ANTIGEN-SPECIFIC CELLS IN MOUSE BONE MARROW

II. Fluctuation of the Number and Potential of Immunocyte Precursors after Immunization*

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 21 December 1970)

Recent experimental evidence strongly suggests that potentially immunocompetent cells of mouse bone marrow are antigen-specific (1–3). These cells are relatively undifferentiated and immature with respect to antibody synthesis, although they are the direct precursors of immunocytes. Differentiation, proliferation, and maturation into functional immunocytes require exposure to antigen and interaction with one or more accessory cells (4–6). With regard to other properties, the marrow precursor cells seem to be rather differentiated or specialized: they are restricted for the class of antibody to be synthesized by descendant immunocytes (7, 8), and committed to specific antigens for sensitization (1, 2) or paralysis (3). Consistent with this view are indications concerning the phenotypic expression in bone marrow cells of determinant-specific immune response genes (9), and the marrow derivation of hapten-specific cells in hapten–carrier cooperation (10). Thus, class and specificity differentiation are antigen-independent processes inherent to marrow cell lines. In the course of immune responses, these cells undergo further differentiation which is antigen-dependent and primarily aimed at expanding the pool of potentially immunocompetent cells and at initiating synthesis of specific antibody. Even the earliest steps of differentiation, from nonrestricted stem cells of the lymphoid system to restricted precursors of immunocytes, can be subjected to experimental scrutiny, since the lymphoid progeny of a few stem cells include populations of restricted cells responding to several antigens (11).

In irradiated-reconstituted mice, immune responses to sheep and chicken erythrocytes are carried out by distinct populations of marrow cells (2). Immunization against sheep erythrocytes (SRBC)[1] results in qualitative and quan-

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

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

[1] *Abbreviations used in this paper:* ARC, antigen-reactive cells of thymic origin; CRBC, chicken erythrocytes; HRBC, horse erythrocytes; PFC, plaque-forming cells; P-PFC, precursors of plaque-forming cells of bone marrow origin; P-PFC^s, precursor cells primed with SRBC, producing larger numbers of PFC; SRBC, sheep erythrocytes.

titative modifications of the cell population responsive to SRBC, but not of that responsive to chicken erythrocytes (CRBC) (2). The present experiments extend these findings by demonstrating antigen specificity of bone marrow cells in a reverse order of antigen presentation, and by assessing the frequency and potential of marrow precursor cells at several intervals after immunization. Direct and indirect hemolytic plaque-forming cells (PFC) induced by SRBC were enumerated in spleens of irradiated mice reconstituted with excess thymocytes from nonimmune donors, and with small graded numbers of bone marrow cells from SRBC or other antigen-primed donors. These limiting dilution assays indicated (*a*) that in the course of immunization the number of marrow precursors of direct and indirect PFC increased greatly; and (*b*) that at a given time after immunization the number of PFC generated by each precursor unit also increased severalfold. Therefore, bone marrow–derived cells may play a major role in the establishment of immunological memory.

Materials and Methods

Mice.—(C3H/He × C57BL/10Cz)F_1 female mice, 3–7 months old were used as recipients and donors of bone marrow cells. Thymocyte donors were 9-wk old mice.

Irradiation.—Mice were exposed to 875–950 rads of ^{137}Cs gamma radiation from two opposite sources, at the rate of 127 rads/min, under conditions of maximum backscatter (Gammacell-20 Irradiator, Atomic Energy of Canada, Ltd., Ottawa, Canada).

Cell Suspensions and Transplantation.—Nucleated bone marrow cells and thymocytes of normal or immunized mice were suspended separately in Eagle's medium, counted, mixed in vitro, and immediately injected into a lateral tail vein of irradiated mice, as previously described (12).

Immunization.—Prospective marrow donors were injected intravenously at the age of 2 months, with 5×10^8 SRBC, or CRBC, or horse erythrocytes (HRBC), or with 1 mg (dry weight) of formalinized *Salmonella typhimurium* bacilli. The xenogeneic erythrocytes and bacteria were washed three times in calcium and magnesium-free phosphate-buffered saline, pH 7.2, and suspended in Eagle's medium. 2–4 months after immunization, bone marrow cells were harvested, mixed in vitro with thymocytes of nonimmune donors, and transplanted into irradiated recipients. 1 day later, these recipients were injected intravenously with 5×10^8 washed SRBC. Fresh erythrocytes in Alsever's solution were purchased from Colorado Serum Co., Denver, Colo., or from Grand Island Biological Co., Microbio Lab, Madison, Wis. The bacteria were grown for 72 hr in Bacto Nutrient broth (Difco Laboratories, Inc., Detroit, Mich.) at 37°C, washed, formalinized, and stored at 4°C.

Assay for Plaque-Forming Cells.—The number of direct and indirect 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. Details of the procedure are described elsewhere (2). Indirect PFC were developed by rabbit anti-mouse gamma globulin antisera (Cappel Laboratories, Downingtown, Pa.) at dilutions inhibitory for 80% of direct PFC.

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 5×10^7 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). The computations and construction of theoretical limiting dilution curves were made with the aid of an IBM 1130 computer (IBM Corp., Data Processing Div., White Plains, N. Y.)

RESULTS

Frequency of Anti-Sheep PFC Responses in Mice Injected with Graded Numbers of Nonspecifically Sensitized Marrow Cells.—Groups of mice were irradiated and injected with a mixture of cells consisting of 5×10^7 thymocytes from nonimmune donors and graded numbers of marrow cells, in a range extending from 1.5×10^4 to 10^6. The thymocytes provided a nonlimiting number of antigen reactive cells (ARC) to each recipient (14). Marrow cells were taken from three different groups of donors: mice injected 3–4 months earlier with *S. typhimurium*, HRBC, or CRBC. 1 day after transplantation of the cells, each recipient animal was given 5×10^8 SRBC. A total of 123 mice were employed. Direct and indirect PFC of recipient spleens were enumerated 9 days after transplantation, at the time of peak response (12). Spleens of control mice injected with antigen and either marrow cells or thymocytes, contained a variable number of PFC, not exceeding 200 direct and 100 indirect. Therefore, spleens of experimental mice were regarded as positive for an anti-sheep response only if the number of PFC was larger than that of the highest controls. Results are presented in Table I and Fig. 1 *B*.

Not every recipient mouse developed PFC responses. The proportion of recipients with positive spleens for direct and indirect PFC increased as the number of transplanted marrow cells was raised from 1.5×10^4 to 10^6. All recipients of 10^6 cells had positive spleens for direct PFC, and three-fourths also for indirect PFC. Direct PFC responses were more frequent than indirect PFC response in every experimental group. None of the antigens used for immunization of marrow donors, i.e. *S. typhimurium*, HRBC, CRBC, had a noticeable effect on the frequencies of anti-SRBC responses or on the numbers of anti-sheep PFC per spleen. The results were identical to those reported earlier for marrow cells of nonimmunized donors (2, 7, 15). For ease of comparison, these data are also shown in Fig. 1 *A*. The relationship between the number of marrow cells transplanted and the proportion of positive spleens did not conform to the predictions of the Poisson model for the nonimmune donor groups and for those immunized with antigens other than SRBC. Increases in frequency of responses with increments of marrow cells grafted were less than expected under the assumption that a homogeneous population of precursor cells participated in PFC production and limited a single-hit event. Nonspecific immunization of marrow cell donors failed to modify the population of cells engaged in anti-sheep responses. The parameters investigated were: numbers of precursors of PFC (P-PFC), functional heterogeneity with respect to cell-to-cell interactions, and class restriction.

Frequency of PFC Responses in Mice Injected with Graded Numbers of Specifically Sensitized Marrow Cells.—Groups of irradiated mice were injected with a mixture of syngeneic cells consisting of 5×10^7 nonimmune thymocytes and graded numbers of immune marrow cells, in a range extending from 10^3 to 1.5×10^6. Marrow cells were taken from donors preimmunized with 5×10^8 SRBC. These donors were used 2, 3, and 3.5 months after immunization. The

TABLE I

Percentage of Recipient Spleens Positive for Anti-Sheep PFC after Infusion of 5×10^7 Thymocytes, 5×10^8 SRBC, and Graded Numbers of Nonspecifically Sensitized Marrow Cells

No. of marrow cells transplanted	Direct PFC		Indirect PFC	
	Percentage of positive spleens*	No. of PFC/positive spleen‡	Percentage of positive spleens*	No. of PFC/positive spleen‡
($\times 10^5$)				
	Marrow donors immunized with *Salmonella typhimurium*			
0.15	50 (8)§	552	25	445
0.62	70 (10)	640 ± 140	30	583
2.50	80 (10)	607 ± 158	40	303
10.00	100 (9)	848 ± 88	78	387 ± 140
	Marrow donors immunized with HRBC			
0.15	30 (10)	412	10	330
0.62	60 (10)	496 ± 151	30	157
2.50	70 (10)	468 ± 194	50	202 ± 52
10.00	100 (9)	757 ± 123	78	286 ± 256
	Marrow donors immunized with CRBC			
0.15	42 (12)	517	17	272
0.62	58 (12)	617 ± 344	25	390
2.50	83 (12)	1128 ± 195	33	429
10.00	100 (11)	1108 ± 230	73	664 ± 205

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

‡ Geometric mean ± standard error. Standard errors were calculated only for means of five or more individual values.

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

day after transplantation of the cell mixtures, each recipient mouse was injected with 5×10^8 SRBC (secondary stimulation of marrow cells). Direct and indirect PFC were enumerated in recipient spleen cell suspensions on the 9th day after grafting. The spleens were classified as positive or negative, depending on whether the number of PFC was larger or smaller than that of controls. Bone marrow cells of immune donors required cooperation with thymocytes for PFC production and exposure to antigen after transplantation as did the cells from nonimmune donors. The number of PFC in spleens of control mice

was not greater than in the preceding series of experiments. Results obtained are presented in Tables II and III and in Fig. 1, sections *C–E*.

TABLE II

Percentage of Recipient Spleens Positive for Anti-Sheep PFC after Infusion of 5 × 10⁷ Thymocytes, 5 × 10⁸ SRBC, and Graded Numbers of Specifically Sensitized Marrow Cells

No. of marrow cells transplanted	Direct PFC		Indirect PFC	
	Percentage of positive spleens*	No. of PFC/ positive spleen‡	Percentage of positive spleens*	No. of PFC/positive spleen‡
($\times 10^5$)				
	Marrow donors immunized 2 months			
0.31	11 (9)§	485	22	1189
0.62	33 (9)	509	45	715
1.25	60 (10)‖	490 ± 55	70‖	523 ± 404
2.50	80 (10)	681 ± 169	90	576 ± 260
	Marrow donors immunized 3 months			
0.01	13 (8)	395	13	360
0.03	40 (10)	391	40	395
0.07	64 (11)‖	644 ± 157	73‖	584 ± 232
0.15	70 (24)	857 ± 258	83	705 ± 162
0.31	83 (24)	1053 ± 265	100	868 ± 248
0.62	83 (6)	810 ± 270	100	218 ± 48
1.25	100 (11)	908 ± 159	100	455 ± 88
	Marrow donors immunized 3.5 months			
0.15	10 (10)	510	30	1191
0.62	38 (8)	1215	75‖	1109 ± 648
0.81	50 (10)	1186 ± 344	80	749 ± 263
1.87	70 (10)‖	1653 ± 486	90	1676 ± 748
2.50	75 (8)	738 ± 1096	100	1004 ± 583
3.75	78 (9)	1281 ± 218	100	829 ± 487
7.50	90 (10)	1895 ± 412	100	1858 ± 584
10.00	100 (10)	906 ± 171	100	2277 ± 428
15.00	100 (10)	1583 ± 524	100	2279 ± 629

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

‡ Geometric mean ± standard error. Standard errors were calculated only for means of five or more individual values.

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

‖ Groups chosen for estimates of the number of PFC generated by one detectable precursor unit (see text).

A total of 38 mice were grafted with marrow cells from mice immunized 2 months earlier. Unlike the preceding limiting dilution assays, in this experiment the proportion of mice with positive spleens for direct and indirect PFC increased sharply as the number of marrow cells grafted was raised from 3.1 ×

10^4 to 2.5×10^5. The distribution of both types of responses in relation to the number of injected marrow cells conformed to the Poisson model (upper section of Table II and Fig. 1 *C*). The numbers of PFC per recipient spleen were similar to those found in recipients of nonimmune marrow cells (2) or of cells from mice

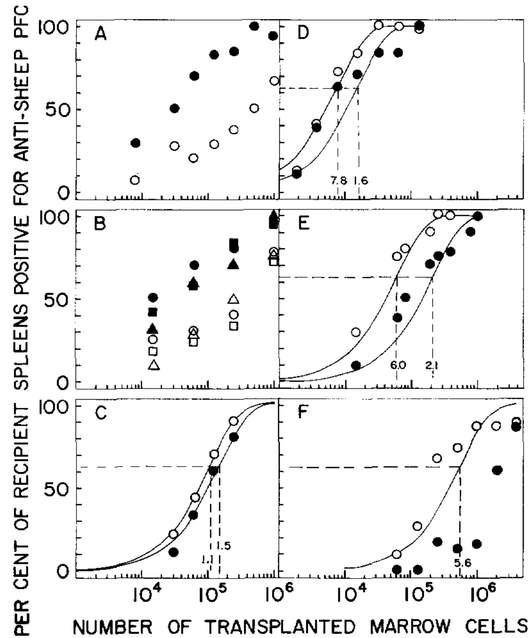

FIG. 1. Percentage of recipient spleens positive for direct PFC (closed symbols) and indirect PFC (open symbols) after injection of irradiated mice with 5×10^7 thymocytes, 5×10^8 SRBC, and graded numbers of marrow cells. Sample sizes for each point are shown in Tables I and II and in reference 2 for sections A and F. Symbols indicate observed percentages and the fitted curves expected percentages according to the Poisson model. The number of transplanted marrow cells for 63% positive spleens (indicated by dashed straight lines) contains, in the average, one detectable precursor cell or unit limiting PFC production. A, nonimmune marrow donors; B, donors immunized with *S. typhimurium* (●○), HRBC (▲△), or CRBC (■□); C–F, donors immunized with SRBC 2, 3, 3.5, and 4–8 months, respectively, before the experiment.

immunized with unrelated antigens (Table I). The frequency of detectable precursor units was $1/1.5 \times 10^5$ nucleated marrow cells for direct PFC and $1/1.1 \times 10^5$ cells for indirect PFC (Table III). The major differences between PFC responses by nonimmune and immune marrow cells were the conversion of flat limiting dilution plots into Poisson plots, and the increased number of indirect PFC responses.

A total of 94 mice were grafted in three experiments with marrow cells taken

3 months after immunization of donors (middle section of Table II, and Fig. 1 *D*). In these assays the distribution of direct and indirect PFC responses in recipients of graded numbers of marrow cells also conformed to the Poisson model. The mean numbers of PFC per spleen were slightly larger than in the preceding assays, but variability was great and the differences were not meaningful. The frequency of detectable precursor units was $1/1.6 \times 10^4$ marrow cells for direct PFC and $1/7.8 \times 10^3$ cells for indirect PFC (Table III). This is a ten- and fourteenfold enrichment of marrow P-PFC, respectively, which occurred during the 3rd month after immunization. Since the number of nucleated cells per femur and tibia did not change greatly, the actual number of P-PFC increased.

TABLE III

Statistical Analysis of Limiting Dilution Assays. Calculated Frequencies of P-PFC in Marrow Cell Suspensions of Adult Immunized (C3H × C57BL/10)F_1 Female Mice*

Time after immunization	Number of marrow cells ($\times 10^5$) containing one detectable precursor of	
	Direct PFC	Indirect PFC
(months)		
2	1.54 (0.97–2.63)‡	1.08 (0.69–1.79)
3	0.16 (0.12–0.21)	0.08 (0.06–0.11)
3.5	2.08 (1.47–3.03)	0.60 (0.40–0.93)
4–8	—	5.60§ (4.46–7.22)

*Only P-PFC which reach the spleens of recipient mice, probably, 4–15% of all P-PFC in the inoculum.

‡ 95% confidence intervals in brackets.

§ Limiting dilution assay reported in reference 2.

A total of 85 mice were grafted in two experiments with marrow cells taken at 3.5 months after immunization of donors (lower section of Table II and Fig. 1 *E*). The end point dilution plots were similar in shape to those of the preceding two assays and conformed to the Poisson model. At this time the frequencies of P-PFC were lower than at 3 months, i.e., $1/2.1 \times 10^5$ marrow cells for direct PFC, and $1/6 \times 10^4$ cells for indirect PFC. Positive spleens contained 2–4 times as many PFC as spleens colonized by nonimmune marrow and marrow taken at shorter intervals after immunization. This was an unexpected finding since the experiments were designed so that recipient spleens would be populated by an excess of thymocytes but by one or a few P-PFC. It was assumed that each P-PFC would form one antigen-sensitive unit in cooperation with thymus-derived cells and generate a fixed number of PFC (12). According

to Poisson statistics, the number of nucleated marrow cells that upon transplantation yield immune responses in 63% of recipients contains, on the average, one P-PFC reaching the spleens and generating PFC. The groups of mice with 63% or fewer positive spleens were those whose spleens were colonized by one, a few, or no P-PFC.[2]

In a preceding study (2), limiting dilution assays were made with marrow cells harvested 4–8 months after immunization. The data are shown in Fig. 1 *F*. The most important finding was the decrease of indirect PFC precursors from 3.5 to 4 months after immunization (Table III), contrasted with the stabilization at $1/5.6 \times 10^5$ cells afterwards. In addition, suppression of direct PFC production, presumably by IgG antibody, became apparent. The suppression of direct PFC made it impossible to estimate the concentration of precursor units.

Estimate of the Number of PFC Generated by Individual Precursor Units.—The numbers of PFC generated by one precursor cell were estimated from the limiting dilution experiments in which frequencies of responses conformed to the Poisson model. The mean numbers of PFC/spleen for groups of mice with 63% positive spleens were regarded as the average yield of PFC per precursor unit. Geometric means were calculated by adding all individual values of PFC/spleen, irrespective of whether the spleens were positive or negative. By choosing the groups whose percentages of positive spleens were nearest to 63% (Table II), the mean values of direct PFC/marrow precursor were 293 and 317 for P-PFC assayed 2 and 3 months after immunization. These values included the background PFC, ranging from 30 to 200; the net mean number of direct PFC per immune precursor unit was very close to the value of 150 PFC per nonimmune precursor, previously estimated by a focus assay (12). Thus, there were no indications of increased efficiency of P-PFC for production of immunocytes up to 3 months after priming. The numbers of indirect PFC/immune precursor were 124 and 197 at 2 and 3 months after immunization, including the background PFC ranging from 10 to 100. Since no data are available for calculating the yield of indirect PFC per nonimmune precursor unit (limiting dilution plots did not conform to the Poisson model and focus assays failed to detect indirect PFC), it was not possible to compare the proliferative potential of indirect PFC precursors before and after immunization.

An increase in the numbers of splenic PFC was observed following transplantation of marrow cells from 3.5-month immune donors. This was not an isolated observation since the numbers of PFC/spleen were found to be unusually large in the three experiments of Table II (lower section) and in several

[2] In a sufficiently large sample of mice injected with the number of marrow cells containing, on the average, one detectable P-PFC, the probability of splenic colonization by P-PFC is as follows: 0 P-PFC, 0.37; 1 P-PFC, 0.37; 2 P-PFC, 0.18; 3 P-PFC, 0.06; and 4–6 P-PFC, 0.02.

others, yet unpublished. It was estimated that 731 direct PFC and 346 indirect PFC (minus background PFC) were generated by individual marrow precursor units.

DISCUSSION

The experiments described in this paper were designed to study the effects of administration of SRBC on precursors of antibody-forming cells. Marrow cells of immunized mice, transplanted into nonprimed irradiated hosts with non-immune thymocytes, were induced to form plaque-forming cells by secondary stimulation. With this protocol only marrow-derived cells were exposed to antigens twice. 2–8 months after immunization with SRBC or unrelated antigens, marrow P-PFC were still dependent on thymocytes and on secondary or additional stimulation with SRBC for full immunocompetence. Apparently, the bone marrow of immunized mice did not contain all the cell types required for a complete antigen-sensitive responding unit, although thymus-derived cells may have immigrated shortly after immunization and persisted in bone marrow for less than 2 months (16). Precursors of PFC, though primarily stimulated, mounted an immune response upon transplantation only if challenged again with SRBC.

Immunization of mice with *S. typhimurium*, HRBC, or CRBC did not alter the population of marrow cells engaged in anti-sheep PFC responses. A similar observation, reported earlier (2), indicated that immunization with SRBC had no effect on the cells participating in anti-chicken PFC responses. Thus, marrow P-PFC were antigen-specific. SRBC immunization modified potentially immunocompetent marrow cells responsive specifically to these antigens in two major ways: P-PFC became more numerous, and the yield of PFC per precursor unit, larger. A third effect of immunization was to reduce functional heterogeneity of anti-sheep P-PFC of nonprimed mice, so that the results of limiting dilution assays (frequencies of positive or negative PFC responses as a function of the number of marrow cells grafted) became interpretable by the Poisson model. Marrow P-PFC were, therefore, not only antigen-specific but also antigen-sensitive.

The concentration of bone marrow P-PFC increased between 2 and 3 months after immunization with 5×10^8 SRBC, decreased during the next month, and remained stable for the following 4 months. P-PFC frequencies varied over two logarithms of nucleated marrow cell numbers; the fluctuations were simultaneous and similar in magnitude for precursors of direct and indirect PFC. 4–8 months after immunization, formation of direct PFC was inhibited, presumably by minute amounts of high affinity IgG antibody, while formation of indirect PFC was not impaired (2). Immunization with 3.5×10^6 instead of 5×10^8 SRBC altered the precursors of direct PFC but not those of indirect PFC (2). Thus, dissociation of secondary IgM and IgG antibody responses

was observed twice in this series of experiments. Antigen-induced changes of P-PFC must have arisen independently in cell lines restricted for IgM and IgG antibody. Other investigators have also found that memory arises independently for antibody responses of different classes and subclasses (17–19). Lowering the antigen dose for priming or shortening the interval between the first and second exposure to antigens favored the detection of specific changes in precursors of direct (IgM) PFC, as in other experimental systems designed for the study of immunologic memory (19, 20).

The quantitative changes of marrow P-PFC thus far described occurred at rather late intervals after immunization. They may simply represent regeneration and overshoot phases after depletion of marrow P-PFC: an earlier burst of activity in the spleen of immunized mice (primary PFC response) could have required intense and sustained supply of marrow-derived cells. However, it is also conceivable that the increased concentration of marrow P-PFC was a primary effect of immunization, perhaps a prerequisite for late-occurring qualitative changes of P-PFC. The selection of cells with increased affinity for antigen (21–23) or with other properties favoring the establishment of memory would be more effective if coupled with proliferation of P-PFC. For example, the production of large numbers of antibody-forming cells during second set responses would be facilitated if primed P-PFC had a greater potential for proliferation.

The yield of PFC per precursor unit did not increase over ~200 direct and ~100 indirect PFC until 3 months after immunization. However, P-PFC generated ~600 direct and ~250 indirect PFC 3.5 months after priming. The proliferative potential of marrow P-PFC increased either by a process of differentiation or selection. The more efficient P-PFC, hereafter indicated P-PFC^{s}, could have generated larger clones of PFC upon interaction with thymic cells, or undergone one or more self-replications before differentiation and maturation into PFC. In the latter case several antigen-sensitive units would have been formed rather than a single one with unusually great potential for proliferation. Experiments designed to distinguish between these possibilities, and to establish whether the thymus played a role before and/or after secondary stimulation of P-PFC^{s}, are in progress.

Synergistic effects of transferred bone marrow and spleen cells for PFC formation have been repeatedly described (24–28). The PFC derived either exclusively from splenic precursors (25, 26), or from both marrow and splenic precursors (27, 28). If primed P-PFC have a greater proliferative potential than nonprimed P-PFC, one can anticipate that after transfer of mixtures of immune and nonimmune cells into irradiated recipients, most antibody-forming cells will be derived from the former (25, 26). The sites of antigen-dependent differentiation and of function of P-PFC are the spleen and lymph nodes rather than the bone marrow. Immune spleen may, therefore, contain more P-PFC than bone

marrow. The probability that PFC descend from splenic or marrow precursors in experiments of this kind, will largely depend on the proportion of splenic and marrow P-PFC and on their respective potential for proliferation. In fact, PFC generated by precursors of both tissues were found when the number of marrow cells greatly exceeded that of spleen cells (27, 28). However, the existence of other factors cannot be ruled out since the mechanism of bone marrow–spleen synergism remains obscure.

Immunologic memory has been associated with long-lived small lymphocytes recirculating in thoracic duct lymph (29). As these lymphocytes do not form antibody themselves, but induce bone marrow–derived cells to do so, they are most likely thymus-derived (30). Antigens induce intense proliferation among thymus-derived cells (31), the resulting population of small lymphocytes (32) being more effective than corresponding unprimed cells in inducing specific antibody formation. Thymic ARC are among the cells proliferating in response to antigenic stimulation (14), they generate in this way the inducer cells which interact with marrow P-PFC. Because of dissemination of inducer cells outside the thymus and readily demonstrable specificity for antigens (14, 30, 32), the thymus-derived carrier of immunologic memory and the inducer of antibody formation in marrow–thymus cooperation may be the same cell. The functions of inducer cells are not yet understood; nevertheless, it has been determined that inducer cells do not dictate serological properties (33), molecular class (14, 19) and specificity of antibody for which marrow precursors of immunocytes are already determined. In view of the specificity and sensitivity to antigens of potentially immunocompetent marrow cells (1–3), immunologic memory cannot be envisioned as being carried exclusively or primarily by thymus-derived cells. Two antigen-specific cell types of marrow and thymus origin, respectively, cooperate in secondary as in primary immune responses, though the initial exposure to antigens may have induced quantitative and qualitative changes in both cell populations. The recognition of marrow-derived carriers of memory is a rather recent and perhaps unexpected development in cellular immunology (1, 2, 19). In summer 1970 a paper on this subject presented at an international meeting (30) concluded that "no such claims for specificity can yet be seriously made with respect to non-thymus derived antibody-forming cell precursors as they exist at a stage prior to contact with antigen and interaction with thymus-derived cells."

SUMMARY

Quantitative and qualitative changes of mouse bone marrow cells were studied by limiting dilution assays 2–3.5 months after immunization of donors with sheep erythrocytes or unrelated antigens (*Salmonella typhimurium*, horse and chicken erythrocytes). Irradiated (C3H × C57BL/10)F_1 mice were reconstituted with an excess of nonprimed thymocytes and small graded numbers

of primed bone marrow cells. Direct and indirect plaque-forming cells (PFC) were induced by secondary stimulation with SRBC and enumerated on the 9th day after cell transplantation.

Marrow precursors of PFC (P-PFC) cooperated with thymocytes in the production of direct and indirect PFC after SRBC priming. The limiting dilution plots, which were not compatible with predictions of the Poisson model before immunization, changed and conformed to this model afterwards, as if the population of P-PFC had become functionally more homogeneous. The concentration of marrow P-PFC increased up to the 3rd month after priming, and decreased during the 4th, varying over two logarithms of nucleated marrow cells. The fluctuation was simultaneous and of the same order of magnitude for precursors of direct and indirect PFC, which were class restricted. A third effect of immunization was detected at 3.5 months: individual precursor units generated 3–4 times more direct and indirect PFC than at earlier intervals. Qualitative and quantitative changes of marrow P-PFC participating in anti-sheep responses were specific, since antigens unrelated to SRBC failed to induce them. The data suggested that marrow-derived cells were the major carriers of immunologic memory, but that they functioned in cooperation with thymus-derived inducer cells during secondary anti-sheep responses.

BIBLIOGRAPHY

1. 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.
2. Miller, H. C., and G. Cudkowicz. 1970. Antigen-specific cells in mouse bone marrow. I. Lasting effects of priming on immunocyte production by transferred marrow. *J. Exp. Med.* **132**:1122.
3. 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.
4. 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.
5. Mosier, D. E., and L. W. Coppleson. 1968. A three-cell interaction required for the induction of the primary immune response in vitro. *Proc. Nat. Acad. Sci. U.S.A.* **61**:542.
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. 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.
8. Miller, H. C., and G. Cudkowicz. 1971. Density gradient separation of marrow cells restricted for antibody class. *Science (Washington).* **171**:913.
9. Mozes, E., G. M. Shearer, M. Sela, and W. Braun. 1970. Conversion with poly-

nucleotides 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, Inc., New York. 162.

10. Mitchinson, N. A., K. Rajewsky, and R. B. Taylor. 1970. Cooperation of antigenic determinants and of cells in the induction of antibodies. *In* Developmental Aspects of Antibody Formation and Structure. J. Šterzl, editor. Academic Press, Inc., New York. In press.
11. Trentin, J., N. Wolf, V. Cheng, W. Fahlberg, D. Weiss, and R. Bonhag. 1967. Antibody production by mice repopulated with limited numbers of clones of lymphoid cell precursors. *J. Immunol.* **98:**1326.
12. 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 by marrow-thymus cell mixtures. *J. Exp. Med.* **129:**935.
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., 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.
15. 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.
16. Chaperon, E. A., J. C. Selner, and H. N. Claman. 1968. Migration of antibody-forming cells and antigen-sensitive precursors between spleen, thymus and bone marrow. *Immunology.* **14:**553.
17. Hamaoka, T., M. Kitagawa, Y. Matsuoka, and Y. Yamamura. 1969. Antibody production in mice. I. The analysis of immunological memory. *Immunology.* **17:**55.
18. L'Age-Stehr, J., and L. A. Herzenberg. 1970. Immunological memory in mice. I. Physical separation and partial characterization of memory cells for different immunoglobulin classes from each other and from antibody-producing cells. *J. Exp. Med.* **131:**1093.
19. Schirrmacher, V., and K. Rajewsky. 1970. Determination of antibody class in a system of cooperating antigenic determinants. *J. Exp. Med.* **132:**1019.
20. Cunningham, A. J. 1969. Studies on the cellular basis of IgM immunological memory. *Immunology.* **16:**621.
21. Steiner, L. A., and H. N. Eisen. 1967. Sequential changes in the relative affinity of antibodies synthesized during the immune response. *J. Exp. Med.* **126:**1161.
22. Siskind, G. W., and B. Benacerraf. 1969. Cell selection by antigen in the immune response. *Advan. Immunol.* **10:**1.
23. Bullock, W. W., and M. B. Rittenberg. 1970. In vitro-initiated secondary anti-hapten response. II. Increasing cell avidity for antigen. *J. Exp. Med.* **132:** 926.
24. Radovich, J., H. Hemingsen, and D. W. Talmage. 1968. The enhancing effect of

bone marrow cells on the immune response of irradiated mice reconstituted with spleen cells from normal and immunized donors. *J. Immunol.* **100**:756.

25. Talmage, D. W., J. Radovich, and H. Hemingsen. 1971. The immunocompetence of the irradiated spleen repopulated with bone marrow. *In* Cellular Interaction in the Immune Response. S. Cohen, G. Cudkowicz, and R. T. McCluskey, editors. S. Karger, AG., Basel, Switzerland. In press.
26. Jacobson, E. B., J. L'Age-Stehr, and L. A. Herzenberg. 1970. Immunological memory in mice. II. Cell interactions in the secondary immune response studied by means of immunoglobulin allotype markers. *J. Exp. Med.* **131**:1109.
27. Cunningham, A. J. 1969. Studies on the cellular basis of IgM immunological memory. The induction of antibody formation in bone marrow cells by primed spleen cells. *Immunology.* **17**:933.
28. Vann, D.C., and P. Campbell. 1970. Plaque-forming cells of two different origins in single hemolytic foci. *J. Immunol.* **105**:1584.
29. Gowans, J. L., and J. W. Uhr. 1966. The carriage of immunological memory by small lymphocytes in the rat. *J. Exp. Med.* **124**:1017.
30. Miller, J. F. A. P. 1971. Origin and differentiation of cells interacting during antibody responses. *In* Cellular Interactions in the Immune Response. S. Cohen, G. Cudkowicz, and R. T. McCluskey, editors. S. Karger, AG., Basel, Switzerland. In press.
31. Davies, A. J. S., E. Leuchars, V. Wallis, and P. C. Koller. 1966. The mitotic response of thymus-derived cells to antigenic stimulus. *Transplantation.* **4**: 438.
32. Miller, J. F. A. P., and G. F. Mitchell. 1970. Interaction between thymus and bone marrow cells in response to antigenic stimulation. *Contr. Process. Multicell. Organisms Ciba Found. Symp.* 238.
33. Orsini, F.R., and G. Cudkowicz. 1971. Thymic antigen-reactive cells do not specify serological properties of antibody. *Cell. Immunol.* **2**. In Press.