CELLULAR DIFFERENTIATION OF THE IMMUNE SYSTEM OF MICE*

VI. Strain Differences in Class Differentiation and Other Properties of Marrow Cells

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Primary immune responses by dissociated spleen cells require participation of at least three distinct interacting cell types in vivo and in vitro (1–6). Experimental recombination of spleen cells separated by density gradient centrifugation (4–6) detected an even greater number of potentially immunocompetent cells with different functions and an unsuspected complexity of interactions. Limiting dilution assays of bone marrow cells, cooperating with thymocytes in an immune response to antigens of SRBC,[1] gave results consistent with the spleen cell separation studies (7). In irradiated mice injected with small numbers of marrow cells and an excess of thymocytes, the production of plaque-forming cells (PFC) was not limited by a single event, but by a multiplicity of events, presumably requiring participation of several marrow cells with different functions or in different stages of maturation. For the purpose of dissecting the immune response, marrow–thymus cell mixtures seemed to be as complex as spleen cell suspensions, although cells of the former organs should have been less differentiated and mature.

In the current study, functions of immunologically relevant marrow cells were analyzed by the limiting dilution approach in mice of three different strains. Dominant genes regulate antibody formation to specific (synthetic) antigens in rodents (8) by influencing in some yet unknown way the competence and/or number of cells necessary for antibody formation (9–11). Less pronounced variations occur in inbred strains of mice also for the PFC response to SRBC and other widely distributed natural antigens (12–16). It is conceivable, therefore, that differentiation and/or maturation of potentially immunocom-

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[1] *Abbreviations used:* ARC, antigen-reactive cells of thymic origin; ASU, antigen-sensitive units; PFC, plaque-forming cells; SRBC, sheep erythrocytes.

petent cells are under genetic control for functions relating to antibody specificity and to other aspects of antibody formation. The experiments to be reported support this view since differences were detected between bone marrow cells of (C57BL/6 × DBA/2)F_1 mice on the one hand, and of (C3H × C57BL)F_1 and (C57BL/10 × WB)F_1 mice on the other, although all three hybrids were good responders to antigens of SRBC. The functional properties under study were restriction of marrow cells for the class of antibody to be synthesized by mature immunocytes, and complexity of cell-to-cell interactions in anti-SRBC responses limited by marrow cells.

Materials and Methods

Mice.—(C3H/He × C57BL/Ha)F_1, (C57BL/6 × DBA/2Cr)F_1, and (C57BL/10Cz × WB/Re)F_1 female mice, 9–12 wk old, were used as donors and recipients in syngeneic or allogeneic cell transfers. The abbreviated designations for the three hybrid strains are $C3BF_1$, BDF_1, and BWF_1.

Irradiation.—Mice to be grafted with marrow cells and/or thymocytes were exposed to 800–850 R of total body X-radiation as described elsewhere (17, 18).

Cell Suspensions, Transplantation, and Immunization.—Nucleated bone marrow and thymus cells of normal mice, suspended in Eagle's medium, were counted and injected into a lateral tail vein of irradiated mice as previously described (17, 18). 5×10^8 washed sheep erythrocytes were injected intravenously 18–24 hr after transplantation.

Assays for Plaque-Forming Cells.—Direct and indirect PFC were enumerated in spleen cell suspensions of recipient mice by the agar gel method of Jerne, as described (17, 18). Indirect PFC were developed by polyvalent rabbit or goat anti-mouse gamma globulin antisera, at dilutions inhibitory for 60–80% of direct PFC.

Statistical Methods.—The Poisson model was used to describe the theoretical probability that known 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 (17, 18). Whenever the results were not adequately described by the Poisson model, the "sign" test (19) was used to compare the proportions of positive spleens by the direct and indirect plaque assays.

RESULTS

Frequency of Direct and Indirect PFC Responses in Syngeneic Recipients of Graded Numbers of BDF_1 Marrow Cells.—The limiting dilution assay of marrow precursors of PFC or of other accessory cells required for PFC production was made as follows. Graded numbers of syngeneic marrow cells in the range of $0.37–6 \times 10^5$ were injected into groups of irradiated (800 R) BDF_1 mice along with a fixed number of 5×10^7 syngeneic thymocytes. A total of 98 mice were grafted in two repeated experiments. 5×10^8 SRBC were given the next day, and individual spleens were assayed for their content of direct and indirect PFC at the time of peak response, i.e., 9–10 days after transplantation (18). The number of BDF_1 thymocytes was chosen on the basis of previous experiments (20), so as to provide every recipient with a large excess of antigen-re-

active cells (ARC) necessary for formation of antigen-sensitive units (ASU). Portion of the recipient spleens amounting to one-fifth of the organ were plated in duplicate for each plaque assay. Spleens of control mice injected with only one cell type (either marrow cells or thymocytes) and antigen contained less than 100 direct and 50 indirect PFC. Responses elicited by adequate numbers of marrow and thymus cells largely exceeded these values. Therefore, spleens

TABLE I

Percentage of Positive BDF_1 Recipient Spleens after Infusion of 5×10^7 Syngeneic Thymocytes, 5×10^8 SRBC, and Graded Numbers of BDF_1 Marrow Cells

No. of marrow cells 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 marrow cells
$\times 10^6$				
			Direct PFC	
0.37	1/15	6.7	235	
0.75	7/20	35.0	292 ± 86	3.49
1.50	8/17	47.1	268 ± 70	(2.57–4.41)‡
3.00	11/15	73.4	447 ± 97	
6.00	25/31	80.6	660 ± 78	
			Indirect PFC	
0.37	2/15	13.3	170 ± 50	
0.75	5/20	25.0	156 ± 33	3.18
1.50	9/17	52.8	176 ± 22	(2.24–3.92)‡
3.00	10/15	66.7	280 ± 18	
6.00	12/17	70.7	250 ± 75	

* More than 100 direct PFC or 50 indirect PFC per spleen. Four-fifths of all spleen cells were plated for the two assays.

‡ 95% confidence intervals in parentheses.

of experimental mice were classified as positive or negative depending on whether the numbers of PFC were above or below 100 direct and 50 indirect PFC. The results are presented in Tables I and II, and in the upper section of Fig. 1.

As the number of marrow cells injected increased, the proportion of recipients with positive spleens also increased for both types of PFC. In most instances, spleens that were positive for direct PFC were also positive for indirect PFC. Hence, the relation between the percentage of responses and the number of marrow cells 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 marrow cells. Presumably, the numbers of ASU (the units of response formed by marrow and thymus-derived cells) were very similar in the different groups.

Probability values for an inoculum of 10^6 BDF_1 marrow cells to produce anti-SRBC responses after transplantation were calculated using the Poisson model. The observed frequencies of responses and the theoretical Poisson curve relating inoculum size to expected frequencies of responses were in close agreement (Fig. 1, upper section). The probability values for direct and indirect PFC responses were 3.49 and 3.18, respectively, with overlapping 95% confidence intervals. The inoculum size required for 63% of the recipient spleens to be positive contains in the average one cell or unit limiting PFC production under the conditions used. This inoculum size was 3×10^5 marrow

TABLE II

Chi-Square Test for Independence of Direct and Indirect PFC Responses in Irradiated Syngeneic Recipients of 5×10^7 BDF_1 Thymocytes, 5×10^8 SRBC, and Graded Numbers of BDF_1 Marrow Cells

No. of marrow cells ($\times 10^5$)		0.75	1.5	3.0	6.0
No. of mice		20	17	15	17
Direct PFC	Indirect PFC				
+	+	5	8	10	12
+	−	2	0	1	1
−	+	0	1	0	0
−	−	13	8	4	4
Chi-square*		8.86	10.10	8.96	8.50

+, positive spleen containing more than 100 direct, or 50 indirect PFC.

−, negative spleen containing fewer PFC.

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

cells for direct and indirect PFC. A single cell or unit may have been responsible for production of both types of PFC; otherwise, two separate class-restricted cells, equally frequent in BDF_1 marrow, may have limited PFC formation. In the first case, direct and indirect PFC responses should have been associated in most positive spleens; in the second case, the two responses should have been independent of each other.

Data concerning the assortment of direct and indirect PFC responses in recipient spleens and the chi-square tests for independence are reported in Table II. The group of mice injected with 0.37×10^5 marrow cells was omitted because of the large proportion of negative spleens, i.e., 93.3% for direct PFC and 86.7% for indirect PFC. For the four other groups, the chi-square values were greater than 6.63, the critical value of chi-square statistic for one degree of freedom at the 0.01 level of significance. The hypothesis that direct and indirect PFC responses are associated in BDF_1 mice was, therefore, not rejected. Statistical evidence favors the interpretation that a single cell not restricted

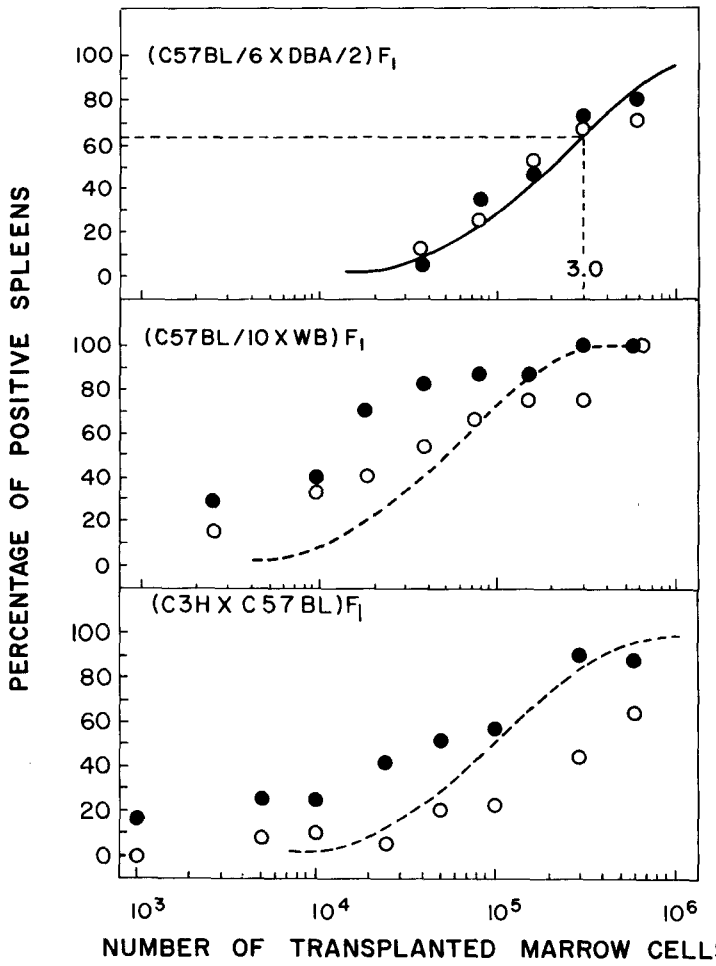

FIG. 1. Percentage of recipient spleens positive for direct PFC (●) and indirect PFC (O) after injection of irradiated mice with 5×10^7 syngeneic thymocytes, 5×10^8 SRBC, and graded numbers of syngeneic marrow cells. Sample sizes for each point are shown in Tables I and III for strains BDF_1 and BWF_1, and in reference 7 for strain $C3BF_1$. In the upper section, symbols indicate observed percentages and the fitted curve expected percentages according to the Poisson model. The number of transplanted BDF_1 marrow cells for 63% positive spleens (indicated by dashed straight lines) contains, in the average, one potentially immunocompetent cell limiting the responses detected by the assays. In the two lower sections, the shape of Poisson curves is indicated by dashed lines (not fitted) and the observed percentages by symbols.

for antibody class limited the two PFC responses by BDF_1 marrow. A preceding study of potentially immunocompetent marrow cells in $C3BF_1$ mice was interpreted in the opposite way (7). The earlier results of limiting dilution assays are shown in the lowest section of Fig. 1 for ease of comparison. Two differences

stand out: the relation between frequencies of responses and number of marrow cells transplanted was not described by the Poisson model for $C3BF_1$ mice; the frequencies of direct PFC responses by $C3BF_1$ marrow were consistently greater than those of indirect PFC.

Frequency of Direct and Indirect PFC Responses in Syngeneic Recipients of Graded Numbers of BWF_1 Marrow Cells.—Since marked differences were observed in the properties of potentially immunocompetent cells of two F_1 hybrid strains, it was of interest to characterize marrow cells from a third F_1 hybrid by the same criteria. $(C57BL/10W \times B)F_1$ mice were chosen because one of the parental strains, C57BL/10, is related to the C57BL/6 and C57BL strains used for breeding the other two hybrids. Limiting dilution assays were made in irradiated (850 R) BWF_1 mice as described for BDF_1 hybrids, except that the numbers of marrow cells injected ranged from 2.5×10^3 to 6×10^5, and that the total number of recipients was 107. The results, reported in Table III

TABLE III

Percentage of Positive BWF_1 Recipient Spleens after Infusion of 5×10^7 Syngeneic Thymocytes, 5×10^8 SRBC, and Graded Numbers of BWF_1 Marrow Cells

No. of marrow cells transplanted	Fraction of positive spleens*	Percentage of positive spleens*	Mean No. of PFC per positive spleen ± SE
$\times 10^5$			
		Direct PFC‡	
0.025	2/7	29.6	190 ± 30
0.093	6/15	40.0	500 ± 219
0.187	12/17	70.5	430 ± 73
0.375	11/13	84.5	400 ± 36
0.75	21/24	87.5	385 ± 51
1.50	16/18	88.8	750 ± 160
3.00	8/8	100.0	753 ± 175
6.00	5/5	100.0	2230 ± 570
		Indirect PFC‡	
0.025	1/7	14.3	145
0.093	3/15	33.3	280 ± 145
0.187	7/17	41.1	310 ± 67
0.375	7/13	53.7	191 ± 56
0.75	16/24	66.7	291 ± 64
1.50	14/18	77.7	482 ± 106
3.00	6/8	75.0	475 ± 240
6.00	5/5	100.0	1170 ± 390

* More than 100 direct PFC or 50 indirect PFC per spleen. Four-fifths of all spleen cells were plated for the two assays.

‡ The frequencies of direct PFC responses were compared with those of indirect PFC responses by the "sign" test (19). Including all experimental groups, the test gave a chi-square value of 4.5. For one degree of freedom, the differences observed were significant ($P < 0.05$).

and in the middle section of Fig. 1, are remarkably similar to those obtained with $C3BF_1$ marrow, and contrast sharply with the PFC responses by BDF_1 marrow. Percentage values of recipient spleens in which PFC were formed, plotted against the number of marrow cells grafted, did not conform to the predictions of the Poisson model. The frequencies of responses increased gradually over three logarithmic increments of marrow cell numbers. The frequencies of responses were greater for direct than for indirect PFC in seven of eight groups tested; most spleens were positive or negative for both types of PFC, or positive for direct but negative for indirect PFC. Using the sign test (19) to compare the percentage of positive spleens for the two classes of PFC, a chi-square

TABLE IV

Number of Direct and Indirect PFC Responses in Spleens of Irradiated BDF_1 and $C3BF_1$ Mice Injected with 5×10^7 Syngeneic Thymocytes, 5×10^8 SRBC, and Graded Numbers of Allogeneic Marrow Cells

Strain of marrow cell donors		$C3BF_1$	BDF_1
No. of marrow cells grafted ($\times 10^4$)		1–4	2–8
Strain of thymocyte donors and recipients		BDF_1	$C3BF_1$
No. of recipient mice		53	58
Direct PFC	Indirect PFC		
+	+	6	24
+	−	10	3
−	+	1	0
−	−	36	31

+, positive recipient spleen containing more than 100 direct, or 50 indirect PFC.
−, negative recipient spleen containing fewer PFC.

value of 4.5 was obtained. For one degree of freedom, the differences between frequencies of direct and indirect PFC responses were statistically significant ($0.01 < P < 0.05$).

Frequency of Direct and Indirect PFC Responses in Allogeneic Recipients of BDF_1 and $C3BF_1$ Marrow Cells.—Association of direct and indirect PFC responses in BDF_1 mice and independence of responses in $C3BF_1$ mice, could have resulted from influences of the host environment on differentiation, rather than from inherent properties of marrow precursor cells. To test this possibility, irradiated BDF_1 and $C3BF_1$ mice were injected with allogeneic $C3BF_1$ and BDF_1 marrow cells, respectively, and with syngeneic thymocytes. In this way, graft-*versus*-host reactions by thymocytes, and SRBC-alloantigen competition for ARC were avoided. The number of thymocytes injected into each mouse was 5×10^7, and the number of marrow cells ranged from 1 to 8×10^4 (Table IV). A total of 53 BDF_1 and 58 $C3BF_1$ were so grafted. SRBC were given 18 hr later to stimulate PFC production. Direct and indirect PFC of recipient spleens were enumerated 9 or 10 days after transplantation. According to pub-

lished reports (21) and to preliminary experiments, allogeneic thymus and marrow cells were not as effective in generating PFC as syngeneic cell mixtures. Responses were rather infrequent in the allogeneic strain combinations used, and were observed primarily in recipients of small marrow inocula. The number of PFC per positive spleen (not shown in Table IV) was about the same in allogeneic as in syngeneic recipients. Although the fraction of positive spleens in $C3BF_1 \rightarrow BDF_1$ cell transfers was only 17/53, direct PFC responses were more frequent than indirect PFC responses, as was seen in syngeneic recipients of $C3BF_1$ marrow (compare Table IV with lowest section of Fig. 1.) 27 of 58 spleens were positive for PFC in $BDF_1 \rightarrow C3BF_1$ cell transfers, but the frequency of responses was about the same for direct and indirect PFC, as was seen in syngeneic recipients of BDF_1 marrow (compare Tables I and IV). It follows that host factors did not detectably influence the assortment of direct and indirect PFC responses limited by BDF_1 and $C3BF_1$ marrow cells.

DISCUSSION

Limiting dilution analysis offers a convenient method for studying the frequency, function, and potential of cells participating in discrete events of an immune response. A desirable prerequisite of such studies is that the smallest number of events, possibly one, limit antibody formation. This may be accomplished in cell transfer systems by providing irradiated test animals with an excess of all required cell types except one. The presence or absence of this cell will then determine whether or not antibody is formed. By choosing the appropriate inoculum size, the number of relevant cells can be limited to one or very few, so that the probability of obtaining immune responses is governed by Poisson statistics. Restriction or potential of the limiting cells for specificity, class, allotype, affinity, or other properties of immunoglobulins, will be reflected by homogeneity or heterogeneity of the antibody formed.

In practice, limiting dilution assays were made in more complex experimental conditions (7, 11, 17, 20, 22, 23). Results were nevertheless predictable by the Poisson model as long as antibody formation depended on availability of a single cell type (as in the case of thymic ARC) or of a multiplicity of cells (as in the case of splenic ASU) participating in one limiting event. Functions and restrictions studied were those of the least frequent cell type, even though additional nonlimiting events may have been necessary for antibody formation. By this approach it has been established that splenic ASU and ASU resulting from marrow-thymus cell mixtures are restricted to produce either anti-SRBC agglutinating cluster-forming cells or anti-SRBC lytic PFC, and that ASU generate either direct (IgM) PFC or indirect (IgG) PFC (17, 18). Class restriction was conferred upon ASU by cells of marrow origin, since thymus-derived cells were not differentiated for such a function, according to statistical (limiting dilution analysis) as well as direct (analysis of splenic foci of PFC) evidence

(23). Class differentiation was later demonstrated in marrow cells (7). However, the only limiting dilution experiments in which results were not predictable by the Poisson model were those in which bone marrow cells of $C3BF_1$ donors were graded in irradiated mice given an excess of thymocytes (7). These exceptional results suggested that $C3BF_1$ marrow may contain several types of cells participating in a multiplicity of cell-to-cell interactions, each one limiting PFC production. The present experiments represent a confirmation and an extension, for it was shown that properties of marrow cells from a different hybrid strain, BWF_1, are similar to those of $C3BF_1$ marrow, while cells of a third hybrid, BDF_1, differ from the former for lack of class differentiation and for greater homogeneity.

The relation between the number of marrow cells grafted into irradiated mice reconstituted with thymocytes, and the number of recipients producing PFC after primary immunization, did not conform to the most plausible and simple Poisson model in BWF_1 mice. As in preceding experiments with animals of the $C3BF_1$ strain (7), a number of recipients responded to antigens of SRBC even if less than 10^4 marrow cells were grafted. However, by increasing the number of transplanted cells, the proportion of recipients positive for PFC increased considerably less than expected, assuming that one kind of marrow precursor of PFC participated in a single-hit limiting event. The shallow limiting dilution plots of Fig. 1 can be explained in a number of ways: one could postulate the existence in bone marrow of precursors of PFC in different stages of differentiation and maturation interacting with thymus-derived inducer cells (20) and with accessory cells of marrow origin (3, 5, 6). Furthermore, each type of PFC precursor engaged in multicellular interactions, could do so either simultaneously or sequentially. The information provided by limiting dilution analysis neither supports nor refutes any of these possibilities; however, the information rules out the simplest hypothesis of single-hit interactions of marrow precursors of PFC of one kind with thymus-derived inducer cells. Physical separation of functionally distinct cells from bone marrow, coupled with limiting dilution assays for each identifiable cell population, may provide answers to the questions raised by the present findings. The current interpretation, by which potentially immunocompetent marrow cells of BWF_1 and $C3BF_1$ mice are regarded as functionally heterogeneous, is likely to be correct for the following reasons: immunocompetent *spleen* cells (derived from bone marrow, thymus, and lymph nodes) are heterogeneous with respect to cell-to-cell interactions and production of PFC in CBA and $(CBA \times C57BL)F_1$ mice (5); preimmunization of $C3BF_1$ mice reduced the functional heterogeneity of marrow cells, since limiting dilution curves conformed to the predictions of the Poisson model when marrow cells of immunized mice were substituted for cells of nonimmune donors (24).

Class restriction of marrow cells and of the units of response to antigens of

SRBC (ASU) has been discussed elsewhere (25). A critique of the limiting dilution approach has also been given in publications of this series. In general, results obtained were incompatible with the hypothesis proposed by Papermaster that direct and indirect PFC belong to the same clone (26). Data supporting the Papermaster hypothesis rest on the frequent association of direct and indirect PFC in spleen fragments of irradiated mice injected with spleen cells. However, the observed association is meaningless because precursors of direct PFC are several times more frequent than precursors of indirect PFC in mouse spleen (17). Under these circumstances, it is inevitable that the less numerous foci of indirect PFC be surrounded by the more numerous foci of direct PFC.

Potentially immunocompetent cells are less heterogeneous in certain mouse strains: spleen cells of C57BL mice are not separable in as many fractions as those of CBA and (CBA × C57BL)F_1 mice (5); and limiting dilution assays of BDF_1 marrow cells give results conforming to the Poisson model. Thus, in BDF_1 mice the bone marrow contains cells participating in a single-hit event limiting the immune response to SRBC. In view of the functional heterogeneity of marrow and spleen cells in other mouse strains and of the existence of accessory cells, the limiting BDF_1 marrow cells could have been the precursors of PFC or accessory cells. The question is of importance because the BDF_1 cells were not differentiated for antibody class. In fact, direct and indirect PFC were generated whenever one or a few of the limiting marrow cells reached spleens on transplantation and interacted with thymic inducer cells. Physical separation of marrow cells and reconstitution of functional units with cells from allogeneic donors should clarify this issue. If BDF_1 marrow precursors of PFC were to lack class restriction, differentiating antibody-forming cell lines rather than mature immunocytes could be used for elucidating control mechanisms of gene expression, in relation to the exclusive synthesis of immunoglobulins of one class and allotype. The general consensus is that antibody-forming plasma cells are restricted, but that peripheral blood cells undergoing blast transformation (27, 28) and long-term tissue culture lymphoid cells (29, 30) may not be restricted and synthesize immunoglobulins of two classes or allotypes.

The observed variation of cellular properties in marrow of three hybrid strains suggests that differentiation and/or maturation of the immune system are under genetic control in mice. "Immune response" genes influencing *specific* immune reactions have been described in several rodent species (8, 9, 11), but these genes do not seem to influence functions unrelated to antibody specificity. The data presented here and preliminary investigations of inbred parental strain marrow (31) point to a broader genetic control of immune responsiveness, by way of influences on cells responsible for one of a series of discrete events leading to antibody synthesis. Conceivably, the great heterogeneity of antibodies results from interactions of extremely specialized accessory cells

with immunoglobulin producing cells. If so, it is to be expected that immune response genes regulating differentiation for a variety of functions will be identified as the cellular basis of antibody formation becomes fully understood.

SUMMARY

Marrow cells and 5×10^7 thymocytes of unprimed (C57BL/6 × DBA/2)F_1, (C57BL/10 × WB)F_1 and (C3H × C57BL)F_1 donor mice were mixed in vitro and transplanted into X-irradiated syngeneic hosts. Upon injection of sheep erythrocytes, splenic plaque-forming cells (PFC) secreting IgM (direct PFC or IgG (indirect PFC) hemolytic antibody were enumerated at the time of peak responses.

By grading the numbers of marrow cells, inocula were found that contained few immunocompetent cells reaching the recipient spleens, interacting with thymocytes or other accessory cells (or both), and generating PFC. The frequency of responses in BDF_1 mice conformed to Poisson statistics, indicating that immunocompetent marrow cells participated in a single-hit interaction *limiting* PFC responses. The marrow cells assayed were not restricted for the antibody class (IgM *versus* IgG) to be secreted by mature PFC. Unrestricted marrow cells could have been either the precursors of PFC or accessory cells. Different results were obtained in BWF_1 and $C3BF_1$ mice. The frequency of responses in relation to the number of marrow cells grafted did not follow Poisson statistics, and the limiting cells were restricted for antibody class. Presumably, immunocompetent cells of these strains were more heterogeneous than those of BDF_1 mice and participated in a multiplicity of cell-to-cell interactions. The strain differences reflected inherent properties of marrow cells and not influences of the environment in which PFC were produced. The results confirmed for bone marrow the heterogeneity of immunocompetent cells reported by others for spleen, and suggested that genetic factors such as "immune response" genes regulate cellular differentiation also for functions other than those related to antibody specificity.

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