IMMUNOLOGICAL MEMORY IN MICE

II. Cell Interactions in the Secondary Immune Response Studied by Means of Immunoglobulin Allotype Markers*

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The immune response elicited by a second injection of antigen differs markedly from that seen after the first injection. The initial contact with antigen results in an altered state of responsiveness, referred to as immunological memory, which enables the animal to produce antibodies more rapidly and in greater quantity. These antibodies are predominantly of the 7S class and are of higher affinity (1) than those produced in the primary response. Memory for different classes of antibody, directed against the same antigenic determinants, has been shown to be carried by different cell lines that arise, independently, during the primary response to antigen (2, 3).

It is now well established that the primary immune response to sheep erythrocytes (SRBC) in mice requires the interaction of two types of lymphoid cells, derived from thymus and bone marrow (4, 5). Thymus derived cells respond to an antigenic stimulus with a burst of mitotic activity (6) but do not appear capable of producing antibody (7). Mitchell and Miller (8) have shown that the cells which produce antibody in the primary 19S response are mainly of bone marrow origin. Thus the antigen-reactive cells (ARC) in the thymus population interact with antibody-forming cell precursors (AFCP) present in the bone marrow, resulting in the differentiation of the latter into antibody-forming cells.

Recognizing that the immune response to SRBC requires the interaction of these two cell types, the difference between a primary and secondary response may be due to a quantitative and/or qualitative change in either or both of these cell types.

Studies of the origin of the antibody-forming cells are greatly facilitated by the use of congenic strains of mice, which differ genetically only at the loci that code for immunoglobulin H chain (or Fc) structure. The Ig-1 allotypic difference between the two strains (C3H.SW$_{SnHz}$ and CWB), provides a marker on the 7S (γG_{2a}) molecules that can be exploited to determine the number and origin of antibody producing cells,

by developing with appropriate anti-allotype sera in the indirect plaque forming cell (PFC) technique (9–11).

In these experiments we first set out to determine whether the thymus cell–bone marrow cell interaction described for γM antibody production, and detected by H-2 (cell membrane antigen) markers (8) is also necessary for γG production, as determined by immunoglobulin allotypic marking of the PFC.

Having established that similar conclusions are reached with both types of cell markers, it was then possible to study the activity of primed cells in the adoptive secondary immune response by injecting, into irradiated recipients, spleen cells from immunized mice or subpopulations of these cells obtained by bovine serum albumin (BSA) gradient centrifugation (3), together with allotypically different normal spleen or bone marrow cells. All the γG_{2a} PFC detected in this adoptive secondary response were found to be of the primed cell allotype.

Materials and Methods

Mice.—C3H·SW$_{\text{SnHz}}$(CSW) mice and their congenic partner strain, CWB mice, were used in these experiments. The genetic background of these mice and the means by which the congenic strain was derived have been described in detail (3, 12). Donor mice were 6–8 wk old at the time of immunization and 9–15-wk-old mice were used as recipients. In those experiments in which thymectomized mice were used, thymectomy was performed at the age of 4–6 wk, using a modification of the method described by Sjodin et al. (13). The mice were irradiated (see below) 1–2 wk after thymectomy and injected intravenously with 10^7 bone marrow cells, obtained from mice of the same strain. The mice were given water containing antibiotic (neomycin-polymyxin B[1]) 1 day prior to irradiation and maintained on this antibiotic throughout the experiment. Absence of thymus remnants was assured at the time of sacrifice by macroscopic, and occasionally microscopic, examination.

Irradiation.—Recipients of primed spleen cells were given 600 rads total body irradiation 4–6 hr prior to cell transfer. Adult thymectomized mice received 800 rads total body irradiation. A Siemens X-ray machine operating under the following conditions was used: 250 kv, 15 ma, 0.25 mm Cu + 1.0 mm Al and a HVL (half-value layer) of 1.10 mm Cu. The dose rate was 80 rads/min, and the focal skin distance was 60 cm.

Immunization.—In most experiments donor mice were given a single intravenous immunizing injection of either 4×10^8 or 4×10^5 SRBC. The mice were sacrificed at various times after SRBC injection (4–118 days) and their spleens were removed. In one set of experiments the mice were given two intravenous injections of 4×10^8 SRBC, spaced 8 days apart, and their spleens were removed 6 months later. Adult thymectomized, irradiated, bone marrow protected mice were injected intravenously with 4×10^8 SRBC together with 5×10^7 thymus cells and sacrificed 8–10 days later. Control mice did not receive thymus cells. In several experiments, a second intravenous injection of 4×10^8 SRBC was given on day 8 and the mice sacrificed 6 days later.

Cell Suspensions.—Single cell suspensions of spleen and thymus were obtained by gently

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[1] 1.13 g neomycin sulfate plus 836.2 units polymyxin B sulfate per liter, diluted in distilled, deionized water.

pressing the tissue through a 50-mesh stainless steel screen into cold MEM-PM[2]. Clumps were broken up by repeated aspiration, using a Pasteur pipette, and the remaining fragments were allowed to settle. The supernatants were transferred, centrifuged for 10 min at 300 g at 4°C, and the cells resuspended in MEM-PM. Thymus cells were given one additional washing. Cell counts were obtained using a Coulter counter, Model B, fitted with a 100 μ aperture and using optimum window settings previously calibrated with hemocytometer counts of nucleated spleen cells.

Bone marrow was obtained from the femurs and tibiae of 6–8-wk old mice. After cutting off the epiphyses, the marrow was extruded by forcing cold MEM-PM through with a syringe. Single cell suspensions were obtained in the manner described above.

Cell Separation.—Cells were separated by means of the BSA density gradient centrifugation method described by Raidt et al. (14). This method is described in detail in the preceding paper (3).

Hemolytic Plaque Assay.—The numbers of direct (γM) and indirect (γG) PFC in spleen cell suspensions were determined using a modification of the hemolysis in gel technique originally described by Jerne et al. (15), adapted for microscope slides by Mishell and Dutton (16), and extended for development of indirect (γG) PFC (9–11). Total γG PFC were developed with polyvalent rabbit anti-mouse Ig. Gamma G_{2a} plaques were developed with mouse anti-allotype sera, which specifically detect cells secreting antibody of this class of the corresponding allotype. The number of total γG and γG_{2a} PFC was determined by subtracting the number of direct PFC from the total number of PFC developed with the antisera. A complete description of the method, and of the antiallotype sera used, has already been published (3).

RESULTS

Thymus-Bone Marrow Interaction in Congenic Mice.—In order to determine whether the thymus-bone marrow relationship described for initiation of the γM hemolysin response (8) is also required for production of γG antibody, the following experiments were set up using congenic mice. Adult thymectomized irradiated CWB mice, protected with 10^7 syngeneic bone marrow cells 1–2 wk previously, were injected with 5×10^7 CSW thymus cells together with 4×10^8 SRBC, and their spleens assayed 8–10 days later. In some of the experiments, reciprocal combinations were used. All of the mice made a good γM primary response as determined by the hemolytic plaque technique. Only a few of the mice showed an increase in γG_{2a} PFC sufficient to determine whether they were of bone marrow or thymus origin, and in these mice all γG_{2a} PFC were of bone marrow allotype. Since no allotypic markers have yet been found for mouse γM, it is essential for this type of study that a large number of γG antibody-forming cells be present in the population. In an attempt to increase the γG_{2a} response, the remaining mice were given a second injection of 4×10^8 SRBC

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[2] Minimum Essential Medium (Eagle) Cat. No. F-12 Instant Tissue Culture Powder Medium without $NaHCO_3$ (Grand Island Biological Co., Oakland, Calif.) made up with $Na_2HPO_4 \cdot 12H_2O$ (358 mg/liter) and $MgCl_2 \cdot 6H_2O$ (200 mg/liter) instead of bicarbonate, in distilled, deionized water.

8 days after the injection of thymus and SRBC, and the spleen cells were assayed 6 days later.

It can be seen in Table I that the thymus cell recipients made an excellent secondary γG_{2a} response, while controls, given two injections of SRBC only, made no response. All of the PFC detected were of the bone marrow allotype. Similar responses were obtained in a few experiments in which neonatally thy-

TABLE I

Origin of PFC in the Secondary Response to SRBC after Thymus-Bone Marrow Interaction, Determined by Ig Allotype Markers

Recipient strain (allotype)	Bone marrow donor (allotype)	Thymus donor (allotype)	Cell No. in recipient spleen ($\times 10^{-6}$)	PFC per recipient spleen ($\times 10^{-2}$)		
				γM	γG_{2a} allotype	
					(a)	(b)
CWB (b)	CWB (b)	CSW (a)	88	23	0	29
"	"	"	130	64	0	102
"	"	"	95	209	0	153
"	"	"	100	221	0	145
"	"	"	65	62	0	126
"	"	"	100	151	0	327
"	"	None	43	0.34	0	0
"	"	"	10	1.2	0	0
CSW (a)	CSW (a)	CWB (b)	100	359	220	0
"	"	None	83	2	0	0
CWB (b)*	CSW (a)	CWB (b)	100	52	53	0
"	"	"	100	126	116	0
"	"	None	65	4.75	0	0
"	"	"	50	0.33	0	0

Adult thymectomized mice were irradiated (800 rads), protected with 10^7 bone marrow cells (intravenous), and injected intravenously 1-2 wks later with 5×10^7 thymus cells together with 4×10^8 SRBC. A second intravenous injection of 4×10^8 SRBC was given 8 days after transfer, and spleen cells were assayed 6 days later.

* These results were taken from a separate experiment in which the recipients had been injected with anti-allotype serum (anti-a) in an attempt to suppress the response, and are included only to show that the irradiated hosts do not contribute to the response.

mectomized mice were used. Thus cells making 7S antibody, at least of the γG_{2a} class, are derived from bone marrow cells both in the primary response (reported above) and in the secondary response. Moreover, both responses are dependent on the initial presence of thymus cells. These results are similar to those reported by Mitchell and Miller for the primary γM response, using H-2 markers (8).

Interaction of Primed Spleen Cells with Bone Marrow Cells.—The following experiments were carried out to determine whether primed cells are capable of interacting with AFCP from unprimed spleen or bone marrow cells and induc-

ing differentiation of these latter cells into antibody forming cells. Mice of one strain were injected intravenously with 4×10^8 SRBC and their spleens were removed 4–118 days after immunization. Cell suspensions obtained from these spleens were injected into irradiated mice of the congenic partner strain (5×10^6 per mouse), either alone or together with 4×10^6 bone marrow cells from normal mice of the recipient strain. In several experiments, 4×10^6 unprimed spleen cells were used in place of bone marrow cells. Control animals

TABLE II

Effect of Bone Marrow Cells on the Adoptive Secondary Immune Response of Spleen Cells from Immunized Mice

Spleen cells injected*	No. of bone marrow cells injected	No. of recipient animals	Cell No. in recipient spleen‡ $\times 10^{-6}$	PFC per recipient spleen‡ $\times 10^{-2}$			
						γG_{2a}	
				Direct	Total γG	Primed allotype	Bone marrow allotype
5×10^6 unfractionated spleen cells	—	19	61 (37–101)	21 (12–35)	89 (34–233)	38 (16–89)	—
	4×10^6	20	129 (89–186)	63 (36–110)	303 (199–461)	84 (36–195)	0.1 (0.01–1.8)
5×10^6 spleen cells from BSA gradient fractions A or B	—	20	67 (48–94)	39 (20–72)	206 (112–378)	95 (45–202)	—
	4×10^6	23	127 (98–163)	85 (51–141)	410 (270–623)	156 (71–342)	0.1 (0.02–1.2)

* Spleen cells were taken from donor mice 4–118 days after immunization with 4×10^8 SRBC. A challenge dose of 4×10^8 SRBC was given at the time of transfer.

‡ Numbers of cells or PFC in recipient spleen 7 days after cell transfer are expressed as geometric means. Values in parentheses indicate the 95% confidence limits computed using appropriate Student t-test values.

were injected with unprimed spleen or bone marrow cells alone. In all of the cell transfers, 4×10^8 SRBC were injected along with these cells.

Bone marrow cells injected with primed spleen cells resulted in much larger spleens. The number of cells recovered from the spleens of these recipients 7 days after transfer was approximately twice that in recipients of primed cells alone (Table II). The mean values for all classes of PFC determined were also increased approximately two-fold when bone marrow was given. However, all of the γG_{2a} PFC found in recipient spleens were of primed cell allotype. Similar results were obtained when cell suspensions which had been enriched for memory cells by BSA density gradient fractionation were used (Table II and see below).

In a few experiments 10^7 primed spleen cells were injected intravenously into adult thymectomized irradiated mice, protected 1 or 2 wk earlier with 10^7

syngeneic bone marrow cells. Nonthymectomized mice, irradiated 4–6 hr before injection of primed cells, were used as controls. As shown in Table III, even bone marrow cells that had resided in the spleens of the recipients for 1 or 2 wk prior to primed cell transfer did not participate in the adoptive secondary response

TABLE III

Adoptive Secondary Immune Response of Primed Spleen Cells* in Mice Injected with Bone Marrow 1 or 2 Wk Previously

Irradiation and cell injection on day:			No. of recipient animals	Cell No. in recipient spleen‡ × 10⁻⁶	PFC per recipient spleen‡ × 10⁻²		
						γG₂ₐ	
0	7	14			Direct	Primed allotype	Bone marrow allotype
800R§ 10⁷ bone marrow	10⁷ primed spleen	—	5	107 (87–132)	25 (5–115)	273 (70–1065)	0
None	800R‖ 10⁷ primed spleen	—	3	32 (16–63)	51 (30–86)	477 (298–764)	0
800R§ 10⁷ bone marrow	—	10⁷ primed spleen	4	90 (60–135)	21 (13–37)	190 (73–495)	0
None	—	800R‖ 10⁷ primed spleen	3	35 (19–64)	28 (17–45)	402 (385–419)	0

* Spleen cell donors were given two injections of 4×10^8 SRBC, spaced 8 days apart, and the primed cells were taken for transfer 6 months later. A challenge dose of 4×10^8 SRBC was given at the time of transfer.

‡ Numbers of cells or PFC in recipient spleen 7 days after primed cell transfer are expressed as geometric means. Values in parenthesis indicate the 95% confidence limits computed using appropriate Student t-test values.

§ Adult thymectomized mice were irradiated 4–6 hr before cell injection.

‖ Adult mice were irradiated 4–6 hr before cell injection.

obtained. All of the γG₂ₐ PFC detected in the spleens of these bone marrow restored mice were of primed cell allotype.

Interaction of Fractionated Spleen Cells with Unprimed Spleen or Bone Marrow Cells.—It was shown in the previous paper (3) that fractionation of primed spleen cells on a BSA density gradient resulted in an enrichment of cells carrying memory for all classes in the fractions of lowest density (A and B). The fraction of greatest density (D) contained cells that gave rise to a γM and γG₁ response, but was totally depleted of cells carrying memory for γG₂ₐ. Injection of 4×10^6 bone marrow cells did not lead to PFC of bone marrow allotype (Table II). Changing the ratio of the two cell types by lowering the total

TABLE IV

Effect of Normal Bone Marrow or Spleen Cells on the Adoptive Secondary Immune Response of BSA Density Gradient Fractions A and D of Spleens from Immunized Mice

Injected cells*	No. of recipient mice	Total cell No. in recipient spleen‖ $\times 10^{-6}$	PFC per recipient spleen§ $\times 10^{-2}$		
			Direct (γM)	γG_{2a} Allotype	
				(a)	(b)
5 $\times$ 10^6 normal spleen cells (a)‡	9	35 (26–48)	3 (1.6–9.1)	0	0
4 $\times$ 10^6 bone marrow cells (b)	8	118 (93–151)	1 (0.2–6.9)	0	0
10^6 A-band cells (a)	8	19 (16–22)	10 (4.3–22)	12 (8.4–18)	0
10^6 A-band cells (a) + 4 $\times$ 10^6 bone marrow cells (b)	8	131 (118–145)	40 (25–63)	33 (22–57)	0.1
10^6 A-band cells (a) + 4 $\times$ 10^6 normal spleen cells (b)	7	35 (23–51)	19 (12–31)	11 (6.3–20)	0.2 (0.01–3.4)
10^6 D-band (a)	6	21 (17–25)	7 (4.6–10)	0	0
10^6 D-band (a) + 4 $\times$ 10^6 bone marrow cells (b)	6	121 (113–130)	29 (15–55)	0	0.1 (0.01–1.5)
10^6 D-band (a) + 4 $\times$ 10^6 normal spleen cells (b)	6	59 (48–72)	26 (21–33)	0	0.03 (0.002–0.5)

* Spleen cells were taken from donor mice 6 days after priming with 4 $\times$ 10^5 SRBC. The cells were injected together with 4 $\times$ 10^8 SRBC into recipient mice.

‡ Letter in parentheses indicates Ig-1 allotype.

§ Numbers of cells or PFC in recipient spleen 7 days after cell transfer are expressed as geometric means. Values in parentheses indicate the 95% confidence limits computed using appropriate student t-test values.

number of spleen cells injected, without greatly decreasing the number of memory cells in the population, might conceivably provide a better opportunity for AFCP in unprimed spleen or bone marrow to interact with these cells. In order to test this, 10^6 cells from fraction A and from fraction D, obtained 6 days after priming with 4 $\times$ 10^5 SRBC, were injected into irradiated recipients with and without 4 $\times$ 10^6 unprimed spleen or bone marrow cells of recipient allotype,

together with 4×10^8 SRBC. As seen in Table IV, injection of 10^6 A-band cells alone resulted in a good γG_{2a} memory response. As previously shown, the simultaneous injection of bone marrow cells had an enhancing effect on recipient spleen cell numbers and total numbers of PFC. This was also found with 4×10^6 unprimed spleen cells, but to a much lesser degree. As in all of the previous experiments, no significant numbers of γG_{2a} PFC of unprimed spleen or bone marrow cell allotype were detected. Transfer of 10^6 D-band cells, together with bone marrow or unprimed spleen cells also resulted in a greater total spleen cell number and an enhanced γM response. The depletion of γG_{2a} memory cells from this fraction was not influenced by the addition of unprimed spleen or bone marrow cells.
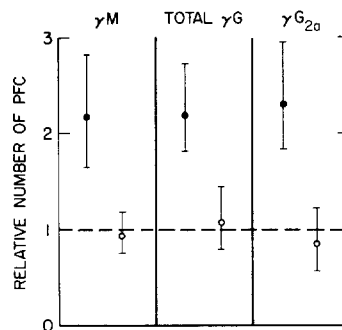

FIG. 1. Number of PFC of different classes, expressed as PFC per 10^6 recipient spleen cells (O) and as PFC per recipient spleen (●) in mice injected with primed spleen cells (5×10^6), alone or together with bone marrow cells (4×10^6), and 4×10^8 SRBC. Points represent the geometric mean of the number of PFC obtained in spleens of recipients receiving bone marrow relative to the numbers obtained from recipients of primed cells alone. Vertical bars indicate 95% confidence limits.

A comparison was made of the number of PFC of each class obtained with and without bone marrow in individual transfer experiments, calculated both per 10^6 recipient spleen cells and per recipient spleen. Since the effect of simultaneously injected bone marrow was similar in all experiments (see Table II), the results were pooled and are shown in Fig. 1, expressed as the values obtained with bone marrow, relative to those obtained without. It can be seen that for all classes, the presence of bone marrow in these recipients resulted in a two-fold increase in PFC when calculated per recipient spleen, while, in contrast, the numbers of PFC per 10^6 cells was not significantly different than in recipients that received primed cells alone.

DISCUSSION

The data presented here, using allotypic markers, provide further evidence for an interaction between thymus-derived and bone marrow derived cells in the

development of an immune response against SRBC in mice. As in the γM response in which the AFCP were identified by means of H-2 markers (8), the γG_{2a} response observed in these studies was due exclusively to cells producing antibodies that carry the allotype of the bone marrow donor.

Since the allotypic markers are present on the H chain (Fc portion) of the immunoglobulin molecules, these results indicate that the interaction between thymus derived ARC and bone marrow derived AFCP is not a result of macromolecular information transfer for the entire H chain, as would be mediated by a mRNA (17). This possibility could not be ruled out in the experiments utilizing H-2 membrane antigenic markers.

Radovich et al. (18) have reported an increase in the number of 19S PFC when primed spleen cells were injected together with syngeneic normal bone marrow cells into irradiated recipients. It was not determined in these studies whether the 19S PFC were of spleen or bone marrow origin, and the results were interpreted to be due to a nonspecific effect of bone marrow cells on the localization or proliferation of antibody-forming cells from the primed spleen inoculum. The results reported in this paper, with markers on the two cell types, support this interpretation. The simultaneous injection of normal bone marrow (or spleen) and primed spleen cells, each of which could be identified by its allotypic marker, resulted in a significant increase in total spleen cell number, and also in the number of PFC per recipient spleen. In no case, however, could any evidence be found that would indicate that this increase in PFC was due to a recruitment of AFCP in the bone marrow inoculum, resulting from an interaction with the primed spleen cells. In every experiment, the γG_{2a} PFC that arose during the adoptive secondary response carried the allotypic determinant of the primed donor cells. This was true even in those experiments in which attempts were made to give the bone marrow cells a selective advantage by using a lower number of primed cells, obtained from BSA density gradient fractions that were either enriched for or depleted of γG_{2a} memory cells.

That the effect of the bone marrow cells is a nonspecific one seems even more probable if one considers that no enhancement of the response is noted when the results are calculated per 10^6 recipient spleen cells (Fig. 1). An enhancement is seen only when the results are calculated per recipient spleen, and thus appears to be merely a reflection of the increase in the total cell number due to the bone marrow inoculum.

The possibility has also been considered that bone marrow cells may not be able to interact specifically with primed cells unless they have previously had time to become established in the lymphoid tissue of the recipient. However, the results obtained indicate that regardless of whether the bone marrow cells are injected together with primed cells or are allowed to previously settle in the recipient spleen, they do not develop into antibody-producing cells.

Moreover, in the latter case, the nonspecific enhancement of the PFC response discussed above is not seen, possibly due to a limitation of biological space.

At least three possible mechanisms for memory at the cellular level may be postulated. These depend on whether the primed cell population is composed of thymus derived cells, bone marrow derived cells, or both cell types. The first possibility is that memory cells are ARC, entirely of thymus origin, which can interact with any specific AFCP present in bone marrow or unprimed spleen to result in a secondary response. This possibility is ruled out, at least for γG_{2a}, by the evidence presented here that primed cells appear to be capable of responding entirely on their own, in the presence or absence of additional bone marrow cells. The second possibility is that memory is carried entirely by the bone marrow cell line, presumably following an interaction with thymus cells in the primary response. This possibility is consistent with the results presented here and accepting it would require that there be a qualitative difference between the primary and secondary immune response: namely, that the cells reacting with antigen in the secondary response be themselves able to become antibody-producing cells, in contrast with the primary response where the ARC are thymus-derived and are not the precursors of antibody-producing cells. However, our results in no way rule out the third possibility: that the primed cell population itself is made up of both thymus-derived and bone marrow-derived cells that interact preferentially with each other, perhaps due to higher affinity receptors on the surface of both cells which compete more effectively for the antigen. If this is the case, then the two cell types are not distinguishable by their behavior on either BSA density gradients or, as shown previously (3), on glass bead columns.

SUMMARY

Congenic mice, differing genetically only at the loci coding for immunoglobulin H chain (or Fc) structures, have been used to study cell interactions in the 7S (γG_{2a}) antibody response to sheep erythrocytes (SRBC), as detected by the Jerne plaque-forming cell (PFC) method.

The interaction between thymus and bone marrow cells was studied in adult thymectomized irradiated recipients, protected with syngeneic bone marrow and injected with thymus cells from the partner congenic strain. All of the γG_{2a} PFC detected in the spleens of these mice were of bone marrow allotype.

Adoptive secondary immune responses were then studied to determine whether a similar interaction between memory cells and bone marrow derived cells could be detected. Primed spleen cells from the partner congenic strain, or a subpopulation of these cells obtained by BSA density gradient fractionation, were injected into irradiated recipients alone, or together with syngeneic nonimmune spleen or bone marrow cells. All γG_{2a} PFC detected in these experi-

ments were of primed cell allotype. There was no evidence that antibody forming cell precursors in normal spleen or bone marrow participate in the adoptive secondary immune response detected 7 days after transfer of primed spleen cells. This was true regardless of whether the bone marrow cells were injected at the time of transfer, or were injected 1–2 wk earlier and allowed to become established in the spleens of recipient mice. Although no specific cell interaction was seen, bone marrow (and, to a lesser degree, normal spleen) cells were found to have a nonspecific enhancing effect on the adoptive secondary response when they were injected together with the primed spleen cells. This enhancement was not evident if the bone marrow cells were injected 1 or 2 wk prior to primed cell transfer.

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