CELLULAR DIFFERENTIATION OF THE IMMUNE SYSTEM OF MICE

III. Separate Antigen-Sensitive Units for Different Types of Anti-Sheep Immunocytes Formed by Marrow-Thymus Cell Mixtures*

By G. M. SHEARER,‡ Ph.D., and G. CUDKOWICZ, M.D.

(From the Department of Experimental Biology, Roswell Park Memorial Institute, Buffalo, New York 14203)

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The immune response to sheep erythrocytes (SRBC) is initiated in mice by functionally distinct cells not directly engaged in specific antibody production. These cells are either capable of reacting with antigens or of generating mature immunocytes by interactions, proliferation, and differentiation. They constitute a functional unit called antigen-sensitive unit (ASU), detectable by transplantation bioassays (1–3). Most ASU are found in spleen and lymph nodes (1–3); each ASU can produce ~100 hemolytic antibody-forming cells detectable by the agar gel assay (1). Thoracic duct lymph contains ASU of lower efficiency (4). Bone marrow and thymus do not contain ASU, since immunocytes are not formed upon transplantation of these individual cell suspensions with antigen (1, 5, 6). However, bone marrow and thymus of unprimed mice do contain potentially immunocompetent cells not yet integrated in functional units. Artificial mixtures of marrow cells and thymocytes complement each other and generate, upon transplantation and exposure to SRBC, hemolytic plaque-forming cells releasing antibody of the IgM class (5). Davies et al. (6–8), Miller and Mitchell (9), Nossal *et al.* (10), and Tyan and Herzenberg (11) have shown that marrow and several embryonic organs, except thymus, provide precursors of cells synthesizing immunoglobulins and specific antibody. The role of the thymus, but not of the marrow, is to provide cells that react with antigen and then interact with immunoglobulin-synthesizing cells or their precursors during the immune response to SRBC. Taken together, these findings suggest that cells of different lineage (myeloid vs. lymphoid) and with specialized functions cooperate during the immune response to SRBC. Therefore, functional ASU of spleen or lymph nodes are either integrated units of more than one cell (each cell type being complementary to the other), or single cells which have acquired immunocompetence following interaction of their precursors with thymus-derived and possibly other elements. The nature of these interactions between cells is not yet understood.

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The kinetics of immunocyte production by splenic ASU reactive with SRBC are well known (1, 3, 12). Splenic ASU of unprimed and primed mice are committed to the exclusive production of one of the following types of immunocytes: direct hemolytic plaque-forming cells (PFC), indirect hemolytic PFC, and hemagglutinating cluster-forming cells (CFC) (3, 13). Thus, splenic ASU engaged in anti-SRBC responses are rather specialized or differentiated cells of the immune system. If these ASU are composed of marrow-derived and thymus-derived elements, then artificial mixtures of marrow and thymus cells may also form specialized ASU. Which of the two cells confers specialization for antibody-class and for antibody-specificity is a fundamental problem of cellular immunology.

The present report describes (*a*) the kinetics of ASU formation after transplantation of marrow cells alone; (*b*) the kinetics of immunocyte production after transplantation of mixtures of marrow cells and thymocytes; and (*c*) the formation of new marrow cells capable of interacting with thymocytes and antigen in irradiated mice. The immune responses were estimated in terms of numbers of direct (IgM) and indirect (IgG) hemolytic PFC, and of hemagglutinating CFC. The similarities of splenic ASU and artificially formed ASU (by marrow-thymocyte mixtures) were striking. We found that the three types of immunocytes were generated by "constituted" ASU. Kinetic parameters differed only slightly. The numbers of direct PFC produced by each splenic ASU and marrow-thymus ASU were approximately equal. Constituted ASU appeared to be as specialized (unipotent) as splenic ASU for the type of progeny immunocytes they generated, i. e., for antibody class. This specialization was conferred to ASU by the marrow-derived component. The possible role of thymus-derived cells was not studied in these experiments.

Materials and Methods

Mice.—(C3H/He × C57BL/Ha)F_1 females, 12–13 wk old, were used in all experiments as recipient mice.

Irradiation.—Mice to be grafted with syngeneic cells were preexposed to 800–925 R of total body X-radiation as described elsewhere (3).

Cell Suspensions.—Bone marrow cells were harvested from femurs, tibias, and humeri of 10–12 wk old (C3H/He × C57BL/Ha)F_1 female donors. The ends of the bones were severed and the marrow cavities were flushed with chilled Eagle's medium. Clumps of marrow cells were dispersed by aspiration through a 23 gauge needle fitted to a 5 ml syringe. Residual particulate matter was removed by filtration through 200 mesh per inch stainless steel cloth.

Thymuses and lymph nodes (mesenteric, inguinal, axillary, brachial) were removed from 8–10 wk old donors. The organs were first rinsed and then minced with scissors in chilled Eagle's medium. The cells in the fragments were dispersed by repeated aspiration through the orifice of a 2.5 ml syringe without needle. The cell suspensions were filtered through stainless steel cloth.

Spleen cells were dispersed as described (3). Nucleated cells of each preparation were counted with a model B Coulter electronic apparatus fitted with a 100 μ aperture. Red blood cells were lysed with saponin for 3 min before counting. The cell suspensions were then adjusted to contain the desired number in 0.5 ml. The mixtures of marrow and thymus cells were made immediately before transplantation.

Transplantation.—Suspensions of one or two cell types were injected into the lateral tail vein of recipient mice within 4 hr after irradiation. In some experiments, SRBC were added to the cell suspensions. The injection volumes did not exceed 1 ml. To prevent embolism, the recipients were given 50 USP units of heparin a few minutes before injection, and the cells were infused slowly.

Immunization.—Defibrinated sheep blood was obtained from Grand Island Biological Co., Grand Island, N. Y., and stored at 4°C for not longer than 3 wk. Immediately before use, SRBC were washed three times in Eagles's medium, counted, and adjusted to a concentration of 10^9/ml. Each recipient mouse received 0.5 ml of the suspension intravenously, either at the time of cell transplantation or 18 hr later.

Assays for Immunocytes and for Hemolytic Foci.—The procedures for enumerating plaque-forming cells (direct and indirect) and cluster-forming cells have already been described (3, 13, 14). The assays were performed on spleen cell suspensions of individual mice in duplicate or triplicate. The average immunocyte count per nucleated spleen cells sampled was multiplied by the total number of cells harvested from the spleen.

Foci of hemolysin-producing cells were detected in slices of spleens laid over agar gel containing SRBC. The procedure used was that described by Kennedy et al. (1).

Abbreviations.—The following symbols are used in the text, tables, and figures. SRBC, sheep erythrocytes: PFC, plaque-forming cells; CFC, cluster-forming cells; ASU, antigen-sensitive units; ARC, antigen-reactive cells of thymic origin; P-PFC, precursors of plaque-forming cells of bone marrow origin.

RESULTS

Formation of New Antigen-Sensitive Units in Irradiated Mice Grafted with Marrow Cells.—After whole body irradiation (925 R), mice are incompetent to elicit an immune response to SRBC because most cells of their immune system have been destroyed.

We have injected irradiated mice with 10^6, or 10^7, or 10^8 syngeneic marrow cells to reconstitute the pool of ASU. At varying intervals, up to 110 days after transplantation, the presence of newly formed ASU was tested by injecting the mice with 5×10^8 SRBC and by determining the number of direct PFC in the spleens 4 days later. Negative controls were marrow chimeras not given SRBC, and positive controls were intact mice (nonirradiated, nongrafted) of the same age given SRBC. The results obtained are shown in Fig. 1.

For the first 5 or 10 days, depending on the size of the marrow graft, there was no increase in the number of PFC per spleen over that of radiation controls, although proliferation and differentiation of myelopoietic cells is known to be conspicuous at this time (15). Thereafter, the PFC response of all chimera groups increased exponentially with a doubling time of 2.7 days until a plateau was reached 25–30 days after marrow transplantation. The magnitude of the plateau response was somewhat lower in all chimera groups than in intact mice, even as late as 190 days after transplantation (data between 110 and 190 days are not shown in Fig. 1). Furthermore, the magnitude of the PFC response at any time after transplantation was not directly proportional to the number of cells grafted. A 100-fold difference in graft size produced only a 9-fold difference in response during the period of exponential rise. Since neither the irradiated recipients nor the grafted marrow cell suspensions contained functional ASU

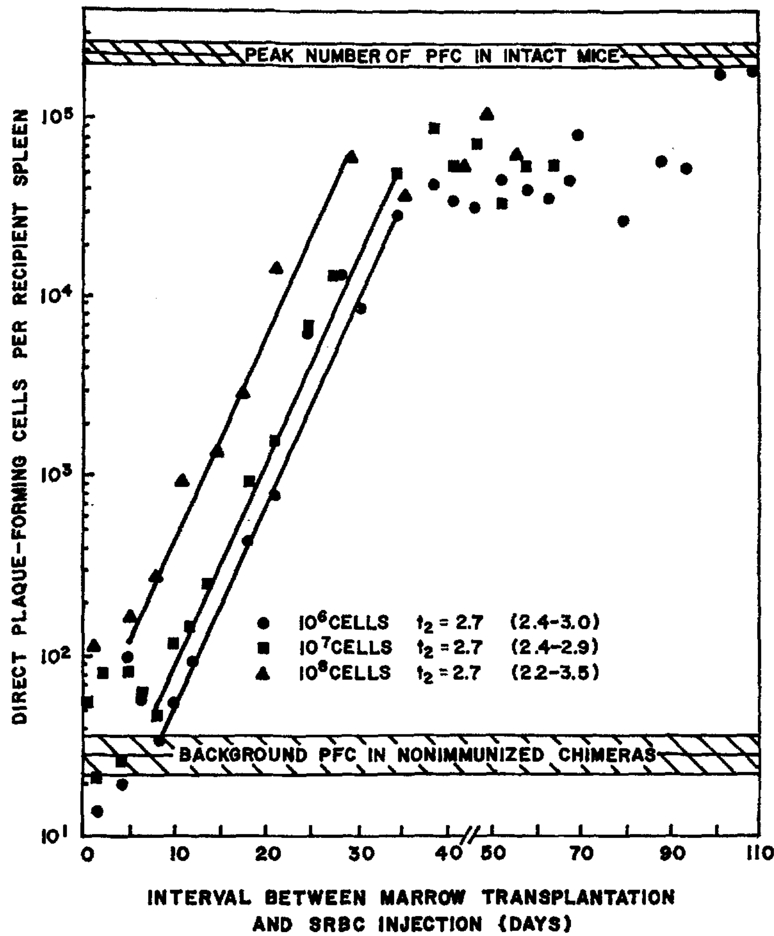

FIG. 1. The direct hemolytic plaque response in irradiated (925 R) mice grafted with graded numbers of marrow cells. SRBC were injected at varying times after transplantation and plaque assays were made on spleen cell suspensions 4 days after SRBC administration. Each point is the mean value of 3 spleens assayed individually. Doubling times (t_2) are indicated in days with 95% confidence intervals.

(lack of response in radiation controls and in experimental mice 0–10 days after grafting), it must be concluded that the PFC response elicited by the chimeras was due to regenerated ASU. Presumably, these ASU were derived from precursors present in the injected marrow, which did not require antigen for differentiation. Similar observations in irradiated mice given 2–5 $\times$ 10^6 marrow cells were reported by Till et al. (16) and by Osoba (17). Our experiments em-

phasize the lack of direct dose-response relation between the number of new ASU (measured by PFC production) and that of grafted marrow cells in the range of 10^6–10^8 cells. Factors other than the size of the graft limited the rate of ASU formation by marrow precursors. Presumably, antigen-reactive cells (ARC) of the thymus (4, 9) were deficient after irradiation and could have limited ASU formation for some time later.

Immunocyte Production by Marrow-Thymus Cell Mixtures.—

A group of 35 irradiated (800 R) mice was given the mixture of 5 × 10^7 thymocytes and 5 × 10^7 marrow cells, followed by 5 × 10^8 SRBC 18 hr later. Other groups of irradiated

TABLE I

Antibody-Forming Cells in Irradiated (800 R) Control Animals

Treatment	Time after X-rays	Fraction of spleens with detectable immunocytes and mean number of immunocytes per positive spleen ± SE*		
		Direct PFC	Indirect PFC	CFC
	days			
Bone marrow cells (1–5 × 10^7) + SRBC	7–12	13/16 43.9 ± 23.4	7/16 14.9 ± 4.3	6/16 7,980 ± 1,870
Thymus cells (5–10 × 10^7) + SRBC	6–9	8/10 57.8 ± 26.6	0/10 —	3/8 2,220 ± 1,320
5 × 10^7 thymus cells + 5 × 10^7 bone marrow cells	8–13	7/13 28.7 ± 3.2	5/13 8.5 ± 3.3	7/13 6,990 ± 1,820

* Two-fifths of all nucleated spleen cells were used for each plaque assay and one-fifth for the cluster assay.

mice (controls) received bone marrow cells and SRBC, thymocytes and SRBC, or marrow and thymus cells without SRBC. Assays for direct and indirect PFC and for CFC were carried out at intervals from the 6th to the 19th day after transplantation. The results are shown in Table I and Fig. 2.

Small numbers of background PFC and CFC were found in every control group, although not in every spleen (Table I). None of the control mice had more than 120 direct PFC, 30 indirect PFC, and 17,000 CFC per spleen.

The spleens of *all* 35 experimental mice contained direct and indirect PFC (Fig. 2); the number of immunocytes increased between days 6 and 10 and then either decreased slowly (direct PFC) or remained near peak values (indirect PFC). In contrast to the PFC responses, which took place in all mice given potentially competent cells and antigen, the CFC response did not take place in

all of the 28 mice assayed between days 7 and 19. Each of the groups of mice killed 7, 9, 11, 13, 15, and 19 days after transplantation included animals whose spleens contained not more than background numbers of CFC. A total of 15 of 28 tested chimeras did not respond to primary SRBC stimulation with CFC, although the chimeras responded with PFC. In Fig. 2, the mean values of CFC per spleen are shown for positive mice only. These values were 10 times greater than those of controls. The observations suggested that transplantation of mix-

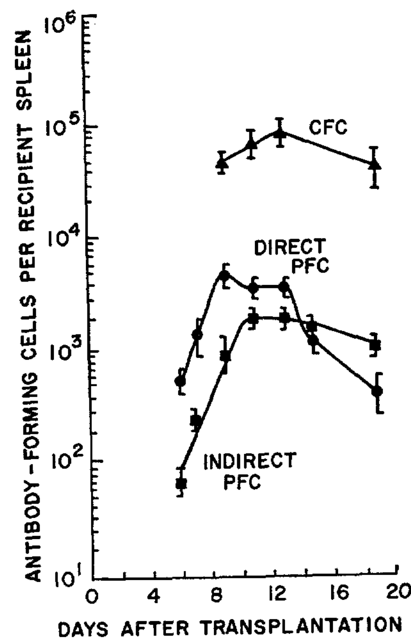

FIG. 2. Mean number of immunocytes in spleens of irradiated (800 R) mice at varying times after the injection of 5×10^7 thymocytes, 5×10^7 marrow cells, and 5×10^8 SRBC. Each point is the mean of five spleens assayed individually. Limits shown are two standard errors. 35/35 positive spleens for direct and indirect PFC. 13/28 positive spleens for CFC.

tures of 5×10^7 thymocytes and 5×10^7 marrow cells resulted in the formation of ASU. The constituted ASU were capable of generating PFC of the direct and indirect type, and CFC. The number of ASU was sufficient to provide each recipient spleen with direct and indirect PFC, but not all recipients with CFC. Therefore, separate ASU may have given rise to PFC and CFC. According to these criteria, splenic ASU of unprimed mice (3) and constituted ASU appear to be similar. However, details of the cytokinetics of the PFC responses elicited by constituted and splenic ASU were different. The production of direct PFC by constituted ASU was delayed (peak value at day 9) and occurred *at the same time* as that of indirect PFC. For splenic ASU, peak numbers of direct PFC ap-

peared at day 7, 5 days *before* the time of peak numbers of indirect PFC (3). Furthermore, the numbers of immunocytes produced by constituted ASU were not greater than 7,000 direct PFC, 4,000 indirect PFC, and 170,000 CFC per recipient spleen. The numbers of immunocytes produced by splenic ASU were about three times greater for grafts of 2×10^7 nucleated spleen cells (3).

Formation of Foci of PFC in Irradiated Mice Grafted with Marrow Cells and Thymocytes, Spleen Cells, or Lymph Node Cells.—The relatively small number of PFC produced by grafts of 5×10^7 marrow cells and 5×10^7 thymocytes raises the following possibilities. The frequency of ASU is greater in spleen cell suspensions than in marrow-thymus cell mixtures, but each type of ASU produces the same number of progeny PFC. Conversely, ASU formed by marrow-thymus mixtures produce fewer PFC than splenic or lymph node ASU, as apparently do the ASU of thoracic duct lymph (4). The PFC progeny of individual ASU form discrete foci in spleens of irradiated mice injected with appropriate numbers of cells (1). Such foci can be visualized because they determine localized areas of hemolysis when spleen slices are laid over semisolid agar containing SRBC and incubated in the presence of complement (1). The focus assay should enumerate, therefore, grafted ASU that lodge in the recipient spleens. By assaying two separate groups of mice (given the same inoculum) for hemolytic foci and for direct PFC, one can estimate the number of immunocytes produced per ASU (1).

We have injected irradiated (850 R) mice with 5×10^6 lymph node or spleen cells mixed with SRBC, or with one of the following cell combinations: 2×10^7 thymocytes mixed with 2×10^7 marrow cells; 10^8 thymocytes mixed with 3×10^5 marrow cells. SRBC were injected 18 hr later. Preliminary studies indicated that 3×10^5 marrow cells and 2×10^7 thymocytes, respectively, were the smallest (limiting) numbers of grafted cells that generated *direct* PFC in *every* recipient mouse when mixed with an excess of cells of the other kind. 10^8 thymocytes and 2×10^7 marrow cells, respectively, are an excess of cells for the *direct* PFC response elicited by marrow-thymus mixtures.[1] 7–9 days after transplantation, at the time of peak responses, the spleens of some recipients were assayed for hemolytic foci, and those of others for their content of direct PFC. The results are shown in Table II.

Hemolytic foci were produced by the marrow-thymus grafts regardless of whether the thymus-derived ARC or the marrow-derived precursors of PFC (P-PFC) were limiting the immune response. The mean number of PFC per focus was approximately the same for constituted ASU and for splenic and lymph node ASU. Hence, the comparatively small number of PFC formed in recipients of marrow and thymus cells (Fig. 2) was due to small numbers of ASU formed. The less frequent, and therefore the limiting cell in PFC production, appears to be the thymus-derived ARC.

Formation of discrete foci, after grafting 67 times the limiting number of

[1] Shearer, G. M., Cudkowicz, G., and Priore, R. L. Cellular differentiation of the immune system of mice. IV. Lack of class differentiation in thymic antigen-reactive cells. Submitted for publication.

marrow cells with limiting numbers of thymocytes, suggests that each ARC interacting with P-PFC forms one or a few antigen-sensitive units. In other words, once the interaction between these cells has occurred, formation of ASU remains localized and does not involve more precursors of PFC in a chain reaction.

The Frequency of PFC and CFC Responses in Irradiated Mice Grafted with 5–10 × 10^7 Thymocytes and 1–5 × 10^7 Marrow Cells.—ASU formed by marrow-thymus cell mixtures may be functionally different, as those of spleen cell suspensions (3, 13). They may be restricted in their potential for differentiation so as to generate a single type of progeny immunocyte. If so, it should be possible to dissociate, upon transplantation of relatively small numbers of cells, not only

TABLE II

*Number of Hemolytic Foci and Direct PFC in Spleens of Irradiated (850 R) Mice Grafted With Marrow-Thymus Cell Mixtures, Spleen Cells, or Lymph Node Cells**

Cells grafted	Day of assay	Hemolytic foci per spleen‡	Direct PFC per spleen‡	Direct PFC per focus‡
2 × 10^7 thymocytes + 2 × 10^7 marrow cells + SRBC	9	5.16 ± 0.31 (6)	845 ± 165 (6)	163 ± 33
10^8 thymocytes + 3 × 10^5 marrow cells + SRBC	9	2.33 ± 0.24 (9)	317 ± 85 (8)	137 ± 40
5 × 10^6 spleen cells + SRBC	7	6.43 ± 0.22 (40)	834 ± 98 (32)	130 ± 15
5 × 10^6 lymph node cells + SRBC	7	6.67 ± 0.53 (21)	575 ± 69 (23)	86 ± 13

* SRBC were mixed with the spleen and lymph node cell suspensions, but not with the marrow-thymus cells. In the latter case SRBC were injected separately 18 hr after the cell grafts.

‡ Mean ± standard error. Number of mice in parentheses.

the production of PFC from CFC (Fig. 2), but also that of direct PFC from indirect PFC.

To obtain such a dissociation (negative spleens for one type of immunocyte, but positive for another) we have chosen to reduce the number of marrow cells to be mixed with thymocytes from 5 × 10^7 per graft to 10^7. Conceivably, fewer ASU would be formed and the probability that only one or two types of ASU reach the recipient spleen should be greater.

The following mixtures were prepared and transplanted into a total of 184 mice: 5 × 10^7 thymocytes with 10^7 or 5 × 10^7 marrow cells; 10^8 thymocytes with 10^7, 2 × 10^7, or 3 × 10^7 marrow cells. SRBC were injected 18 hr later. 9–12 days after transplantation, at the time of expected maximum responses (Fig. 2), the mice were killed and their spleens were assayed for direct and indirect PFC. Spleens of 128 mice were also assayed for CFC. In all instances, duplicate samples representing one-fifth of the recipient spleen each, were plated for plaque assays. Results obtained with the different mixtures were similar and therefore pooled (except for statistical analysis, Table III). The frequencies of recipient spleens with given values of immunocytes are presented in Fig. 3.

The spleens of almost all mice (177 of 184) were positive for direct PFC, indicating that ASU were formed by the cell mixtures and that immunization had occurred. Significant numbers of indirect PFC were found in 98 of 184 spleens. In the remaining 86 spleens the number of indirect PFC was less than 50. We consider, therefore, that these spleens were negative for indirect PFC. Presumably, cells competent to generate this particular immunocyte were relatively infrequent in the grafts and hence, did not lodge in all recip-

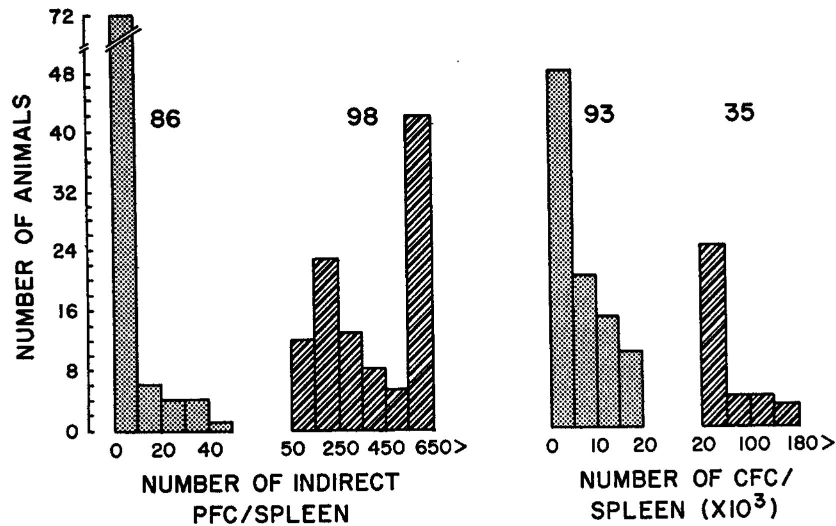

FIG. 3. Number of mice from which spleens—assayed 9–12 days after transplantation of marrow-thymus cell mixtures—were negative (stippled bars) or positive (striped bars) for indirect PFC and for CFC. The total number of positive and negative spleens is indicated above the bars, and the number of immunocytes per spleen below the bars. Each mouse was exposed to 800 R and grafted with 5–10 $\times$ 10^7 thymocytes mixed with 1–5 $\times$ 10^7 marrow cells. SRBC were injected 18 hr later.

ient spleens. It should be noted, however, that cells competent to generate direct PFC did lodge and produce immunocytes in most of these spleens.

Significant numbers of CFC were found in 35 of 128 spleens. The remaining 93 spleens were indistinguishable from controls, i.e., they were negative. Most of the spleens that were negative for CFC were positive for direct PFC, and some were also positive for indirect PFC. The proportion of mice with positive spleens was 97% for direct PFC, 53% for indirect PFC, and 27% for CFC. This distribution resembles that obtained in comparable experiments with splenic ASU (3). Likewise, it suggests that ASU formed after grafting marrow-thymus mixtures are unipotent and independent in their ability to generate immunocyte

progeny in recipient spleens. The frequencies of the unipotent ASU appear to be unequal. Both in marrow-thymus mixtures (Fig. 3) and spleen cells of unprimed mice (3), they decrease in this order: ASU for direct PFC, indirect PFC, and CFC. Since dissociation of responses was obtained primarily by reducing the number of marrow cells, it follows that the number and types of ASU formed depended on the availability of particular marrow cells for interaction with ARC.

Test for Independence of Specialized ASU.—If the ASU of marrow-thymus cell mixtures are unipotent, reach the spleens of recipients independently, and generate progeny immunocytes without interacting, then we expect chance

TABLE III

Results of χ^2 Tests for Independence of Antigen-Sensitive Units Producing Indirect PFC and CFC on Transplantation

No. of thymocytes ($\times 10^7$)		5	5	10	10
No. of marrow cells ($\times 10^7$)		1	5	1	2
No. of recipient mice		24	33	63	8
Indirect PFC	CFC	Number of recipient spleens			
+	+	2	13	11	0
+	−	7	13	20	2
−	+	0	0	8	3
−	−	15	7	24	3
χ^2*		1.31	3.87	0.40	0.18

+, positive spleen containing more than 50 indirect PFC, or 2×10^4 CFC. −, negative spleens containing fewer immunocytes.

* Chi-square values in the table were compared with 3.84, the critical value of χ^2 statistic at the 0.05 level of significance. Only one of the comparisons (2nd column) was incompatible with the hypothesis that antigen-sensitive units for different types of immunocytes were independent. However, the significance of this comparison was marginal.

dissociation of cellular responses in the spleens that we have analyzed. All treatment groups described in the preceding paragraph included spleens that were positive or negative for indirect PFC or CFC. Therefore, the data of spleens assayed for both types of immunocytes were subjected to a chi-square (χ^2) test for independence of cellular responses (Table III). The χ^2 values of three comparisons were below the critical value of 3.84, the χ^2 statistic at 0.05 level of significance for one degree of freedom. Therefore, there was no statistical evidence for dependence between the indirect PFC and CFC responses elicited by 95 grafts of 1–2 $\times 10^7$ marrow cells. The χ^2 value of a fourth comparison (33 grafts) reached the level of significance, but was only 3.87, suggesting marginal association, if any, between indirect PFC and CFC responses.

Production of New P-PFC after Transplantation of Marrow Cells into Irradi-

ated Mice.—The marrow cells that cooperate with thymocytes during the immune response to SRBC may have been differentiated for antibody class before ASU formation. It was of interest, therefore, to establish whether regeneration of P-PFC, committed to either direct or indirect PFC, precedes regeneration of ASU in the spleens of marrow chimeras.

A group of 54 mice was exposed to 900 R of X-rays and each was injected with 10^7 nucleated marrow cells. At several intervals between days 1 and 46 after cell transfer, groups

TABLE IV

*Production of New Precursors of PFC in Bone Marrow of Irradiated (900 R) Mice Grafted With 10^7 Marrow Cells**

Time after primary transplantation	Fraction of mice whose marrow contained P-PFC and mean number of immunocytes ± SE‡ per spleen of secondary test recipients. Fraction of positive test recipients in parentheses.	
	Direct PFC	Indirect PFC
days		
1–4	0/12 — (0/23)	0/12 — (0/23)
5–6	18/19 673 ± 79 (24/28)	8/19 357 ± 87 (11/28)
8–9	17/17 756 ± 102 (43/46)	10/17 297 ± 145 (18/46)
46	6/6 1570 ± 271 (12/12)	6/6 428 ± 104 (11/12)

* Newly formed P-PFC of marrow were assayed as follows: Cells of six long bones were divided into two or three equal parts each of which was mixed with 5 × 10^7 thymocytes from intact donors and regrafted into individual irradiated (850 R) test recipients. SRBC were given 18 hr later. Plaque tests were made on spleen cells of test recipients 9 days later.

‡ Mean weighted for the number of primary marrow recipients whose cells were assayed on retransplantation.

of 6–17 mice were killed to determine whether their marrow and spleens contained P-PFC. Marrow cells were harvested from six long bones (femurs, tibias, humeri). Marrow and spleen cell suspensions from chimeras were mixed with thymocytes from intact (nonirradiated, nongrafted) donors and transplanted into irradiated (850 R) secondary test recipients. SRBC were injected 18 hr later.

One-half of the marrow or spleen cells of each chimera was given to each of two secondary recipients from day 1 to 6 after primary transplantation; and one-third to each of three secondary recipients on days 8, 9, and 46. Each test animal received 5 × 10^7 thymocytes mixed with the chimera cells. The spleens of the test recipients were assayed for direct and indirect PFC 9 days after retransplantation. The results are shown in Tables IV and V. For each group of primary recipients of marrow we report the proportion of mice that had de-

tectable P-PFC in marrow or spleen, the mean number of immunocytes generated by the P-PFC in positive test recipients, and the proportion of test recipients that had positive spleens, i.e., spleens with > 100 direct and > 50 indirect PFC. Controls for the presence of complete ASU in thymus cell suspensions and in chimera spleens were irradiated mice grafted with thymocytes only and SRBC (not shown in the tables), or with chimera spleen cells and SRBC without thymocytes. The latter confirmed the lack of ASU in spleens of chimeras during the first 10 days after marrow transplantation (Fig. 1).

P-PFC were not detected in marrow and spleens in 11 of 12 irradiated mice tested 1–4 days after transplantation of marrow cells. The numbers of splenic

TABLE V

*Production of New Precursors of PFC in Spleens of Irradiated (900 R) Mice Grafted With 10^7 Marrow Cells**

Time after primary transplantation	Fraction of mice whose spleens contained P-PFC or ASU and mean number of immunocytes ± SE‡ per spleen of secondary test recipients. Fraction of positive test recipients in parentheses.		
	Spleen cells tested for*	Direct PFC	Indirect PFC
days			
1–4	P-PFC	1/5 310 (1/7)	0/5 — (0/7)
5–6	P-PFC	5/6 631 ± 93 (9/11)	4/6 425 ± 28 (6/11)
8–9	ASU	1/10 900 (1/21)	1/10 655 (1/21)

* Newly formed P-PFC of spleens were assayed as follows: One-half or one-third of the spleen cells was mixed with 5×10^7 thymocytes from intact donors and regrafted into individual irradiated (850 R) test recipients. SRBC were given 18 hr later. ASU were assayed by regrafting one-third of the spleen cells with SRBC but without thymocytes. Plaque tests were made on spleen cells of test recipients 9 days later.

‡ Mean weighted for the number of primary marrow recipients whose cells were assayed on retransplantation.

PFC of test recipients did not exceed background values. However, by day 5 marrow and spleens of chimera mice contained P-PFC. The marrows of 42 mice were tested between days 5–46, and the spleens of 6 of them were tested between days 5–6. Precursors of *direct* PFC were found in all but one chimera. Since P-PFC were not detected during the first 4 days, new formation of P-PFC is likely to have occurred, either by self-replication of grafted P-PFC or by differentiation from more primitive precursors. By days 5 and 6 the frequency of P-PFC was such that one-half of the harvested marrow and spleen cells generated direct PFC in almost all test recipients. In contrast, only 1 of 10 spleens contained complete ASU by days 8–9. Furthermore, not all of the 42 chimera

mice also had precursors of *indirect* PFC in their marrow and spleens. The cells of only one-half of the chimeras tested between days 5 and 9 generated indirect PFC. Moreover, indirect PFC were found in less than 40% of the test recipients, suggesting that these precursors were less frequent than those of direct PFC. By day 46 after transplantation, the marrow of all tested chimeras contained precursors of both direct and indirect PFC.

P-PFC were detectable in spleens of chimeras at a time in which newly formed ASU were not, e.g., during the first 10 days posttransplantation (Fig. 1, Table V). Hence, the delay in ASU formation of marrow chimeras cannot be ascribed to lack of marrow or splenic P-PFC. Similarly, precursors of direct PFC were detectable in chimeras in which precursors of indirect PFC were not. Possibly two types of P-PFC exist: one for direct PFC only, and one either capable of generating both direct and indirect PFC, or indirect PFC only.

DISCUSSION

Bone marrow of adult mice is the source of the more primitive progenitor cells of the immune system (18, 19). It is not known, however, whether a single pluripotent, and/or separate unipotent progenitors differentiate into ARC, P-PFC, macrophages, and other hemopoietic cells. We have verified that marrow contains cells that upon transplantation generate P-PFC in spleens and bone cavities of recipients. P-PFC could have arisen either by self-replication of preexisting P-PFC, or by differentiation and proliferation of more primitive cells. Our experiments were not designed to elucidate this point. We found that not all newly formed P-PFC were alike. Some appeared earlier and could have been restricted to the exclusive production of direct PFC descendents. Others appeared a few days later and could have been capable of producing either direct and indirect PFC, or indirect PFC only. If so, we expect that each ASU formed upon interaction of such P-PFC with ARC and SRBC should be accordingly restricted in its potential (see later in discussion).

P-PFC were formed several days before functional ASU appeared in the spleens of irradiated mice grafted with marrow. Such mice were incompetent to elicit anti-SRBC immune responses for about 10 days, although P-PFC were available in the bone cavities and in the spleens for ASU formation. It follows that the missing elements were, presumably, functional ARC. Grafts of thymocytes added to marrow cells were immunologically competent without delay. This supported the view that new ARC become available for ASU formation later than P-PFC in marrow chimeras, specifically 10–30 days after transplantation, but not during the first 10 days.

ARC are marrow-derived cells like P-PFC, since they are formed in heavily irradiated, thymectomized mice grafted with marrow cells and implanted with thymus enclosed in a cell-impermeable chamber (17). In such mice new ARC were formed in sites other than the thymus. However, the kinetics of splenic ASU formation did not differ greatly from those we described in nonthymec-

tomized marrow chimeras, and others described in chimeras with thymuses implanted under the kidney capsule (20). It is possible that a humoral factor elaborated by the thymus controlled differentiation of marrow cells into ARC (17). The process is rather slow and appears to be the rate-limiting step in the reconstitution of the immune system in irradiated mice by grafted marrow. Both the formation of P-PFC and ARC occurred in the absence of SRBC.

In most of our other experiments, relatively large numbers of thymocytes were mixed with marrow cells for transplantation, exposure to SRBC (18 hr later) and in vivo formation of ASU. This was done to ensure that thymic ARC would not limit the processes leading to ASU formation. One of these processes, the priming or activation of ARC by antigen, could take place in the absence of marrow cells (21). Another process, the interaction between activated ARC and one or more marrow cells in vivo, also required the presence of antigen (unpublished observations) and was a nonrepetitive event, i.e., the same ARC did not continue to interact in succession with several P-PFC. ASU formed in recipients of marrow cells and thymocytes were similar to those found in intact mice. They were capable of generating direct and indirect PFC, as well as CFC, and the number of direct PFC produced by one ASU was 137–163, about the same as that produced by splenic ASU. The most important similarity, however, was the specialization of ASU, since our results strongly suggest that each ASU generated either indirect PFC or CFC, but not both. Furthermore, in a number of mice we found ASU producing direct PFC only. In general, dissociations of PFC and CFC responses were enhanced by reducing the number of marrow cells mixed with 5×10^7 or 10^8 thymocytes. This suggests (*a*) that P-PFC and/or other marrow cells were specialized for the molecular class of antibody to be secreted by P-PFC–derived immunocytes, and (*b*) that the existence of such marrow cells accounted for class differentiation of ASU. Limiting dilution experiments for *restricted* P-PFC and *unrestricted* ARC, to be reported in the next papers of this series,[1] have confirmed these interpretations. The possibility that two or more different marrow-derived cells cooperate with ARC and SRBC in forming functional splenic ASU has been raised by our own results[1] and by the reconstitution of ASU by mixing spleen cell fractions separated by physical means (22, 23).

The only differences we noticed between marrow-thymus and splenic ASU were the kinetics of appearance of direct and indirect PFC (3). In recipients of marrow-thymus grafts, direct PFC responses were comparatively late and indirect PFC responses early, so that peak values for both PFC were reached at the same time rather than in succession. This could reflect qualitative differences between thymic ARC and ARC residing in the spleen and body fluids (4). We have not observed differences between splenic and marrow P-PFC, the other components of ASU. Results of the following experiments suggest that thymic ARC may be relatively immature: tolerogenic injections of antigens

seem to abrogate or impair the function of thymocytes (24, 25), with a latent period of only one day or less (25), but of four days for spleen cells (26).

SUMMARY

Marrow cell suspensions of unprimed donor mice have been transplanted into X-irradiated syngeneic hosts. 5–46 days later, bone cavities and spleens contained regenerated cells of the immune system which required interaction with thymocytes (from intact donors) and antigen (SRBC) to form antigen-sensitive units (ASU) and to generate mature immunocytes. These cells were capable of differentiating either into direct or indirect hemolytic plaque-forming cells (PFC). The precursors of PFC regenerated earlier than the other cell type necessary for immunocompetence, the antigen-reactive cell (ARC). The latter was not found until 10 or more days after transplantation. Availability of ARC was inferred from PFC responses elicited by grafted mice challenged with SRBC at varying intervals.

In a second series of experiments, graded numbers of marrow cells (ranging from 10^7 to 5×10^7) were transplanted with 5×10^7 or 10^8 thymocytes into irradiated mice, and SRBC were given 18 hr later. After 9–12 days the recipient spleens contained all or some of the following immunocytes: direct and indirect PFC, and hemagglutinating cluster-forming cells. The frequency of each immune response varied independently of the others, but in relation to the number of marrow cells grafted. This was interpreted to indicate that ASU formed in irradiated mice by interaction of marrow and thymus cells were similar to those of intact mice. In particular, they were specialized for the molecular class (IgM or IgG) and function (lysis or agglutination) of the antibody to be secreted by their descendent immunocytes. Hence, class-differentiation appeared to be conferred upon ASU by their marrow-derived components.

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