Immunology, 1973, **25**, 33.

Studies on the Differentiation of B Lymphocytes in the Mouse

G. J. V. Nossal and Beverley L. Pike

The Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria 3050, *Australia*

(*Received* 17*th October* 1972; *accepted for publication* 19*th December* 1972)

Summary. The capacity of CBA mouse B lymphocytes to bind polyvalent rabbit anti-mouse immunoglobulin antibody, even when this reagent is present in low concentration, was used as an index of a lymphocyte's B cell status. Various mouse tissues were held for 30 minutes at 0° with 0·2 μg/ml of ^{125}I-labelled anti-Ig, and smear preparations of washed cells were examined, following radioautography, for their content of B cells. The survey covered two areas of B cell differentiation, namely the spontaneous emergence of B cells in the mouse foetus, and the development of B cells in lethally irradiated mice that had received early foetal liver as a source of haematogenous stem cells.

The foetal survey suggested a multifocal origin of B cells, commencing 3 days before birth, in all major sites of erythromyelopoiesis, namely liver, spleen and bone marrow. Lymph nodes showed B cells later than did these organs, and thymus contained virtually no B cells at any stage. A rapid influx of B cells into lymph nodes took place shortly after birth. The CBA mouse is born with somewhat over 10^5 B lymphocytes *in toto*, of which the majority are in liver, spleen and blood.

The irradiation-recovery study showed: (1) that B cells are, statistically, more sensitive to high dose irradiation than T cells; (2) that a B cell-free stem cell source can repopulate the B cell pool; (3) that repopulation is surprisingly slow; and (4) that numerical and functional recovery of B cells parallel each other to a reasonable degree.

The significance of the results is discussed from the viewpoints of lymphocyte differentiation and immunological tolerance.

INTRODUCTION

The definition of separate functional roles for thymus-derived (T) and bone marrow-derived (B) lymphocytes (Claman and Chaperon, 1969; Miller and Mitchell, 1969), and the availability of lymphocyte surface markers capable of discriminating between mouse T and B cells (Raff, 1970; Basten, Miller, Sprent and Pye, 1972; Nossal, Warner, Lewis and Sprent, 1972), have led to an extensive re-examination of lymphocyte physiology. Origins, life-span, homing and recirculation characteristics, and sensitivity to noxious agents all differ between T and B lymphocytes. As regards the foetal origins of lymphocytes, this has been well studied in the mouse for T cells (Owen and Raff, 1970), and in the chicken for B cells (Kincade and Cooper, 1971), but few details of B cell differentiation are known for the mouse.

It is known that, like all blood cells, lymphocytes are ultimately the progeny of multipotential haematogenous stem cells (Wu, Till, Siminovitch and McCullogh, 1967), which in their turn are derived from primitive cells in the blood islets of the embryonic yolk sac (Moore and Owen, 1967). In the adult mouse, bone marrow contains both stem cells and B lymphocytes, relatively uncontaminated by T lymphocytes in some strains, and is frequently used as a B cell source for adoptive transfer experiments. It is generally assumed that the differentiation of stem cells to B cells occurs in the marrow and the life-span characteristics of marrow lymphocytes as studied in the rat (Everett and Tyler, 1967), and guinea-pig (Osmond, 1967; Osmond, Miller and Yoshida, 1973), are consistent with this view. However, this picture is neither proven nor complete.

This paper seeks to define the sites and tempo of appearance of B lymphocytes in two situations of differentiation. First, the spontaneous appearance of B cells in foetal and young mice was studied. Secondly, a B cell-free source of stem cells, namely early foetal liver, was used to repopulate lethally irradiated syngeneic mice, and the emergence of B lymphocytes was measured in these hosts. Various markers for B lymphocyte status are available in the mouse, such as high capacity to bind anti-immunoglobulin antibody (Raff, 1970), Fc receptor (Basten *et al.*, 1972) or the mouse B lymphocyte antigen (Raff, 1970). As we have had extensive experience with the first of these markers (Nossal, Warner, Lewis and Sprent, 1972) we preferred it for both projects. Cell suspensions were washed, held at 0° with a low concentration of ^{125}I-anti-immunoglobulin known to be capable of binding detectably to B but not to T lymphocytes, and after further washing, radioautographs were prepared and labelled lymphocytes were sought in various organs.

MATERIALS AND METHODS

Animals

CBA/H/Wehi mice were used. For embryological studies, females were examined daily for vaginal plugs and impregnation was deemed to have occurred at the preceding midnight. Pregnant mice were killed between 09.00 and 12.00, and the period of gestation was taken as the number of days between detection of a plug and killing plus 0·5. Birth occurred at 19·5 days on the average. For irradiation-restoration studies, recipients of foetal liver cells were male mice aged 10–11 weeks.

X-irradiation

Adult mice were given a whole body dose of 800 rads of X-rays, using a Philips RT 250 machine, 250 kV, HVL 0·8 mm Cu, under maximum backscatter conditions. Restored mice received 2×10^6 foetal liver cells i.v. 2–6 hours after irradiation. Non-restored mice died between 8 and 12 days after irradiation.

Preparation of cell suspensions

Foetal liver cells acting as repopulating stem cells were obtained from 12·5 to 13·5 day foetuses.

Cell suspensions for determination of B cell content were obtained as described in detail previously (Nossal, Warner, Lewis and Sprent, 1972). A key feature of experimental design was the use of Shortman's method for removal of dead or damaged cells (Shortman, Williams and Adams, 1972). Unfortunately, this could not be applied to some of the early foetal cell sources because of paucity of cell numbers. In such cases, cell smudges (which

may label non-specifically with many proteins) were ignored in the final scoring of smears.

Special techniques had to be adopted for some of the very early foetal stages, e.g. 11·5-day thymus or 14·5-day spleen. Here, organs or organ anlagen were removed under a dissecting microscope and teased apart into small droplets of medium containing labelled anti-Ig using fine, hand-drawn glass needles or the points of fine hypodermic needles. Subsequently, labelling was stopped by greatly diluting the small droplets, and cell smears were prepared by cytocentrifuge. As it turned out, these special procedures proved unrewarding, as no B cells were found before 16·5 days, at which stage the organs examined had reached a sufficient size for processing by conventional procedures.

In most cases, each cell suspension of foetal or young mouse origin represented a pool of one pregnancy or litter, and in the case of irradiated-restored adults, a pool of from two to four mice.

Blood was collected into foetal calf serum (FCS) containing 100 units/ml of preservative-free heparin and samples of buffy coat were prepared by centrifugation in narrow tubes. In early foetuses, blood was taken from the severed umbilical vessels. From later foetuses, decapitation was found more satisfactory. Subsequently, blood was taken from severed axillary vessels.

Labelling of cell suspensions with ^{125}I-antiglobulin

The procedure previously described for direct labelling was followed exactly (Nossal *et al.*, 1972). Briefly, cells at a concentration of $2–10 \times 10^6$ nucleated cells/ml were held for 30 minutes at 0° in HEPES-buffered Eagle's minimal essential medium (HEM) containing 10 per cent foetal calf serum (10 per cent FCS-HEM) and 0·2 μg/ml of a ^{125}I-labelled antiglobulin. The antiglobulin reagent used (R19NS) was a γ-globulin fraction prepared by starch block electrophoresis in Veronal buffer, pH 8·2, from an antiserum derived from a rabbit that had been multiply immunized with an IgM-containing fraction of normal NZB mouse serum. This was a strong polyvalent antiglobulin, the highest activities being against mouse κ and μ chains. At the concentration used, and with a 10-day radioautographic exposure period, R19NS labels >90 per cent of cells from the thoracic duct of congenitally athymic mice, 0·2 per cent of adult mouse thymus cells, and in other respects follows the expected behaviour of a B cell marker (Nossal *et al.*, 1972; Wilson, Nossal and Lewis, 1972). The Ig fraction was kindly supplied by Dr N. L. Warner. Iodinations, kindly performed by Mr J. Pye, were by the chloramine T method, substitution being 6–8 μCi/μg of γ-globulin or 0·5–0·7 atoms of ^{125}I/mol. The labelled protein was not stored beyond 1 week.

Following the labelling period, the cells were washed twice through discontinuous density gradients consisting of successive layers of 100 per cent FCS; 75 per cent FCS–25 per cent HEM; and 50 per cent FCS–50 per cent HEM. They were then given a final wash in FCS and smeared, usually by hand, but sometimes by cytocentrifuge.

Radioautographic procedure and cell counting

Air-dried cell smears were fixed in 90 per cent methanol, dipped in 'Kodak' NTB/2 emulsion (Eastman Kodak Co., Rochester, N.Y.), and exposed for 10 (or occasionally 11) days. They were developed, stained with Giemsa, and examined for labelled lymphocytes.

The following entities other than B lymphocytes exhibited a variable tendency to display labelling: dead or damaged cells, recognized as pink smudges; platelet aggregates;

small pieces of cell debris; polymorphonuclear leucocytes (rarely); macrophages; a small proportion of large blast cells (possibly primitive B cells); a proportion of plasma cells (Nossal and Lewis, 1972). None of these caused any trouble in interpretation. If there was the slightest possibility on morphological grounds that a labelled cell was, e.g. a monocyte or macrophage, it was not counted. Attention was confined to cells that were (a) indubitably small or medium lymphocytes by morphology and (b) labelled with fifteen or more grains. Generally, 30–100 grains were present over and around the labelled lymphocyte.

Usually, 300–1000 nucleated cells were examined per smear, but if this failed to reveal B cells, 5,000–10,000 cells were counted.

The intensity of labelling of B cells of adult peripheral blood was found to vary from experiment to experiment, being unsatisfactorily light on some occasions. Unfortunately, we did not realize the reason for this until late in the series of experiments. Erythrocytes have a slight but definite capacity to bind antiglobulin (Nossal *et al.*, 1972), and as their number in buffy coat preparations greatly exceeded that of the leucocytes, competition for antiglobulin must have occurred, to an extent varying with the red cell content. This problem was partially solved by raising the antiglobulin concentration for labelling to 1 μg/ml, but ammonium chloride treatment (which we did not use in these experiments) represents a better way out. Foetal erythrocytes were less able to absorb out radioactivity and foetal B lymphocytes could be detected satisfactorily.

Assessment of immunological capacity of irradiated-restored mice

As a test of functional restoration of B cells in irradiated mice, the response to a conjugate of dinitrophenol (DNP) and the flagella (FLA) of *Salmonella adelaide* was measured. DNP–FLA was donated by Dr J. W. Schrader; it had a mean of 1·5 DNP groups per monomeric unit of flagellin, and mice received a single i.p. injection of 25 μg at various times after irradiation and restoration. They were killed 5 days after antigen injection and the DNP-specific plaque-forming cell response in the spleen was measured. The relevant techniques were as described by Feldmann (1972), who has shown that the 5-day IgM (direct plaque) response to DNP–FLA is essentially T cell-independent.

RESULTS

APPEARANCE OF B CELLS IN THE CBA MOUSE FOETUS

For the sake of brevity, a cell with the morphology of a small or medium lymphocyte displaying fifteen or more grains will be termed a B cell, and the validity of the marker will be taken up in the Discussion.

Foetuses were examined from 11·5 days gestation to birth. The tissues studied were liver, thymus, spleen, femoral bone marrow, mesenteric lymph node and blood. Cell labelling was performed at the first practicable stage, which was 11·5 days for liver, 12·5 days for thymus, 14·5 days for spleen and blood, 17·5 days for bone marrow and 19·5 days for the mesenteric node. Peyer's patches were not detectable in foetuses of this strain. The appendix was identified in foetuses nearing birth, but significant numbers of lymphocytes could not be obtained from it.

B cells were first detected, in very small numbers, at 16·5 days gestation. All samples from earlier embryos were negative, even after prolonged scanning. At 16·5 days, a mean of 0·2 per cent of nucleated spleen cells, 0·2 per cent of nucleated blood cells and 0·005 per cent of foetal liver cells had the characteristics of B cells. The proportions and total

numbers of B cells rose in later foetal and post-natal life as shown in Figs 1 and 2. Not graphed on Fig. 2 are the values for blood, which increased from a mean of 0·2 per cent at day 16·5 to 2·2 per cent at day 17·5, 3·5 per cent at day 18·5 and 4·8 per cent at birth. In the spleen, the absolute number of B cells increased as shown in Fig. 1, with the event of

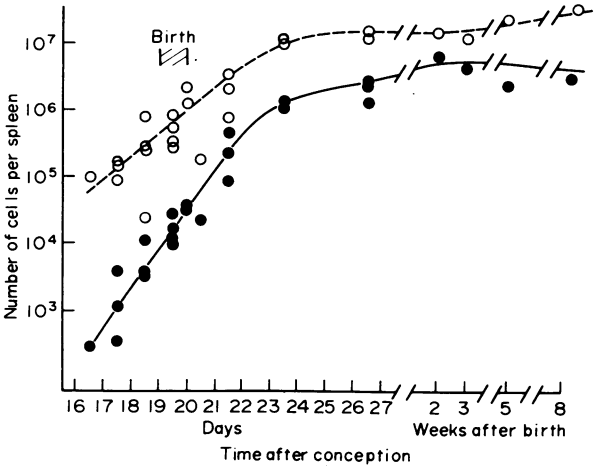

FIG. 1. Total numbers of nucleated cells and B cells recovered from the spleens of foetal and young mice. (○—○) Total cells. (●—●) B cells.

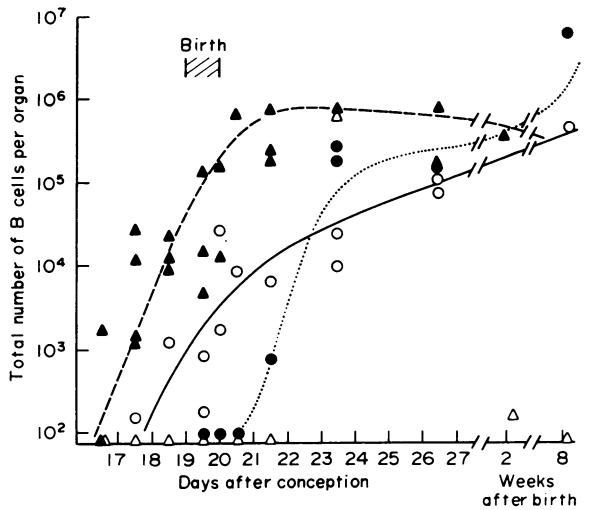

FIG. 2. Total number of B lymphocytes present in various organs in foetal and young mice. (●) Mesenteric node. (▲) Liver. (○) Bone marrow (one femoral shaft). (△) Thymus.

birth producing no detectable change in the rate of appearance as plotted on a semi-logarithmic scale. The adult proportion of 40–50 per cent of B cells was reached about 2 weeks after birth.

Femoral bone marrow showed small proportion (0·7 per cent) of B cells at 17·5 days, the first stage at which it was feasible to collect cells. Mesenteric lymph nodes were virtually alymphoid before birth. The reticular framework could be made out well before birth, but the only cells released on teasing at day 18·5 of gestation or before, were either dead or

non-lymphoid. At 19·5 days, a few lymphocytes (presumably T cells) and macrophages were isolated (*ca* 10^4 nucleated cells per node) but no B cells were found. Shortly after birth, there was a sharp rise in the proportion and absolute number (Fig. 2) of B cells in the node.

The thymus was virtually free of B cells throughout embryonic life. Values varied from <0·01 per cent at day 16·5 or before to between <0·01 per cent and 0·1 per cent at later stages of gestation. After birth, the proportion of B cells was found to vary from 0·03 to 0·2 per cent.

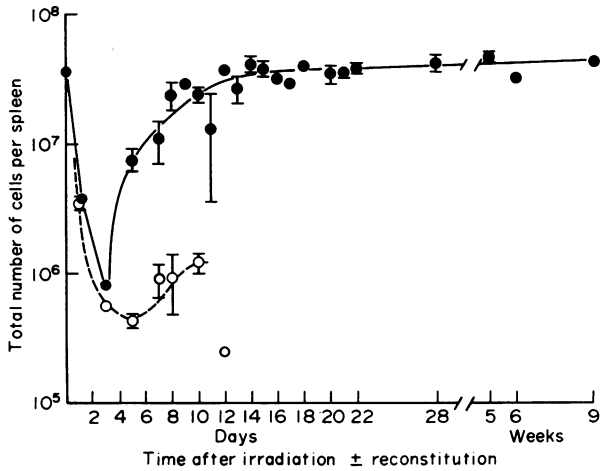

FIG. 3. Total number of nucleated cells recovered from spleens of lethally irradiated mice. (●—●) Reconstituted. (○—○) Non-reconstituted.

At 19·5 days, i.e. at or near birth, the mean absolute number of B cells was $1·6 \times 10^4$ in the spleen, $7·1 \times 10^4$ in the liver, and $4·9 \times 10^2$ in one femoral shaft. It has been estimated for adult animals that the marrow from one femoral shaft represents about 5 per cent of total marrow, and in the newborn, technical factors may have precluded full recovery of marrow cells. The blood volume was not determined, but if this approximates to 50 μl, the total blood content of B cells would have been about $2·4 \times 10^4$ at birth, so as a first approximation, it appears that a CBA mouse is born with somewhat over 10^5 B cells altogether.

Overall, the results showed the first appearance of B cells recognizable by the anti-Ig marker at 16·5 days gestation, more or less simultaneously in all the major sites of erythro-myelopoiesis, but a delayed appearance in lymph node and an absence from the thymus. While such studies cannot reveal the site of B cell genesis in the mouse foetus, they are consistent with a multifocal origin in liver, spleen and bone marrow.

DIFFERENTIATION OF B LYMPHOCYTES IN X-IRRADIATED, REPOPULATED MICE

As 12·5–13·5-day foetal liver was consistently negative for B cells, this tissue could confidently be used as a source of haemopoietic stem cells for repopulation studies. Figs 3 and 4 give some results relevant to the cell content of the spleen after irradiation. Adult mice were lethally irradiated and transfused with 2×10^6 foetal liver cells, or left un-reconstituted. Fig. 3 shows the total number of nucleated cells, which fell by a factor of 50 or more in non-reconstituted animals, some of which made a feeble effort at endogenous

reconstitution just before death. In transfused animals, cellular reconstitution was minimal at day 3 but well advanced by day 5, and the total cellularity of the spleen was approaching normal by day 8. In Fig. 4, the changes in splenic numbers of B lymphocytes are shown. The first point of interest relates to the non-reconstituted control mice. During the first day, while the total cellularity of the spleen fell by a factor of 10, the number of B cells fell by a factor of over 200. In other words, the proportion of B lymphocytes fell from over 40 per cent to 1–2 per cent. While the proportion of macrophages and plasma cells (relatively radio-resistant cells) rose somewhat, and that of polymorphonuclear lymphocytes rose considerably, lymphocytes still constituted the majority cell population. This suggested that a proportion of T lymphocytes was more resistant to high-dose irradiation than were B lymphocytes. A further fall in B lymphocyte levels occurred over the next

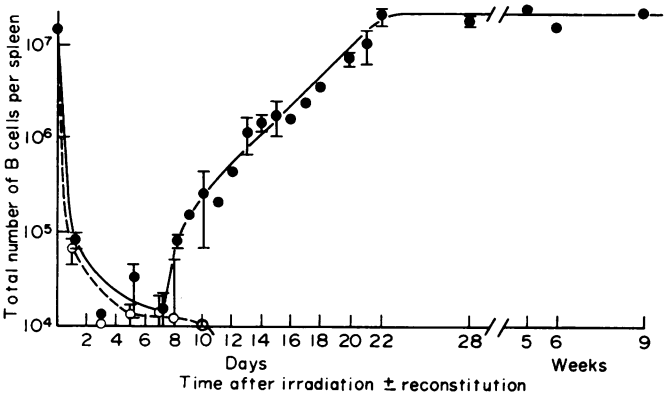

FIG. 4. Total number of B cells recovered from spleens of lethally irradiated mice. (●—●) Reconstituted. (○—○) Non-reconstituted.

few days, and non-reconstituted mice died with <10,000 residual B cells in their spleen. This provided a satisfactorily low background for a study of B cell regeneration.

Fig. 4 shows that by day 7 after irradiation and restoration, detectable new formation of B lymphocytes had not yet begun. This was of interest, as by that time erythroid and myeloid regeneration was well under way. Small numbers of B cells, still representing less than 1 per cent of the nucleated cells, were present at day 8. Following this, B cell neogenesis continued exponentially, but at a rate that was slow relative to the rate of restoration of erythrocytes and myelocytes. The total number of B cells in the spleen doubled each 1·9 days, and reached normal levels only 22 days after irradiation and restoration.

We also examined the re-appearance of B cells in femoral bone marrow, mesenteric lymph node, thymus and peripheral blood. The results with peripheral blood, as noted in Materials and Methods, were not satisfactory, but were broadly consistent with the pattern shown for spleen. Fig. 5 gives the regeneration patterns for mesenteric lymph node, bone marrow and thymus. It shows that, again, total cellularity of lymph node and marrow was regenerated earlier than B cell content. Moreover, it shows that B cell reappearance was certainly not earlier and perhaps a little later in bone marrow than in spleen. It took 4 weeks after restoration for the B cell content of the organs to return to pre-irradiation levels. The thymus did not contain significant numbers of B cells at any stage. Its total cellular repopulation (not shown) was also slow, reaching about half the original cell content by 3 weeks after irradiation.

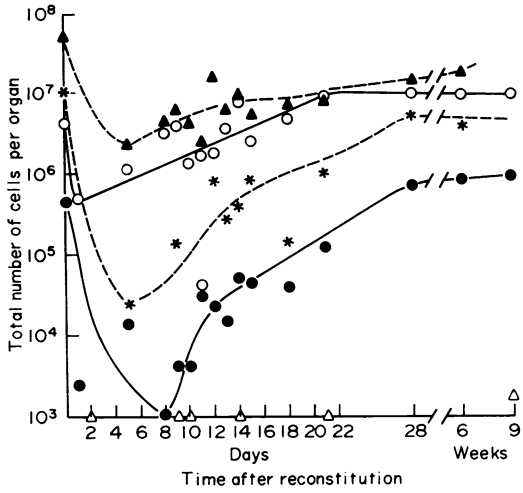

FIG. 5. Total number of nucleated cells and B cells recovered from various organs of lethally irradiated mice given 2×10^6 foetal liver cells. (▲) Mesenteric node total. (○) Bone marrow total. (●– – ●) Mesenteric node B cells. (●—●) Bone marrow B cells. (△) Thymus.

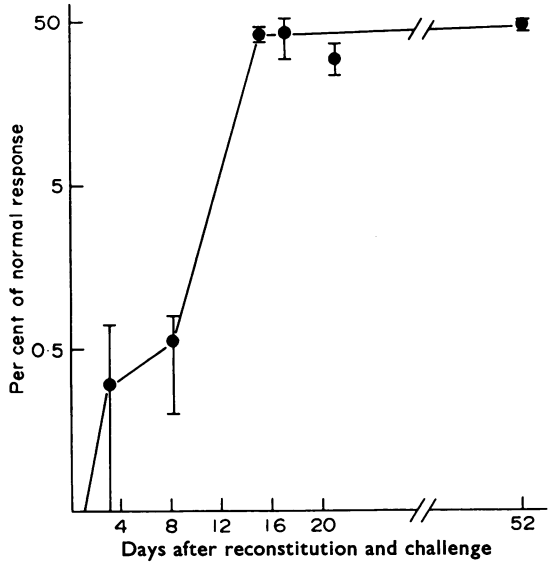

FIG. 6. DNP-specific PFC response of lethally irradiated foetal liver reconstituted mice as a function of time after irradiation at which antigen was given.

RESTORATION OF IMMUNE RESPONSE CAPACITY

We tested the capacity of the spleens of irradiated, restored mice to respond to DNP coupled to *Salmonella* flagella (DNP–FLA). The study was confined to the early, IgM response as measured by the number of direct DNP-specific plaque-forming cells. This was because we wished to study recovery of B cell function unencumbered by problems of T cell regeneration. Four experiments were performed, characterized by a considerable degree of individual variation amongst restored mice during the early recovery phase.

Control, non-irradiated mice produced about 10^4 DNP-specific PFC per spleen. A typical experiment is summarized in Fig. 6. It shows virtually no residual immune capacity in animals challenged during the first 8 days after irradiation and reconstitution, followed by a rapid recovery. It must be borne in mind that the mice were given a maximally immunogenic challenge on a particular day, and were killed 5 days later. The number of PFC seen at killing would have represented a compounded value reflecting some functional B cells present at challenge and some recruited during the next few days. Therefore, recovery of B cell function was not grossly discordant with recovery in B cell numbers as shown in

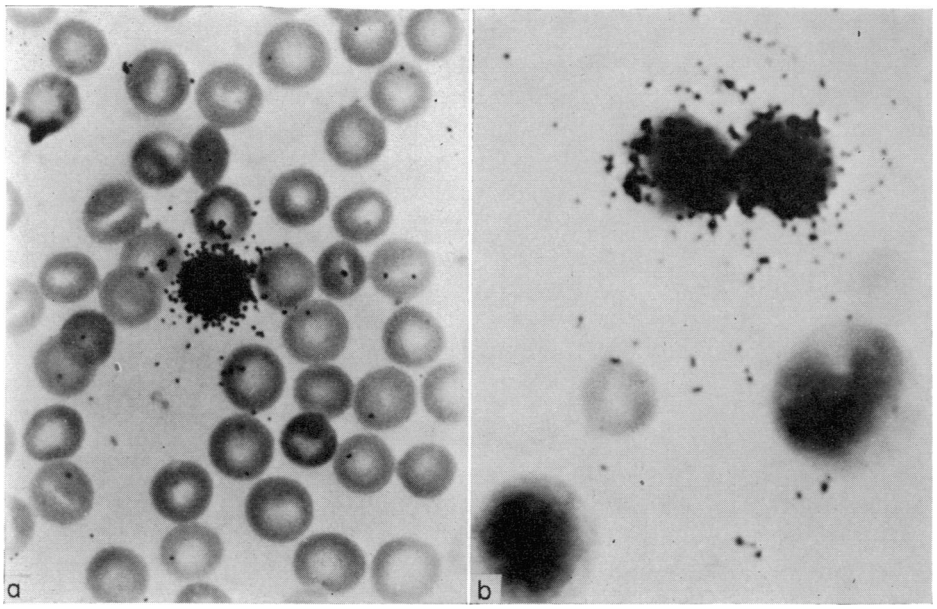

FIG. 7. Radioautographs of B cells as detected by ^{125}I-antiglobulin binding ×1280. (a) A heavily labelled B cell from 17·5-day gestation foetal blood. (b) Two heavily labelled B cells (top) with (from left to right) unlabelled erythroblast, platelet and macrophage. The smear is from a spleen of an irradiated mouse 15 days after repopulation with foetal liver.

Fig. 4. The three other experiments (not shown) substantiated the absence of functional recovery during the first week, considerable mouse to mouse variation, and a 30–80 per cent restoration by 3–9 weeks after irradiation and transfusion.

DISCUSSION

Considerable knowledge has accumulated on the differentiation of erythroid and myeloid cells in recent years (Metcalf and Moore, 1971), and by contrast the information on lymphocyte differentiation is still rather primitive. The elegant cloning studies of Till and McCullogh's group (Wu *et al.*, 1967) have made it clear that a single stem cell can act as the precursor of essentially all dividing cells in lymphatic tissues of irradiated, repopulated animals, thus acting as a common precursor for erythroid, myeloid and lymphoid cells. As regards T lymphocyte differentiation, there is now consensus that stem cells populate the thymus, divide and differentiate, after which some leave the thymus to colonize the peripheral lymphoid tissues as T lymphocytes (Moore and Owen, 1967; Owen and Raff,

1970; Weissman, 1967). Speculations and experimentation about sequential maturation stages of T cells are well advanced (Cantor, 1972).

In contrast, and in the absence of an obvious mammalian equivalent of the avian bursa of Fabricius, the stages of B lymphocyte differentiation in mammals are poorly understood. In some species, the bone marrow has been shown to contain a population of small lymphocytes which becomes labelled rapidly after repeated ^{3}H-thymidine injections, and which can be regarded as short-lived by comparison with thoracic duct lymphocytes (Everett and Tyler, 1967; Osmond, 1967). Following the localized infusion of ^{3}H-thymidine into the guinea-pig bone marrow, labelled small lymphocytes soon emerge in blood and secondary lymphoid organs (Brahim and Osmond, 1970). This is consistent with the view that marrow is a source of peripheral B cells.

The present study describes the appearance of B lymphocytes as a natural event in ontogeny, and as a consequence of repopulation of irradiated animals. The mouse was chosen, despite the small size of foetuses, because much more is known about T and B lymphocytes in this species than in any other. As a marker for a cell's B lymphocyte status, we used the known high capacity of B cells to bind ^{125}I-labelled antiglobulin. Previous work (Nossal *et al.*, 1972; Wilson *et al.*, 1972) had shown that conditions of labelling, using dilute antiglobulin, could be found which failed to label T cells but labelled the great majority of B cells. We stress that we do not take this finding to imply an absence or paucity of immunoglobulin on the T cell surface. In fact, more direct chemical techniques (Marchalonis and Cone, 1973) have shown considerable amounts of an IgM-like molecule on T cells. The poor labelling of T cells by anti-Ig is probably due to a limited accessibility of the receptor to the antiglobulin (Greaves, 1970). We have no information on how soon in the differentiation of the B lymphocyte from stem cells the accessible surface Ig appears. There could well be partially differentiated 'pre-B' cells, lacking receptors but already committed to B cell development. Such cells would not have shown up in our survey.

The foetal survey can be regarded essentially as providing factual background information, without having resolved any deep problems. It showed a multifocal first appearance of B cells 3 days before birth in all the chief sites of erythromyelopoiesis. The mesenteric lymph node contained lymphocytes significantly later than did spleen, liver or marrow. This is consistent with the view that B lymphocytes did not arise locally, but were seeded into the node. The first lymphocytes appearing in the lymph node lacked the B cell marker, and were probably T cells. However, a rapid influx of B cells occurred soon after birth, and at age 4 days the proportion of B lymphocytes had almost reached the adult level. The studies were against the notion that gut-associated lymphoid tissue subserved a bursal function in this species. In fact, Peyer's patches could be identified only 1–2 days after birth, and the appendix was too minuscule in foetal life to have been a likely source of large-scale B cell differentiation, at least in the foetus. The multifocal first appearance of B cells in organs of eythromyelopoiesis suggested, but of course could not prove, a similar multifocal differentiation of stem cells to B cells.

The irradiation experiments raised some interesting issues. First, the irradiated, non-restored mice showed a residuum of radio-resistant lymphocytes, but the proportion of these with accessible surface Ig was very low. In other words, it appeared that most B cells were radio-sensitive while a proportion of presumed T cells was radio-resistant. The kinetics of B cell death have not been investigated in sufficient detail to determine whether interphase death or simple failure of replacement (mitotic death of precursors) was involved, but the dramatic drop over the first day strongly suggests that the former process

dominated. The fall of about 1000-fold in total B cell content of the spleen provided an unexpectedly low background from which to study B cell re-emergence.

The capacity of foetal liver entirely free of B cells to reconstitute the B cell population was not unexpected, but it did provide useful and direct confirmation of the capacity of haematogenous stem cells to generate B cells. Perhaps the most intriguing feature of the results was the rather slow emergence of new B cells in the irradiated hosts. For the first 7 days after irradiation, the total number of B cells did not differ between restored and non-restored animals. At 5–9 days, the paucity of B cell neogenesis stood in marked contrast to the vigorous erythropoiesis occurring in the spleen. Even after 10 days, when new B cell formation had clearly started, the rate of return to normal B cell numbers was slow by comparison with both the restoration of the other formed elements of the blood, and the known mitotic cycle times of lymphoid cells. Clearly, the restoration process is a very complex one, involving not only neogenesis of B cells from stem cells, but also antigenic stimulation of, and mitosis amongst, newly-formed B cells. Nevertheless, we wonder whether the observed 3–4-week period for numeric and functional B cell restoration may have reflected some kind of homeostatic priority selection by the repopulating stem cells. These are multipotent, and it is possible that the balance of regulatory factors in the post-irradiation state is such as to 'tell' the stem cells that they must restore the granulocyte, platelet and erythrocyte levels before diverting their efforts to the perhaps less urgent task of lymphopoiesis. This hypothesis could be tested by watching B lymphocyte emergence in irradiated-restored animals that were also given transfusions of platelets, granulocytes and erythrocytes. Other causes of a delayed lymphocyte restoration are possible. First, it could be that B cell neogenesis occurs in some organ other than the spleen and that the spleen receives B cells seeded out from this source. The failure to find earlier B cell repopulation in any other tissue makes this unlikely. Secondly, there may be obligatory intermediate stages in B cell neogenesis, with several generations of receptorless (pre-B) intermediate cells. Thirdly, the cell type involved in producing the tissue inducers needed to convert stem cells to B cells may itself have suffered some irradiation damage.

The delay in B cell emergence seen in these studies stands in contrast to two prior studies on B cell regeneration in irradiated hosts (Gregory and Lajtha, 1970; Lafleur, Miller and Phillips, 1972), which showed a more rapid functional recovery. Both of these studies used adult bone marrow as a source of repopulating cells, and it is likely that both stem cells and more differentiated progeny (pre-B) cells contributed to the results observed. We think that we have avoided contamination of our foetal liver stem cell source with either pre-B or B cells by using the donor tissue 3–4 days before B cell emergence.

We had hoped to study B cell re-emergence in a clonal fashion, but this proved impracticable. When numbers of stem cells ideal for the subsequent counting of splenic haemopoietic foci were used (e.g. 10^5 foetal liver cells), restored mice died before significant B cell recovery had occurred. Though it may be worthwhile to reinvestigate this point using germ-free mice (perhaps with concurrent erythrocyte transfusions), it is probable that by the time workable numbers of new B cells had appeared (14–16 days) foci would have become indistinct or confluent. Cells with typical lymphocyte morphology have not been reported in spleen haemopoietic colonies, which may reflect their late development, an early migratory tendency, or the need for some inductive influence to be found only outside the colony.

A simultaneous and independent study with many similarities to the present one has been performed by Stutman (1973). He also used anti-immunoglobulin binding as a B cell

marker, except that blast transformation rather than radioautography was taken as evidence of binding. B cells were first found at 16 days gestation in the foetus (in very small numbers), and at 8–9 days after foetal yolk sac or liver reconstitution of lethally irradiated animals. Stutman also performed re-transplantation experiments in which tissues from irradiated-restored hosts were re-injected into irradiated animals. Cells which could reconstitute lymphoid organs were found in spleen or bone marrow, but not in lymph node or Peyer's patches. In all, the studies agree well with our own.

We embarked on these studies partly in the hope that a sharply-defined stage of rapid B cell neogenesis would allow us to test the postulate that newly-differentiated or primitive B cells go through a phase of immaturity during which any contact with antigen causes immunological tolerance. We intend to pursue these studies, but the slow tempo of regeneration and the individual variation in immune response capacity of recently restored mice present complications. Specific pathogen free mice, which may be healthier in the immediate post-irradiation period, will be preferable for such experiments.

ACKNOWLEDGMENTS

This work was supported by the National Health and Medical Research Council, and the Australian Research Grants Committee, Canberra, Australia; and by Grant No. AI-O-3958 and Contract No. NIH-NCI-G-72-3889 from the National Institutes of Health, U.S. Public Health Service. This is Publication Number 1823 from the Walter and Eliza Hall Institute of Medical Research.

REFERENCES

Basten, A., Miller, J. F. A. P., Sprent, J. and Pye, J. (1972). 'A receptor for antibody on B lymphocytes. I. Method of detection and functional significance.' *J. exp. Med.*, **135**, 610.

Brahim, F. and Osmond, D. G. (1970). 'Migration of bone marrow lymphocytes demonstrated by selective bone marrow labelling with thymidine-H^3.' *Anat. Rec.*, **168**, 139.

Cantor, H. (1972). 'Two stages in development of lymphocytes.' *Cell Interactions* (Ed. by L. G. Silvestri). Proc. 3rd Lepetit Colloquium, London, p. 172. North-Holland Publishing Co., Amsterdam, London.

Claman, H. N. and Chaperon, E. A. (1969).'Immunologic complementation between thymus and marrow cells—a model for the two cell theory of immunocompetence.' *Transplant Rev.*, **1**, 92.

Everett, N. B. and Tyler, R. W. (1967). 'Lymphopoiesis in the thymus and other tissues: Functional implications. *Int. Rev. Cytol.*, **22**, 205.

Feldmann, M. (1972). 'Induction of immunity and tolerance *in vitro* by hapten-protein conjugates.' *J. exp. Med.*, **135**, 735.

Greaves, M. (1970). 'Biological effects of anti-immunoglobulins. Evidence for immunoglobulin receptors on "T" and "B" lymphocytes.' *Transplant. Rev.*, **5**, 45.

Gregory, C. J. and Lajtha, L. G. (1970). 'Recovery of immune responsiveness in lethally irradiated mice protected with syngeneic marrow cells.' *Int. J. Radiat. Biol. Related Stud. Physchem. Med.*, **17**, 117.

Kincade, P. W. and Cooper, M. D. (1971). 'Development and distribution of immunoglobulin-containing cells in the chicken.' *J. Immunol.*, **196**, 371.

Lafleur, L., Miller, R. G. and Phillips, R. A. (1972). 'A quantitative assay for the progenitors of bone marrow-associated lymphocytes.' *J. exp. Med.*, **135**, 1363.

Marchalonis, J. J. and Cone, R. E. (1973). 'Biochemical and physiological properties of lymphocyte surface immunoglobulin.' *Transplant. Rev.* (In press).

Metcalf, D. and Moore, M. A. S. (1971). *Haemopoietic Cells*. North-Holland Publishing Co.

Miller, J. F. A. P. and Mitchell, G. F. (1969). 'Thymus and antigen reactive cells.' *Transplant. Rev.*, **1**, 3.

Moore, M. A. S. and Owen, J. J. T. (1967). 'Experimental studies on the development of the thymus.' *J. exp. Med.*, **126**, 715.

Nossal, G. J. V. and Lewis, H. (1972). 'Variation in accessible cell surface immunoglobulin amongst antibody-forming cells.' *J. exp. Med.*, **135**, 1416.

Nossal, G. J. V., Warner, N. L., Lewis, H. and Sprent, J. (1972). 'Quantitative features of a sandwich radio-immunolabeling technique for lymphocyte surface receptors.' *J. exp. Med.*, **135**, 405.

Osmond, D. G. (1967). 'Lymphocyte production in the bone marrow: radioautographic studies in polycythaemic guinea pigs.' *The Lymphocyte in Immunology and Haemopoiesis*. (Ed. by J. M. Yoffey), p. 120. Edward Arnold Ltd, London.

Osmond, D. G., Miller, S. C. and Yoshida, Y. (1973). 'Kinetic and haemopoietic properties of lymphoid cells in the bone marrow.' In: *CIBA Foundation Symposium on Haemopoietic Stem Cells*. (In press).

Owen, J. J. T. and Raff, M. C. (1970). 'Studies on the differentiation of thymus-derived lymphocytes.' *J. exp. Med.*, **132**, 1216.

RAFF, M. C. (1970). 'Two distinct populations of peripheral lymphocytes in mice distinguishable by immunofluorescence.' *Immunology*, **19**, 637.

SHORTMAN, K., WILLIAMS, N. and ADAMS, P. B. (1972). 'Separation of different cell classes from lymphoid organs. V. Simple procedures for removal of cell debris, damaged cells and erythroid cells from lymphoid cell suspensions.' *J. Immunol. Meth.*, **1**, 273.

STUTMAN, O. (1973). 'Haemopoietic origin of B cells in the mouse.' In: *Fourth International Conference on Lymphatic Tissue and Germinal Centres in Immune Reactions*. (Ed. by B. Jankovic.) Plenum Press, New York.

WEISSMAN, I. L. (1967). 'Thymic cell migration.' *J. exp. Med.*, **126**, 291.

WILSON, J. D., NOSSAL, G. J. V. and LEWIS, H. (1972). 'Metabolic characteristics of lymphocyte surface immunoglobulins.' *Eur. J. Immunol.*, **2**, 225.

WU, A. M., TILL, J. E., SIMINOVITCH, L. and MCCULLOGH, E. A. (1967). 'A cytological study of the capacity for differentiation of normal hemopoietic colony-forming cells.' *J. cell. Physiol.*, **69**, 177.