ALLOGENEIC CARRIER-SPECIFIC ENHANCEMENT OF HAPTEN-SPECIFIC SECONDARY B-CELL RESPONSES*

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One of the consequences of immunization with "T-dependent" antigens is the generation of immunologic memory. This is evidenced by an increase in the number of B cells responsive to the stimulating antigen and by the generation of a new type of B cell (secondary B cells), which is qualitatively different from the type that predominates in nonimmune animals (primary B cells) (1).

Included among the characteristics that discriminate secondary from primary B cells are their prolonged half-life (2), their lymphatic recirculation (2), the immunochemical nature of their antigen receptors (3, 4), and the parameters of their stimulation and tolerance induction (1, 5–7). In a previous report it was demonstrated that spleen cells from nonimmune donors could be stimulated by T-dependent antigens in collaboration with T cells which differ at the *H-2* locus as long as these T cells recognized the carrier portion of the antigen (8). Antibody-forming cell clones resulting from primary B cells stimulated in the allogeneic carrier-primed environment made antibody solely of the IgM heavy chain isotype, while the response of the same cells in syngeneic recipients was predominantly IgG_1 or IgG_1 and IgM. The ability of carrier-primed T cells and hapten-specific B cells to collaborate to yield clones of IgG_1 antibody-producing cells was dependent on the genetic identity of the collaborating cells in the *I* region of the *H-2* complex.

In this report we have extended this study to an analysis of the response of secondary B cells stimulated in carrier-primed syngeneic and allogeneic environments to determine if secondary B cells require *I*-region identity with collaborating carrier-specific T cells to be stimulated to IgG_1 antibody synthesis. The results of these studies demonstrate that, in contrast to primary B cells which produced only IgM antibody in allogeneic carrier-primed recipients, the majority of secondary B cells can be stimulated to produce IgG_1 antibody-forming B-cell clones in allogeneic carrier-primed recipients. The ability to be stimulated to IgG_1 antibody synthesis by a collaborative interaction with allogeneic T cells would appear to be a characteristic of secondary B cells which further distinguishes secondary from primary B cells.

Materials and Methods

Antigens. Limulus polyphemus hemocyanin (Hy),[1] 2,4-dinitrophenylated-Hy (DNP_{10}-Hy), phosphorylcholine-Hy (PC_{10}-Hy), DNP_{10}-bovine serum albumin (DNP_{10}-BSA), PC_{10}-BSA, and 3-

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; CFA, complete Freund's adjuvant; HS, horse serum; Hy, hemocyanin; PBS, phosphate-buffered saline; PC, phosphorylcholine.

(*p*-azophenylphosphorylcholine)-*N*-acetyl-L-tyrosylglycylglycine Boc hydrazide-Hy, a phosphorylcholine-tripeptide-spacer-Hy conjugate, were prepared as described elsewhere (5, 9).

Animals. 6- to 8-wk old BALB/cJ mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. BALB/cAnN mice were acquired through the Institute for Cancer Research, Philadelphia, Pa. Mice from both sources were used interchangeably. AKR/J, C3H/J, and A/J strains of mice were purchased from The Jackson Laboratory.

Immunizations. Animals used as carrier-primed recipients of cell transfers received an intraperitoneal injection of 0.1 mg of Hy in complete Freund's adjuvant (CFA) 6–12 wk before use. BALB/c, C3H/J, and AKR/J mice were injected with 0.1 mg DNP-Hy or PC-Hy in CFA intraperitoneally 8 wk before use as secondary B-cell donors (5).

Cell Transfers and Fragment Cultures. The methodology for obtaining monoclonal antibody responses in splenic fragment cultures and the detection of antibody in culture fluids by a radioimmunoassay have been previously described (1, 5, 10). Anti-DNP and anti-PC antibodies were also detected using a modification of this solid-phase radioimmunoassay. DNP-BSA or PC-BSA was used to coat wells of disposable polyvinyl microtiter plates (Cooke Laboratory Products Div., Dynatech Laboratories, Inc., Alexandria, Va.) by incubating 0.1 ml of a solution of DNP_{10}-BSA or PC_{10}-BSA [10^{-4} M hapten in phosphate-buffered saline, (PBS)] in each well for 2 h at room temperature (11, 12). Antigen was removed by suction and the plates were washed with PBS containing 1% agamma horse serum (PBS-HS) by adding 0.1 ml of PBS-HS to each well and removing by inverting the plates and flicking out the PBS-HS. 0.1 ml PBS containing 10% HS was added to each well, and incubated for 30 min at room temperature to saturate the protein-binding capacity of the plastic. This solution was removed and the plates were washed once in PBS-HS. 25 μl of culture fluid was added to each well and the plates were incubated for 2 h at room temperature. The culture fluids were removed and the plates were washed twice with PBS-HS. 0.1 ml ^{125}I-labeled anti-Fab, anti-γ1, or anti-μ purified antibodies (5, 13) was added to each well and incubated overnight at 4°C. The ^{125}I-labeled antibody was removed by suction and the plates washed twice with PBS-HS and eight times with water. The plates were dried and cut into individual wells which were then counted in a gamma counter.

Assays for the TEPC 15 idiotype of anti-PC antibody and the heavy chain isotype of monoclonal antibodies have been described previously (9, 13).

Depletion of T Lymphocytes. Donor spleen cells were treated with anti-theta antiserum and complement (C) as previously described (14). The anti-theta antiserum was prepared from the ascites fluids of AKR mice injected with C3H mouse thymocytes. The effectiveness of this antiserum against T cells and its lack of toxicity for B cells has been previously demonstrated (14, 15). Rabbit serum was absorbed with BALB/c spleen cells, frozen, and used as a source of C.

Results

The ability of secondary B cells to respond to DNP-Hy in syngeneic and allogeneic environments was investigated using the splenic fragment culture system which permits estimates of the efficiency of antigenic stimulation at the level of individual antigen-specific B cells. Previous studies have indicated that: (*a*) 5% of the transferred B cells are lodged in the cultured fragments, (*b*) 80% of the antigen-specific B cells lodged in the fragment cultures can be stimulated to yield detectable responses, and (*c*) these responses represent the clonal progeny of a single stimulated donor B cell and yield homogeneous antibody (1, 10). Table I summarizes the data obtained from an analysis of the culture fluids from fragments of Hy-primed and nonprimed syngeneic and allogeneic recipients which had received spleen cells from DNP-Hy-immunized mice. As previously reported, 10–20% of secondary B cells can be stimulated in noncarrier-primed recipients (5, 14). As much as one half of this response has been shown to be dependent on carrier-specific donor T cells in that anti-theta antiserum and C treatment of the donor cell inoculum reduced the number of secondary B cells responding to the hapten-carrier complex used for immunization to the level of

TABLE I

The Frequency of Secondary B-Cell Anti-DNP Responses in Syngeneic and Allogeneic Recipients

Donor*	Recipient	Recipient carrier primed‡	No. of donor cells analyzed × 10^{-6}	No. of clones per 10^6 spleen cells transferred
BALB/c(d)	BALB/c(d)	–	84	1.14
	BALB/c(d)	+	20	5.40
BALB/c(d)	A/J(a)	–	30	0.58
	A/J(a)	+	18	4.17
BALB/c(d)	AKR(k)	–	18	0.70
	AKR(k)	+	16	3.50

* All donor mice had been immunized with 0.1 mg DNP-Hy in CFA 6–8 wk before cell transfer, 2 × 10^6 donor cells were transferred to carrier-primed recipients and 6 × 10^6 donor cells were transferred to noncarrier-primed recipients.

‡ Recipient mice were carrier-primed by immunizing with 0.1 mg Hy in CFA 6–8 wk before cell transfer.

the response to the hapten on heterologous or nonimmunogenic carriers (14). This is illustrated in Table II, which shows that treatment of the donor cell inoculum with anti-theta antiserum and C before transfer, reduced by half the number of secondary B cells responding in noncarrier-primed syngeneic recipients. It is of interest that the response of anti-theta-treated donor B cells approximated the frequency of the secondary B-cell responses in allogeneic noncarrier-primed recipients. Table II also demonstrates that anti-theta antiserum and C treatment of donor cells did not reduce the frequency of secondary B cells responding in allogeneic noncarrier-primed recipients. These findings imply that carrier-primed donor T cells may be incapable of enhancing donor secondary B-cell responses in an allogeneic environment.

The effect of carrier priming of the syngeneic and allogeneic recipients on the donor secondary B-cell responses is shown in Table I. As can be seen, carrier priming the syngeneic or allogeneic recipients increased the number of responding secondary B cells by at least fivefold. The increase in the number of B cells responding in allogeneic recipients was somewhat variable and generally less than the increase observed for syngeneic recipients. In both instances this enhancement is dependent on specific T-cell-B-cell interaction since it was found that equivalent concentrations of the carrier protein used to immunize the recipients did not enable enhancement of the response of secondary B cells to the hapten on a heterologous carrier.

Table III summarizes the data obtained from an analysis of the heavy chain isotype of antibody produced by secondary B cells stimulated in syngeneic and allogeneic recipients. Secondary B cells stimulated in nonprimed syngeneic or allogeneic recipients yielded almost entirely IgG_1-producing antibody-forming cell clones. Carrier priming of syngeneic recipients increased the number of IgG_1-producing clones by approximately fourfold. Carrier priming of the allogeneic recipients increased the number of IgG_1 antibody-producing secondary B-

TABLE II

The Effect of Donor T-Cell Depletion on Secondary B-Cell Responses

Donor*	Anti-theta anti-serum and C treatment	Noncarrier-primed recipients	*H-2* identity	No. of donor cells analyzed $\times 10^{-6}$	No. of clones per 10^6 spleen cells transferred
BALB/c(d)	–	BALB/c(d)	+	24	1.08
	+	BALB/c(d)	+	24	0.50
BALB/c(d)	–	AKR(k)	–	24	0.42
	+	AKR(k)	–	24	0.50

* All donor animals were immunized with 0.1 mg DNP-Hy in CFA 6–8 wk before cell transfer.

TABLE III

The Heavy Chain Isotype of Anti-DNP Antibody Derived from Secondary B cells Transferred to Syngeneic and Allogeneic Recipients

Donor*	Recipient	Recipient carrier primed‡	*H-2* identity	Frequency IgG_1§	% Total response IgG_1	Frequency IgM‖	% Total response IgM
BALB/c(d)	BALB/c(d)	–	+	1.12	98	0.02	2
AKR(k)	AKR(k)	–	+	1.50	93	0.11	7
BALB/c(d)	BALB/c(d)	+	+	5.10	94	0.30	6
AKR(k)	AKR(k)	+	+	6.18	87	0 95	13
C3H(k)	C3H(k)	+	+	4.67	90	0 50	10
BALB/c(d)	A/J(a)	–	–	0 58	>99	<0.01	<1
	AKR(k)	–	–	0.50	>99	<0 01	<1
	C3H(k)	–	–	0.70	>99	<0.01	<1
AKR(k)	BALB/c(d)	–	–	0.95	>99	<0.01	<1
BALB/c(d)	A/J(a)	+	–	3.67	88	0.50	12
	AKR(k)	+	–	2.75	79	0 75	21
AKR(k)	BALB/c(d)	+	–	3.92	90	0.42	10
C3H(k)	BALB/c(d)	+	–	3.00	75	1.00	25

* Donor mice had been immunized intraperitoneally with 0.1 mg DNP-Hy in CFA 8 wk before cell transfer

‡ Recipient mice were carrier-primed by immunizing with 0.1 mg Hy in CFA 8 wk before cell transfer.

§ These figures represent the frequency of IgG_1 or IgG_1 plus IgM antibody-producing B-cell clones per 10^6 spleen cells transferred

‖ These figures represent the frequency of B-cell clones producing only IgM antibody per 10^6 spleen cells transferred

cell clones by at least threefold. The frequency of IgM antibody-producing clones was higher (12–25%) in allogeneic carrier-primed recipients than in syngeneic carrier-primed recipients (6–13%). This may be indicative of primary B-cell populations present within the immunized donor cell population.

The above data implies that the same secondary precursor cells can be stimulated to IgG_1 antibody production in either the syngeneic or allogeneic carrier-primed environment. In addition, it is clear from this data that secondary B cells differ markedly from primary B cells in that primary B cells, while capable of IgG_1 antibody responses in carrier-primed syngeneic recipients are capable of only IgM antibody production in allogeneic carrier-primed recipients. Since it would be of interest to know if this difference between primary and secondary B cells can be expressed within B cells of the same clonotype, these studies were extended to the analysis of a second antigenic determinant, PC. It has been previously shown that the majority of B cells responding to this determinant in BALB/c mice are of a single identifiable idiotype which is

identical to the idiotype of the TEPC 15 myeloma protein (13, 16–18). It has been possible to establish radioimmunoassays that permit the identification of monoclonal antibodies of this idiotype within the anti-PC response of BALB/c mice (9). In a previous report it was demonstrated that PC-specific primary B cells of the TEPC 15 idiotype yielded IgM antibody responses in allogeneic carrier-primed recipients, but yielded IgG_1 antibody responses in syngeneic carrier-primed recipients (8).

Mice were immunized with PC coupled directly to Hy, which is a T-dependent antigen. After 2 mo, cells from these mice were analyzed to determine if they displayed secondary B-cell characteristics. The results indicated that B cells of the TEPC 15 idiotype from immunized mice yielded IgG_1 responses in either syngeneic or allogeneic carrier-primed recipients. This implies that the same secondary B cells can be stimulated to IgG_1 antibody production through collaboration with either carrier-primed allogeneic or syngeneic T cells. The ability to produce IgG_1 antibody under these stimulatory conditions would thus appear to be a property of the majority of secondary B cells, distinguishing secondary B cells from primary B cells, which produce only IgM antibody in carrier-primed allogeneic recipients.

Discussion

The experiments presented here were carried out to further define the requisites of stimulation of secondary B cells and in particular to determine if the enhancement of secondary B-cell antigenic stimulation provided by carrier-specific T cells is restricted to cells that share genes in the *H-2* gene complex. These experiments were initiated in light of previous findings which demonstrated that syngeny in the *I* region of the *H-2* complex between collaborating hapten-specific primary B cells and carrier-specific T cells was necessary for IgG_1 but not IgM antibody responses (8). The findings presented here indicate that this is not the case for secondary B cells, the majority of which can be stimulated to IgG_1 antibody production in collaboration with T cells which do not share genes in the *I* region, as long as the collaborating T cells were previously immunized with the carrier portion of the antigen used for in vitro stimulation.

These results contradict findings obtained from other experimental approaches which indicate that T-cell-B-cell interactions are restricted to cells syngeneic in the *H-2* complex (19, 20). However T-cell-B-cell collaboration has been observed previously in secondary B-cell responses in chimeric mice in which allogeneic T and B cells were in contact for both primary and secondary immunization (21–23). These results, together with analogous findings, indicating that primed T cells may recognize antigen only in conjunction with macrophages syngeneic to those present during the initial immunization (24, 25), have led these investigators to postulate that the specificity of T-cell recognition may be "determined at the time of primary immunization by the specific combination of antigen and cell surface molecules (23)." Such constraints on T-cell triggering may have been obviated in the experiments reported here, since recipient T cells were carrier primed in the presence of syngeneic macrophages and B cells and were restimulated in the presence of these syngeneic cells to collaborate with

allogeneic B cells. Regardless of the mode of their triggering, these carrier-specific T cells clearly interact with hapten-specific allogeneic secondary B cells to enhance IgG_1 antibody responses in this in vitro system.

In evaluating the positive antigen-specific collaborative interactions reported here, it is important to emphasize that we have observed allogeneic T-cell-B-cell collaboration in an in vitro system in which antigen-specific T cells are irradiated, and positive and negative allogeneic effects seem to play no role (8). Recently collaboration has also been observed in secondary B-cell responses to sheep erythrocytes when alloreactivity to the responding B cells had been eliminated from the T-cell population (Swain and Dutton, personal communication). These and our findings may indicate that collaborative interactions can be maximized only when allogeneic recognition is minimized. It should be re-emphasized, however, that in this same in vitro system there is a genetic restriction in certain aspects of the collaborative interactions of primary B cells and allogeneic carrier-primed T cells. Since, in the stimulation of secondary B cells, little evidence of similar restrictions is observed, it would appear that the requisites of stimulation of secondary B cells differ in a fundamental way from those of primary B cells.

A variety of diverse findings over the past several years have had the intriguing implication that products of the *I* region of the *H-2* locus may play a critical role in the collaborative interactions of T and B cells. These findings include (*a*) the localization to the *I* region of much of the responder-nonresponder status of T-cell-B-cell collaboration in responses to a variety of antigens (26), (*b*) the observation of T-cell stimulation by *I*-region-controlled antigenic determinants (Ia antigens) of allogeneic B cells (27), (*c*) the characterization of T-cell factors bearing Ia antigens which are capable of replacing T-cell helper function (28, 29), (*d*) the observation that B-cell stimulation can be inhibited with anti-Ia antibodies (30), (*e*) the identification of Ia-"negative" primary B cells which yield antibody of only the IgM heavy chain isotype when stimulated with T-dependent antigens (31), and (*f*) the observation of the apparent need for Ia syngeny between collaborating T and B cells to yield secondary immune responses in some systems and primary IgG_1 responses in the fragment culture system (8). This latter finding has led us to postulate that both Ia expression on the B cells' surface and its homology with syngeneic antigen-specific T cells may be essential elements in the triggering events responsible for IgG_1 antibody expression by the clonal progeny of primary B cells (8). It would appear that *I*-region gene products do not function in the same manner in secondary B-cell triggering, in that secondary B cells can be triggered to IgG_1 antibody production via collaborative interaction with allogeneic T cells. Since the fragment cultures may contain an excess of carrier-specific T cells, it is possible that this collaboration involves a subset of primed T cells which recognize the carrier moiety per se rather than the carrier moiety plus *I*-region products. Regardless of the triggering signal employed, it appears that secondary B cells may be precommitted to IgG_1 antibody synthesis. This would contrast with primary B cells which appear to vary in their ability to produce antibody of different isotypes depending on the mode of antigenic stimulation (8). Recent reports have indicated that T-independent antigens, which stimulate primary B cells

only to IgM antibody production, stimulate secondary B cells to IgG_1 antibody synthesis (32). Theoretically, these antigens represent another means of B-cell stimulation without the involvement of the *I*-region genes.

It is important to emphasize that the difference observed in primary and secondary B-cell responses in an allogeneic environment reported here, by capitalizing on the differences in their respective triggering requisites, allows for a quantitation of secondary B cells in any lymphoid cell population. This means of quantitation should be extremely useful in assessing the role of antigenic stimulation in the generation of B-cell subpopulations and specificities.

Summary

We have analyzed the capacity of carrier-specific T cells to enhance the immune response of hapten-specific secondary B cells which do not share genes in the *H-2* complex with the T cells. For this analysis we have used the in vitro splenic focus technique which allows assessment of monoclonal responses of B cells isolated in splenic fragment cultures of irradiated reconstituted carrier-primed mice. A previous report from this laboratory demonstrated that syngeny in the *I* region of the *H-2* complex was necessary between collaborating hapten-specific primary (nonimmune) B cells and carrier-specific T cells for responses yielding IgG_1 but not IgM antibody. These findings lead us to postulate that the expression of *I*-region gene products on the surface of primary B cells and *I*-region syngeny with collaborating carrier-specific T cells were essential elements in the triggering events leading to IgG_1 synthesis by primary B cells. The results presented in the present report indicate that, unlike primary B cells, the majority of secondary B cells can be stimulated to produce IgG_1 antibody in carrier-primed allogeneic recipients. Although the enhancement of secondary IgG_1 responses is slightly greater with syngeneic T cells, the allogeneic collaborative interaction requires both carrier priming of recipient mice and stimulation with the homologous hapten-carrier complex and thus appears to be specific. These findings clearly discriminate secondary from primary B cells and indicate that the mechanism of stimulation of secondary B cells to yield IgG_1-producing clones differs fundamentally from the stimulation of primary B cells in that the requisite for *I*-region syngeny is obviated.

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