DISTINCT EVENTS IN THE IMMUNE RESPONSE ELICITED BY TRANSFERRED MARROW AND THYMUS CELLS

I. Antigen Requirements and Proliferation of Thymic Antigen-Reactive Cells*

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The production of antibodies against antigens of sheep erythrocytes and bovine serum albumin depends on the cooperation of at least two major cell types in mice (1–4). Bone marrow contains the precursors of immunocytes which are induced to differentiate and mature by cells of thymic origin. Little is known about the mechanism of this induction process, except that thymocytes are not ancestral to immunocytes (2, 5–8). They are, however, the cells that react with, or respond to, antigen to initiate events leading to antibody formation (5, 9). For this reason, the relevant thymocytes have been designated antigen-reactive cells (5). Although the way in which ARC[1] interact with immunocyte precursors is not understood, it has been established that ARC do not specify the molecular class of antibodies to be produced by marrow-derived cells (10, 11). In cell transfer experiments the number of ARC available for interaction with marrow cells was limited to 1 or a few. Under such conditions, interaction of individual ARC (or of their descendent cells) occurred with several marrow precursors of plaque-forming cells (P-PFC) without regard to the class of antibody to be produced by the latter. In other words, under the influence of antigen, each individual ARC induced the formation of more than one antigen-sensitive unit (ASU). These are functional units committed to the production of immunocytes secreting antibody of a single molecular class and specificity (12–15). Accordingly, among the ASU resulting

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[1] *Abbreviations used in this paper:* ARC, antigen-reactive cells of thymic origin; ASU, antigen-sensitive units; PFC, plaque-forming cells; P-PFC, precursors of plaque-forming cells of bone marrow origin; RRBC, rat erythrocytes; BRBC, burro erythrocytes; SRBC, sheep erythrocytes.

from exposure of 1 ARC to marrow cells and sheep erythrocytes, some generated direct PFC and others indirect PFC (10).

Thymic ARC might be capable of interacting with more than one P-PFC, either simultaneously or in rapid succession (10). However, it is also possible that ARC proliferate upon exposure to antigen and that the descendant cells interact with marrow P-PFC. If each progeny cell were to interact with 1 P-PFC, formation of more than 1 ASU/ARC would still be expected. These possibilities can be subjected to verification by two-step experiments in which 1 or a few ARC are first exposed to antigen in the absence of marrow cells, and several days later allowed to interact with marrow cells and with antigen again. One-step experiments are those in which marrow and thymus cells are mixed and exposed to antigen at the same time. If thymic ARC were the cells that interact with marrow P-PFC without having to undergo proliferation, the number of ASU formed per ARC should be equal in one-step and two-step experiments. However, were ARC to proliferate under the influence of antigen before interaction with P-PFC, then the number of ASU per initial thymic ARC should increase in two-step experiments, assuming that under these conditions the proliferative potential of ARC is fully realized. The results reported in this paper support the second possibility. In the course of establishing the requirements for antigen in two-step experiments, it was also found that PFC production is dependent on the presence of SRBC at two critical stages of the immune response: first, when thymic ARC are stimulated, and second, when ARC progeny and marrow cells are mixed for interaction.

Materials and Methods

Mice.—(C3H/He × C57BL/Ha)F_1 and (C57BL/6 × DBA/2Cr)F_1 females, 9–12 wk old, were used as donors and recipients in syngeneic cell transfers. The abbreviated designations for the two hybrid strains are C3BF_1 and BDF_1, respectively.

Irradiation.—Mice to be grafted with hemopoietic cells were exposed to 825–850 R of total body X-radiation as described elsewhere (12).

Cell Suspensions and Transplantation.—Nucleated bone marrow and thymus cells of normal mice, and spleen cells of irradiated-reconstituted mice suspended in Eagle's medium, were counted and injected into a lateral tail vein of irradiated syngeneic mice as previously described (16).

Immunization.—Either at the time of cell transplantation or 18 hr later, each irradiated mouse was injected intravenously with 5×10^8 washed sheep erythrocytes (SRBC) in 0.5 ml of Eagle's medium. Fresh defibrinated sheep blood was obtained every 2nd wk from Grand Island Biological Co., Grand Island, N. Y.

Assays for Plaque-Forming Cells.—Direct and indirect PFC were enumerated in spleen cell suspensions of test recipient mice by the agar gel method of Jerne, as previously described (12).

Statistical Methods.—Limiting dilution assays were performed to estimate the frequencies of ARC in thymus cell suspensions of normal donors and in spleen cell suspensions of irradiated donor mice reconstituted with thymocytes and SRBC. The experimental design for such limiting dilution assays and the statistical analysis of results have been described elsewhere (10, 12).

TABLE I

*Production of PFC in Irradiated C3BF$_1$ Mice Injected with 10^7 Marrow Cells, SRBC, and Retransplanted Thymocytes Exposed in Vivo to SRBC**

Thymocyte residence in primary recipient	Cell mixture residence in test recipient	Fraction of positive spleens in test recipients and mean No. of PFC per positive spleen ± SE‡	
		Direct PFC	Indirect PFC
days	*days*		
3	7	5/7	0/7
		245 ± 81	
5	7	20/21	12/22
		985 ± 478	320 ± 48
5	10	6/6	6/6
		735 ± 208	490 ± 91
5	12	4/5	4/5
		335 ± 72	750 ± 215
6	7	5/5	4/5
		855 ± 210	390 ± 116
7	7	4/5	2/5
		345 ± 61	290 ± 145
7	8	3/3	3/3
		1380 ± 333	868 ± 390
7	10	3/3	2/3
		410 ± 91	240 ± 10

* 10^8 thymocytes mixed with SRBC injected into mice exposed to 850 R. All cells lodging in one recipient spleen were recovered 3–7 days later and retransplanted with marrow cells and SRBC into one irradiated test recipient.

‡ Spleens were regarded as positive if they contained more than 100 direct or 50 indirect PFC (11, 16).

RESULTS

Reaction of Thymocytes with SRBC Prior to Interaction with Marrow Cells.—

It has been demonstrated by Mitchell and Miller (5) that thymic ARC can react with SRBC in the absence of viable marrow cells (first step) and that they maintain for at least 7 days the ability to interact with marrow precursors of direct PFC (second step). The immune response to SRBC was thereby presumably dissected into two discrete sequential steps, thus permitting analysis of the effects of antigen on ARC, on the one hand, and of the requirements for effective marrow-thymus interaction, on the other. Data are not available on indirect PFC production by marrow-thymus cell mixtures in two-step experiments, or on the necessary duration of the first step. Therefore, 56 irradiated mice were injected with 10^8 thymocytes mixed with SRBC. These mice were used 3, 5, 6, and 7 days later as donors of thymus-derived

cells. The thymocytes lodging in the spleens of one primary recipient were suspended and mixed with 10^7 marrow cells and SRBC. This mixture was injected into one irradiated test mouse to produce anti-sheep PFC. Plaque assays were made 7–12 days later on test recipient spleens.

The results are presented in Table I.

After 3 days in primary recipients, thymocytes recovered from the spleen were relatively ineffective for interaction with P-PFC. Test recipients contained few direct PFC and no indirect PFC in their spleens. After 5–7 days in primary recipients, the thymocytes recovered were capable of inducing direct and indirect PFC production by marrow cells in almost every test recipient, provided that the plaque assays were done 8 days after retransplantation or later. For this reason, retransplantation of thymus-derived cells was done in all subsequent two-step experiments on the fifth day, and plaque assays on the eighth day, after retransplantation.

Antigen Requirements for Marrow-Thymus Interaction in Two-Step Experiments.—In the preceding experiment SRBC were given twice: at the time of thymocyte transplantation and at the time of thymocyte retransplantation with marrow cells. Mitchell and Miller have shown that immunocytes are not produced if SRBC have been omitted or substituted with non–cross-reacting erythrocytes *in the first step* (5). However, it was not reported whether SRBC are necessary during the first and second steps, or whether they could be replaced in the second step by non–cross-reacting erythrocytes. Also, it is not known whether PFC could be produced in a reversed two-step experiment in which marrow cells are transplanted first with antigen, and then retransplanted with thymocytes from normal donors and SRBC.

Groups of irradiated mice were injected with either 10^8 thymocytes or 10^8 bone marrow cells, with or without SRBC. After 5 days in irradiated host mice, cells that lodged in one spleen were retransplanted into one test recipient together with either 10^7 marrow cells, or 10^8 thymocytes, respectively, with or without SRBC. In a few experiments, spleen cells of one marrow-injected primary recipient were mixed for retransplantation with spleen cells of one thymus-injected mouse. Finally, SRBC were replaced by burro or rat erythrocytes in the second step of an experiment in which thymus-derived cells were retransplanted with marrow. Negative controls were set up by omitting thymocytes or SRBC (but not both) in primary recipients. Positive controls were set up by injecting thymocytes and SRBC into primary recipients, and by retransplanting thymus-derived cells with marrow and SRBC.

The 8-day production of direct PFC by the different cell mixtures is reported in Table II.

The positive and negative control groups that were set up (Nos. 1–5 of Table II) confirmed the results of Mitchell and Miller (5). To obtain PFC in two-step experiments, thymocytes had to be injected with SRBC into primary recipients, and retransplanted with marrow and SRBC into test recipients. Our experi-

mental groups indicated, furthermore, that SRBC were necessary in *both* the first and second steps (Nos. 6–9 of Table II). Retransplanted thymocytes mixed with marrow cells from normal donors or with retransplanted marrow cells failed to generate PFC if SRBC were omitted at the time of cell mixing. It is possible

TABLE II

Antigen Requirements for Anti-Sheep PFC Production by Marrow and Thymus cells in Two-Step Experiments

Group	Cells injected into		Mean No. of direct PFC per spleen of test recipients ± SE	Fraction of positive spleens§
	Primary recipients*	Secondary recipients‡		
1	SRBC	SP‖ + BM + SRBC	46 ± 20	1/6
2	TY	SP_{TY} + BM + SRBC	35 ± 18	1/9
3	TY + SRBC	SP_{TY} + BM + SRBC	760 ± 97	31/33
4	TY + SRBC (2×)	SP_{TY} + BM + SRBC	585 ± 161	9/9
5	TY + SRBC	SP_{TY} + BM + SRBC (2×)	1320 ± 210	11/11
6	TY + SRBC	SP_{TY} + BM	28 ± 8	0/11
7	TY + SRBC (2×)	SP_{TY} + BM	29 ± 15	1/14
8	TY + SRBC; BM + SRBC	SP_{TY} + SP_{BM} + SRBC	577 ± 57	5/5
9	TY + SRBC; BM + SRBC	SP_{TY} + SP_{BM}	87 ± 22	1/4
10	BM + SRBC	SP_{BM} + TY + SRBC	358 ± 150	8/11
11	BM	SP_{BM} + TY + SRBC	820 ± 264	6/6
12	BM + SRBC	SP_{BM} + TY	35 ± 18	0/6
13	TY + SRBC	SP_{TY} + BM + BRBC	64 ± 19	4/22
14	TY + SRBC	SP_{TY} + BM + RRBC	15 ± 3	0/13

* Primary recipients were irradiated and injected with either 10^8 thymocytes or 10^8 bone marrow cells, with or without SRBC. 5 days later the spleen cells of one mouse were retransplanted into one test recipient.

‡ Test recipients were irradiated and injected with one of the following cell mixtures: spleen cells of one primary recipient and 10^7 marrow cells with or without antigen (groups 1–7, 13, and 14); one primary recipient spleen and 10^8 thymocytes with or without antigen (groups 10–12); cells of two different primary recipient spleens with or without antigen (groups 8 and 9).

§ Containing more than 100 direct PFC.

‖ Abbreviations: BM, bone marrow cells; SP, SP_{TY}, SP_{BM}, spleen cells from a primary recipient injected with SRBC, thymocytes, or marrow cells, respectively; TY, thymocytes.

that two-step experiments require two antigen injections at critical times, e.g. at the time of initial stimulation of ARC and at the time of interaction between thymic inducer cells and marrow P-PFC. To verify this possibility, groups of mice were injected twice with SRBC, but not at the time of cell mixing (Nos. 7 and 9 of Table II); in neither instance were PFC produced. Thus, antigen was required first for ARC stimulation and again at the time of cell mixing and re-

transplantation. The second antigen injection could have mediated the interaction between cells or stimulated marrow cells before, after, or during the interaction. It was of interest, therefore, to establish whether incubation of marrow cells with SRBC resulted, as in the case of thymocytes, in specific activation. Marrow cells that were injected into primary recipients with or without SRBC were equally competent on retransplantation to interact with thymocytes (Nos. 10–12 of Table II). Hence, SRBC had no detectable effect on marrow cells. As before, antigen was required in the second step of the experiment. In this case, the second injection of antigen initiated and completed the response; i.e. it stimulated ARC and either mediated cell interactions or stimulated marrow cells.

The requirement for the second injection of SRBC could have been nonspecific. If so, other xenogeneic erythrocytes might substitute effectively for SRBC. However, anti-sheep PFC were not formed when burro or rat erythrocytes were injected with marrow and thymocytes preexposed to SRBC (Nos. 13 and 14 of Table II). Thus, whatever is the nature of the events following the second antigen injection, the triggering of the second step of the PFC response appears to be as antigen-specific as the initial reaction of thymocytes.

In summary, these experiments have shown that thymic ARC are the minimal requirements for *initiation* of the antibody response to SRBC, since activation of ARC occurred in the absence of viable marrow cells, and activation of marrow cells did not occur in the absence of viable thymocytes. At least one other subsequent event in the PFC response is dependent on the same antigen complex used for initiation, since the response did not occur unless SRBC were added to SRBC-activated thymocytes at the time of retransplantation with marrow.

Frequency of ARC-Derived Inducer Cells in C3BF$_1$ Mice.—

Following transplantation of 10^7 thymocytes and SRBC, an average of 1 ARC lodges in the spleens of irradiated recipient mice (10). In an attempt to establish whether ARC proliferated after exposure to SRBC, we injected graded numbers of thymocytes with antigen into irradiated mice. 5 days later, each primary recipient spleen was retransplanted into *one* test recipient with 10^7 marrow cells and SRBC to assess its content of ARC-derived inducer cells by formation of direct and indirect PFC. The number of marrow cells was chosen to provide adequate numbers of PFC precursors (11). The spleens of the groups of mice injected with 10^7 or fewer thymocytes received either 1 or a limited number of ARC, or none (Poisson distribution). Had ARC proliferated in such spleens, enough new cells could have arisen to provide, on retransplantation, test recipient spleens with at least 1 inducer cell. Had ARC not proliferated extensively, the probability that small numbers of retransplanted ARC could reach test recipient spleens would be very low, since through dilution only 4–15% of immunocompetent cells injected into the blood stream lodge in spleens (17–19). Hence, if each primary recipient spleen (containing 1 or 2 ARC) is to repopulate test spleens with inducer cells, the dilution must be compensated for by a 10–100-fold increase in the number of inducer cells.

All negative primary spleens (containing no ARC) should yield negative test spleens on retransplantation.

The results are presented in Table III and Fig. 1.

As the number of thymocytes grafted into primary recipients increased from 1.2 to 25 $\times 10^6$, the proportion of mice with spleens positive for ARC-derived cells increased. 22 of 24 spleens that were positive for inducer cells yielded both direct and indirect PFC on retransplantation with marrow. Most test spleens

TABLE III

*Percentage of Positive Spleens in C3BF$_1$ Test Recipient Mice after Infusion of 10^7 Marrow Cells, 5 $\times 10^8$ SRBC, and Retransplanted Thymocytes**

No. of thymocytes to primary recipients ($\times 10^6$)	Fraction of positive spleens‡	Percentage of positive spleens‡	Mean No. of PFC per positive test spleen ± SE	Pooled probability of positive spleens per 10^6 transplanted thymocytes
		Direct PFC		
1.2	2/10	20.0	273 ± 138	
2.5	2/8	25.0	380 ± 220	
5.0	3/8	37.5	290 ± 75	
10.0	3/5	60.0	625 ± 194	
12.5	10/11	91.0	670 ± 169	
25.0	7/7	100.0	1360 ± 245	0.12
		Indirect PFC		(0.09–0.16)§
1.2	2/10	20.0	187 ± 87	
2.5	1/8	12.5	470	
5.0	2/8	25.0	277 ± 112	
10.0	3/5	60.0	453 ± 225	
12.5	7/8	87.5	350 ± 135	
25.0	7/7	100.0	575 ± 110	

* Graded numbers of thymocytes mixed with SRBC to primary recipients. All cells lodging in one recipient spleen were retransplanted 5 days later into one test recipient.

‡ More than 100 direct PFC or 50 indirect PFC (10).

§ 95% confidence intervals in parentheses.

were therefore either positive or negative for both types of PFC. It follows that the relation between the percentage of positive test spleens and the number of thymocytes grafted into primary recipients did not vary significantly for direct and indirect PFC. This confirms previous results and the conclusion that ARC lack specialization for antibody class (10). For a given number of thymocytes, the proportion of positive spleens was similar in this two-step and in a preceding one-step limiting dilution assay (10). The probability value that 10^7 thymocytes would yield primary spleens positive for ARC was 1.0 (10). The corresponding value for positive test spleens on retransplantation was 1.2. The curves of Fig. 1,

relating inoculum size to the expected frequencies of positive spleens, were plotted using the Poisson model. The observed frequencies in one-step and two-step experiments fit the model, and the values are not significantly different. This result implies that negative test spleens in the two-step experiment were due entirely to lack of ARC in the retransplanted primary spleens. It also implies that all positive primary spleens yielded positive test spleens, in spite of the 10–100-fold dilution of ARC on retransplantation. Hence, the results

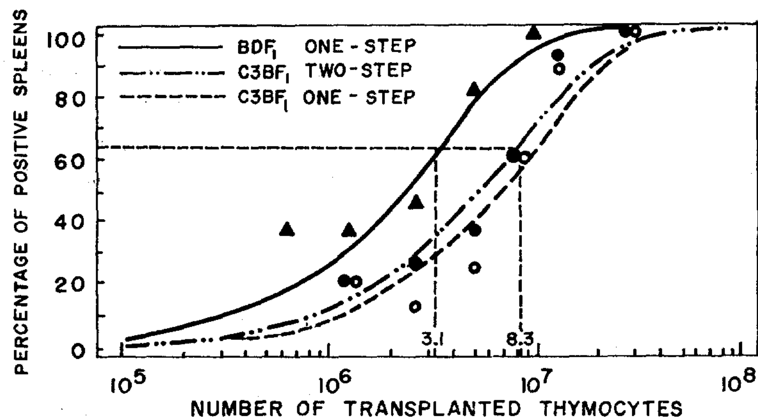

FIG. 1. Percentage of spleens positive for direct (▲, ●) and indirect (○) PFC after injection of irradiated test mice with marrow cells, SRBC, and graded numbers of thymocytes (one-step experiments) or thymus-derived cells (two-step experiments). The numbers of spleens assayed are given in Table III and IV. Symbols indicate observed percentages, and curves expected percentages according to the Poisson model. The $C3BF_1$ two-step curve was fitted to the pooled percentages of positive spleens for direct and indirect PFC, since probability values were overlapping. The $C3BF_1$ one-step curve was taken from reference 10. The numbers of transplanted thymocytes for 63% positive spleens contain an average of 1 detectable ARC and are indicated by dotted lines.

strongly suggest that under the influence of SRBC, ARC proliferated during the 5 days between transplantations.

Frequency of Thymic ARC in BDF_1 Mice.—

Quantitative variations of the plaque response to SRBC have been described in several strains of mice (20–22). Conceivably, the results obtained with $C3BF_1$ hybrids could have reflected the particular immunocompetence determined by the genomes of C3H and C57BL mice. It was desirable, therefore, to study ARC in at least another hybrid mouse. A limiting dilution assay for thymic ARC was made in BDF_1 mice using the one-step design (10). Direct PFC were enumerated in the spleens of irradiated recipients 8–9 days after transplantation of graded numbers of thymocytes mixed with 2×10^7 marrow cells.

Results are shown in Table IV and Fig. 1.

As in previous assays of this type, the percentage of recipient spleens receiving 1 or more ARC, and consequently containing significant numbers of PFC, depended on the number of thymocytes grafted. The relationship between these two variables was predicted by the Poisson model. It was possible, therefore, to estimate the number of ARC per unit number of thymocytes (10). As the frequencies of positive spleens increased in recipients of the larger inocula of thymocytes, the mean number of PFC per positive spleen did not increase in proportion. This suggested that once spleens contained 1 or a few ARC, nearly equal numbers of ASU were formed, provided that adequate numbers of P-PFC were available. The interpretation was supported by the fact that P-PFC were

TABLE IV

Percentage of Positive BDF_1 Recipient Spleens after Infusion of 2×10^7 Marrow Cells, 5×10^8 SRBC, and Graded Numbers of Thymocytes

No. of thymocytes transplanted ($\times 10^6$)	Fraction of positives spleens	Percentage of positive spleens*	Mean No. of direct PFC per positive spleen ± SE	Probability of positive spleen per 10^6 transplanted thymocytes
		One-Step Experiments		
0.62	3/8	37.5	342 ± 24	
1.25	6/16	37.5	450 ± 60	
2.50	7/15	46.6	518 ± 235	0.33
5.00	14/17	82.4	515 ± 116	(0.23–0.47)‡
10.00	10/10	100.0	857 ± 199	
		Two-Step Experiments		
8.00§	31/35	88.6	700 ± 102	

* More than 100 direct PFC/spleen. One-half of all spleen cells were plated.

‡ 95% confidence intervals in parentheses.

§ To primary recipients. Cells lodging in one spleen were retransplanted 5 days later with marrow cells into one test recipient.

present in excess numbers by design, and that under the proper conditions (e.g. low ARC/P-PFC ratio) 1 ARC induced more than 1 marrow cell to generate PFC (10). The only difference noted between thymic ARC of BDF_1 and $C3BF_1$ mice was quantitative: 1 ARC/$3 \pm 1 \times 10^6$ BDF_1 thymocytes, and 1 ARC/$10 \pm 2 \times 10^6$ $C3BF_1$ thymocytes (10). Since the number of nucleated cells per organ was similar in the two strains, the thymus of BDF_1 mice contained 3 times as many ARC as the thymus of $C3BF_1$ mice.

To verify that ARC also proliferated in BDF_1 mice under the influence of antigen, 8×10^6 thymocytes were grafted with SRBC into irradiated BDF_1 hosts. This provided each spleen with an average of 2–3 ARC. 5 days later, cells of one spleen were mixed with 10^7 marrow cells and retransplanted into one test recipient with SRBC for PFC production. As was the case in $C3BF_1$ mice, the percentage of PFC response in the two-step experiment was similar to that found in one-step experiments (Table IV).

Frequency of ARC-Derived Inducer Cells in BDF_1 Mice.—

In the preceding two-step experiments, whole spleens of irradiated mice injected with thymocytes and SRBC were individually retransplanted into test recipients. All ARC progeny cells in each primary spleen were given to one test recipient. This was done to increase the probability of recovering such cells in spleens of secondary hosts despite cell dilution. Since the results clearly indicated antigen-dependent expansion of inducer cells, it was of interest to estimate the magnitude of this expansion and to verify that it was due to proliferation. By retransplanting graded fractions of primary spleens containing the progeny of 1 or a few ARC,

TABLE V

*Percentage of Positive Spleens in BDF_1 Test Recipient Mice after Infusion of 1.5×10^7 Marrow Cells, 5×10^8 SRBC, and Retransplanted Thymocytes**, ‡

Portion of primary spleen retransplanted with marrow	Fraction of positive spleens‡	Percentage of positive spleens‡	Mean No. of direct PFC per positive spleen ± SE	Probability of positive test spleens per 1/100th of retransplanted primary spleens
		Retransplantation 4 Days after SRBC		
1/128	2/12	18.2	185 ± 55	
1/64	11/25	44.0	345 ± 72	
1/32	13/24	54.2	325 ± 38	0.25
1/16	24/29	82.7	325 ± 60	(0.19–0.31)§
1/8	22/24	91.7	628 ± 82	
1/4	19/19	100.0	790 ± 100	
		Retransplantation 7 Days after SRBC		
1/256	5/8	62.5	335 ± 40	
1/128	6/9	66.7	263 ± 43	
1/64	10/10	100.0	695 ± 187	
1/32	7/7	100.0	424 ± 71	

* 10^7 thymocytes to primary recipients and SRBC 18 hr later. Portions of cells lodging in spleens were retransplanted 4 or 7 days after SRBC.

‡ More than 100 direct PFC/spleen. One-half of all test spleen cells were plated.

§ 95% confidence limits in parentheses.

and a fixed number of marrow cells, it should have been possible to limit the number of ARC progeny cells involved in ASU formation. It was assumed that such cells were capable of reaching the test spleens and of interacting with marrow P-PFC independently of each other. If so, the relationship between the number of retransplanted cells and the number of test spleens positive for PFC should conform to Poisson statistics.

10 million thymocytes were injected into groups of irradiated mice to provide each recipient spleen with an average of 3 ARC. SRBC were given 18 hr later. 4 or 7 days after immunization, cells of primary spleens were pooled in suspension to average the number of ARC-derived cells per spleen equivalent. Graded fractions of one spleen equivalent of the suspension were mixed with 1.5×10^7 marrow cells for retransplantation with SRBC into test recipients.

8-day direct plaque responses pooled from four separate experiments are shown in Table V and Fig. 2.

As the retransplanted fraction of 4 day spleens containing ARC-derived in-

ducer cells increased from 1/128 to 1/4, the proportion of positive test spleens increased from 18.2 to 100%. The observed frequencies of positive spleens plotted as a function of the fraction of retransplanted spleen (log number of inducer cells) fell within the predictions of the Poisson model. The probability value that 1% of the cells of one primary spleen would yield positive test spleens for PFC was 0.25. This means that 0.04 (1/25) of each primary 4 day spleen contained 1

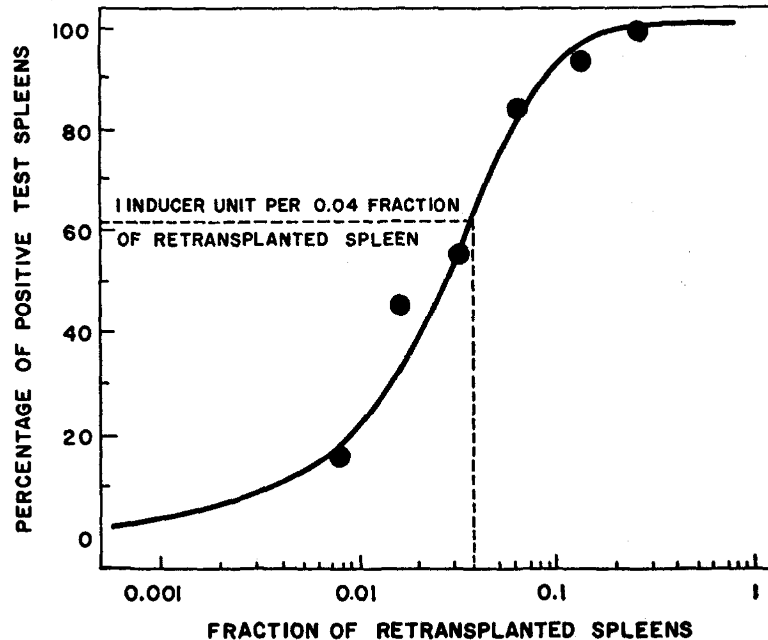

FIG. 2. Percentage of spleens positive for direct PFC after injection of irradiated test mice with 1.5×10^7 marrow cells, 5×10^8 SRBC, and graded fractions of spleens repopulated by 3 thymic ARC (see the text). The number of test spleens assayed is given in Table V. Symbols indicate observed percentages, and the curve expected percentages according to the Poisson model. The fraction of retransplanted spleens for 63% positive test spleens contains an average of 1 detectable inducer unit and is indicated by the dotted line.

unit of ARC-derived inducer cells lodging in the test spleen on retransplantation and interacting with marrow P-PFC. Inasmuch as each primary spleen received 3 ARC and presumably only 1–10% of the retransplanted cells were recovered (17–19), each ARC may have generated from 80 to 800 units of inducer cells in 4 days. Assuming that no cells were lost from the spleens by migration, a minimum of 6–10 cell divisions could have occurred, with a doubling time of 10–16 hr. Expansion of the population of inducer cells continued beyond this time. 7 days after immunization, 1/256 of a primary spleen (instead of 1/25 at 4 days) contained 1 unit of ARC-derived inducer cells (Table V, lower section).

Contrary to what had been noted before in one-step experiments (10) (Table IV), the mean number of PFC per test spleen varied in relation to the number of inducer cells regrafted and, hence, to the percentage of positive spleens in the different groups. Whenever more than 63% of the mice in a group had positive spleens, the mean number of PFC was 3–5 multiples of 150, the mean number of PFC generated by 1 ASU (16). Presumably, such spleens received several inducer cells, each of which interacted with 1 P-PFC to form several ASU. Whenever less than 63% of the mice in a group had positive spleens, the mean number of PFC per positive spleen was smaller, and could have been generated by 1 or 2 ASU. In these groups, spleens must have received either 1 or 2 inducer cells, or

TABLE VI

*Percentage of Positive Spleens in BDF_1 Test Recipient Mice after Infusion of 1.5×10^7 Marrow Cells, 5×10^8 SRBC, and Retransplanted Thymocytes Exposed to Vinblastine**

Portion of primary spleens retransplanted with marrow	Fraction of positive spleens‡	Percentage of positive spleens‡	Mean No. of direct PFC per positive spleen ± SE
	Vinblastine 3.75 Days after SRBC		
1/16	9/13	69.3	375 ± 45
1/8	12/14	85.8	330 ± 59
1/4	2/2	100.0	910 ± 690
	Vinblastine 1 Day after SRBC		
1/16	1/8	12.5	725
1/8	2/10	20.0	300 ± 10

* 10^7 thymocytes to primary recipients and SRBC 18 hr later. Portions of cells lodging in spleens were retransplanted 4 days after SRBC and either 3 days or 7 hr after vinblastine. Untreated controls are included in Table V.

‡ More than 100 direct PFC/spleen. One-half of all test spleen cells were plated.

none. If so, individual inducer cells would have interacted with 1 P-PFC, while all the progeny of 1 ARC would have interacted with several P-PFC, as previously shown (10).

Effect of Vinblastine on Generation of Inducer Cells by ARC.—

To test whether the increase of inducer cells was due to proliferation of ARC, 0.5 mg of vinblastine sulfate (Velban, Eli Lilly and Co., Indianapolis, Ind.) dissolved in saline was injected into the peritoneum of irradiated BDF_1 mice grafted with 10^7 thymocytes and SRBC. The drug specifically inhibits cells entering mitosis and has been used to study the proliferative state of ASU (23, 24). Vinblastine was given either 3 days or 7 hr before retransplantation of thymus-derived cells of the primary spleens. This corresponded to 1 and 3.75 days after the first exposure to antigen, respectively. Graded fractions of pooled spleens were mixed with 1.5×10^7 marrow cells and injected into test recipients with SRBC.

8-day PFC responses are shown in Table VI.

Vinblastine did not reduce significantly the frequency or magnitude of PFC responses when given 3.75 days after stimulation of thymocytes with SRBC, i.e. when production of inducer cells by proliferation of ARC could have already occurred to a great extent. If most inducer cells were also proliferating, the time of exposure to vinblastine, although short (7 hr), should have been sufficient to arrest a measurable proportion of such cells, their estimated doubling time being 10–16 hr. Vinblastine possibly carried into the test recipients with retransplanted spleen cells did not interfere with interaction between inducer cells and P-PFC. In contrast to the lack of effect at 3.75 days, vinblastine markedly reduced the frequency of PFC responses when given 1 day after stimulation of thymocytes by SRBC. One-eighth of such treated spleens was approximately equivalent to $1/128$ of untreated spleens with respect to inducing marrow P-PFC. It is likely, therefore, that the drug reduced formation of inducer cells by inhibiting proliferation of ARC that had just been stimulated and were entering mitosis.

DISCUSSION

The finding of Claman et al. (1) that bone marrow and thymus cells act synergistically in antibody formation against SRBC gave impetus to studies aimed at identifying (*a*) the events occurring during the lag phase of the response (2, 5, 9) and (*b*) the sources of restrictions for synthesis of one molecular species of antibody by individual immunocytes (10, 11, 16). The experiments reported here have indicated that one of the first detectable events of the immune response after administration of antigen to repopulated mice is proliferation of thymic ARC. This was demonstrated in two-step experiments by exposing transferred thymocytes and marrow cells to SRBC in separate irradiated host mice, and by assessing antigen-dependent changes of their number and/or function by retransplantation. Thymic ARC generated "inducer" cells capable of interacting with marrow precursors of immunocytes so as to render them fully immunocompetent. Under the same conditions, potentially competent marrow cells did not appear to gain a new function. This is to say that, in contrast to thymus, transferred mouse marrow did not contain antigen-reactive cells capable of *initiating* the response to SRBC. It was not excluded, however, that quantitative changes of marrow P-PFC had occurred prior to interaction with thymus-derived cells.

These results and interpretations are consistent with, and extend, the previous work of Davies et al. (2, 9) and of Mitchell and Miller (3, 5). A mitotic response by thymus-derived cells preceding one by marrow-derived cells was seen in spleens of repopulated mice 1–5 days after a single injection of SRBC (2, 9). Since the thymic origin of mitotic cells was recognized by chromosomal markers, and since ARC are a minute fraction of thymocytes (10), Davies et al. could not decide whether ARC proliferated in response to SRBC or other thymic

cells not necessarily relevant for the development of the immune response. In fact, nonspecific thymus-dependent proliferation has been observed in mesothelial cells of the omentum of mice injected with SRBC (25). In contrast, the experiments reported here were designed to assess only the population of thymus-derived cells that interacts with marrow P-PFC. Mitchell and Miller have shown that an antibody response could be obtained by first reacting thymic cells with antigen and then with marrow cells and again with the same antigen (3, 5). Our experiments have indicated that thymic ARC first proliferate in response to SRBC to generate specific inducer cells, which then interact with marrow cells.

The next steps of the immune response detected by these experiments were the interaction of inducer cells with marrow precursors, and the production of PFC. These steps are rather complex and can only be studied in detail if the distinct events composing them become separable; their occurrence required a second administration of the same antigen complex employed for stimulation of ARC. Antigen could have facilitated the interaction between specific inducer cells and P-PFC, or stimulated P-PFC to generate immunocytes once the interaction had occurred, or both. If potentially immunocompetent cells possessed antibody-like receptors at their surface, as postulated by Rajewsky et al. (26), one would expect that antigenic determinants play an essential role in bringing together inducer cells and P-PFC to form ASU. Laskov has shown that marrow cells of unprimed mice can attract SRBC and form rosettes in vitro (27). Miller and Phillips have suggested that rosette-forming cells possess receptors for antigens and that some of them participate in production of immunocytes (28). However, the reactivities of marrow cells and of thymocytes with antigens of SRBC are of a different nature: marrow cells, but not thymocytes, form rosettes (27), and cells of thymus, but not of marrow, generate specific inducer cells.

Do the findings of cell transfer experiments conducted under rather unusual conditions provide information pertinent to the functioning of the intact immune system? It is reasonable to assume that each distinct event of the immune response which can be experimentally separated for analysis can also occur in the intact animal. However, one has to be reminded that under experimental conditions, cell functions could be expressed to a degree that is normally not required or not permitted by homeostatic controls. With these reservations, we shall attempt to delineate the sequence of some cellular events during the primary immune response to antigens of SRBC.

Antigen-reactive cells of thymic origin and immunocyte precursors of marrow origin are dispersed throughout the lymphoid system of adult mice. Since bacteria of the intestinal flora and SRBC share some antigens (29), it is likely that ARC generate inducer cells under the influence of cross-reacting antigens prior to immunization of mice with SRBC. The injection of SRBC will be followed promptly by interaction of existing inducer cells with precursors of immunocytes, and by sustained formation of new inducer cells from stimulated ARC.

The latter cells may be precommitted to react with one or a small number of antigenic determinants. If so, antigen-dependent proliferation of ARC would lead to expansion of the "selected" population of cells that will interact with marrow-derived cells. However, there is no compelling evidence against the possibility that ARC are not restricted. In this case, their "instructed" progeny cells would have to undergo specificity differentiation for the antigen initiating the immune response. In fact, inducer cells are specific for antigen (Table II). This may be a necessary condition either for their contact (directed by antigen ?) with specific marrow-derived P-PFC or for conferring specificity to uncommitted precursors of immunocytes. An important consideration is the number of cell divisions occurring after stimulation of ARC. In transfer experiments and in the absence of marrow cells, 6–10 divisions can occur in 4 days. It is likely, however, that under more usual circumstances interaction with marrow-derived cells removes inducer cells from the proliferating pool, thus limiting or arresting further expansion. One could anticipate that the number of generations in each ARC-derived clone is variable and dependent upon the concentration of marrow-derived cells and of the latter's availability for interaction. Although ARC are known to descend from marrow progenitor cells under thymic influence (16, 30), it is not yet possible to say whether ARC differentiate or simply mature into inducer cells, or whether they can self-replicate. Each of these possibilities is presently being subjected to experimental test.

The elementary units for immune responses result from interaction of inducer cells of thymic origin with marrow-derived precursors of immunocytes, and perhaps with other cell types (11, 31). All cells participating in the interaction constitute an antigen-sensitive unit which is monospecific, since it generates immunocytes restricted to a single molecular class and specificity of antibody (10–16). The information for class restriction of ASU resides in one of the marrow-derived components (11), and this may also be the case for specificity restriction. Restriction strongly suggests that 1 marrow-derived precursor cell becomes part of each ASU in combination with at least 1 inducer unit or cell. ASU thus formed are independent of each other (13) and generate clusters of immunocytes detectable by focus assays (16). They are the units responsible for all-or-none responses in limiting dilution assays (10, 12, 13). The large number of inducer cells that ARC can generate accounts for multiple ASU formation by individual ARC (10). If these considerations are correct, the number of immunocytes generated by each ASU should remain constant when different cell types limit the formation of ASU in cell transfer experiments. The number of direct PFC/ASU is approximately 150 when thymocytes or marrow cells are the limiting cell types (16). In spleen cell suspensions of unprimed mice, ARC and/or inducer cells are presumably in relative excess over marrow P-PFC (32). Individual splenic ASU also yield about 150 direct PFC. Available evidence would then suggest that one inducer cell interacts with one marrow precursor

(and perhaps another cell type) to form the elementary responding unit specialized for antibody class and specificity.

One would expect that dose-response curves for PFC produced by transferred spleen should be linear and have a slope of 1.0, since (*a*) the yield of direct PFC/ASU is constant in marrow-thymus cell mixtures and in spleen cell suspensions (16), and (*b*) the number of splenic ASU is a linear function of the number of nucleated cells (12, 13, 15, 17, 33). Instead, it has been observed that such dose-response curves are biphasic, with a linear upper portion having a slope of 1.0, and a lower portion that is either nonlinear or having a slope greater than 1.0 (19, 33, 34). To explain such observations, Groves et al. (34) proposed that each ARC may interact with a variable but finite number of marrow precursors, thus forming ASU differing with respect to their cellular composition. The number of PFC/ASU should then be variable and directly related to the number of P-PFC entering the interaction. As P-PFC may not saturate ARC in spleen cell suspensions until a critical P-PFC concentration has been reached, such a model could reproduce the biphasic dose-response curves. However, the model has little predictive value, since it is inconsistent with the following realities and ignores other possibilities: the number of PFC/ASU is not variable (16); ARC undergo proliferation and perhaps differentiation into inducer cells prior to interaction; each of the 80–800 inducer cells derived from 1 ARC can form 1 ASU yielding the constant number of approximately 150 direct PFC[2]; graded numbers of inducer cells transferred with a nonlimiting constant number of marrow cells yield linear dose-response lines for direct PFC[2]; progeny cells of ARC can interact with precursors of direct and indirect PFC, but each ASU is restricted to generate either direct or indirect PFC, but not both (10, 16); the lower portion of biphasic dose-response curves characteristic of transferred spleen cells can be accounted for by variable numbers of cell divisions in ARC-derived clones rather than by multiple ARC interactions.

SUMMARY

Marrow cells and thymocytes of unprimed donor mice were transplanted separately into X-irradiated syngeneic hosts, with or without sheep erythrocytes (SRBC). Antigen-dependent changes in number or function of potentially immunocompetent cells were assessed by retransplantation of thymus-derived cells with fresh bone marrow cells and SRBC; of marrow-derived cells with fresh thymocytes and SRBC; and of thymus-derived with marrow-derived cells and SRBC. Plaque-forming cells (PFC) of the direct (IgM) and indirect (IgG) classes were enumerated in spleens of secondary host mice at the time of peak responses. By using this two-step design, it was shown (*a*) that thymus, but not

[2] Shearer, G. M., T. Ito, and G. Cudkowicz. Distinct events in the immune response elicited by transferred marrow and thymic cells. II. Maturation of thymic antigen-reactive cells into specific inducer cells. Manuscript in preparation.

bone marrow, contained antigen-reactive cells (ARC) capable of *initiating* the immune response to SRBC (first step), and (*b*) that the same antigen complex that activated thymic ARC was required for the subsequent interaction between thymus-derived and marrow cells and/or for PFC production (second step).

Thymic ARC separated from marrow cells but exposed to SRBC proliferated and generated specific inducer cells. These were the cells that interacted with marrow precursors of PFC to form the elementary units for plaque responses to SRBC, i.e. the class- and specificity-restricted antigen-sensitive units. It was estimated that each ARC generated 80–800 inducer cells in 4 days by way of a minimum of 6–10 cell divisions. On the basis of the available evidence, a simple model was outlined for cellular events in the immune response to SRBC.

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