

## ANTIGEN-SPECIFIC CELLS IN MOUSE BONE MARROW

### I. LASTING EFFECTS OF PRIMING ON IMMUNOCYTE PRODUCTION BY TRANSFERRED MARROW\*

By HAROLD C. MILLER,† Ph.D. AND GUSTAVO CUDKOWICZ, M.D.

*(From the Department of Pathology, School of Medicine, State University of New York at Buffalo, Buffalo, New York 14214)*

(Received for publication 16 June 1970)

The realization that antibody-forming cells descend from bone marrow precursors (1-3), and that complex interactions between thymus- and marrow-derived cells precede antibody formation (1-6), gave impetus to attempts aimed at establishing which cell type was antigen specific (7-12). The issue is of great importance; should one of the cell types cooperating in an immune response be differentiated so as to react with a restricted number of antigenic determinants, this cell could perhaps dictate the specificity of immunoglobulins synthesized by marrow-derived immunocytes. On the other hand, if cells of thymic and marrow origin were both antigen-specific, cell-to-cell interactions would probably not occur at random but between cells sharing receptors of appropriate specificity.

Results of several cell transfer experiments indicated that antigen-reactive cells (ARC)<sup>1</sup> from mouse thymus or their progeny cells were antigen specific. The evidence was obtained either by sensitizing (10-12) or by paralyzing (7-9) thymus-derived cells prior to their interaction with marrow cells. Both induced changes of reactivity were specific. In contrast, potentially immunocompetent marrow cells were neither paralyzed nor sensitized upon exposure to antigens under the same experimental conditions (8-11). The failure to specifically alter the reactivity of marrow cells could have been due to nonexistence of antigen-specific cells in this tissue. However, it was also possible that such a cell existed but went undetected because it lacked a role in the establishment of tolerance or could not be sensitized upon short-term cell transfer.

In the experiments to be described, hemolytic plaque-forming cells (PFC) induced by antigens of sheep or chicken erythrocytes (SRBC, CRBC) were studied in irradiated mice reconstituted with excess thymocytes and small,

---

\* Research supported by Grants AM-13,969 from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, and T-476 from the American Cancer Society.

† Conducted during the tenure of American Cancer Society Postdoctoral Fellowship PF-501.

<sup>1</sup> *Abbreviations used in this paper:* ARC, antigen-reactive cells of thymic origin; CRBC, chicken erythrocytes; PFC, plaque-forming cells; P-PFC precursors of plaque-forming cells of marrow origin; SRBC, sheep erythrocytes.

graded numbers of bone marrow cells. While thymus donors were always non-immune mice, marrow cell donors were either nonimmune or SRBC-primed mice. The results of limiting dilution assays differed markedly for responses to SRBC and CRBC by cells of untreated donor animals. Responses to SRBC by marrow cells of immune and nonimmune donors also differed in several respects. The *induced* changes in anti-SRBC responses were detectable for several months after immunization during which responses to antigens of CRBC were not altered. It was, therefore, apparent that mouse marrow contained cells which were antigen specific and antigen sensitive.

### *Materials and Methods*

*Mice.*—(C3H/He × C57BL/Ha)F<sub>1</sub> females, 7–10 months old, and (C3H/He × C57BL/10Cz)F<sub>1</sub> females, 6–7 months old, were used as donors of bone marrow cells. The abbreviated designations for the two hybrid strains are C3BF<sub>1</sub> and C3B10F<sub>1</sub>, respectively. In all experiments, the donors of thymocytes were 2-month old C3B10F<sub>1</sub> mice, and the irradiated recipients were 3-month old C3B10F<sub>1</sub> mice. Preliminary tests indicated that transplanted C3BF<sub>1</sub> marrow cells were as effective as C3B10F<sub>1</sub> cells in generating PFC in C3B10F<sub>1</sub> recipients.

*Irradiation.*—Mice were exposed to 750–850 R of total body X-irradiation, as described elsewhere (13), a few hours prior to marrow and thymus cell transplantation.

*Cell Suspensions and Transplantation.*—Nucleated bone marrow cells and thymocytes of normal or immunized mice were suspended in Eagle's medium, counted, and injected into a lateral tail vein of irradiated mice, as previously described (13, 14).

*Immunization.*—Prospective marrow donors were injected intravenously at the age of 2 months, with either  $3.5 \times 10^6$  or  $5 \times 10^8$  SRBC. The erythrocytes were washed three times in calcium and magnesium-free phosphate-buffered saline, pH 7.2, and suspended in Eagle's medium. Several months after immunization, bone marrow cells were harvested, mixed *in vitro* with thymocytes of nonimmunized donors, and transplanted into irradiated recipients. 1 day later, these recipients were injected intravenously with  $5 \times 10^8$  washed SRBC or CRBC. Fresh erythrocytes in Alsever's solution were purchased from Colorado Serum Co., Denver, Col., or from Grand Island Biological Co., Microbio Lab, Madison, Wis.

*Assays for Plaque-Forming Cells.*—The number of PFC in spleens of irradiated, reconstituted mice was determined by the Jerne hemolytic plaque method in gel, as modified for use with glass microscope slides (15–17). Agarose (L'Industrie Biologique Francaise S.A., Gennevilliers, France, lot No. 9822), 0.5% in Eagle's minimal essential medium with Hank's salts, 0.4 ml; erythrocytes, 10%, 0.05 ml; and the spleen cell suspensions, 0.1 or 0.2 ml, were mixed with the aid of a Vortex mixer at 44°C in 10 × 75 mm tubes and poured onto microscope slides. The slides were previously dipped into 0.1% agarose in water and dried.

Portions of recipient spleens, each amounting to one- or two-tenths of the organ, were plated on a minimum of four slides for duplicate enumeration of direct and indirect PFC. After solidification, the slides were inverted and placed on special trays (16). For development of direct PFC (presumably releasing IgM antibody) the slides were kept 1 hr at 37°C in a humid atmosphere containing 5% CO<sub>2</sub>. Guinea pig complement (Grand Island Biological Co., Grand Island, N. Y.), diluted 1:10 in Eagle's medium, was added and incubation was continued for 2 hr. For facilitation of indirect PFC (presumably releasing IgG antibody), the slides were kept for the 1st hr as indicated above. Polyvalent rabbit or goat anti-mouse gamma globulin antisera (Cappel Laboratories, Downingtown, Pa., and Hyland Laboratories, Los Angeles, Calif.) were added. The sera were diluted to give approximately 80% inhibition of

direct PFC and maximum development of indirect PFC. After another hour of incubation, the slides were transferred to a clean tray, diluted guinea pig complement was added, and incubation was continued for another 3 hr (anti-SRBC plaques) or 4 hr (anti-CRBC plaques). Developed slides were fixed by immersion for 15 min into 5% glutaraldehyde in water. The slides were then rinsed three times in tap water and dried at 45°C. Plaques were counted at  $7\times$  magnification with direct or indirect illumination.

As the antiglobulin sera used partially inhibited the development of direct PFC, corrections were necessary to calculate the number of indirect PFC in each slide (total number of PFC minus noninhibited direct PFC). Correction factors were applied according to Wortis et al. (18). The average PFC count per spleen fraction sampled, i.e. 0.1 or 0.2 of the organ, was multiplied by 10 or 5, respectively, to calculate the number of PFC per spleen.

*Experimental Design and Statistical Methods.*—The Poisson model was used to describe the theoretical probability that given numbers of marrow cells would produce PFC in limiting dilution assays. Marrow cells were transplanted along with  $5\times 10^7$  thymocytes to ensure that only the former cells would limit immune responses. The method of maximum likelihood was used to estimate probability values and 95% confidence intervals, as described in preceding publications (13, 14). The computations and construction of theoretical limiting dilution curves were made with the aid of an IBM 1130 computer. Whenever the results were not adequately described by the Poisson model, the "sign" test (19) was used to compare the proportions of responses detected by the direct and indirect plaque methods.

## RESULTS

### *Frequency of Anti-Sheep PFC Responses in Mice Injected with Graded Numbers of Marrow Cells.*—

Groups of C3B10F<sub>1</sub> mice were exposed to whole body X-irradiation and injected with a mixture of cells consisting of  $5\times 10^7$  syngeneic thymocytes (from nonimmune donors) and graded numbers of C3BF<sub>1</sub> or C3B10F<sub>1</sub> marrow cells, in a range extending from  $8\times 10^5$  to  $4\times 10^7$ . The thymocytes provided each recipient mouse with a nonlimiting number of ARC (11). Marrow cells were taken from three different groups of donors: nonimmune mice, or mice injected 4–8 months earlier with either  $3.5\times 10^6$  or  $5\times 10^8$  SRBC. 1 day after transplantation of the cells, each animal was given  $5\times 10^8$  SRBC. Direct and indirect PFC of recipient spleens were enumerated 9 days after transplantation, at the time when peak numbers of PFC are generated by transferred marrow and thymus cells (20). Spleens of 85 control mice injected with antigen and either marrow cells or thymocytes, contained a small but variable number of PFC, not exceeding 150. The variability was primarily related to the number of marrow cells transplanted and to previous exposure of marrow donors to SRBC. Spleens of experimental mice were regarded as positive if the number of PFC exceeded the highest number found in control mice. Even after immunization of donors with  $5\times 10^8$  SRBC, marrow cells interacted synergistically with thymocytes in generating PFC. The results are presented in Table I and Fig. 1.

A total of 112 mice were grafted with marrow cells from nonimmune C3BF<sub>1</sub> donors in two experiments. The donors' age was matched with that of immunized mice to be used in subsequent experiments. As the number of marrow cells was increased, the proportion of recipients with positive spleens increased for both direct and indirect PFC (upper sections of Table I and Fig. 1). The relationship between these two variable parameters did not conform to the predictions of the Poisson model. Some of the recipients generated PFC even if

less than  $10^4$  marrow cells were grafted. However, upon transplantation of higher numbers of cells, the increase in frequencies of PFC response was less than expected under the assumption that a homogeneous population of potentially immunocompetent marrow cells participated in, and limited a single-hit event. Direct PFC responses were consistently more frequent than indirect PFC

TABLE I  
Percentage of Recipient Spleens Positive for Anti-Sheep PFC After Infusion of  $5 \times 10^7$  Thymocytes,  $5 \times 10^8$  SRBC, and Graded Numbers of Marrow Cells

No. of marrow cells transplanted ( $\times 10^6$ )	Direct PFC		Indirect PFC	
	Percentage of positive spleens*	No. of PFC per positive spleen†	Percentage of positive spleens*	No. of PFC per positive spleen‡
<i>Nonimmune marrow donors</i>				
0.08	29 (14)§	300	7	465
0.31	50 (18)	433 $\pm$ 70	28	141
0.62	70 (10)	311 $\pm$ 26	20	255
1.25	82 (17)	444 $\pm$ 279	29	452
2.50	85 (13)	489 $\pm$ 105	38	246
5.00	100 (22)	466 $\pm$ 61	50	230 $\pm$ 72
10.00	94 (18)	457 $\pm$ 78	67	200 $\pm$ 116
<i>Marrow donors immunized with <math>3.5 \times 10^6</math> SRBC</i>				
0.31	13 (16)	1035	13	794
0.62	25 (28)	1304 $\pm$ 487	25	520
1.25	57 (27)	456 $\pm$ 109	41	200 $\pm$ 59
2.50	73 (34)	628 $\pm$ 113	47	282 $\pm$ 135
5.00	87 (30)	421 $\pm$ 62	50	246 $\pm$ 96
10.00	88 (27)	720 $\pm$ 226	70	373 $\pm$ 146
20.00	91 (33)	720 $\pm$ 133	71	294 $\pm$ 110
40.00	100 (11)	612 $\pm$ 178	73	266 $\pm$ 165
<i>Marrow donors immunized with <math>5 \times 10^8</math> SRBC</i>				
0.62	0 (10)	—	10	165
1.25	0 (14)	—	27	371
2.50	17 (18)	404	67	576 $\pm$ 174
5.00	13 (17)	497	74	744 $\pm$ 252
10.00	16 (31)	327	87	465 $\pm$ 190
20.00	61 (23)	493 $\pm$ 183	87	624 $\pm$ 181
40.00	90 (10)	535 $\pm$ 287	90	974 $\pm$ 238

\* For marrow grafts of nonimmune donors and of donors immunized with  $3.5 \times 10^6$  SRBC, recipient spleens with  $>200$  direct and  $>100$  indirect PFC were scored as positive. For marrow grafts of donors immunized with  $5 \times 10^8$  SRBC, spleens with  $>150$  direct PFC and  $>100$  indirect PFC were scored as positive.

† Geometric mean  $\pm$  standard error. Standard errors were calculated only for means of six or more individual values.

§ Numbers in brackets indicate the number of mice in the groups.

responses. Using the sign test (19) to compare the percentage of positive spleens for the two classes of PFC, a  $\chi^2$  value of 5.14 was obtained. For one degree of freedom, the differences were statistically significant ( $P < 0.05$ ). These results confirmed earlier experiments in which nonimmune C3BF<sub>1</sub> marrow cells were grafted into fully syngeneic recipients, and PFC were enumerated by the

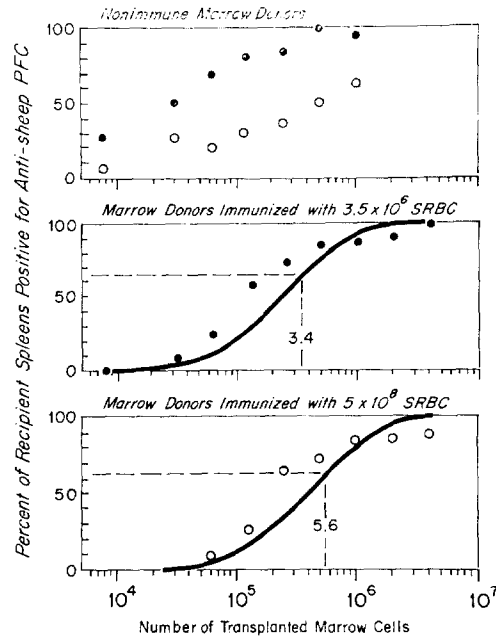


FIG. 1. Percentage of C3B10F<sub>1</sub> recipient spleens positive for direct PFC (symbol, ●) and indirect PFC (symbol, ○) after irradiation and injection with  $5 \times 10^7$  syngeneic thymocytes,  $5 \times 10^8$  SRBC, and graded numbers of marrow cells. Sample sizes for each point are shown in Table I. In the middle and lower sections, symbols indicate observed percentages and the computer fitted curves expected percentages according to the Poisson model. The numbers of transplanted marrow cells for 63% positive spleens are indicated by dashed straight lines. They contain, on the average, one detectable precursor unit limiting the immune responses studied.

original Jerne plaque assay in dishes (21, 22). The non-Poisson distribution and the unequal frequencies of the two PFC responses suggested that potentially immunocompetent cells of C3BF<sub>1</sub> marrow were class differentiated and heterogeneous for other properties as well. PFC may have been generated via a multiplicity of cell-to-cell interactions, conceivably by marrow cells in different stages of differentiation and maturation.

A total of 206 mice were grafted with marrow cells from C3BF<sub>1</sub> donors immunized with  $3.5 \times 10^6$  SRBC, in three experiments. As in the preceding limit-

ing dilution assay, the proportion of recipient mice with spleens positive for PFC was a function of the number of marrow cells grafted (middle sections of Table I and Fig. 1). The frequencies of indirect PFC responses and the mean number of indirect PFC per positive spleen were unaffected by donor preimmunization. In sharp contrast, the yield of direct PFC responses was changed by donor preimmunization so that their distribution conformed to the Poisson model. The close fit of the observations to the theoretical Poisson curve substantiated this selective change within the marrow cell population. The calculated probability value ( $P$ ) for an inoculum of  $10^6$  marrow cells to produce positive spleens for direct PFC was 2.91 with 95% confidence intervals of 2.34–3.58. According to Poisson statistics, the inoculum size required for 63% of the recipient spleens to be positive ( $DP$ ), contains on the average one marrow cell or unit limiting PFC production. This inoculum size  $DP$  is the reciprocal of  $P$  or  $1/P$ . Hence,  $3.4 \times 10^6$  primed marrow cells (with 95% confidence intervals of 2.7–4.3) contained on the average one detectable precursor unit interacting with thymocytes and generating direct PFC.

A total of 133 mice were grafted with marrow cells from C3BF<sub>1</sub> or C3B10F<sub>1</sub> donors immunized with the higher number of  $5 \times 10^8$  SRBC, in two experiments (lower sections of Table I and Fig. 1). In this assay, it was the frequency distribution of indirect PFC responses that changed upon donor immunization, to conform to the Poisson model. The probability value for  $10^6$  cells to yield positive recipient spleens for indirect PFC was 1.78 with 95% confidence intervals of 1.38–2.28. Consequently, the inoculum size containing an average of one precursor unit was  $5.6 \times 10^5$  cells with 95% confidence intervals of 4.4–7.2. The frequencies of direct PFC responses were considerably lower in several groups of this limiting dilution assay than in the two preceding assays with marrow cells from nonimmune donors and donors immunized with a smaller number of SRBC. Only the highest numbers of 2 and  $4 \times 10^6$  grafted cells from fully primed donors gave rise to direct PFC in 60–90% of recipients. The absence of direct IgM PFC in spleens of mice receiving the smaller marrow inocula may have resulted from inhibition by cells differentiating along the pathway leading to indirect (IgG) PFC, or by other marrow cells of primed mice. In turn, the inhibition could have been effected by cells themselves or by minute amounts of IgG antibody.

*Inhibition of Direct PFC Responses by Marrow Cells of Primed Mice.*—The competence of marrow cells and admixed thymocytes from nonimmune donors for direct PFC production was tested in the presence of primed marrow cells.

Irradiated C3B10F<sub>1</sub> mice were injected with  $5 \times 10^7$  thymocytes,  $10^6$  marrow cells from donors primed with  $5 \times 10^8$  SRBC, and graded numbers of marrow cells from nonimmune donors, in a range extending from  $3.1 \times 10^4$  to  $10^6$ . All donors and recipients were syngeneic. After 1 day, each animal was given  $5 \times 10^8$  SRBC; direct and indirect PFC were enumerated 9 days after transplantation.

All recipient spleens contained relatively large numbers of indirect PFC, presumably because precursors of the two marrow cell populations, grafted along with thymocytes, were additive in generating PFC (Table II). Few spleens contained direct PFC. This was anticipated since  $10^6$  primed marrow cells grafted by themselves generated direct PFC in no more than 16% of recipients (Table I, lower section), and may have contributed cells capable of inhibiting direct PFC production by precursors of the nonimmune marrow. Direct PFC were found in spleens of only 8 of 46 recipient mice (17%), whereas indirect PFC were found in spleens of 42 (Table II). Of the eight direct PFC responses, three were barely above control values. Among comparable recipients of thymocytes and nonimmune marrow cells (without added primed cells), direct PFC

TABLE II  
*Percentage of Recipient Spleens Positive for Anti-Sheep PFC After Infusion of  $5 \times 10^7$  Thymocytes,  $5 \times 10^8$  SRBC,  $10^6$  Anti-SRBC Immune Marrow Cells, and Graded Numbers of Nonimmune Marrow Cells*

No. of non-immune marrow cells transplanted	Direct PFC			Indirect PFC	
	Fraction of positive spleens*	Percentage of positive spleens*	No. of PFC per positive spleen	Percentage of positive spleens*	No. of PFC per positive spleen†
( $\times 10^5$ )					
0.31	0/6	0		100	1363 $\pm$ 1017
0.62	1/8	13	200	88	366 $\pm$ 91
1.25	3/10	30	350, 320, 360	100	405 $\pm$ 205
2.50	2/11	18	200, 205	73	444 $\pm$ 200
10.00	2/11	18	345, 1065	100	511 $\pm$ 247

\* Recipient spleens with  $>200$  direct and  $>100$  indirect PFC were scored as positive.

† Geometric mean  $\pm$  standard error.

responses were found in 58 of 76 mice (76%, Table I). The results indicated that production of direct PFC by normal marrow cells was inhibited by  $10^6$  added primed marrow cells. This experiment, however, could not provide information on the mechanism of the inhibitory effect.

*Frequency of Anti-Chicken PFC Responses in Mice Injected with Graded Numbers of Marrow Cells.—*

Groups of irradiated C3B10F<sub>1</sub> mice were injected with a mixture of syngeneic cells consisting of  $5 \times 10^7$  thymocytes (from nonimmune donors) and graded numbers of marrow cells, in a range extending from  $3.1 \times 10^4$  to  $2 \times 10^6$ . Marrow cells were taken from two different groups of donors: nonimmune mice, or mice injected 4 months earlier with  $5 \times 10^8$  sheep erythrocytes. 1 day after transplantation, each animal was given  $5 \times 10^8$  chicken erythrocytes. Hemolytic anti-chicken PFC were enumerated in recipient spleens on the 9th day. Spleens of 39 control mice given either marrow or thymus cells with CRBC contained less than 200 direct and 100 indirect anti-chicken PFC, regardless of whether the marrow donors were immunized against antigens of SRBC. The results are presented in Table III and Fig. 2.

A total of 114 mice were grafted with marrow cells from nonimmune donors in three experiments. As the number of marrow cells was increased, the proportion of recipients with positive spleens increased for direct and indirect PFC. Spleens were either positive for both types of PFC or for direct PFC only. Therefore, the direct PFC responses were more frequent. The mean number of PFC per positive spleen did not increase with the number of marrow cells grafted, as in limiting dilution assays for anti-sheep responses. The distribution of positive spleens in the different treatment groups followed closely the predic-

TABLE III  
Percentage of Recipient Spleens Positive for Anti-Chicken PFC After Infusion of  $5 \times 10^7$  Thymocytes,  $5 \times 10^8$  CRBC, and Graded Numbers of Marrow Cells

No. of marrow cells transplanted ( $\times 10^6$ )	Direct PFC		Indirect PFC	
	Percentage of positive spleens*	No. of PFC per positive spleen†	Percentage of positive spleens*	No. of PFC per positive spleen†
<i>Nonimmune marrow donors</i>				
0.31	0 (9)§	—	0	—
0.62	9 (11)	265	0	—
1.25	35 (20)	722 $\pm$ 313	15	202
2.50	52 (23)	501 $\pm$ 82	35	290 $\pm$ 181
5.00	69 (26)	608 $\pm$ 86	42	467 $\pm$ 123
10.00	93 (15)	646 $\pm$ 168	80	410 $\pm$ 126
20.00	100 (10)	842 $\pm$ 144	80	321 $\pm$ 134
<i>Marrow donors injected with <math>5 \times 10^8</math> SRBC</i>				
0.62	17 (6)	415	0	—
1.25	29 (17)	742	18	455
2.50	56 (16)	635 $\pm$ 154	38	403 $\pm$ 99
5.00	78 (14)	660 $\pm$ 209	50	430 $\pm$ 184
10.00	93 (16)	1155 $\pm$ 174	81	440 $\pm$ 140
20.00	100 (10)	953 $\pm$ 221	80	312 $\pm$ 136

\* Recipient spleens with  $> 200$  direct and  $> 100$  indirect PFC were scored as positive.

† Geometric mean  $\pm$  standard error. Standard errors were calculated only for means of six or more individual values.

§ Numbers in brackets indicate the number of mice in the groups.

tions of the Poisson model (Fig. 2). The probability values for direct and indirect PFC responses per unit number of  $10^6$  grafted marrow cells were 2.52 and 1.24, respectively. The 95% confidence intervals did not overlap (Fig. 2). The inoculum sizes containing an average of one precursor unit were  $3.9 \times 10^6$  marrow cells (with 95% confidence intervals of 3.0–5.4) for direct PFC, and  $8.1 \times 10^5$  cells (with 95% confidence intervals of 5.9–11.2) for indirect PFC. It was remarkable that the numbers of precursor units for anti-chicken and anti-sheep PFC responses were so close, though donors were not primed for the former limiting dilution assay and were specifically immunized for the latter.



Most likely this is a coincidence. It must be noted that anti-chicken and anti-sheep PFC responses by nonimmune marrow cells differed with respect to the shape of the limiting dilution plots, which conformed to the Poisson model for the former but not for the latter responses. This suggested that at least one of the bone marrow cells required for PFC production was antigen specific.

In two other experiments, a total of 79 mice were grafted with marrow cells from donors immunized against SRBC. The anti-chicken PFC responses were undistinguishable from the responses elicited by marrow cells of mice not injected with SRBC, both with regard to frequencies of positive spleens and numbers of PFC per spleen (compare upper and lower sections of Table III).

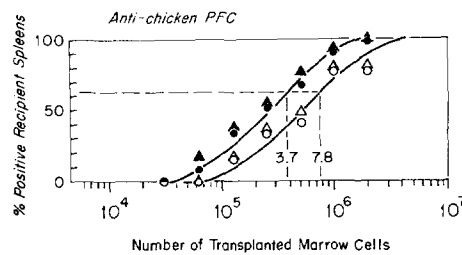


FIG. 2. Percentage of C3B10F<sub>1</sub> recipient spleens positive for direct PFC (closed symbols) and indirect PFC (open symbols) after irradiation and injection with  $5 \times 10^7$  syngeneic thymocytes,  $5 \times 10^8$  CRBC, and graded numbers of marrow cells. Donors of marrow cells were either untreated mice (symbols, ● and ○) or mice injected 4 months earlier with  $5 \times 10^8$  SRBC (symbols, ▲ and △). Sample sizes for each point are shown in Table III. Symbols indicate observed percentages and curves expected percentages according to the Poisson model. The curves were computer fitted to the pooled percentages of positive spleens in recipients of nonimmune and immune (anti-SRBC) marrow cells. The *P* values were 2.71 (95% confidence intervals, 2.16–3.36) for direct PFC, and 1.29 (95% confidence intervals, 1.01–1.62) for indirect PFC. The numbers of transplanted marrow cells containing on the average one detectable precursor unit are indicated by the dashed straight lines.

Accordingly, the calculated probability values for positive anti-chicken spleens per  $10^6$  marrow cells did not differ significantly for the two kinds of donors (Fig. 2). The effects of SRBC priming were apparently restricted to marrow cells engaged in anti-sheep responses. The production of direct anti-chicken PFC was not inhibited upon transplantation into irradiated mice of marrow cells from donors immunized against SRBC. Cells of such marrow strongly inhibited the production of direct anti-sheep PFC under identical experimental conditions (Table I, lower section). Hence, SRBC immunization induced functional changes in a subpopulation of bone marrow cells endowed with antigen specificity.

#### DISCUSSION

The peculiar organ distribution of potentially immunocompetent cells in the mouse has proven to be of great advantage for understanding cellular mecha-

nisms and controls of antibody formation. The advantage stems from natural separation of cells endowed with distinct functions in thymus and bone marrow. Thus, the thymus contains and releases ARC, i.e., cells which react with antigens *in vivo* and *in vitro* during an early phase of the immune response (3, 5, 6, 9–12). Subsequently, ARC proliferate and generate inducer cells, i.e., cells capable of interacting with marrow derived cells and of inducing specific antibody formation (11). Although thymus-derived cells are not engaged directly in synthesis of immunoglobulins (23, 1), their function as inducers is essential for bone marrow-derived cells to produce specific antibodies against a variety of naturally occurring and synthetic antigens (1–3, 8, 24). The induction process requires the presence of viable inducer cells (12) and of the antigens that have initiated the immune response by reacting with ARC (10–12). Therefore, antigens play an essential role during at least two discrete events (11). Bone marrow contains and releases cells which do not share with thymic ARC the same kind of antigen reactivity, so that the former cells cannot substitute for thymocytes in early events of immune responses (11). The possibility that marrow cells are antigen reactive or antigen sensitive in some other way has not been excluded. However, such functional properties would have to be recognized by manifestations different from those elicited by ARC. The best known function of marrow-derived cells is the synthesis of antibody molecules (1–3, 6). Several lines of evidence suggest that cells with other accessory immune functions, yet to be defined, are also derived from bone marrow (5, 6, 21, 22, 25). Genetic information concerning molecular class of immunoglobulins is expressed differentially in marrow-derived cells (precursor or accessory cells) but not in thymic ARC (11, 21, 22).

Little is known about the respective role of thymus- and marrow-derived cells in the determination of other properties of antibody to be synthesized by mature immunocytes. Of primary interest are the controls of antibody specificity and the related cross-reactivity and affinity for antigens. Inducer cells of thymic origin are antigen specific (10–12). Commitment to given antigenic determinants may have preexisted exposure to antigens in the ARC, or may have arisen as a consequence of reaction with antigens. The first possibility is generally preferred, primarily because induced specific unresponsiveness is rather difficult to understand under the assumption that ARC are not capable of discriminating among antigenic determinants. In fact, several experiments have indicated that tolerogenic treatments of mice incapacitate thymus-derived cells in a specific way, i.e. without affecting either the ability of marrow cells to participate in immune reactions against the same antigens, or the competence of both thymus and marrow cells for reactions against other antigens (7–9). This type of evidence, though suggestive, falls short of formally proving antigen specificity of thymic ARC prior to immunization, since other cell types involved in primary antigen handling could have been the targets of tolerogenic treatments. However, the progeny cells of ARC are specific inducer cells as a matter

of fact (10-12); hence, one can conclude that one of the sources of specificity restriction for mature antibody-forming cells has to be traced to thymus-derived inducers. Then, the pertinent question is whether there are other sources of information for antibody specificity, e.g., in marrow cells, or whether thymus derived inducer cells have to confer specificity upon uncommitted precursors of immunocytes by transfer of information.

Two experimental approaches were used to investigate whether marrow-derived cells are antigen specific: (a) anti-sheep and anti-chicken primary PFC responses elicited by limiting numbers of transplanted marrow cells (in the presence of excess thymocytes) were compared with regard to shape and position of limiting dilution plots; (b) anti-sheep and anti-chicken PFC responses elicited by marrow cells from donors immunized against SRBC (with the cooperation of nonimmune thymocytes), were compared with primary responses to identify specific changes in marrow cells. The primary anti-SRBC and anti-CRBC responses by marrow cells of C3B10F<sub>1</sub> mice differed sharply from each other under the conditions of limiting dilution assays. Furthermore, immunization of marrow donors against antigens of SRBC altered the population of cells engaged in PFC responses to SRBC but not that engaged in anti-CRBC responses.

Limiting dilution analysis is a method for studying the frequency, function, and potential of the least frequent cell type of a heterogeneous population of cells participating in an immune response. In cell transfer systems, irradiated test animals can be provided with an excess of all required cell types, while limiting the availability of one (11, 13, 14, 21, 22, 24). If an antibody response depends entirely on the presence or absence of the rarest cell type or functional unit of cells (all other cells or events being nonlimiting), the distribution of immune responses among mice given graded numbers of limiting cells will be dictated by Poisson statistics. Conditions such as these were established by transplanting into several groups of irradiated mice  $5 \times 10^7$  thymocytes with graded numbers of marrow cells. The distribution of anti-chicken PFC responses in the various treatment groups conformed to the expectations of the Poisson model, indicating that the assumptions made in designing the experiments (21, 22) were presumably correct. The most important assumption concerned the existence in marrow of a cell type which alone limited the occurrence of an event essential for PFC formation. Such an event could have been cell-to-cell interaction with thymus derived inducer cells or with other marrow derived accessory cells. This assumption does not restrict the number of cells with distinct functions which may participate in immune responses during discrete subsequent events. It requires, however, that one of the cell types and one event limit PFC production.

The distribution of primary anti-sheep PFC responses in recipients of graded numbers of C3BF<sub>1</sub> and C3B10F<sub>1</sub> marrow cells was not predictable by the

Poisson model. These results indicated that the assumptions made were not correct for marrow cells engaged in formation of anti-sheep PFC, although they were correct for marrow cells producing anti-chicken PFC. If marrow contained several types of potentially immunocompetent cells participating in a multiplicity of cell-to-cell interactions, each one limiting anti-sheep PFC production, one would expect non-Poisson limiting dilution plots. Whatever the interpretation of the plots, certain types of marrow cells, precursors of PFC (P-PFC), accessory cells, or both, were antigen specific since their function was expressed differentially during responses to SRBC and CRBC. Although the present data gave no indication of the functional cell type which was antigen specific, nor of the proportion of unispecific cells, they ruled out the possibility that the same cells effected anti-SRBC and anti-CRBC responses. The results were consistent with the observations of Playfair (26): in the fetal liver of NZB mice there were more P-PFC for SRBC than for CRBC, while in the liver of BALB/c mice the numbers of the two types of P-PFC were similar. Thus, the existence of antigen-specific immunocompetent cells other than those derived from thymus was strongly suggested by qualitative as well as quantitative differences between cells engaged in anti-SRBC and anti-CRBC responses.

The second approach to demonstrate antigen specificity in marrow derived cells made use of SRBC-immune donor mice. Marrow cells of such donors, cooperating with normal thymocytes, were undistinguishable from nonimmune marrow cells in anti-chicken PFC responses. In contrast, specifically immune marrow cells differed markedly from nonimmune cells in anti-SRBC responses: the frequencies of positive spleens after transplantation of limiting numbers of marrow cells and  $5 \times 10^7$  nonimmune thymocytes conformed to the Poisson model; and immune marrow cells specifically inhibited direct PFC production. The conversion of shallow limiting dilution plots to Poisson plots (Fig. 1) indicated that antigen-dependent differentiation or maturation occurred after SRBC injection, so as to drive into a particular functional state the heterogeneous population of potentially immunocompetent marrow cells of nonimmune mice. As a result, PFC responses depended on the availability of one critical cell type, the rarest, participating in a single-hit event. The data gave no indication of which cell types, P-PFC or accessory cells, underwent differentiation or maturation. It was of interest that immunization of marrow donors with the suboptimal number of  $3.5 \times 10^6$  SRBC did not induce functional changes in precursor units of indirect PFC, while it did in precursor units of direct PFC. Dissociation of the two responses with regard to the shape of limiting dilution plots strengthened the interpretation of earlier experiments according to which marrow precursor units of PFC are restricted for the molecular class of antibody to be made by descendent PFC (21, 22). Since limiting dilution plots for anti-CRBC responses conformed to the Poisson model regardless of whether marrow donors were immunized against SRBC or not, the present data cannot

rule out the possibility that antigen-dependent differentiation or maturation of marrow cells was nonspecific, or affected cell types with functions not related to antibody specificity. Limiting dilution assays for anti-SRBC responses by marrow cells of mice immunized with non cross-reacting antigens are underway, and preliminary results suggest that this is not the case.

Immunization of marrow donors with the optimal number of  $5 \times 10^8$  SRBC gave rise to cells which either inhibited precursors of direct PFC by cell-to-cell interactions, or by diffusible substances. In view of the specificity of inhibition (not detected in anti-CRBC responses), and of the small number of primed marrow cells required ( $10^6$  cells per recipient), it is most likely that minute amounts of IgG antibody released by differentiating cells inhibited direct PFC production (27, 28). The mechanism of direct P-PFC inhibition, although interesting in its own merit, was not of importance in the present studies. The specificity of the inhibition effect constituted evidence for the existence in mouse marrow of another antigen-specific and antigen-sensitive cell, perhaps involved in regulation of antibody responses.

In conclusion, bone marrow of mice contains antigen-specific and antigen-sensitive cells detectable in experimental systems involving immunization against SRBC. Possibly, marrow cells of more than one functional type are endowed with these properties, since their existence was demonstrated by antigen-dependent differentiation or maturation of precursor units, and by the appearance of cells responsible for inhibition of direct PFC production. In studies of others, evidence for the existence of antigen-specific marrow cells is as follows: (a) certain humoral antibody responses are not dependent on marrow-thymus cell cooperation and can be elicited by marrow cells transplanted by themselves into irradiated mice (29); (b) genetic low responder mice to the synthetic polypeptide antigen (T, G)-Pro-L are defective in marrow-derived cells cooperating with thymocytes for this particular immune response (24). The defective phenotype, correctable by administration of the synthetic ribonucleotide poly A:U, is determinant specific. For all these evidences it can be postulated that transfer of informational molecules from thymus-derived to marrow-derived cells may not be required for a model describing specificity differentiation of immunocytes. In fact, marrow cells appear to be antigen specific prior to engagement in cell-to-cell interactions.

#### SUMMARY

Graded numbers of marrow cells and  $5 \times 10^7$  thymocytes were mixed in vitro and transplanted into X-irradiated (C3H  $\times$  C57BL/10) $F_1$  mice. Upon injection of sheep or chicken erythrocytes, splenic plaque-forming cells secreting IgM (direct PFC) or IgG (indirect PFC) hemolytic antibody were enumerated at the time of peak responses.

Anti-sheep and anti-chicken primary PFC responses elicited by nonimmune marrow cells differed sharply from each other under the conditions of limiting

dilution assays. The frequencies of anti-chicken responses in recipients of different numbers of marrow cells conformed to the predictions of the Poisson model, while the frequencies of anti-sheep responses did not. Hence, the function of certain marrow-derived cells was expressed differentially during the two immune responses, to exclude that the same precursor units generated anti-sheep or anti-chicken PFC. The former precursor cells or units were functionally more heterogeneous than the latter.

Immunization of marrow donors against sheep erythrocytes did not alter the population of cells engaged in anti-chicken responses, since limiting dilution assays with immune and nonimmune marrow cells gave identical results. However, anti-sheep immunization altered specifically the cell population engaged in anti-sheep responses, in two ways: (a) potentially immunocompetent marrow cells underwent antigen-dependent differentiation or maturation, to become functionally homogeneous. Consequently, the frequencies of PFC responses in limiting dilution assays conformed to the Poisson model; the changes occurred independently in class-restricted precursors of direct and indirect PFC. (b) marrow cells capable of inhibiting precursors of direct anti-sheep PFC arose in primed mice. The inhibition, which was specific, could have been effected directly by marrow cells or by a diffusible product such as IgG antibody. Results indicated that potentially immunocompetent cells of mouse marrow with distinct functions were antigen specific and antigen sensitive.

*Note Added in Proof:* After submission of the manuscript, we read two new publications describing the existence of antigen-specific and antigen-sensitive cells in mouse bone marrow. Such cells were incapacitated by tolerogenic treatments in vivo (30) and sensitized by immunization (31).

We thank Dr. C. W. Pierce, National Institute of Allergy and Infectious Diseases, Bethesda, Md., for demonstrating the plaque technique in agar, as modified for glass slides. We also acknowledge the assistance of Dr. R. L. Priore of the Computer Center, Roswell Park Memorial Institute, Buffalo, N. Y., in the statistical analysis of data.

#### BIBLIOGRAPHY

1. Mitchell, G. F., and J. F. A. P. Miller, 1968. Cell to cell interaction in the immune response. II. The source of hemolysin-forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes. *J. Exp. Med.* **128**:821.
2. Nossal, G. J. V., A. Cunningham, G. F. Mitchell, and J. F. A. P. Miller. 1968. Cell to cell interaction in the immune response. III. Chromosomal marker analysis of single antibody-forming cells in reconstituted, irradiated, or thymectomized mice. *J. Exp. Med.* **128**:839.
3. Davies, A. J. S., E. Leuchars, V. Wallis, N. S. C. Sinclair, and E. V. Elliott. 1968. The selective transfer test—an analysis of the primary response to sheep red cells. *In* Advance in Transplantation. J. Dausset, J. Hamburger, and G. Mathé, editors. Ejnar Munksgaard, Copenhagen. 97.

4. Claman, H. N., E. A. Chaperon, and R. F. Triplett. 1966. Immunocompetence of transferred thymus-marrow cell combinations. *J. Immunol.* **97**:828.
5. Moiser, D. E., F. W. Fitch, D. A. Rowley, and A. J. S. Davies. 1970. Cellular deficit in thymectomized mice. *Nature (London)*. **225**:276.
6. Haskill, J. S., P. Byrt, and J. Marbrook. 1970. In vitro and in vivo studies of the immune response to sheep erythrocytes using partially purified cell preparations. *J. Exp. Med.* **131**:461.
7. Gershon, R. K., V. Wallis, A. J. S. Davies, and E. Lenchars. 1968. Inactivation of thymus cells after multiple injections of antigen. *Nature (London)*. **218**:381.
8. Taylor, R. B. 1969. Cellular cooperation in the antibody response of mice to two serum albumins: specific function of thymus cells. *Transplant. Rev.* **1**:114.
9. Miller, J. F. A. P., and G. F. Mitchell. 1970. Cell to cell interaction in the immune response. V. Target cells for tolerance induction. *J. Exp. Med.* **131**:675.
10. Mitchell, G. F., and J. F. A. P. Miller. 1968. Immunological activity of thymus and thoracic-duct lymphocytes. *Proc. Nat. Acad. Sci. U.S.A.* **59**:296.
11. Shearer, G. M., and G. Cudkowicz. 1969. Distinct events in the immune response elicited by transferred marrow and thymus cells. I. Antigen requirements and proliferation of thymic antigen-reactive cells. *J. Exp. Med.* **130**:1243.
12. Claman, H. N., and E. A. Chaperon. 1969. Immunological complementation between thymus and marrow cells. A model for the two-cell theory of immunocompetence. *Transplant. Rev.* **1**:92.
13. Shearer, G. M., G. Cudkowicz, M. S. J. Connell, and R. L. Priore. 1968. Cellular differentiation of the immune system of mice. I. Separate splenic antigen-sensitive units for different types of anti-sheep antibody-forming cells. *J. Exp. Med.* **128**:437.
14. Shearer, G. M., G. Cudkowicz, and R. L. Priore. 1969. Cellular differentiation of the immune system of mice. II. Frequency of unipotent splenic antigen-sensitive units after immunization with sheep erythrocytes. *J. Exp. Med.* **129**:185.
15. Mishell, R. I., and R. W. Dutton. 1967. Immunization of dissociated spleen cell cultures from normal mice. *J. Exp. Med.* **126**:423.
16. Plotz, P. H., N. Talal, and R. Asofsky. 1968. Assignment of direct and facilitated hemolytic plaques in mice to specific immunoglobulin classes. *J. Immunol.* **100**:744.
17. Pierce, C. W. 1969. Immune responses in vitro. I. Cellular requirements for the immune response by nonprimed and primed spleen cells in vitro. *J. Exp. Med.* **130**:345.
18. Wortis, H. H., R. B. Taylor, and D. W. Dresser. 1966. Antibody production studied by means of the LGH assay. I. The splenic response of CBA mice to sheep erythrocytes. *Immunology*. **11**:603.
19. Snedecor, G. W., and W. G. Cochran. 1967. Statistical Methods. The Iowa State Univ. Press, Ames, Iowa. 6th edition. 125.
20. Shearer, G. M., and G. Cudkowicz. 1969. 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. *J. Exp. Med.* **129**:935.
21. Cudkowicz, G., G. M. Shearer, and R. L. Priore. 1969. Cellular differentiation of

- the immune system of mice. V. Class differentiation in marrow precursors of plaque-forming cells. *J. Exp. Med.* **130**:481.
22. Cudkowicz, G., G. M. Shearer, and T. Ito. 1970. Cellular differentiation of the immune system of mice. VI. Strain differences in class differentiation and other properties of marrow cells. *J. Exp. Med.* **132**:623.
  23. Davies, A. J. S., E. Leuchars, V. Wallis, R. Marchant, and E. V. Elliott. 1967. The failure of thymus-derived cells to produce antibody. *Transplantation.* **5**:222.
  24. Mozes, E., G. M. Shearer, M. Sela, and W. Braun. 1970. Conversion with polynucleotides of a genetically controlled low immune response to a high response in mice toward a synthetic polypeptide antigen. *In* Symposium on Biological Effects of Polynucleotides. R. F. Beers and W. Braun, editors. Springer-Verlag, New York. In press.
  25. Shortman, K., E. Diener, P. Russell, and W. D. Armstrong. 1970. The role of nonlymphoid accessory cells in the immune response to different antigens. *J. Exp. Med.* **131**:461.
  26. Playfair, J. H. L. 1968. Strain differences in the immune response of mice. II. Responses by neonatal cells in irradiated adult hosts. *Immunology.* **15**:815.
  27. Henry, C., and N. K. Jerne. 1968. Competition of 19S and 7S antigen receptors in the regulation of the primary immune response. *J. Exp. Med.* **128**:133.
  28. Haughton, G., and D. O. Adams. 1970. Specific immunosuppression by minute doses of passive antibody. II. The site of action. *J. Reticuloendothel. Soc.* **7**:500.
  29. Armstrong, W. D., E. Diener, and G. R. Shellam. 1969. Antigen-reactive cells in normal, immunized, and tolerant mice. *J. Exp. Med.* **129**:393.
  30. Chiller, J. M., G. S. Habicht, and W. O. Weigle. 1970. Cellular sites of immunologic unresponsiveness. *Proc. Nat. Acad. Sci. U.S.A.* **65**:551.
  31. Kennedy, J. C., P. E. Treadwell, and E. S. Lennox. 1970. Antigen-specific synergism in the immune response of irradiated mice given marrow cells and peritoneal cavity cells or extracts. *J. Exp. Med.* **132**:353.