CELLULAR DIFFERENTIATION OF THE IMMUNE SYSTEM OF MICE

II. Frequency of Unipotent Splenic Antigen-sensitive Units after Immunization with Sheep Erythrocytes*

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Antibody-forming cells produce, as a rule, only one of several possible species of immunoglobulins at any one time. Apparently, immunocytes are differentiated so as to synthesize exclusively antibody of one given antigen-specificity (1–10), biological activity (11, 12), and immunoglobulin class or subclass (4, 12–15). Specialization of immunocytes is so extreme that only one allotypic variant of a particular immunoglobulin subclass is produced by cells possessing the genetic information for two variants (16–18). Identification of the source of the diversity among immunocytes is a major goal in immunology, since it bears upon the instructive and selective theories of antibody formation. One way of studying the mechanism by which this diversity is generated, is to recognize the stages and cell types along the lymphoid and/or myeloid differentiation pathways at which specialized functions arise, and possibly, the sequential development of different levels of specialization.

The immediate precursors of antibody-forming cells are units of one or more highly integrated cell types that do not release antibody, but interact with antigen and generate functional immunocytes by proliferation and/or differentiation (2, 3, 12, 19). Such integrated units, called antigen-sensitive units (ASU), are found in spleen, lymph nodes (19, 20), and peripheral blood (unpublished results), and are detected by transplantation methods. Some of the cellular components of ASU are found separately in marrow and in thymus, since artificial mixtures of cells from these and other organs yield functional ASU (21, 22). Like antibody-forming cells, splenic ASU have undergone antigen-specificity differentiation (8, 9, 23, 24), antibody-class differentiation (12) and presumably allotypic restriction (18) *before* contact with antigen. Hence, diversity had occurred at least at the time of splenic ASU assembly. In a preceeding paper of this series (12) we demonstrated that splenic ASU reacting with the antigen complex of sheep erythrocytes were specialized, for they generated either hemolytic plaque-forming cells (PFC) or hemagglutinating cluster-forming cells (CFC), but not both. Furthermore, some of the precursors of hemolysin-forming cells generated direct

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PFC but not indirect PFC. The frequencies of specialized ASU in the spleens of *unimmunized* mice differed greatly and decreased in this order: ASU for direct PFC, for indirect PFC, and for cluster-forming cells. Because of a sevenfold difference in the frequencies of ASU for direct and indirect PFC, it was not possible to ascertain whether indirect PFC secreting IgG antibodies arose directly from unipotent precursors or from pluripotent precursors also capable of generating direct PFC, secreting IgM antibodies.

In the present study we have used *preimmunized* donor mice to reexamine the potential of splenic antigen-sensitive units for differentiation into one or more types of immunocytes, and the relative frequency values of these ASU. Although it was understood that immunization might have changed ASU qualitatively, it was our objective to preferentially increase the frequency of ASU for IgG-producing cells, so as to reverse the order of frequencies of splenic ASU, or reduce the differences between them. We, therefore, transplanted graded numbers of primed spleen cells with sheep erythrocytes into irradiated syngeneic mice to quantitate the secondary immune responses elicited by the grafted cells in terms of direct hemolytic PFC, indirect PFC, and agglutinating CFC. If the production of indirect PFC would occur in the absence of direct PFC, one could conclude that separate differentiating cell lines generate immunocytes synthesizing IgG and IgM antibodies of the same specificity. If, however, the production of indirect PFC would be always preceded by direct PFC, one could conclude that IgM-forming cell lines progressively shift to IgG antibody production. In addition, changes in relative frequencies of ASU induced by immunization would virtually rule out the possibility that the frequency values previously found reflected different sensitivities of the assays for PFC (direct and indirect) and CFC.

Materials and Methods

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

Irradiation.—Mice to be grafted with syngeneic spleen cells were preexposed to 800 R of total body X-radiation as described elsewhere (12).

Spleen cell suspensions were prepared, counted, and transplanted as indicated in the initial paper of this series (12). The procedures for enumerating plaque-forming cells (direct and indirect) and cluster-forming cells in recipient spleens were already described (11, 12).

Immunization.—Defibrinated sheep blood was obtained from Grand Island Biological Co., Grand Island, N. Y., and stored at 4°C. Immediately before use, erythrocytes (SRBC) were washed three times in Eagle's medium and counted with a Coulter Particle Counter. 10-wk-old mice were primed with a single intravenous injection of 2×10^6 SRBC; this number of cells was chosen to induce a submaximal primary response followed by sustained immunological memory. Primed mice were used as spleen cell donors 122–138 days later. Their spleens contained $\backsim 170 \times 10^6$ nucleated cells. Equal volumes of spleen cell and SRBC suspensions were mixed so as to inject into the tail vein of each recipient mouse a variable number of primed spleen cells with 5×10^8 SRBC.

Statistical Methods.—Limiting dilution assays were performed to measure the frequencies

of ASU in spleen cell suspensions of primed mice. The Poisson model was used to describe the theoretical probability that ASU, contained in a given number of nucleated spleen cells, reach the recipient spleens and generate antibody-forming cells. The procedure followed for calculating this probability value is reported elsewhere (12).

The hypothesis that specialized splenic ASU generate different types of immunocytes independently was tested in two-by-two tables using the Yates corrected chi-square procedure.

RESULTS

Primary Response in Prospective Donor Mice.—The spleens of three to six mice were tested for the presence of direct and indirect PFC, and of CFC

TABLE I

Antibody-Forming Cells in Control Animals

Treatment	Time after transplantation	Fraction of spleens with detectable immunocytes and mean number of immunocytes per positive spleen ± standard error		
		Direct PFC	Indirect PFC	CFC
	days			
Spleen cells* ($2.5–10 \times 10^5$)	7–11	20/27 24.5 ± 10.5	11/27 25.8 ± 12.6	6/22 5830 ± 3620
Spleen cells* ($1.25–5 \times 10^5$) + SRBC	5–7	18/26 15.0 ± 2.2	15/26 17.8 ± 3.5	11/22 3840 ± 1270
Spleen cells* (10×10^5) + SRBC	8–9	15/15 714.0 ± 82.0	15/15 574.0 ± 82.6	14/14 400,000 ± 45,900

* Donor mice were injected with 2×10^6 SRBC 4 months before spleen cell transplantation. The cells were infused into irradiated recipients with or without 5×10^8 SRBC.

at each of the following intervals: 5, 6, 7, 10, 18, 31, 53, 77, and 100 days after intravenous immunization with 2×10^6 SRBC. Peak numbers of immunocytes were 41,200 ± 4,930 direct PFC at day 5, 2970 ± 720 indirect PFC at day 10, and 195,000 ± 38,500 CFC at day 7. At days 77 and 100, usual background values of direct and indirect PFC were found in eight spleens tested, but a few spleens contained 50,000 CFC. The total number of nucleated cells per spleen was $176 \pm 13.7 \times 10^6$, the same as that of spleens of unimmunized mice. The magnitude and time sequence of the response was similar to that described by others (25). The tests assured us that donors to be taken from these groups of mice had experienced a primary immune response to SRBC with production of hemolysins and hemagglutinins, but did not contain large numbers of immunocytes in their spleens at the time of the experiments.

Secondary Response Elicited by Spleen Cells Transplanted into Irradiated Mice.—Groups of animals were given graded numbers of spleen cells mixed with 5×10^8 SRBC to verify whether the time of appearance of immunocytes in recipient spleens was similar to that reported for recipients of unprimed cells (12). Negative control groups received spleen cells, but not SRBC, to enumerate background immunocytes. Assays were carried out 7–11 days after transplantation on individual spleens. The results obtained are shown in Table I.

Relatively small numbers of background PFC and CFC were found in a proportion of irradiated mice grafted with $2.5–10.0 \times 10^5$ spleen cells *without* SRBC. None of these negative control mice had more than 50 direct PFC, 50 indirect PFC, and 2×10^4 CFC per spleen. During the first 7 days posttransplantation, the mice that received $1.25–5.0 \times 10^5$ spleen cells *with* SRBC did not contain more immunocytes in their spleens than the controls. In other words, these recipient spleens were negative. If the number of grafted spleen cells was greater (e.g. 10^6 cells), then a few, but not all mice had positive spleens within 7 days, i.e., spleens with more immunocytes than the negative controls (data not shown). At days 8 and 9 posttransplantation, all recipients of 10^6 spleen cells *with* SRBC were positive for direct and indirect PFC and for CFC (Table I). Therefore, recipient spleens were not assayed earlier than 8 days posttransplantation in all subsequent experiments. It is noteworthy that grafts of 10^6 *unprimed* cells gave positive spleens for indirect PFC and CFC in a small fraction of recipients, i.e., in 15.6 and 10.7%, respectively (12). Hence, these findings suggest that immunization caused a severalfold increase in frequency of the relevant ASU.

Frequency of Response in Irradiated Mice Grafted with $2.5–5.0 \times 10^5$ Primed Spleen Cells.—If splenic ASU of immunized mice differ from each other with respect to the types of progeny immunocytes they can generate, then it should be possible to dissociate the production of direct PFC, indirect PFC, and CFC (negative spleens for one type of immunocyte, but positive for another) by reducing the size of spleen cell grafts below 10^6 cells so that only one or two ASU reach the recipient spleens.

2.5 to 5.0×10^5 spleen cells mixed with SRBC were injected into a series of mice. 8–9 days later, about one-half of the mice were killed and their spleens assayed for direct and indirect PFC, and for CFC. The remaining mice were killed 34–35 days after transplantation and their spleens were also assayed for the three types of immunocytes. In all instances, duplicate samples, representing one-fifth of the recipient spleens each, were plated for plaque assays. Results are presented in Fig. 1.

Only the spleens of a proportion of the mice contained significant numbers of immunocytes above control values. This was so for each type of immunocyte assayed, at the earlier as well as the later interval. The number of animals with given values of immunocytes per recipient spleen are plotted on a fre-

quency distribution diagram (Fig. 1). For each type of antibody-forming cell, a second group of spleens could be identified containing numbers of immunocytes not different from those of negative control mice (up to 50 direct or indirect PFC, and 2 × 10^4 CFC). We regard these spleens as negative, presumably because grafted ASU were too few to lodge in each of them. Early after transplantation, negative spleens were not due to delayed responses, since negatives were found 35 days after grafting as well. Alt ough some spleens were either positive or negative for all three types of immunocytes, other spleens were

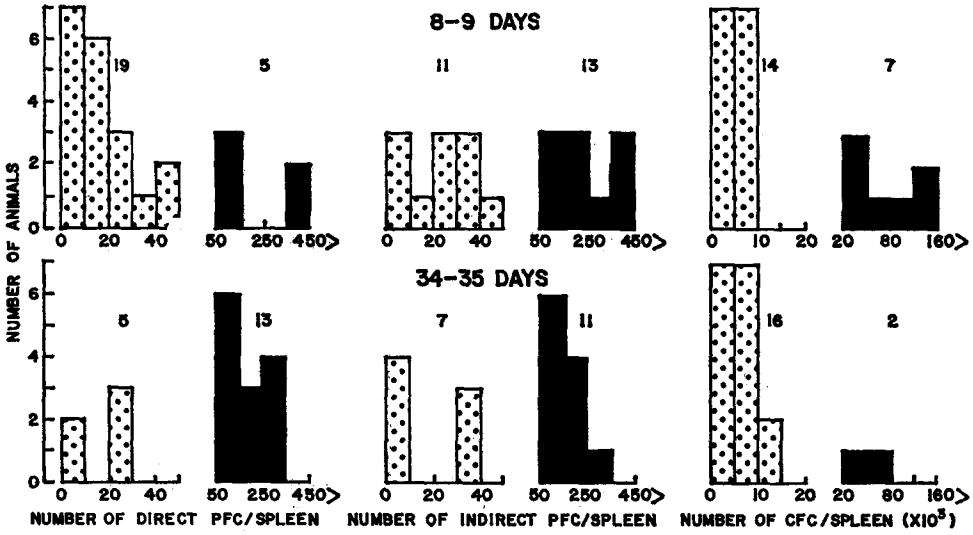

FIG. 1. Number of mice from which spleens assayed 8–9 or 34–35 days after antigenic stimulation were negative (stippled bars) or positive (solid bars) for direct PFC, indirect PFC, and CFC. The total number of positive and negative spleens is indicated above the bars. Each mouse was exposed to 800 R of X-rays and grafted with 2.5–5 × 10^5 primed spleen cells mixed with 5 × 10^8 SRBC.

negative for one or two types, but positive for the remaining. Every possible combination was found among the 42 spleens analyzed (see more extensive data of Table IV). It seems then that the precursors of indirect PFC and of CFC are more frequent in spleens of immunized than in those of unimmunized mice (12), since the number of primed cells necessary for equivalent responses was considerably lower. Furthermore, the dissociation of the three cellular responses studied suggests that grafted ASU have different potentials and reach the recipient spleens independently.

Limiting Dilution Assays.—To measure the frequency of ASU in spleens of primed mice, a wider range of graded numbers of spleen cells mixed with SRBC was injected into groups of irradiated mice. After 8–11 days, individual spleens

were assayed for their content of direct and indirect PFC, and of CFC. As before, aliquots of the recipient spleen cells amounting to two-fifths of the organ were plated for each plaque assay. Spleens were regarded as positive if the number of immunocytes exceeded 50 direct or indirect PFC, and 2×10^4 CFC.

TABLE II

Percentage of Recipient Spleens Containing Significant Numbers of Immunocytes as a Function of the Number of Donor Cells

Type of immunocyte assayed	Number of cells transplanted	Fraction of spleens containing significant numbers of immunocytes*	Percentage of positive spleens	Probability of positive spleen per 10^6 transplanted cells
	($\times 10^6$)			
Direct PFC	1.25	1/9	11.1	
	1.85	2/8	25.0	
	2.50	5/18	27.7	
	3.75	4/16	25.0	1.25
	5.00	9/20	45.0	(0.90–1.68)‡
	6.67	6/14	42.8	
	8.33	6/9	66.7	
	10.00	10/10	100.0	
Indirect PFC	1.25	3/9	33.3	
	1.85	2/8	25.0	
	2.50	5/18	27.7	
	3.75	10/16	62.5	2.34
	5.00	15/20	75.0	(1.77–3.04)
	6.67	10/14	71.4	
	8.33	8/9	88.9	
	10.00	10/10	100.0	
CFC	1.25	2/9	22.2	
	1.85	3/8	37.5	
	2.50	5/15	33.3	
	3.75	6/14	42.8	1.69
	5.00	12/19	63.2	(1.24–2.25)
	6.67	10/14	71.4	
	8.33	4/9	44.4	
	10.00	6/6	100.0	

* More than 50 direct PFC, or 50 indirect PFC, or 2×10^4 CFC per spleen. Four-fifths of all nucleated spleen cells were used for the two plaque assays.

‡ 95% confidence intervals in parentheses.

Otherwise the spleens were regarded as negative. The results are presented in Table II.

As the number of grafted cells increased from 1.25 to 10×10^5, the proportion of mice with positive spleens increased for all three types of immunocytes.

Except for recipients of the largest cell inoculum, the spleens of some mice of each group were positive while others were negative. The relation between the percentage of positive spleens and the number of potentially competent cells grafted varied for each type of antibody-forming cell, but not nearly as much as in similar experiments with spleen grafts from unprimed donors (12).

The probability values P for ASU to give positive spleens after transplantation were calculated per unit number of donor cells using the Poisson model. Differences in these P values were statistically significant at the 0.05 level for direct and indirect PFC. The P value for CFC was intermediate between the other two values, and the 95% confidence intervals were overlapping. The curves relating inoculum size to the expected frequency of positive spleens

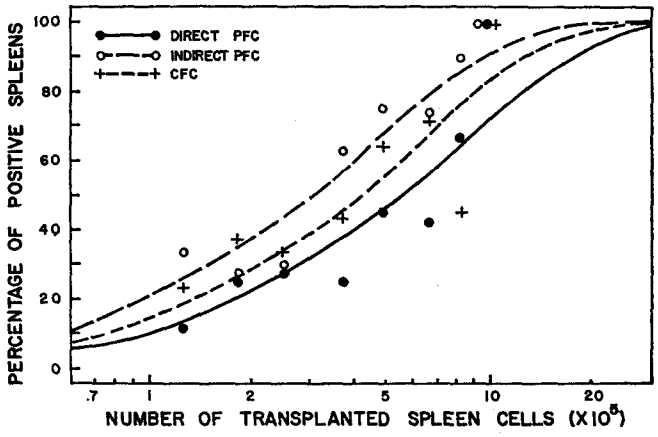

FIG. 2. Percentage of recipient spleens positive for antibody-forming cells following X-irradiation and injection of graded numbers of spleen cells mixed with 5×10^8 SRBC. The number of spleens assayed for each inoculum size is given in Table II. Curves indicate expected percentages (Poisson model) and symbols observed percentages.

are shown in Fig. 2, together with the observed frequencies. Results of this analysis provided an estimate of the number of nucleated donor spleen cells containing one ASU that reached the recipient spleen on transplantation. These estimates are reported in Table III along with the corresponding estimates of splenic ASU in unprimed donor mice (12). A critique of the use of limiting dilution assays to measure ASU frequency was made in the preceding paper (12). The data of Table II and their statistical analysis (Fig. 2 and Table III) indicate that the frequencies of ASU for direct and indirect PFC differ significantly in spleens of primed mice. Furthermore, immunization greatly increased the frequencies of ASU for indirect PFC ($\times$ 15.7) and CFC ($\times$ 32.7), but it did not appreciably alter the frequency of ASU which generate direct PFC.

Tests for Independence of Specialized ASU.—If splenic ASU of primed donor

mice are unipotent and reach the spleens of recipients independently as well as generate progeny immunocytes without interacting, then we expect chance dissociation of cellular responses in the recipient spleens that we have analyzed for the limiting dilution experiment. This is likely to be so because the frequencies of the three specialized ASU studied did not differ greatly. All data of the groups in which there were positive and negative spleens were subjected to a chi-square test for independence of cellular immune responses. Table IV summarizes the tests in which direct PFC responses were compared with indirect PFC responses; direct PFC with CFC; and indirect PFC with CFC. All χ^2 values were below the critical value of 3.84, the chi-square statistic at

TABLE III

Results of Statistical Analysis of Limiting Dilution Assays. Calculated Frequencies of Antigen-Sensitive Units in Spleen Cell Suspensions of Adult Unimmunized and Immunized (C3H × C57BL)F_1 Female Mice*

Type of immunocyte assayed	Number of spleen cells (× 10^6) containing one detectable antigen-sensitive precursor*	
	Unimmunized‡	Immunized
Direct PFC	0.98 (0.81–1.19)§	0.80 (0.60–1.11)
Indirect PFC	6.90 (5.85–8.17)	0.44 (0.33–0.57)
CFC	19.3 (14.8–25.0)	0.59 (0.45–0.81)

* Only antigen-sensitive units which reach the spleens of recipient mice (4–15% of all antigen-sensitive units in the inoculum).

‡ Limiting dilution assays for splenic antigen-sensitive units of unimmunized mice are reported in reference 12.

§ 95% confidence intervals in parentheses.

0.05 level of significance for one degree of freedom. Therefore, there is no statistical evidence for dependence between the different cellular responses.

DISCUSSION

Information concerning the processes of differentiation of potentially immunocompetent cells would provide some of the essential facts necessary for understanding the great heterogeneity of antibodies produced against given antigens, and the broad spectrum of immune reactions elicited by mammals. A large number of experiments has dealt with the possibility that single mature immunocytes either produce antibodies of more than one specificity (1–10), or antibodies belonging to more than one immunoglobulin class (4, 12–15). The general consensus is that the great majority of immunocytes are engaged in

the exclusive synthesis of one molecular subclass of immunoglobulin endowed with a given specificity and biological property.

Another series of experiments has dealt with the possibility that antigen-sensitive precursors generate more than one kind of immunocyte. If this were

TABLE IV

Results of Chi-Square Tests for Independence of Antigen-Sensitive Units Producing Direct PFC, Indirect PFC, and CFC on Transplantation

Number of cells transplanted, ($\times 10^5$)		1.25	1.85	2.50	3.75	5.00	6.67	8.33
Number of recipients		9	8	15	14	19	14	9
Direct PFC	Indirect PFC							
+	+	0	1	1	3	7	5	6
+	−	1	1	2	0	2	2	0
−	+	3	1	3	6	7	4	2
−	−	5	5	9	5	3	3	1
	χ^{2*}	0.14	0	0.19	0.60	0.02	0	0.14
Direct PFC	CFC							
+	+	1	1	2	0	5	4	3
+	−	0	1	1	3	4	3	3
−	+	0	2	3	6	4	6	1
−	−	8	4	9	5	6	1	2
	χ^2	1.72	0.18	0.47	1.07	0.05	0.35	0.06
Indirect PFC	CFC							
+	+	0	0	1	3	9	7	6
+	−	3	2	3	6	5	2	0
−	+	1	3	4	3	0	3	2
−	−	5	3	7	2	5	2	1
	χ^2	0.14	0.56	0.04	0.16	3.80	0.01	0.14

+, positive spleen containing more than 50 direct PFC, or 50 indirect PFC, or 2×10^4 CFC.

−, negative spleen 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. None of the comparisons is incompatible with the hypothesis that antigen-sensitive units for different types of immunocytes are independent.

so, the extreme specialization of antibody-forming cells would have occurred during the terminal stages of differentiation. Playfair et al., Nakano and Braun, and Celada and Wigzell have demonstrated the independent clonal assortment of direct plaque-forming cells reacting against two unrelated antigens (2–4), and that of direct and indirect plaque-forming cells reacting against the same

antigen (4). The results of these experiments have clearly indicated that hemolytic antibody of a given specificity and molecular class was produced by immunocytes descending from individual antigen-sensitive units. However, the experiments did not test competition between the two antigens, and hence, did not establish whether the antigens addressed themselves to committed or uncommitted ASU. Gell and Sell found that the blastogenic responses of rabbit blood leukocytes to mixtures of monospecific antiallotype sera were additive (18). They concluded that the responding leukocytes were producing only one of the two IgG subclasses. It is questionable, however, whether the peripheral blood cells which undergo blastogenic transformation are comparable to ASU.

Mishell and Dutton (8, 9) and O'Brien and Coons (24) have induced primary or secondary immune responses in vitro to two noncross-reacting antigens that were added either to separate or to the same cultures, with or without mitotic inhibitors. They found that two unrelated antigens did not compete for ASU, but rather stimulated differentiated ASU with a narrow range of reactivities. Furthermore, the expansion of one of the two populations of progeny immunocytes could be suppressed by appropriate timing of stimulation with the second antigen and inhibition of mitosis. Nossal et al. were able to separate by physical means ASU reactive with SRBC from those reactive with *Salmonella* flagellin (23), thereby confirming that splenic ASU have gone through specificity differentiation before immunization of the spleen cell donors. The same experiments suggested also the occurrence of class-differentiation of ASU, for secondary responses to *Salmonella* flagellin could be initiated by fractionated spleen cell populations incompetent to initiate primary responses.

Estimates of the number of splenic ASU of unprimed mice and studies of their potential for differentiation into anti-SRBC hemolytic PFC (direct and indirect) and hemagglutinating CFC have been reported in the first paper of this series (12). Our results indicated that the three types of immunocytes could not have arisen from proliferation and differentiation of pluripotent ASU, at least under the circumstances of spleen cell transplantation. It became apparent that splenic ASU were specialized for antibody class and function, and that ASU for CFC and for *direct* PFC were unipotent. Since the frequency of specialized ASU differed greatly in spleens of unprimed mice, it was not possible to establish whether ASU for *indirect* PFC were also unipotent, or capable, instead, of generating both direct and indirect PFC. Due to the greater frequency of the precursors of direct PFC, spleen cell grafts containing limiting numbers of ASU for indirect PFC always had excessive numbers of ASU for the former type of PFC. This prevented us from testing in a stringent way the independence of direct and indirect PFC formation by grafted ASU.

Results of experiments reported here show that immunization of mice did

not increase the frequency of splenic ASU for direct (IgM) PFC assessed 122–138 days after SRBC administration. This finding is consistent with the generally accepted view that the IgM antibody responses do not display immunological memory (26, 27), and with several detailed studies of the primary and secondary responses to SRBC, humoral and cellular (28–32). In contrast, immunization markedly increased the frequencies of splenic ASU for indirect (IgG) PFC and for CFC. These latter findings are also consistent with the view that IgG responses do display immunological memory, and with the descriptions of anti-SRBC responses. For example, the secondary increase of IgG hemolysin titers is not as great as that of IgG hemagglutinin titers (28–32). Accordingly, we have found that ASU for indirect hemolytic PFC increased upon immunization by a factor of 15.7, while ASU for hemagglutinating CFC increased by a factor of 32.7.

By virtue of immunization, the spleen cell grafts used contained the three types of ASU under study in the following proportions: 0.53 (direct PFC), 1.00 (indirect PFC), and 0.72 (CFC). These relative frequencies were close enough so that results of limiting dilution assays could be used to test independence of production of particular immunocytes. The results obtained indicated that there was only chance association between positivity for direct and indirect PFC, or for PFC and CFC in the same recipient spleens. The inescapable conclusion is that splenic ASU that were primed with SRBC are differentiated for specificity, class, and biological function (agglutination or lysis) of the antibody to be secreted by their progeny cells. It remains to be established whether marrow-derived and/or thymus-derived components (21, 22) conferred the observed specialization to splenic ASU. In any event, our results do not support the view that ASU-derived clonal populations shift from IgM to IgG antibody production.

Since the conclusion of our experiments answers an important immunological question and calls for further studies with more primitive or immature cells than splenic ASU, it is desirable to discuss the possibilities of error arising from the particular experimental protocol we have chosen. Upon intravenous injection of spleen cells into irradiated mice, only a small fraction of ASU lodges in the recipient spleen (2, 19). This fraction is variable in time and, furthermore, has been assessed only for precursors of direct PFC. The possibility exists, therefore, that differences in estimated numbers of ASU per unit number of unprimed spleen cells were due to differences in "homing" among injected ASU. Likewise, the relative sensitivities of assays for direct and indirect PFC, and for CFC (defined as the number of immunoglobulin molecules necessary to detect a single immunocyte) are not fully known. Consequently, it is also possible that estimated frequencies of unprimed ASU reflected decreasing assay sensitivities. Neither of the two possible causes of error has been formally excluded; nevertheless, they appear very improbable in view of the effect of

immunization on the frequencies of splenic ASU. Using identical procedures, we found the order of estimated frequencies reversed, so that the rarer types of ASU became the more frequent. It could still be argued however, that the priming changed ASU qualitatively as well as quantitatively. Haskill has compared density gradient profiles of splenic anti-SRBC ASU from nonimmune and immune (90 days) rats, and found that most peaks were common, albeit with a shift toward the low-density regions in the immune spleens (33). Recourse to primed mice may possibly be avoided by working with more primitive populations of potentially immunocompetent cells (as thymus-marrow combinations or certain fetal tissues may be), in which the differences in frequencies of ASU may not be so great. Other critical aspects of our experimental design were discussed in the preceding paper (12).

The extensive specialization of ASU and possibly of their precursors in unprimed and primed mice could have resulted from exposure to antigens of enteric bacteria cross-reacting with SRBC (34). This possibility could eventually be excluded by studies in colostrum-deprived, antigen-free gnotobiotic animals. It is unlikely, however, that differentiation of ASU is directed by bacterial antigens, since we have found that following radiation injury (550 R whole-body), regeneration of new ASU for direct PFC is quantitatively undistinguishable in germ-free and conventional mice (35).

SUMMARY

Spleen cell suspensions of primed donor mice containing precursors of immunocytes have been transplanted into X-irradiated recipient mice 122–138 days after immunization. Following secondary stimulation with antigen (sheep erythrocytes), these precursors, called antigen-sensitive units (ASU), gave rise to progeny cells secreting specific antibody in the spleens of recipients. Single cells releasing IgM hemolysins (direct plaque-forming cells or PFC), IgG hemolysins (indirect PFC), and hemagglutinins (cluster-forming cells or CFC) were enumerated.

By transplanting graded and limiting numbers of primed spleen cells, inocula were found which contained one or a few ASU reaching the recipient spleens. We estimated, thereby, the frequency of ASU detectable by our procedures in donor cell suspensions. The values obtained from direct and indirect plaque assays, and from cluster assays were 1 in $\sim 8.0 \times 10^5$, 1 in $\sim 4.4 \times 10^5$, and 1 in $\sim 5.9 \times 10^5$ nucleated spleen cells, respectively. The number of splenic ASU for direct PFC was not greater than that of unimmunized mice; however, immunization greatly increased the number of splenic ASU for indirect PFC and for CFC.

By applying to each recipient spleen direct and indirect plaque tests and cluster tests, we found that positivity for each type of immunocyte was independent from that of the other two types. These results confirm the unipotent

nature of splenic ASU in general, and document the commitment of ASU primed with SRBC to generate progeny cells secreting antibody of a single molecular (IgM or IgG) or functional (lysin or agglutinin) class.

We concluded that splenic ASU are composed of relatively differentiated cells of the immune system of mice. With respect to specificity and class differentiation, ASU appear to be as specialized as antibody-producing cells themselves. Our results did not support the view that ASU-derived clonal populations shift from IgM to IgG antibody production.

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