The analysis of serum Ig by the radial immunodiffusion technique, revealed that antibodies of the IgG-type predominate in mice with and without spleen immunized with SE (Fig. 1), thus confirming the results obtained by determining the amount of 2-ME sensi-

tive antibodies. The majority of IgG present in the serum of normal mice was of γ-2 type, whereas the concentration of IgG1 and IgG2 were found to be approximately equal in the serum of asplenic animals. It is worth mentioning that the proportional levels of IgG2 were similar in asplenic and normal littermates. A significantly higher concentration of IgG1 was found after splenectomy as well as in asplenic mice than in normal animals. Splenectomized mice had in addition, a marked decrease of serum IgG2 levels compared

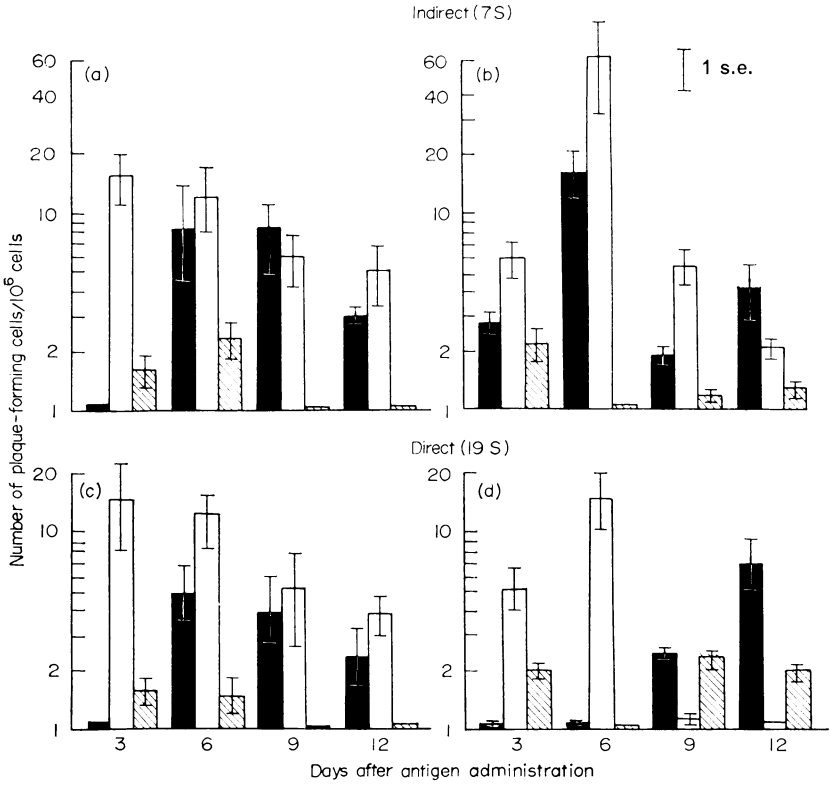

FIG. 2. Plaque-forming cells (PFC) produced by (a) and (c) leucocytes and (b) and (d) lymph node cells during the primary response to sheep erythrocytes. (a) and (b) indirect 7S. (c) and (d) direct 19S. Each column is the mean $\pm$ 1 s.e. of ten to fifteen mice. Counts up to 1·5 PFC per 10^6 cells were considered the normal background as determined in non-immunized normal and sham-splenectomized mice. Solid columns represent normal mice; blank columns represent asplenic mice; hatched columns represent splenectomized mice.

to normal or asplenic mice. There was a decrease and delay of IgM production in asplenic mice which reached a nearly normal concentration between 9 and 12 days after immunization. No significant alterations of Ig levels could be demonstrated 2 months after sham-splenectomy as compared to non-operated littermates.

As can be seen in Table 1, a large number of 19S ppm was found in the spleen of normal mice between 3 and 6 days after immunization. The number of 19S antibody-forming cells subsequently fell precipitously and on the 9th and 12th days had decreased by a factor of 6–7. The response of 7S PFC exhibited a different trend in values. Their number was low from the 3rd to the 9th day and abruptly rose between two and three times 12 days after

antigen stimulation. Further data pertaining to PFC in leucocytes and lymph node cells are illustrated on the bottom (19S) and top (7S) of Fig. 2.

Maximum levels of PFC were detected among peripheral leucocytes coincidentally with the corresponding 19S and 7S peaks in the spleen and lymph nodes. The number of 19S ppm was particularly high 3 days after immunization and gradually decreased to low levels 12 days later. The characteristic event was the significantly larger number of 19S and 7S ppm in the blood of asplenic mice than that of normal or splenectomized littermates. The highest levels were demonstrated 3 and 6 days after SE, when 12–16 ppm of either 19S or 7S type were found in the circulation of asplenic mice.

Direct plaque counts in the lymph nodes of asplenic mice showed a significant increase over the background level on the 3rd day after immunization, when 5·3 ppm were demonstrated. At this time, the number of 19S ppm within lymphoid cells from normal and splenectomized mice was slightly over the background count. This difference was substantially greater on the 6th day, when 15 ppm were observed in asplenic animals and none were demonstrated in normal and splenectomized mice. Counts of indirect PFC produced by lymph node cells, followed a similar pattern, with asplenic mice having three to four times more 7S ppm, than normal animals, on the 3rd day (6 ppm) and on the 6th day (63 ppm) after the antigen. Both 19S and 7S ppm diminished in lymph nodes and peripheral leucocytes on the 19th and 12th day after immunization. The number of PFC in the lymphoid tissues of splenectomized mice was extremely low compared with normal and asplenic mice.

The response of bone marrow lymphoid cells to antigenic stimulation with SE was minimal compared with that of the spleen, lymph nodes and blood leucocytes. It exhibited less than one 19S or 7S ppm throughout the course of the experiment in all mice studied, regardless of sex, genotype and splenectomy.

The number of PFC was also determined in twenty-five sham-splenectomized mice. As expected, the number of 19S and 7S PFC found in the tissues of sham-operated mice was similar to that reported earlier for normal (untreated animals).

SECONDARY IMMUNE RESPONSE

The serum antibody titres and type of immunoglobulins present during the secondary immune response are presented in Table 2. Total serum agglutinin levels of asplenic and splenectomized mice were significantly lower than those of mice with spleens, and more than 90 per cent of serum antibodies were of 7S type as determined either with the ME method or by analysis of radial immunoprecipitates. The proportional levels of IgG1 of asplenic and splenectomized mice were higher than that of littermates with spleen. On the other hand, a significantly decreased concentration of IgG2 was demonstrated in asplenic and splenectomized mice compared to normal or sham-operated animals. No major differences in the proportional levels of serum IgM were observed among the three groups of mice studied. The reduction of the proportional amounts of IgG2 was not seen during the primary response of asplenic mice.

The data acquired from PFC determination and level of serum immunoglobulin elicited 5 days after a secondary stimulation with SE are presented in Fig. 3.

The characteristic event in the secondary immune response was the large number of 7S PFC. The number of 19S antibody-forming cells in the spleen was almost half of that observed in the primary response at similar intervals after immunization (Table 2, Fig. 3).

An average of 6880±1630 direct PFC per spleen or 33 ppm spleen cells was found in mice with spleen. In marked contrast, the number of 7S PFC rose sharply and reached an average number of 126,000±27,300 PFC per spleen 5 days after a secondary antigenic

TABLE 2

SERUM ANTIBODY LEVELS AFTER A SECONDARY IMMUNIZATION WITH SHEEP ERYTHROCYTES

Groups	Number of mice	Agglutinin titre (log_2)*		Immunoglobulins (per cent)		
		Untreated	2-ME	IgG1	IgG2	IgM
Normal (+/+)	10	12	11	58·9	34·4	6·8
Asplenic (Dh/+)	10	9	7	67·3	25·1	7·6
Splenectomized (+/+)	5	8	8	70·9	23·7	5·5
Sham-splenectomized (+/+)	5	11·5	10	60·4	32·4	7·2

2-ME = serum titre after treatment with 2-mercaptoethanol.

* Mean titre of sera of mice 5 days after a second injection of SE, which was given 30 days after the primary immunization. Splenectomized and sham-splenectomized mice were used 2 months after surgery.

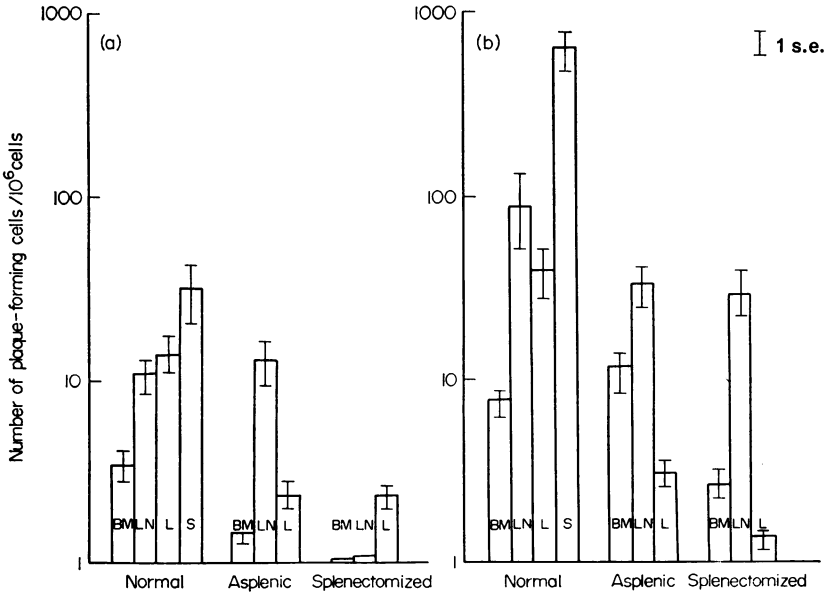

FIG. 3. Plaque-forming cells produced during the secondary immune response (a) direct 19S and (b) indirect 7S to sheep erythrocytes by bone marrow (BM), lymph node (LN), leucocytes (L) and spleen (S) cells of five normal, ten asplenic and five splenectomized mice. Each column represents the mean± 1s.e. 5 days after a second injection of SE, which was given 30 days after the primary immunization. Splenectomized mice were used 2 months after surgery. Sham-splenectomized mice had a value similar to normal littermates.

stimulation with a yield of almost 560 indirect ppm cells in either normal or sham-operated mice.

An extremely low number of 19S ppm was demonstrated within the narrow cells in normal, asplenic and splenectomized mice. At identical periods of the secondary response,

marrow cells from normal and asplenic mice exhibited a significantly higher number of 7S PFC than those produced by marrow cells of splenectomized littermates. Plaque counts in lymph node cells showed a substantial increase over background levels with normal and asplenic mice producing a similar number (12–14) of 19S ppm. Further, lymph node cells from asplenic and splenectomized mice produce a similar number of 7S PFC (29–34 ppm), but far below the 7S PFC number (86 ppm) found among lymph node cells from normal mice. It should be emphasized, however, that there was a marked increase of the number of 19S and 7S ppm lymph node cells from normal and splenectomized mice but not in those cells from asplenic mice during the secondary immune response. This observation is apparent from comparing the number of PFC 6 days after a single immunization (Fig. 2) with those of Fig. 3 illustrating the number of PFC in the secondary response.

ALLOGRAFT REJECTION

The mean rejection time (10–13 days) of skin allografts was very similar in ten normal, five sham-splenectomized, and ten asplenic mice, no matter if they were recipients of homografts or isografts.

Essentially, the same results were obtained when a spleen was implanted in the peritoneal cavity of ten asplenic, nine splenectomized and five sham-operated mice. The rejection time was about 8–10 days as judged by leucocytes counts and at autopsy. The grafting of the spleen did not alter the elevated leucocyte count (19,000–23,000/mm^3) of mice without spleen for at least 6–8 days. At that time, a marked leucocytosis (30,000–45,000/mm^3 was present. Mice killed 2 days after the sharp rise of leucocytes (day 10) had an atrophic and avascular spleen mass weighing approximately one-third of the normal spleen transplanted.

DISCUSSION

The results of immunological studies demonstrate basic differences, resulting from congenital asplenia, in the cellular events and their relationship to circulating antibodies produced in the primary and secondary immune responses to SE, a thymus-dependent antigen. A comparison of the total number of 19S ppm leucocytes, lymph node and spleen cells, 3 and 6 days after the primary immunization, demonstrated that asplenic mice had about 25 per cent of the number of IgM producing cells found in normal mice. Splenectomized mice had almost 4 and 13 per cent of the number of 19S ppm of normal and asplenic littermates, respectively. The disparity in the number of IgM antibody-forming cells was due to the large number of 19S PFC produced by spleen cells of normal mice in contrast to the small number of similar PFC produced by lymph node cells and leucocytes of asplenic mice, or compared to the severe immunological deficiency of animals splenectomized in adult life.

The situation was reversed when the number of 7S ppm leucocytes, lymph nodes and spleen cells were combined and compared among mice of different phenotypes 3 and 6 days after a single injection of SE. Asplenic mice had from 2- to 3-fold increase in the number of IgG ppm cells over normal mice. Leucocytes and lymph node cells largely accounted for the majority of 7S PFC produced in asplenic mice. It should be emphasized however, that the absolute number of PFC in lymph nodes and peripheral leucocytes represent only a fraction of the total number of PFC seen in normal mice. The production of 7S antibodies changed again by the 12th day after SE when spleen cells from normal mice

began to produce a large number of IgG antibodies, and very few were detected among lymph node cells and leucocytes of asplenic mice. The difference was 3:1 indirect ppm in normal *versus* asplenic mice. Once again, lymphoid cells from splenectomized animals had an extremely low number of IgG-producing cells.

The serum antibody titres paralleled the cellular events. Thus, the diminished number of antibody-forming cells during the primary response, due to the lack of the spleen cells, accounted for the low antibody titres and was associated with an initial decrease of the concentration of serum IgM in asplenic and splenectomized littermates. The serum levels of IgM rose steadily from the 3rd day to the 9- to 12-day period, when similar proportional amounts were found in the serum of asplenic mice, thus indicating a delay in the synthesis of IgM. On the other hand, the serum levels of IgG1 were higher in asplenic and splenectomized than in normal mice, hence suggesting that IgG1 synthesis did not require spleen cells. The analysis of Ig established on a quantitative basis preliminary qualitative studies of Ig in asplenic mice made by Battisto *et al.* (1969) who by use of the passive cutaneous anaphylaxis technique were able to demonstrate homocytotropic (IgG1) but not heterocytotropic (IgG2) antibodies during the secondary immune response. We could not demonstrate a decrease of IgG2 in asplenic mice during the primary immune response, but we did find a diminished IgG2 level 5 days after a secondary immunization.

The cellular events of the secondary immune response demonstrated the marked deficiency of immunoglobulin synthesis of asplenic and splenectomized mice, which had approximately 7 and 4 per cent, respectively, of the number of 7S ppm found in normal mice. These results are not surprising in view of the findings that the anamnestic response to i.v. injection of SE largely depends on a 7S memory cell compartment that is built up in the spleen as a result of primary antigenic stimulation (Campbell and La Via, 1967; Mellbye, 1970; Rowley, 1950; Taliaferro and Taliaferro, 1950, 1951). The serological studies demonstrated once again that asplenic mice had a significantly lower haemagglutinin titre than normal mice, owing to the lack of the large mass of splenic lymphopoietic tissue, and to a diminished proportional concentration of IgM. We cannot estimate the absolute amount of Ig with the data obtained in this study. Furthermore, the increased amount of IgG1 probably represented a compensation for the diminished serum level of IgG2 during the primary or secondary immune response of splenectomized and asplenic mice, respectively.

It has been found in spleen cell transfer experiments that there is a linear relationship between the number of spleen cells transferred and agglutinin titre (Perkins, Robinson and Makinodan, 1961) after a primary and secondary immunization. In our experiment, asplenic mice have a profound reduction of the number of 19S and 7S ppm cells with a significant but not equally marked decrease of serum antibody titres. The relatively large mass of lymphopoietic tissue in the bone marrow and lymphatic system, which is difficult to measure in terms of total cells, surely accounted for a major part of the serum antibody titres observed.

An interesting finding of this study was the large number of antibody-forming cells found within peripheral leucocytes. The enumeration of antibody-forming cells in peripheral leucocytes has been the subject of much recent study. The majority of investigations in this field demonstrated that there is no doubt that immunocompetent cells developing in any part of the lymphoreticular system, can leave the original site of formation and differentiation and circulate in the lymph (Cunningham, Smith and Mercer,

1966; Halasa, 1968; Hulliger and Sorkin, 1963) and blood stream (Hulliger and Sorkin, 1963; Kearney and Halliday, 1965; Landy, Sanderson, Berstein and Jackson, 1964; Sorkin and Landy, 1965). Despite the circumstantial evidence, it has been postulated that antibody-forming cells found in peripheral blood may be 'false' PFC arising from the release of antibody adsorbed to cell–platelet aggregates (Roseman, Leserman, Fitch and Rowley, 1969). We have also examined several PFC of 19S and 7S type produced by leucocytes under magnification $\times 100$, and found no evidence of central platelet or cell–platelet aggregates, despite the fact that the majority of asplenic mice have thrombocytosis (Lozzio, 1972). In the present study leucocytes obtained on the 3rd and 6th day after a single immunization produced a large number of 19S and 7S PFC. The number of PFC gradually decreased after the 6th day of the primary response. Peripheral leucocytes from asplenic mice produced a significantly greater number of 19S and 7S ppm than those of normal and splenectomized mice early in the primary response. Although asplenic mice have twice as many leucocytes as normal mice (Lozzio, 1972), the number of PFC observed appears not to be related to leucocyte counts, because asplenic mice had fewer PFC in the blood during the secondary response and the leucocytosis was very similar in both primary and secondary responses. Furthermore, splenectomized mice had a very low number of PFC in blood even though they have a nearly identical leucocytosis and differential counts to those of asplenic mice.

Another type of 'false' PFC could be produced by cytophilic antibodies, which in the mouse are of IgG (γ-2) type (Brown and Carpenter, 1971; Likaj, 1968). Preliminary experiments made in our laboratory indicate that no PFC developed when peripheral leucocytes from asplenic mice were incubated with an isologous mouse anti-SE serum from either primary or secondary immune responses. The failure to demonstrate 'false' PFC formation in this experiment, and the fact that there was a significantly lower number of PFC during the secondary response, when between 75 and 90 per cent of antibodies present were of 7S type, suggest that the majority of PFC observed may have been true antibody-forming cells. They may appear in larger number in the blood of asplenic than normal mice because of the absence of a trapping mechanism and appropriate environment normally provided by the spleen to circulating antibody-forming cells. Recently, Rabin and Rose (1973) have reported that there is a selective release of antibody-forming cells into the blood stream, suggesting that the immunological active cells are retained within the lymphopoietic tissue while newly produced antibody-forming cells are released into the general circulation.

The observations reported herein confirm and extend those of Battisto, Borek and Bucsi (1971), Battisto *et al.* (1969) and Bucsi *et al.* (1972) who have demonstrated a deficiency of IgM synthesis and the thymus-marrow cell co-operation in antibody formation in hereditarily asplenic and neonatally splenectomized mice.

Our data and those of others mentioned earlier do allow the deduction that agenesis of the spleen is markedly detrimental for a normal level of Ig synthesis, and that other areas of the lymphopoietic system do not compensate for the diminished antibody formation. Thus, the harmonic embryological development of central and peripheral lymphopoietic organs is essential to achieve normal immunocompetence in adult life.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the helpful criticism and suggestions provided by

Jack R. Battisto, Ph.D., Department of Microbiology and Immunology, Albert Einstein College of Medicine, Yeshiva University, New York City and Eugene Perkins, Ph.D., Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee. This research was supported in part by Grant FR5541 from the National Institutes of Health, U.S. Public Health Service.

REFERENCES

ARVY, L. (1965). 'La genese des anticorps.' *Splenologie* (Ed. by L. Arvy), p. 396. Gauthier-Villars, Paris.

BATTISTO, J., BOREK, F. and BUCSI, R. (1971). 'Splenic determination of immunocompetence: influence on other lymphoid organs.' *Cell. Immunol.*, **2,** 627.

BATTISTO, J., CANTOR, L., BOREK, F., GOLDSTEIN, A. and CABRERRA, E. (1969). 'Immunoglobulin synthesis in hereditarily spleenless mice.' *Nature (Lond.)*, **222,** 1196.

BOSMA, M. J., PERKINS, E. H. and MAKINODAN, T. (1968). 'Further characterization of the lymphoid cell transfer system for the study of antigen-sensitive progenitor cells.' *J. Immunol.*, **101,** 963.

BROWN, G. and CARPENTER, P. (1971). 'Characteristics of cytophilic antibody in the mouse.' *Inf. Immunol.*, **3,** 637.

BUCSI, R., BOREK, F. and BATTISTO, J. (1972). 'Splenic replenishment of synergistic ability to bone marrow and thymic cells of neonatally splenectomized CBA mice.' *J. exp. Med.*, **136,** 761.

CAMPBELL, P. and LA VIA, M. (1967). 'Effect of splenectomy on primary and secondary response to sheep erythrocytes in rats.' *Proc. Soc. exp. Biol. (N.Y.)*, **124,** 571.

CROSBY, W. H. (1963). 'Hyposplenism: an inquiry into normal function of the spleen.' *Ann. rev. Med.*, **14,** 349.

CUNNINGHAM, A. J., SMITH, J. B. and MERCER, E. H. (1966). 'Antibody formation by single cells from lymph nodes and efferent lymph of sheep.' *J. exp. Med.*, **124,** 701.

DAMESHEK, W. (1955). 'Hypersplenism.' *Bull N.Y. Acad. Med.*, **31,** 113.

DRESSER, D. W. and WORTIS, H. H. (1965). 'Use of antiglobulin serum to detect cells producing antibody with low hemolytic efficiency.' *Nature (Lond.)*, **208,** 859.

FICHTELIUS, K. E. and BACK, O. (1970). 'The afferent pathways of lymphocytes to the spleen.' *The Spleen* (Ed. by K. Lerner and D. Harms), p. 118. Springer-Verlag, Berlin.

HALASA, J. (1968). 'Plaque forming cells in the spleen, lymph and blood.' *Folia Microbiol. (Praha)*, **13,** 253.

HAUPTFELDOVA-DOLEJSKOVA, V., NOUZA, K., MATOUSEK, V. and HAUPTFELD, M. (1969). 'Neonatal splenectomy in mice.' *Folia Biol. (Praha)*, **15,** 288.

HULLIGER, L. and SORKIN, E. (1963). 'Synthesis of antibodies by blood leucocytes of the rabbit.' *Nature (Lond.)*, **198,** 299.

HULLIGER, L. and SORKIN, E. (1965). 'Formation of specific antibody by circulating cells.' *Immunology*, **9,** 391.

JERNE, N. K., NORDIN, A. A. and HENRY, O. (1963). 'The agar plaque technique for recognizing antibody forming cells.' *Cell Bound Antibodies* (Ed. by B. Amos and H. Koprowski), p. 109. Wistar Institute Press, Philadelphia.

KEARNEY, R. and HALLIDAY, W. J. (1965). 'Enumeration of antibody-forming cells in the peripheral blood of immunized rabbits.' *J. Immunol.*, **95,** 109.

LANDY, M., SANDERSON, R., BERNSTEIN, M. and JACKSON, A. (1964). 'Antibody production by leukocytes in peripheral blood.' *Nature (Lond.)*, **204,** 1320.

LIKAJ, J. (1968) 'Cytophilic antibodies in mice immunized with sheep red cells.' *Folia Microbiol. (Praha)*, **13,** 97.

LOZZIO, B. B. (1972). 'Hematopoiesis in congenitally asplenic mice.' *Amer. J. Physiol.*, **222,** 290.

LOZZIO, B. B. and COMAS, F. V. (1969). 'Biological effects of pokeweed mitogen.' *Int. Arch. Allergy*, **36,** 266.

MANCINI, G., CARBONARA, A. O. and HEREMANS, J. F. (1965). 'Immuno-chemical quantitation of antigens by single radial immunodiffusion.' *Immunochemistry*, **2,** 235.

MEIER, H. and HOAG, W. G. (1962). 'Blood proteins and immune response in mice with hereditary absence of spleen.' *Naturwissenshaften*, **49,** 329.

MELLBYE, O. J. (1970). 'Spleen and immunity.' *The Spleen* (Ed. by K. Lerner and D. Harms), p. 139. Springer-Verlag, Berlin.

NAKOV, L., SEJKOROVA, J. and NOUZA, K. (1969). 'Neonatal splenectomy in rats.' *Folia Biol. (Praha)*, **15,** 439.

PERKINS, E. J. (1970). 'Digestion of antigen by peritoneal macrophages.' *Mononuclear Phagocytes* (Ed. by R. Van Furth), p. 562. Blackwell Scientific Publications, Oxford and Edinburgh.

PERKINS, E. J., ROBINSON, M. A. and MAKINODAN, T. (1961). 'Agglutinin response, a function of cell number.' *J. Immunol.*, **86,** 533.

RABIN, B. S. and ROSE, N. R. (1973). 'Selective release of antibody forming cells into the blood.' *Cell. Immunol.*, **7,** 389.

ROSEMAN, J., LESERMAN, L., FITCH, F. and ROWLEY, D. (1969). 'Do antibody-forming cells circulate in blood?' *J. Immunol.*, **102,** 1002.

ROWLEY, D. A. (1950). 'Effect of splenectomy on formation of circulatory antibody in adult male albino rat.' *J. Immunol.*, **64,** 289.

SORKIN, E. and LANDY, M. (1965). 'Antibody production by blood leucocytes.' *Experientia (Basel)*, **21,** 677.

TALIAFERRO, W. H. (1956). 'Functions of the spleen in immunity.' *Amer. J. trop. med. Hyg.*, **5,** 391.

TALIAFERRO, W. H. and TALIAFERRO, L. G. (1950). 'The dynamics of hemolysin formation in intact and splenectomized rabbits.' *J. infect. Dis.*, **87,** 37.

TALIAFERRO, W. H. and TALIAFERRO, L. G. (1951). 'Role of the spleen and non-splenic sites in antibody formation.' *Science*, **113,** 473.