CELLULAR DIFFERENTIATION OF THE IMMUNE SYSTEM OF MICE

V. Class Differentiation in Marrow Precursors of Plaque-Forming Cells*

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Precursors of immunoglobulin-synthesizing cells are found in bone marrow of adult mice (1). Upon injection of sheep erythrocytes or bovine serum albumin, immunocompetent marrow cells generate specific antibody-forming descendents with the cooperation of thymic antigen-reactive cells (2–6). Such cooperation is not necessary when immunocyte production is initiated by other antigens, e.g., purified *Salmonella adelaide* flagellin (7). Precursors of immunocytes have been studied by transplantation of marrow into irradiated mice. Mature progeny cells can be recognized either by genetic markers of immunoglobulins (8) or by the specificity of the antibody produced (2–7, 9).

The frequencies of precursors of anti-sheep hemolytic plaque-forming cells (PFC)[1] and of hemagglutinating cluster-forming cells differ in marrow of unprimed adult mice (9). The frequency values decrease in the following order: precursors of direct or IgM PFC, of indirect or IgG PFC, and of cluster-forming cells. Under the conditions of transplantation, precursors of PFC (P-PFC) and of cluster-forming cells generate their progeny immunocytes independently of each other. Furthermore, P-PFC often generate direct but not indirect PFC. These findings suggest that the marrow precursors are differentiated or specialized for molecular class of antibody. This suggestion is strengthened by the realization that thymic antigen-reactive cells (ARC) are not specialized for the molecular class of antibody to be produced by descendents of the cooperating marrow cells (10). It is well established that the functional unit resulting from interaction of marrow- and thymus-derived cells (antigen-sensitive unit or ASU) is restricted to produce antibody of a single molecular class

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[1] The following abbreviations are used: PFC, plaque-forming cells; P-PFC, precursors of plaque-forming cells; ARC, antigen-reactive cells of thymic origin; ASU, antigen-sensitive unit; SRBC, sheep erythrocytes.

(9, 11, 12). The source of this restriction of ASU could have been class differentiation of marrow precursors of immunocytes. Such a characteristic of P-PFC may result from differentiation within marrow cell lines or may be acquired through interaction with a class-restricted cell other than thymic ARC.

In the present study we have reinvestigated class differentiation of P-PFC. An estimate of the frequencies of specialized precursors of immunocytes secreting either IgM or IgG hemolytic antibody was attempted. Graded and limiting numbers of marrow cells were mixed with a large nonlimiting number of thymocytes and transplanted into heavily irradiated mice. The cell mixtures were then exposed to SRBC and the immune responses elicited were assessed in terms of direct and indirect PFC. If marrow P-PFC were not specialized for antibody class, the probabilities of formation of direct or indirect PFC should be equal. Otherwise, the probabilities of direct and indirect PFC formation could be unequal and would then reflect different frequencies of specialized 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 (11).

Transplantation, Immunization, and Plaque Assays.—The methods used for preparing and transplanting cell suspensions, for immunization against SRBC, and for enumerating hemolytic plaque-forming cells were described in the preceding papers of this series (9–11).

Experimental Design and Statistical Methods.—Limiting dilution assays were performed to estimate the frequencies of P-PFC in marrow cell suspensions. Since marrow and thymus cells are both necessary for ASU formation and PFC production, graded numbers of marrow cells were added to a fixed nonlimiting number of thymocytes for transplantation. This was done to reduce the number of marrow-P-PFC to about one per recipient spleen and to provide, at the same time, adequate numbers of thymocytes. If all P-PFC had the same probability of reaching recipient spleens on transplantation, interacting with ARC and antigens, and, consequently, of generating PFC, then the relationship between percentage of spleens with PFC and number of marrow cells grafted should be described by the Poisson model. The statistical procedure followed for comparing observed and expected results has been already described (11). In addition, the "sign" test (13) was used to compare the proportions of spleens which were positive by the direct and indirect plaque assays in mice grafted with graded numbers of marrow cells.

RESULTS

PFC Responses in Control Mice.—Mouse marrow and thymus contain a relatively small number of ASU reactive with antigens of SRBC (9-10). These "background" ASU may have been formed in response to cross-reacting antigens of the intestinal flora, or may represent contamination of marrow cell suspensions by blood-borne thymus derived cells and vice versa. For this reason we assessed the PFC responses elicited either by grafts of 0.5–2 × 10^7

marrow cells, or of 2×10^8 thymocytes in irradiated control mice. Responses elicited by 4×10^7 marrow cells or 5×10^7 thymocytes have been reported earlier (10). SRBC were injected 18 hr after cell transplantation, and plaque assays (direct and indirect) were performed on recipient spleen cells 9–11 days after grafting. Results are presented in Table I.

Spleens of all the mice injected with marrow cells or thymocytes contained direct PFC, whereas only a fraction contained indirect PFC. The numbers of PFC per spleen were rather small in recipients of marrow cells and did not exceed 90 direct or 30 indirect PFC. In recipients of 4×10^7 marrow cells the number of "background" PFC per spleen was greater (10), as in recipients of

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 ±SE*	
		direct PFC	indirect PFC
$0.5\text{–}2 \times 10^7$	0	20/20 45 ± 7	5/20 14 ± 5
0	2×10^8	20/20 211 ± 33	13/20 135 ± 40

* Two-fifths of all nucleated spleen cells were used for each assay, 9–11 days after transplantation

‡ Background PFC values after transplantation of 5×10^7 thymocytes or 4×10^7 marrow cells were reported earlier (10).

2×10^8 thymocytes. In the latter spleens, direct PFC were usually less than 300, but occasionally (3 spleens of 20) rose to 450. Indirect PFC were less than 200 per spleen. In recipients of 5×10^7 thymocytes, the number of background PFC per spleen was considerably smaller (10).

Frequency of Direct and Indirect PFC Responses in Recipients of Graded Numbers of Marrow Cells.—To assess P-PFC in marrow of unprimed mice, a range of graded numbers of nucleated marrow cells mixed with 5×10^7 thymocytes from syngeneic donors were injected into groups of irradiated recipients totaling 594 mice (limiting dilution assays). 5×10^8 washed SRBC were given to each mouse 18 hr later. Individual spleens were assayed for their content of direct and indirect PFC either 9–11 days after grafting, at the time of expected peak responses (9), or 25 days after grafting. The number of thymocytes was chosen so as to provide every recipient with a multiple of the number of ARC necessary for ASU formation. Portions of the recipient spleen cells amounting to one-fifth of the organ were plated in duplicate for each plaque assay. Spleens were regarded as positive if the number of PFC exceeded the maximum number

of background PFC found in control mice. Otherwise, the spleens were regarded as negative. The results are presented in Tables II and III and in Fig. 1.

As the number of grafted marrow cells increased from 5×10^2 to 5×10^7, the proportion of mice with spleens positive for PFC also increased. Virtually

TABLE II

Percentage of Recipient Spleens Positive for Direct PFC 9–11 Days After Infusion of Thymocytes, 5×10^8 SRBC, and Graded Numbers of Marrow Cells

No. of thymocytes transplanted	No. of marrow cells transplanted	Fraction of positive spleens	Percentage of positive spleens	Mean no. of PFC per positive spleen ±SE
5×10^7	5×10^2	2/15*	13.3*	125 ± 15
	1×10^3	2/12	16.7	260 ± 90
	5×10^3	3/12	25.0	200 ± 30
	1×10^4	8/32	25.0	160 ± 13
	2.5×10^4	7/16	43.7	260 ± 37
	5×10^4	10/19	52.7	315 ± 62
	1×10^5	12/21	57.2	255 ± 49
	3×10^5	45/50	90.0	600 ± 74
	3×10^5	11/15‡	73.4‡	340 ± 59
	6×10^5	32/36	88.8	770 ± 87
	6×10^5	9/9‡	100.0‡	360 ± 88
	1×10^6	47/52	90.4	534 ± 262
	2.5×10^6	53/53	100.0	1150 ± 135
	5×10^6	36/36	100.0	1410 ± 160
	1×10^7	99/100	99.0	700 ± 49
	2×10^7	30/31	96.7	1480 ± 125
	3×10^7	34/34§	100.0§	2450 ± 228
	4×10^7	26/26	100.0	2150 ± 164
	5×10^7	24/25	96.0	2810 ± 259
2×10^8	5×10^3	2/10‖	20.0‖	615 ± 110
	1×10^4	8/21	38.1	464 ± 45
	2.5×10^4	2/8	25.0	342 ± 32
	5×10^4	10/14	71.5	956 ± 120
	1×10^5	18/25	72.0	973 ± 210
	2×10^5	13/14	92.8	1250 ± 403

* For grafts of 5×10^2–2×10^7 marrow cells, recipient spleens with >100 direct PFC were regarded as positive (Table I).

‡ Assayed 25 days after cell transplantation.

§ For grafts of 3–5 $\times 10^7$ marrow cells, spleens with >200 direct PFC were regarded as positive (10).

‖ For grafts of 2×10^8 thymocytes, spleens with >300 direct PFC were regarded as positive (Table I).

all spleens were positive for direct PFC when 3 or 6×10^5 marrow cells were grafted. However, such grafts did not yield as many positive spleens for indirect PFC. The inoculum size necessary for nearly 100% positive spleens for indirect PFC was about 20 times greater, i.e., $\sim 10^7$ marrow cells. In each group of

mice receiving an inoculum of less than 4×10^7 marrow cells, the spleens of some recipients were positive, in that they contained significant numbers of PFC, while others were negative. Most spleens were either positive or negative for both types of PFC, or positive for direct but negative for indirect PFC.

TABLE III

Percentage of Recipient Spleens Positive for Indirect PFC 9–11 Days After Infusion of Thymocytes, 5×10^8 SRBC, and Graded Numbers of Marrow Cells

No. of thymocytes transplanted	No. of marrow cells transplanted	Fraction of positive spleens	Percentage of positive spleens	Mean No. of PFC per positive spleen ±SE
5×10^7	5×10^2	0/15*	0*	—
	1×10^3	0/12	0	—
	5×10^3	1/12	8.3	150
	1×10^4	3/32	9.4	370 ± 122
	2.5×10^4	1/16	6.3	200
	5×10^4	4/19	21.1	325 ± 210
	1×10^5	5/21	23.8	110 ± 17
	3×10^5	22/50	44.0	340 ± 76
	3×10^5	5/15‡	33.3‡	154 ± 59
	6×10^5	23/26	63.9	290 ± 39
	6×10^5	6/9‡	66.7‡	202 ± 37
	2.5×10^6	41/53	77.3	330 ± 52
	5×10^6	26/36	72.3	470 ± 23
	2×10^7	21/31	67.7	1040 ± 341
	3×10^7	26/34§	76.5§	2400 ± 282
	4×10^7	26/26	100.0	1440 ± 109
	5×10^7	24/25	96.0	1480 ± 187
2×10^8	5×10^3	1/10‖	10.0‖	305
	1×10^4	1/21	4.8	785
	2.5×10^4	0/8	0	—
	5×10^4	4/14	28.7	690 ± 89
	1×10^5	10/25	40.0	913 ± 190
	2×10^5	4/14	28.7	894 ± 418

* For grafts of 5×10^2–2×10^7 marrow cells, recipient spleens with >50 indirect PFC were regarded as positive (Table I).

‡ Assayed 25 days after cell transplantation.

§ For grafts of 3–5×10^7 marrow cells, spleens with >100 indirect PFC were regarded as positive (10).

‖ For grafts of 2×10^8 thymocytes, spleens with >200 indirect PFC were regarded as positive (Table I).

Only 2 of 442 spleens contained indirect but not direct PFC. It follows that the proportion of positive spleens for direct PFC exceeded that of positive spleens for indirect PFC in 13 of 15 groups. In the remaining two groups, these proportions were equal. Therefore, the relation between the percentage of positive spleens and the number of marrow cells grafted differed for the two types of

antibody-forming cells studied (Fig. 1). Using the sign test (13) to compare the percentages of positive spleens for direct and indirect PFC after transplantation of 15 different cell inocula, a chi-square (χ^2) value of 9.6 was obtained. For one degree of freedom, this indicated that the differences between frequencies of direct and indirect PFC responses were statistically significant ($P < 0.01$).

Mice injected with 3 and 6 $\times$ 10^5 marrow cells were assayed 25 days after grafting as well as at 9–11 days. This was done to ascertain whether the proportion of negative spleens changed during this interval. Immune responses could have been delayed in mice grafted with relatively few immunocompetent cells. However, the results obtained (Tables II and III) ruled out this possibility, since the proportion of negative spleens was about the same at both intervals.

The mean number of PFC per positive spleen increased with the number of grafted marrow cells, but not in linear fashion. This was probably due to the fact that (*a*) similar numbers of ASU were formed after grafting several graded numbers of marrow cells, and (*b*) each ASU produced a fixed number of PFC. For direct PFC, this number has been estimated to be $\sim$ 150 (9). The data of Table II suggest that in spleens of mice grafted with up to 10^6 marrow cells one or two ASU were formed, but multiples thereof in spleens of mice grafted with the larger inocula. We expected and actually verified that the rate of increase of the number of PFC per spleen should have been greatest in groups of mice with nearly 100% positive spleens. Each thymic ARC can interact with more than one P-PFC (10). When the latter become numerous, as in recipients of the larger marrow inocula, considerably more ASU and, hence, PFC will be formed.

The results of limiting dilution assays indicated that marrow of unprimed mice contains at least two types of P-PFC differing in potential and frequency. The P-PFC more frequently found generated direct but not indirect PFC upon interaction with thymic ARC and antigen. The less frequently found P-PFC generated indirect PFC, but the assays used could not determine whether the latter P-PFC were unipotent or competent to generate both indirect and direct PFC. The greater frequency of the precursors of direct PFC made it impossible to assess the progeny of the precursors of indirect PFC in the absence of the former.

It was assumed in designing the experiments that: (*a*) P-PFC are single cells independent of each other and of other types of cells in generating PFC; and (*b*) after transplantation, P-PFC reach the recipient spleen and interact with available ARC at random, i.e., with equal probability. If these assumptions were correct, the frequency distribution of positive spleens in relation to the number of marrow cells grafted should have been described by the Poisson model. Fig. 1 indicates that the observed frequencies of positive spleens did not

conform to the Poisson model for both direct and indirect PFC. The observed increments in percentage of positive spleens with increasing numbers of marrow cells grafted were far below expectation. Hence, at least one of the assumptions made must have been incorrect.

The random distribution of potentially immunocompetent cells reaching the spleens of irradiated mice and the all-or-none response to antigen have been demonstrated in at least three different laboratories (10–12, 14, 15). The availability of ARC for interaction with P-PFC was ensured by adding to marrow cells five times as many thymocytes as necessary to provide each recipient

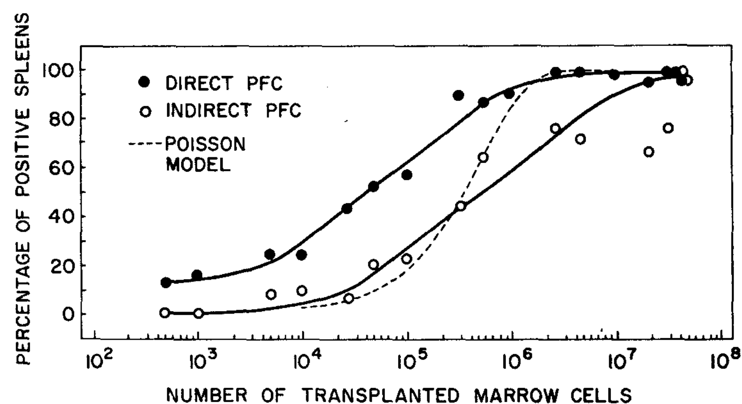

FIG. 1. Percentage of recipient spleens positive for direct and indirect PFC after injection of irradiated mice with 5×10^7 thymocytes, 5×10^8 SRBC, and graded numbers of marrow cells. The numbers of spleens for each point are shown in Tables II and III. Symbols indicate observed percentages and solid lines the curves eye-fitted to the data. The shape of Poisson curves is indicated by the dashed line.

spleen with an average of one ARC (10). In addition, we grafted a few groups of mice with 2×10^8 thymocytes instead of 5×10^7, and added graded numbers of marrow cells. This was done to determine whether the divergence of the curves from the Poisson model was due to inadequate numbers of thymocytes (Tables II and III, lower sections). Even under these conditions, the frequencies of positive spleens as a function of marrow cells number differed from those predicted by Poisson statistics. The remaining assumption made was that P-PFC were independent of each other and of other types of cells. This is to say that the interaction between ARC and P-PFC required only one marrow cell to form ASU. It is possible that this was an incorrect assumption, since it is known that two and possibly more distinct cell types are necessary for in vitro anti-SRBC responses by fractionated spleen cells (16, 17).

If ASU formation involved more than one marrow cell, only one cell type might have been present in adequate numbers at the time of grafting. We

therefore delayed the injection of 5×10^7 thymocytes and antigen. The latter cells were given to 31 irradiated mice 2, 3, or 4 days after the injection of 5×10^4 marrow cells. 12 control mice received thymocytes and marrow cells simultaneously. Assays for direct and indirect PFC were performed 9 days after immunization. The percentage of positive spleens did not increase as a result of delayed thymocyte injection. Thus, marrow cells, within 4 days of grafting, do not produce by differentiation or maturation cells that facilitate ASU formation.

DISCUSSION

We have previously presented evidence that restricted ASU were formed upon administration of SRBC to irradiated mice reconstituted with grafts of marrow cells and thymocytes (9). The restriction studied concerned the ability of ASU to generate either hemolytic PFC or hemagglutinating cluster-forming cells, but not both. In addition, ASU were often competent to generate direct PFC, but not indirect PFC, suggesting that these ASU were unipotent. ASU generating indirect PFC always did so in the presence of direct PFC. The two classes of immunocytes could have arisen either from the same ASU or from separate unipotent ASU. In the latter case, the simultaneous production of direct and indirect PFC could be explained by greater frequency of direct PFC precursors (9). However, by analogy with splenic ASU, it is likely that both classes of marrow-thymus ASU were unipotent (12).

Under the conditions of transplantation, ASU were formed from injected thymocytes and marrow cells without participation of reproductively viable cells of the irradiated host. Therefore, ASU restriction for antibody class could have originated from specialization of viable precursor cells residing in one or both donor tissues, or from specialization of some irradiated host cell participating in ASU formation. Experiments described in the preceding paper of this series demonstrated that thymic ARC interacted with marrow P-PFC without regard to class, and failed to confer class restriction upon ASU thus formed (10). If thymic ARC are not differentiated for class of anti-SRBC antibody, it is inescapable that P-PFC and/or other interacting cells must be so differentiated. The present experiments were designed to answer the questions of (*a*) whether separate and specialized marrow cells give rise to direct and indirect PFC after interaction with thymocytes and antigen, and (*b*) whether ASU formation involves a single event, or more complex interactions.

Limiting dilution assays have provided answers to questions concerning the potential of splenic ASU and thymic ARC (10-12). By this procedure we have now found that marrow precursors of PFC are indeed specialized cells for antibody class. In marrow cell suspensions of adult unprimed donors, precursors of direct or IgM PFC were unipotent and $\sim$ 15 times more frequent than precursors of indirect or IgG PFC. We have not formally proved that the latter P-PFC were also unipotent. However, by analogy with splenic ASU (12), which

are composed of marrow- and thymus-derived cells, marrow precursors of indirect PFC are likely to be unipotent. We were unable to estimate the absolute frequency of P-PFC because the relation between the number of marrow cells grafted and the number of responding mice did not conform to the most plausible and simple Poisson model. This is interpreted to mean that the cellular interactions leading to PFC formation are more complex than assumed in designing these limiting dilution assays. Since thymic ARC conform to the predictions of the Poisson model in limiting dilution assays (10), it is probable that the participation of more than one kind of marrow cell or of irradiated host cells, or more than one discrete event are required for productive interaction with ARC. Several possibilities can be visualized if it is only postulated that sequential involvement of more than one cell of the same or of different types takes place. Furthermore, the sequence of events in these experiments could have been influenced by the contact of ARC and marrow cells in vitro. For example, ARC could have interacted in vitro with variable numbers of marrow cells so as to yield similar numbers of positive spleens on subsequent transplantation, even when numbers of marrow cells were increased. Although none of these complex models have yet been tested, the simple one-event interaction has been ruled out by the failure of the Poisson model to predict the results of limiting dilution assays for P-PFC. It remains to be established whether cells of the irradiated host, although unable to proliferate, play some role in ASU formation, perhaps by influencing class differentiation of P-PFC.

Regardless of the nature of the interaction between thymic ARC, immunocompetent marrow cells, and possibly other cell types, information for the molecular class of antibody resides in cells of bone marrow, presumably in the immediate precursors of immunocytes. The specialized marrow cells interact with thymic ARC without regard to the class of PFC they can generate, and under appropriate conditions precursors of direct and indirect PFC interact with the same ARC (10). If differentiation for antibody specificity were independent of class-differentiation, then molecular class profiles and allotypic markers should assort independently of immunoglobulin specificities. Weiler and Weiler (18) found, however, that the commitments of cells to synthesize immunoglobulins of given allotype and specificity are associated in cell lines rather than independent. This finding emphasizes the importance of assessing specificity differentiation in thymic and marrow immunocompetent cells, so as to uncover possible relationships with class differentiation in a system requiring cell-to-cell interactions. Ultimately, such studies should provide insight into the mechanisms by which heterogeneity of antibodies and of immune responses is generated.

SUMMARY

Marrow cells and thymocytes of unprimed donor mice were mixed in vitro and transplanted into X-irradiated syngeneic hosts. 18 hr later sheep erythro-

cytes 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 marrow cells with 5×10^7 thymocytes, inocula were found that contained few precursors of PFC (P-PFC) reaching the recipient spleens, interacting with thymocytes, and generating PFC. However, the frequency of responses in relation to the number of grafted marrow cells did not follow Poisson statistics, presumably because the interaction of marrow cells with thymocytes was more complex than a single or a one-to-one cell event. The frequency of direct PFC responses was greater than that of indirect PFC responses in 13 of 15 groups of mice tested. This was interpreted as evidence for the existence of two classes of P-PFC, each of which was restricted to generate either direct or indirect PFC. The precursors of direct PFC were $\sim$ 15 times more frequent than those of indirect PFC. Since thymic antigen-reactive cells were not differentiated for antibody class, it follows that antigen-sensitive units reactive to sheep erythrocytes owe their class restriction to specialized marrow cells. Specialization of P-PFC may have arisen within marrow cell lines by differentiation, or may have been conferred upon P-PFC by interaction with other cells, including those of the irradiated host.

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