CELLULAR DIFFERENTIATION OF THE IMMUNE SYSTEM OF MICE

IV. Lack of Class Differentiation in Thymic Antigen-Reactive Cells*

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The immune response of mice to antigens of sheep erythrocytes (SRBC) requires the cooperation of at least two distinct cell types. Thymic-derived cells react with antigen and possibly process it, but do not produce antibody (1–3). Marrow-derived cells are capable of synthesizing nonspecific immunoglobulins (4), but generate large numbers of specific immunocytes in the presence of thymocytes and antigen (2, 3, 5–7). The cooperating cells constitute what has been called an antigen-sensitive unit (ASU) in the spleen and lymph nodes of intact mice and in those of heavily irradiated mice reconstituted with syngeneic grafts of marrow and thymus cells (8–10). Complete ASU, which are regarded as functional and not necessarily anatomical units, are not found in bone marrow and thymus of unprimed mice. ASU are restricted to the generation of immunocytes of one kind by way of antigen-dependent proliferation and differentiation. Thus, cells derived from a single ASU are engaged in the exclusive synthesis of antibody of one molecular class (8–12) and one antigen specificity (13–15).

In a preceding study (10) and in experiments to be published (16), we found that the restriction of ASU for antibody class depended on specialization of marrow cells. After transplantation of graded numbers of marrow cells mixed with a fixed number of thymocytes and SRBC, ASU were formed that generated immunocytes secreting one of the following antibodies: IgM or IgG hemolysins or hemagglutinins. The frequency of formation of each type of ASU was unequal and, furthermore, varied independently of the others, but in direct relation to the number of grafted marrow cells. Hence, dissociation of the three immune responses mediated by three types of ASU was obtained by reducing the number of marrow cells. Presumably, this limited the availability of specialized precursors of immunocytes for ASU formation.

In the present study we have investigated whether thymic antigen-reactive cells also restrict the potential of ASU for antibody class. Graded and limiting numbers of thymocytes were mixed with a large nonlimiting number of marrow cells and transplanted into heavily irradiated mice. The cell mixtures were then

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exposed to SRBC and the immune responses elicited were assessed in terms of direct (IgM) and indirect (IgG) hemolytic plaque-forming cells (PFC). If antigen-reactive cells (ARC) of murine thymus were not specialized for antibody class, then the probability that they participate in ASU for direct or indirect PFC should be equal. Depending on whether each ARC interacts with one type of specialized PFC precursor (P-PFC), or with precursors of direct and indirect PFC simultaneously, one or two types of ASU should be formed, respectively. We have ascertained that thymic ARC are not specialized themselves and do not restrict ASU for antibody class. Furthermore, several unipotent ASU for direct and indirect PFC are formed upon interaction of one or two ARC with antigen and relatively large numbers of marrow P-PFC.

Materials and Methods

Mice.—(C3H/He × C57BL/Ha)F_1 females, 10–12 wk old, were used as donors and recipients in all experiments.

Irradiation.—Mice to be grafted were exposed to 850 R of total body X-irradiation as described elsewhere (8).

Cell Suspensions and Transplantation.—Bone marrow, thymus, or spleen cells were suspended in Eagle's medium, counted, and injected into a lateral tail vein of irradiated mice as described in preceding papers of this series (8, 10).

Immunization.—18 hr after transplantation of nucleated cells, each irradiated mouse was injected intravenously with 5×10^8 washed SRBC suspended in 0.5 ml of Eagle's medium.

Assays for Plaque-Forming Cells.—Direct and indirect PFC were enumerated in spleen cell suspensions by the agar gel method of Jerne, as already described (8).

Assay for Hemolytic Foci.—Discrete foci of direct plaque-forming cells, presumably derived from single ASU, were detected in slices of spleens laid over agar gel containing SRBC. The procedure has been described in detail by Kennedy et al. (17).

Experimental Design and Statistical Methods.—Limiting dilution assays were performed to estimate the frequencies of ARC in thymus cell suspensions. Since marrow and thymus cells are both necessary for formation of ASU and production of PFC, graded numbers of thymocytes were added to a fixed, nonlimiting number of marrow cells for transplantation. This was done to reduce the number of thymic ARC to about one per recipient spleen. The Poisson model was used to predict the probability that ARC contained in a given number of nucleated thymocytes reach the recipient spleens, interact with marrow cells, and generate PFC. The procedure followed for calculating this probability value is reported elsewhere (8).

The hypothesis that ASU of only one kind are formed upon interaction of specialized thymic ARC with marrow P-PFC was tested by subjecting the results of limiting dilution assays to Chi-square analysis. If the hypothesis were correct, direct and indirect PFC responses should be independent of each other, since they would have been initiated by separate ARC.

The number and kinds of ASU formed upon exposure of one ARC to several P-PFC were also studied by transplanting the appropriate numbers of marrow and thymus cells. ASU were assessed either by the focus method or by separately assaying pieces of recipient spleens for direct and indirect PFC. If more than one ASU resulted from interaction of one ARC with different kinds of P-PFC, several hemolytic foci should be found in spleens receiving one ARC. Furthermore, direct and indirect PFC should be frequently found in such spleens.

RESULTS

PFC Responses in Control Mice.—Natural anti-sheep immunocytes occur in the spleens of intact mice and in those of irradiated mice grafted with hemopoietic cells (8–10). Groups of animals exposed to 850 R of X-rays were injected either with 4×10^7 marrow cells, or with 5×10^7 thymocytes. Both cell suspensions were not expected to contain large numbers of complete ASU. The grafted mice were immunized 18 hr later with SRBC, and the number of PFC was determined 9–11 days after cell transplantation. This is the time of expected peak PFC responses in experimental mice grafted with marrow cells and thymocytes (10). The results are shown in Table I. Many but not all spleens of mice

TABLE I

Plaque-Forming Cells in Spleens of Irradiated Control Mice Grafted With Marrow Cells or Thymocytes

No. of marrow cells + SRBC	No. of thymocytes + SRBC	Fraction of spleens with detectable PFC and mean number of PFC per spleen ± standard error*	
		Direct PFC	Indirect PFC
4×10^7	—	21/21	6/21
		65 ± 13	46 ± 14‡
—	5×10^7	46/56	8/56
		36 ± 5	18 ± 3

* Two-fifths of the cells of individual spleens were used for each plaque assay, 9–11 days after transplantation.

‡ Only spleens with five or more PFC were used for calculating mean values.

which were given thymocytes contained small numbers of immunocytes, not exceeding 110 direct PFC and 30 indirect PFC. However, the number of immunocytes was slightly greater in spleens of mice injected with marrow cells. In a few animals this number was $\sim$ 200 direct PFC and $\sim$ 100 indirect PFC. The "background" immunocytes could have resulted from immunity to antigens of the bacterial flora cross-reacting with SRBC and from occasional contamination of the marrow and thymus cell suspensions with complete ASU.

Frequency of ARC in Thymus Cell Suspensions.—To measure the frequency of ARC in thymus of unprimed mice, a range of graded numbers of thymocytes mixed with 4×10^7 marrow cells from the same donors were injected into groups of irradiated recipients (limiting dilution assay). SRBC were given to each mouse 18 hr later. 9–11 days after grafting, individual spleens were assayed for their content of direct and indirect PFC. This interval was chosen because it was the time of peak anti-SRBC response in recipients of marrow-thymus cell mixtures (10). The number of marrow cells was chosen so as to provide every recipient with several multiples of the number of PFC precursors necessary for

ASU formation. Portions 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 PFC exceeded that found in spleens of control mice (Table I). Otherwise, the spleens were regarded as negative. The results are presented in Table II and Fig. 1.

TABLE II

Percentage of Positive Recipient Spleens after Infusion of 4 × 10^7 Marrow Cells, 5 × 10^8 SRBC, and Graded Numbers of Thymocytes

Number of thymocytes transplanted	Fraction of positive spleens*	Percentage of positive spleens*	Mean No. of PFC per positive spleen ± SE	Probability of positive spleens per 10^6 transplanted thymocytes
× 10^6		*Direct PFC*		
0.62	1/24	4.2	265	
1.25	5/24	20.8	1220 ± 470	
2.50	7/24	29.2	394 ± 27	
5.00	11/25	44.0	727 ± 225	0.12
7.00	19/36	52.7	565 ± 59	(0.09–0.15)‡
10.00	22/26	84.6	772 ± 136	
20.00	14/16	87.6	900 ± 245	
40.00	28/29	96.6	2530 ± 310	
		Indirect PFC		
0.62	0/24	0	—	
1.25	5/24	20.8	488 ± 245	
2.50	7/24	29.2	170 ± 26	
5.00	8/25	32.0	454 ± 67	0.08
7.00	12/36	33.3	509 ± 40	(0.06–0.10)
10.00	18/26	69.7	433 ± 70	
20.00	11/16	68.8	324 ± 130	
40.00	28/29	96.6	1280 ± 154	

* More than 200 direct PFC or 100 indirect PFC per spleen. Four-fifths of all nucleated cells were used for the two plaque assays.

‡ 95% confidence intervals in parentheses.

As the number of grafted thymocytes increased from 6.2 × 10^5 to 4 × 10^7, the proportion of mice with positive spleens increased for both types of PFC. The spleens of some mice of every group were positive while others were negative; in most instances, spleens were either positive or negative for both direct and indirect PFC. The relation between the percentage of positive spleens and the number of thymocytes grafted did not vary greatly for the two classes of antibody-forming cells. The mean number of PFC per positive spleen did not increase proportionately to the number of grafted thymocytes, suggesting that similar numbers of ASU were formed in all positive spleens of the different groups.

The probability values for thymic ARC to give positive spleens after trans-

plantation with marrow cells were calculated per 10^6 donor thymocytes, using the Poisson model. The probability values were not significantly different for direct and indirect PFC and the 95% confidence intervals overlapped. The curve relating inoculum size to the expected frequencies of positive spleens is shown in Fig. 1, together with the observed frequencies. It was estimated that $\sim 10^7$ thymocytes contained one ARC that reached the recipient spleen on transplantation and formed anti-sheep ASU. The data of Table II and their statistical analysis indicate that the frequencies of ARC for direct and indirect

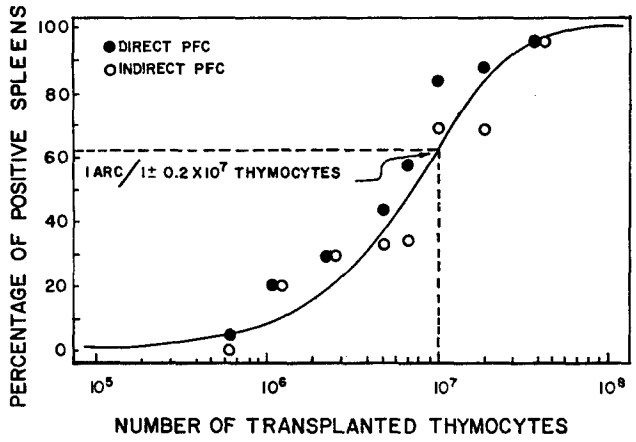

FIG. 1. Percentage of recipient spleens positive for direct and indirect PFC after injection of 4×10^7 marrow cells, 5×10^8 SRBC, and graded numbers of thymocytes into irradiated mice. The number of spleens assayed is given in Table II. Symbols indicate observed percentages and curve expected percentages according to the Poisson model. The curve was fitted to the pooled percentages of positive spleens for direct and indirect PFC, since probability values were overlapping.

PFC were equal and, therefore, suggest that the same ARC could form ASU for direct and/or indirect PFC. Whether each ARC formed one or more kinds of ASU will be inferred from dissociation or association of direct and indirect PFC in individual spleens (see next section). A critique of the use of limiting dilution assays to measure frequencies of potentially immunocompetent cells was made in two preceding papers (8, 9).

Lack of Class Differentiation of ARC.—If thymic ARC were specialized for antibody class and would thereby restrict the potential of ASU, then we would expect chance dissociation or independence of direct and indirect PFC responses in the spleens that were analyzed for the limiting dilution experiment. However, the same result would be expected if ARC were not specialized for antibody class but would interact at random with one kind of marrow P-PFC only, e.g., with marrow cells restricted to generate either direct or indirect PFC (10).

The ASU thus formed by a single ARC would be of one kind. On the other hand, lack of independence or association of the two immune responses in recipient spleens would suggest that ARC are not specialized for antibody class and that each ARC could interact with more than one kind of P-PFC. The number of ASU thus formed by a single ARC would be at least two, one for direct PFC and the other for indirect PFC.

Of the data presented in Table II, those of groups having substantial numbers of positive and negative spleens were subjected to a Chi-square test for independence of direct and indirect PFC responses. Table III summarizes the

TABLE III

Chi-Square Tests for Independence of Direct and Indirect PFC Responses in Recipients of 4×10^7 Marrow Cells, 5×10^8 SRBC, and Graded Numbers of Thymocytes

No. of thymocytes ($\times 10^6$)		1.25	2.5	5.0	7.0	10.0
No. of mice		24	24	25	36	26
Direct PFC	Indirect PFC					
+	+	5	6	8	11	18
+	−	0	1	3	8	4
−	+	0	1	0	1	0
−	−	19	16	14	16	4
χ^{2*}		18.32	11.67	11.81	8.70	7.14

+, positive spleen containing more than 200 direct, or 100 indirect PFC.

−, negative spleen containing fewer PFC.

* Chi-square values in the table were compared with 6.63, the critical value of χ^2 statistic at the 0.01 level of significance. All the comparisons are compatible with the hypothesis that direct and indirect PFC responses are not independent from each other.

tests. The values of 5 comparisons, including 135 mice, were above the critical value of 6.63, the Chi-square statistic at 0.01 level of significance for one degree of freedom. Therefore, there was statistical evidence for *lack of independence* or *association* of the two cellular responses. This was due, of course, to the relatively rare occurrence of spleens positive for one and negative for the other class of PFC. It follows that the results are compatible with the idea that ARC are not specialized and are capable of interacting with two kinds of P-PFC simultaneously or within a short interval.

Distribution of Direct and Indirect PFC in Recipient Spleens.—By transplanting 7×10^6 thymocytes into irradiated mice, 52.7% of recipient spleens were positive for direct PFC (Table II and Fig. 1). According to the Poisson model, this means that grafts of 7×10^6 thymocytes did not provide on the average one ARC to each recipient spleen. 4×10^7 marrow cells were added to the thymocytes to provide each recipient spleen with an excess of P-PFC (10). P-PFC are cells specialized for antibody class. The precursors of direct PFC are about 10

times more frequent than those of indirect PFC (16). If ARC could interact with more than one P-PFC, each ARC should be capable of forming more than one ASU. The spleens of mice given 7×10^6 thymocytes and marrow cells should then contain PFC in several focal areas, although only one or two ARC lodged in them. By definition, a *focal* area contains the progeny of one ASU, i.e., of one P-PFC that has interacted with ARC. In view of the 10-fold difference in the number of direct and indirect PFC precursors, the number of positive areas containing direct PFC (the progeny of the more frequent P-PFC) should be greater than that of areas containing indirect PFC.

Two experiments were performed to test these possibilities. First, groups of irradiated mice were grafted with 7×10^6 thymocytes and 1.6×10^7 marrow

TABLE IV

*Hemolytic Foci of Direct PFC in Spleens of Irradiated Mice Grafted with Thymocytes and Marrow Cells**

Cells transplanted + SRBC	No. of foci in each recipient spleen	Fraction positive spleens
1.6×10^7 marrow cells	0 0 0 0 0 0 0 0 0 0 0 1 2 2 3	(Controls)
1.6×10^7 marrow cells and 7×10^6 thymocytes	0 1 1 1 1 1 1 2 2 2 2 2 2 2 2 2 3 3 3 3 4 4 4 4 4 4 5 5 6	13/29‡ (44.8%)

* The numbers of cells grafted were chosen so as to provide recipient spleens either with one or two ARC, or with none. SRBC were injected 18 hr after the nucleated cells.

‡ Spleens with more than 2 foci. Compare with result of limiting dilution assay in Table II and Fig. 1.

cells for the determination 9 days later of the number of hemolytic foci per spleen containing direct PFC. SRBC were injected 18 hr after cell transplantation. The cells grafted provided recipient spleens with an average of less than one ARC (see in discussion), but with large numbers of P-PFC. Control mice were grafted with marrow cells only, to assess the number of "background" foci. Results are presented in Table IV.

One or two hemolytic foci were produced in some but not all spleens of irradiated mice grafted with marrow cells alone. Such foci were presumably due to rare, complete ASU, reactive with SRBC which are naturally present in murine marrow. They are regarded as equivalent to "background" PFC (Table I). The spleens of mice grafted with marrow cells and thymocytes fell into two categories: about half contained no foci or one or two, like control spleens; the others contained a greater number of foci ranging from three to six. We regarded the former spleens as negative; their frequency was as expected on the basis of

the preceding limiting dilution assay. The positive spleens contained more foci than would have been expected had each ARC generated one ASU by interaction with one P-PFC. In fact, the majority of positive spleens contained more than one focus above maximum "background" value. Several spleens contained at least three foci above background. Since the distribution of ARC in recipient spleens followed the Poisson model (Fig. 1), it is extremely improbable that so many spleens contained two or three ARC after transplantation of 7×10^6 thymocytes. Hence, ARC must have interacted with more than one precursor of direct PFC. We had already excluded in a preceding experiment (10) the possibility that interaction between ARC and P-PFC could be a repetitive

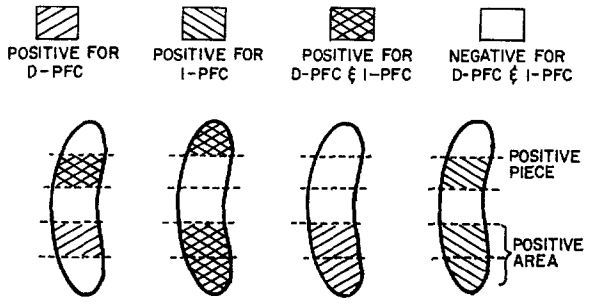

FIG. 2. Schematic representation of the distribution of direct and indirect PFC in discrete splenic areas. Irradiated mice were grafted with 7×10^6 thymocytes and 4×10^7 marrow cells. Each spleen was cut into 5 pieces as indicated, and the cells of every piece were assayed separately. D-PFC and I-PFC indicate direct and indirect plaque-forming cells, respectively.

event, similar to a chain reaction. Formation of discrete foci took place after the grafting of limiting numbers of thymocytes with 67 times the limiting number of marrow cells. Had ARC continued to interact with the excess of P-PFC, immunocytes would have been distributed throughout the spleen, rather than in focal areas.

The purpose of the second experiment was to determine whether one ARC, lodging in spleens after transplantation of thymocytes, could form ASU for direct and indirect PFC. Since the focus method can be used only to detect areas of hemolysis due to clusters of direct PFC, localized distribution of indirect PFC had to be verified by a different approach. The spleens of 36 mice grafted with 7×10^6 thymocytes and 4×10^7 marrow cells (included in Tables II and III) were cut transversally into five nearly equal pieces, as shown in Fig. 2. The cells of each piece ($1.5–2 \times 10^7$) were suspended and assayed separately for direct and indirect PFC. In 17 of the 20 spleens that were positive (Table III), direct and indirect PFC were localized in some but not in all pieces of a spleen (Table V). Within pieces, every possible combination of PFC was found, i.e.,

direct PFC only, indirect PFC only, and both types of PFC, as illustrated in Fig. 2. In some spleens, two or three contiguous pieces were positive; in others, individual positive pieces were separated by negatives. Contiguous positive pieces were regarded as one discrete area (Fig. 2), although it is likely that such

TABLE V

*Distribution of Direct and Indirect PFC in Different Splenic Areas after Transplantation of Thymocytes and Marrow Cells**

Mouse No.	No. of positive areas per spleen‡	No. of adjacent pieces per positive area	No. of PFC per positive area	
			direct PFC	indirect PFC
1	1	1	147	—
2	1	1	176	—
3	1	2	177	—
4	2	1	117	—
		1	132	—
5	1	2	236	—
6	1	3	306	—
7	1	2	441	—
8	2	2	372	—
		1	148	104
9	2	1	138	—
		1	204	324
10	2	1	108	—
		3	300	552
11	1	1	136	120
12	1	3	351	84
13	1	4	516	288
14	2	1	366	60
		2	456	234
15	2	1	156	76
		2	392	980
16	2	2	360	396
		2	—	372
17	2	1	—	294
		2	—	532

* 9–11 days after 7×10^6 thymocytes and 4×10^7 marrow cells. SRBC 18 hr after cell grafts.

‡ Only spleens containing a total of more than 200 direct PFC or 100 indirect PFC were regarded as positive and, therefore, could contain positive areas.

areas contained more than one hemolytic focus of PFC. This was, in fact, suggested by the number of direct PFC per area, which was often considerably greater than 150, the average number of direct PFC per focus (10). In 3 of the 20 spleens, all 5 pieces were positive for direct PFC. These spleens were excluded from further consideration because discrete positive areas could not be iden-

tified. The distribution of direct and indirect PFC in discrete positive areas of 17 spleens is shown in Table V.

In 9 spleens (No. 8–16), 11 discrete areas were positive for both direct and indirect PFC, 3 for direct PFC, and 1 for indirect PFC. In seven other spleens (No. 1–7), eight discrete areas were positive for direct but not for indirect PFC. One spleen (No. 17) contained two discrete areas positive for indirect PFC only. Altogether 25 areas were identified, 22 containing direct PFC, and 14 indirect PFC. 14 positive areas contained a multiple of 150 direct or indirect PFC, suggesting more than one hemolytic focus per such area. An estimate of the total number of foci in the positive areas was made under the assumption of 150 PFC/focus: 35 foci of direct PFC and 28 of indirect PFC. Since these foci were formed in 17 spleens, the multiplicity factor was 3.7. The results confirm that several discrete areas with PFC are formed after immunization in spleens seeded by one ARC and numerous P-PFC. The results also indicate that areas either with direct or indirect PFC can be formed under these conditions. The greater frequency of areas with direct PFC or with both types of PFC was anticipated, since the precursors of direct PFC are more frequent than those of indirect PFC in marrow. It follows that ARC can interact with more than one P-PFC without regard to class differentiation of the latter.

DISCUSSION

We have previously presented evidence that unipotent ASU were formed upon administration of SRBC to irradiated mice reconstituted with grafts of marrow cells and thymocytes (10). Such ASU generated cells which synthesize antibody of one molecular class. The restriction appeared to have originated in specialized marrow precursors of immunocytes (10, 16). However, thymic ARC could also have played a role in determining class restriction of ASU. The present experiments were designed to answer the questions whether (*a*) ARC are specialized for antibody class, and (*b*) if they interact with one or more marrow, immunocompetent cells without regard to class restriction of the latter.

It has been shown that transplantation of small numbers of lymphoid cells in limiting dilution assays provided a reproducible method for enumerating potentially immunocompetent cells in heterogeneous populations (8, 9, 17). By such a procedure, we have estimated that $\sim 10^7$ thymocytes of 10–12 wk old (C3H $\times$ C57BL)F_1 female mice contain one ARC reaching the recipient spleen (after transplantation) and interacting with marrow P-PFC. In view of the dilution of ARC in the recipient's body, the number of ARC of thymus cell suspensions must have been greater, perhaps by 10–100 fold (17, 18). Even so, the frequency of thymic ARC was rather low when measured by immune responses requiring cooperation with marrow cells. This has also been noted by Mitchell and Miller (6). After immunization with SRBC, the number of foci of direct PFC produced by grafts of marrow-thymus cells was considerably smaller than that of foci

produced by grafts of marrow-thoracic duct cells. In our experiments, the frequency of thymic ARC has been estimated from the direct and indirect PFC responses elicited by thymus-marrow cell mixtures. The values obtained were so close to suggest that the same ARC participated in both responses.

Direct and indirect PFC were frequently associated in spleens of the limiting dilution experiment. This observation was important because one, or at most two, ARC should have lodged in such spleens. Association of the two antibody classes was interpreted to mean that individual ARC were not restricted to participate in interactions leading to a single molecular class of antibody. On the contrary, ARC interacted with P-PFC capable of generating IgM or IgG hemolytic antibody. Since the majority of marrow precursors of PFC are restricted either to direct or to indirect PFC (16), individual ARC or their descendant cells must have interacted with more than one kind of P-PFC in several instances. Had ARC been restricted for antibody class, most recipient spleens should have contained either direct or indirect PFC, but not both. Furthermore, had nonrestricted ARC interacted with one P-PFC or exclusively with P-PFC of one kind, most recipient spleens should also have contained one type of PFC only. The results obtained were obviously incompatible with the last two possibilities.

The above conclusions rested mainly on Chi-square tests for independence of immune responses, i.e., on statistical evidence. Another critical consideration was the following: the frequency distribution of ARC in spleens of mice grafted with graded numbers of thymocytes conformed very closely to the Poisson model. This meant that after grafting $1 \times 10^7 \pm 0.2$ thymocytes into several mice, the spleens of 37% did not receive even one ARC (negative spleens) while those of 63% received one or more ARC, and became positive for PFC under our experimental conditions. The Poisson model also predicts that 37% of the spleens would contain one ARC, 18% two ARC, and 6% three ARC. The latter predictions were not testable by the limiting dilution assay nor by the test of independence which was done. To strengthen our conclusions, the multiple focal distribution of PFC was observed directly in spleens of two groups of mice grafted with the appropriate number of thymocytes and marrow cells. The hemolytic foci per spleen and the areas positive for direct and for indirect PFC were considerably more than one in most spleens that should have contained one or two ARC. Thus, visible evidence was provided for the multiplicity of P-PFC triggered into PFC production by one thymic ARC or their progeny.

To date, only immune responses to heterologous erythrocytes (2, 3, 6, 7, 10) and to bovine serum albumin (19) have been shown to require cooperation of thymus-derived and marrow-derived cells in mice. Responses to other antigens, such as those of *Salmonella adelaide* flagellin, do not require thymocytes and can be effected by marrow cells alone (20). The list of antigens that either require or do not require thymic ARC for immunogenicity is likely to become

longer in the near future. It may overlap with the list of antigens that are either weakly or fully immunogenic in neonatally thymectomized mice (21). At the present time it is reasonable to postulate that thymic ARC are involved in the initiation of the immune response so as to offer information concerning specificity to marrow precursors of immunocytes. For antigens of SRBC, thymocytes must be viable to cooperate effectively with marrow cells (22), and perhaps engage in proliferative activity (23). Davies et al. have observed a wave of mitosis in thymus-derived cells preceding antibody formation against SRBC. However, it remains to be established that ARC have participated in the mitotic response to antigens, since they represent such an infrequently found cell among thymocytes. To understand the function of thymocytes it would be extremely useful to know whether ARC are committed to react with particular antigens and eventually confer specificity restriction to ASU. Available evidence is indirect and ambiguous, since thymic ARC are the targets of specific tolerogenic injections of antigens (19, 24) and of nonspecific immunosuppressive injections of antilymphocyte serum (25, 26). Such serum abrogates the response in mice to SRBC, but not that to hemocyanin (27), an antigen which elicits normal responses in thymectomized mice (21).

Whatever the function of thymic ARC may be, they interacted with several marrow P-PFC without controlling the molecular class of the antibody to be secreted. Conceivably, progeny cells of thymic ARC could have interacted with individual P-PFC. The availability of specialized P-PFC may have solely determined the kind of ASU formed. In our chimeras, the number of P-PFC supplied was far greater than necessary for ASU formation, so that each ARC was enabled to interact not only with many P-PFC, but also with both kinds of P-PFC under study. Several ASU were therefore formed by each ARC. Had the number of P-PFC been not so great, relative to that of ARC, fewer ASU would have been formed by each ARC and interaction would have occurred more frequently with precursors of direct PFC only. The latter are about 10 times more numerous than the precursors of indirect PFC (16). The situation postulated above actually occurs in the spleens of intact mice: P-PFC are relatively infrequent, since mixtures of marrow and spleen cells produce more PFC than do spleen cells alone (28), and ASU for direct PFC are seven times more numerous than those for indirect PFC (8).

SUMMARY

Thymocytes and marrow cells of unprimed donor mice were mixed in vitro and transplanted into X-irradiated syngeneic mice. 18 hr later, sheep erythrocytes were injected to induce immune responses. Splenic plaque-forming cells (PFC) secreting IgM (direct PFC) or IgG (indirect PFC) hemolytic antibody were enumerated at the time of peak responses.

By transplanting graded and limiting numbers of thymocytes with 4×10^7

marrow cells, inocula were found which contained one or a few thymic antigen-reactive cells (ARC) reaching the recipient spleens, interacting with marrow cells, and inducing PFC formation. The frequency values of ARC inferred from direct and indirect plaque assays were very similar, 1 in $\sim 10^7$ thymocytes. Furthermore, statistical analysis indicated that the formation of direct PFC was not independent of the formation of indirect PFC. This was interpreted to mean that ARC were not specialized themselves and did not determine the molecular class of antibody to be secreted after interaction with marrow cells.

Spleens of thymus-marrow grafted mice containing one or two ARC and non-limiting numbers of marrow precursors of PFC (P-PFC), had direct and indirect PFC clustered in several focal areas. Assuming that each focal area represented the progeny of one P-PFC that had interacted with ARC, these results confirmed the statistical evidence for lack of class differentiation in thymic ARC, and also indicated that each ARC or its progeny cells interacted with more than one P-PFC of either class.

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