

## T LYMPHOCYTE-DEPENDENT B LYMPHOCYTE PROLIFERATIVE RESPONSE TO ANTIGEN

### I. Genetic Restriction of the Stimulation of B Lymphocyte Proliferation\*

BY HARLEY Y. TSE,‡ JAMES J. MOND, AND WILLIAM E. PAUL

*From the Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205; and the Department of Medicine, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20014*

The activation of B lymphocytes by antigenic stimuli can be divided into two stages. These are (a) their transformation into blasts and subsequent cell division and (b) their differentiation into antibody-secreting cells. Recent studies suggest that both of these events can be regulated by T lymphocytes or their products (1-5). In general, T lymphocyte-B lymphocyte interactions have been studied by the measurement of antibody production. This approach, when used with a clonal analysis, can give information about both the proliferative and differentiative components of the B cell response. However, it is severely limited in that any B lymphocyte proliferation that does not immediately lead to antibody synthesis cannot be detected.

Some efforts have been made to measure *in vitro* B lymphocyte proliferation in response to antigenic stimulation (6-8). It is known that in mixed cell populations obtained from primed donors, antigen stimulation will lead to thymidine uptake and cell division by both T and B lymphocytes, although relatively little characterization of the B cell component of this cell division has been carried out. The present study was undertaken to determine the requirements for this T lymphocyte-dependent B lymphocyte proliferation. Our results indicate that the B lymphocyte proliferation depends upon the presence in the culture of T lymphocytes derived from a donor primed to the antigen used for *in vitro* challenge. In contrast, the B lymphocytes may be obtained from primed or unprimed donors and the magnitude of the response of B lymphocytes from these two sources is not distinguishable. This suggests that T lymphocyte-dependent proliferation of B cells is polyclonal. However, the interaction of the two cell types is regulated by genes encoded in the major histocompatibility complex (MHC).<sup>1</sup> Our results thus suggest that an MHC-restricted recruitment of nonspecific B lymphocyte proliferation can be mediated as a result of antigen-specific activation of T lymphocytes.

\* Supported by grant R08315 from the Uniformed Services University of the Health Sciences Research. The opinions and assertions contained herein are the private views of the authors and should not be construed as official or as necessarily reflecting the views of the Uniformed Services University of the Health Sciences or the Department of Defense. There is no objection to its presentation and/or publication.

‡ Recipient of a fellowship from the Arthritis Foundation. Present address: Department of Immunology, Merck, Sharp, and Dohme Research Laboratories, Rahway, N. J. 07065.

<sup>1</sup> *Abbreviations used in this paper:* CFA, complete Freund's adjuvant; Con A, concanavalin A; DNP-OVA, dinitrophenyl derivative of ovalbumin; FCS, fetal calf serum; GAT, Glu<sup>60</sup>-Ala<sup>30</sup>-Tyr<sup>10</sup> polymer; LPS, lipopolysaccharide; MHC, major histocompatibility complex; PPD, purified protein derivative of tuberculin; SRBC, sheep erythrocytes; (T,G)-A-L, poly(Tyr,Glu)-poly, D,L-Ala--poly Lys; TNP, trinitrophenyl.

### Materials and Methods

**Animals.** C57BL/10 SgSN and B10.A mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. BALB/c mice were obtained from Charles River Breeding Laboratories Inc., Wilmington, Mass. Breeding pairs of B10.S mice were kindly provided by Dr. D. Sachs, National Cancer Institute, National Institutes of Health, Bethesda, Md. B10.S and (B10.S × B10)F<sub>1</sub> mice were bred in our own facilities.

**Antigens.** A dinitrophenyl derivative of ovalbumin (DNP-OVA) was prepared as previously described (9). The batch used for these experiments had approximately 7 M DNP per 1 M of protein. A Glu<sup>60</sup>-Ala<sup>30</sup>-Tyr<sup>10</sup> polymer (GAT) (lots 7 and 8) was purchased from Miles Laboratories Inc., Miles Research Div., Elkhart, Ind. GAT was first dissolved in 1% Na<sub>2</sub>CO<sub>3</sub> in saline and then adjusted to pH 7.2 with 1 N HCl. Purified protein derivative of *Mycobacterium tuberculosis* (PPD) was purchased from Connaught Laboratories, Toronto, Ontario.

**Immunization.** DNP-OVA or GAT was emulsified in complete Freund's adjuvant (CFA; Difco Laboratories, Detroit, Mich.) containing 1 mg/ml of killed *M. tuberculosis* H37Ra. 100 μl, containing 50 μg of either antigen, was injected subcutaneously at the base of the tail and in the hind footpads (10).

**Preparation of Lymph Node T Cells.** 7–20 d after immunization, inguinal, para-aortic, and popliteal lymph nodes were removed and teased into single cell suspensions. For enrichment of T cells, lymph node cells ( $4 \times 10^8$  or fewer) in 8 ml of RPMI-1640 (Media Unit, Division of Research Services, National Institutes of Health) containing 5% fetal calf serum (FCS; Grand Island Biological Co., Grand Island, N. Y.) were applied on a packed nylon wool column. Each column consisted of 3–5 g of recycled nylon wool in a 35-ml syringe (11). The nylon wool had originally been obtained from Fenwal Laboratories, Division of Travenol Laboratories, Morton Grove, Ill. Cells were incubated on these columns for 1 h at 37°C and nonadherent cells were eluted through a 23-gauge needle with warm RPMI-1640 containing 5% FCS.

**Preparation of B Lymphocytes from Lymph Nodes and Spleens.** Cells were teased with forceps from lymph nodes or spleens and resuspended to  $2 \times 10^7$  cells/ml. Cells were incubated for 1 h on an anti-immunoglobulin-coated culture dish and adherent cells were then removed (12) and resuspended to  $2 \times 10^7$  cells/ml in a monoclonal anti-Thy-1.2 antibody (NEI-001; New England Nuclear, Boston, Mass.) for 45 min at 4°C. Cells were then washed once in balanced salt solution and resuspended to the same volume in guinea pig serum that had been absorbed with spleen cells to remove any nonspecific cytotoxic activity. After 45 min at 37°C, cells were washed twice and resuspended in modified Mishell-Dutton medium (13) containing 10% FCS and  $5 \times 10^{-5}$  M 2-mercaptoethanol.

**Cell Cultures.** Cells were cultured in an 0.2-ml volume in modified Mishell-Dutton culture medium in flat-bottomed microculture dishes (Costar, Data Packaging, Cambridge, Mass.). DNP-OVA and GAT were added to cultures at a final concentration of 100 μg/ml. PPD was used at 20 μg/ml. Cultures were maintained in 5% CO<sub>2</sub> at 37°C for 4 d. 16–20 h before harvesting, 1 μCi of tritiated thymidine (sp act 5 Ci/mmol; Amersham/Searle Corp., Arlington Heights, Ill.) was added to each culture. Incorporation of tritiated thymidine was determined with a Mash II automatic harvester (Microbiological Associates, Walkersville, Md.) and in a Beckman liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). All cultures were established in triplicate and arithmetic means were determined.

**Preparation of Irradiated Spleen Cells.** Spleens from unprimed animals were teased into single cell suspensions with forceps and washed twice. Cells were resuspended in complete Mishell-Dutton culture medium and irradiated with 2,000 rad from a Cesium source (Gammator M., Isomedix, Inc., Parsippany, N. J.).

**Removal of Dead Cells with a Ficoll-Hypaque Gradient.** Dead cells in cultures were removed by a Ficoll-Hypaque gradient centrifugation technique after antisera plus complement treatment. Briefly, cells ( $4 \times 10^7$  or fewer) in 1 ml of medium were layered over 3 ml of Ficoll-Hypaque (Pharmacia Fine Chemicals, Division of Pharmacia Inc., Piscataway, N. J.) in a Falcon 2001 tube (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.; 14). The tube was centrifuged at 400 g for 30 min at room temperature. Viable lymphocytes formed a diffuse white band at the interface, whereas most of the dead cells were found at the bottom of the tube. The viable cell band and the medium, except for the bottom 0.5 ml, was aspirated with

TABLE I  
*T Lymphocyte Dependency of B Lymphocyte Proliferative Response*

Experiment	Primed T cells	Primed B cells	Proliferative response				
			Medium	GAT	PPD	LPS*	Con A*
			<i>cpm</i>			<i>cpm</i>	
1	+	-	175	160	155	8,358	47,889
	-	+	1,151	1,541	3,600	60,803	3,568
	+	+	9,922	42,010	37,808	NT‡	NT
2	+	-	74	250	170	NT	NT
	-	+	1,187	1,435	3,590	NT	NT
	+	+	10,949	85,179	95,596	NT	NT

B10.A mice were primed with GAT in CFA. 7 d later, T and B lymphocytes were prepared from lymph nodes as described in Materials and Methods. T lymphocytes were used at  $1 \times 10^5$  cells per well whereas B lymphocytes were present at  $4 \times 10^5$  cells per well. Cultures were assayed on day 4.

\* Responses to LPS and Con A were those of  $2 \times 10^5$  cells.

‡ Not tested.

a Pasteur pipette. This procedure consistently recovered >70% of the input viable cells. The viability of the recovered cells was between 95 and 100%.

## Results

*Dependency of B Lymphocyte Proliferation on Stimulation of Antigen-primed T Lymphocytes.* In an effort to develop a system that could measure antigen-specific B lymphocyte proliferation, B lymphocytes from primed lymph nodes were cultured with antigen in the presence or absence of T lymphocytes from the same lymph nodes. In this series of experiments, T and B lymphocytes were obtained from lymph nodes of animals that had been primed at the base of the tail and in the hind footpads. T lymphocytes were prepared by nylon wool-column purification. B lymphocytes were obtained by adherence to anti-immunoglobulin-coated dishes and then by anti-Thy-1.2 plus complement treatment of the released adherent cells. Such cell populations contained >90% Ig<sup>+</sup> and <5% Thy-1.2<sup>+</sup> cells, as determined by immunofluorescence. Testing with mitogens showed that the cells resuspended well to lipopolysaccharide (LPS) but poorly to concanavalin A (Con A) (Table I). These B cells failed to mount a significant proliferative response when cultured with antigen alone. The addition of  $10^5$  nylon wool-column-purified primed T cells to this B cell population caused the development of a significant proliferative response, which was highest on day 4 of culture.

It could be argued that the sole role of the added B lymphocyte population in augmenting the observed proliferation was to provide a source of antigen-presenting cells. Because the antigen-presenting function of spleen cells has been shown to be radio-resistant (15), we compared the effect of irradiated and nonirradiated B lymphocytes on the uptake of thymidine by cultures containing  $10^5$  T lymphocytes from primed donors. Table II shows that cultures of T lymphocytes and nonirradiated B lymphocytes show a marked enhancement in the degree of thymidine uptake when compared with x-irradiated B lymphocytes alone, or B lymphocytes and irradiated T lymphocytes. Cultures of irradiated B lymphocytes and T lymphocytes yielded relatively small responses. These results strongly suggest that antigen presentation was

TABLE II  
*T Lymphocyte Dependency of B Lymphocyte Proliferative Response*

Primed T cells	Primed B cells	Proliferative response		
		Medium	GAT	PPD
			<i>cpm</i>	
+	+ (irradiated)	106	7,460	20,274
+	+	497	58,297	115,445
-	+	173	193	843
+ (irradiated)	+	138	769	3,269

Experimental details were the same as in Table I. Irradiation of cells was at 2,000 rad.

TABLE III  
*Participation of B Lymphocytes in Proliferation*

	Percentage of total cells stained		Percentage of blast cells stained	
	Thy-1.2 <sup>+</sup>	Ig <sup>+</sup>	Thy-1.2 <sup>+</sup>	Ig <sup>+</sup>
Before culture	15.8	79.4	—	—
After culture	32.6	70.0	37.0	54.2

BALB/c mice were primed with DNP-OVA as described. Lymph node cells were prepared 9 d after priming. See text for experimental details.

not the sole function of the added B lymphocytes. As a control in all subsequent experiments, cultures containing primed T cells and irradiated B lymphocytes were included.

Direct proof that B lymphocytes participated in the proliferation came from examination of the blast cells that appeared in culture. Cultures were established with  $4 \times 10^5$  B lymphocytes and  $10^5$  nylon column-purified T lymphocytes in the presence of antigen. The proportions of Ig-bearing and Thy-1.2-bearing cells at the initiation of culture were verified using fluoresceinated rabbit anti-mouse Ig and fluoresceinated anti-Thy-1.2 reagents. At the end of a 3-d culture period, cells were stained with the same reagents and the percentages of total cells and blast cells that were fluorescent were scored. A summary of the results is presented in Table III. It can be seen that the frequencies of Thy-1.2- and Ig-bearing cells in the initial culture were reasonably close to the expected 1:4 ratio, based on the number of T and B lymphocytes added. After 3 d in culture, ~25% of the recovered viable cells were blasts. Of these, more than half were identified as B lymphocytes by the presence of immunoglobulin on their surface. It was concluded from these experiments that B lymphocytes were induced to proliferate in the presence of primed T lymphocytes and antigens.

*Linear Relationship between the Proliferative Response and the Number of B Lymphocytes Cultured.* Having thus shown that B lymphocytes make a substantial contribution to the proliferating cell population, we next examined the effects of adding varying numbers of B lymphocytes to a constant number of T lymphocytes. The proliferative response to GAT and to PPD was found to be directly related to the number of B lymphocytes present in the culture. When these results were plotted logarithmically, the slope of the straight lines relating added numbers of B lymphocytes and response

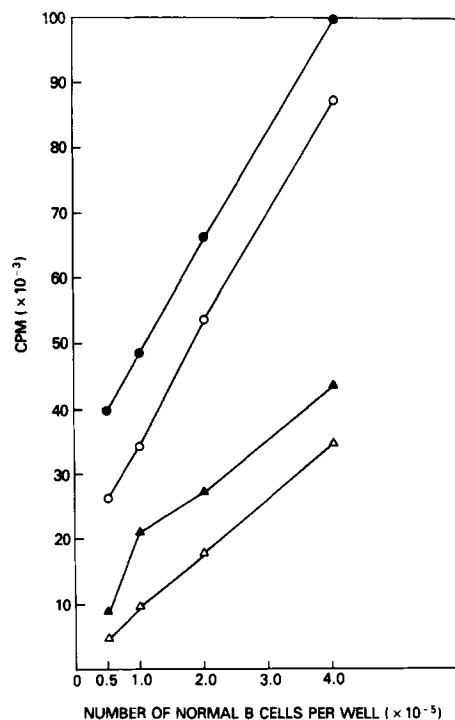


FIG. 1. Linear relationship between the proliferative response and the number of B lymphocytes in culture. B10 mice were primed to GAT in CFA. T and B lymphocytes were isolated as described in Materials and Methods. Various numbers of lymph node B lymphocytes were then titrated against either  $1 \times 10^5$  or  $0.75 \times 10^5$  T lymphocytes. (▲),  $1 \times 10^5$  and (△),  $0.75 \times 10^5$  T cells responding to GAT. (●),  $1 \times 10^5$  and (○),  $0.75 \times 10^5$  T cells responding to PPD. The responses for  $4 \times 10^5$  irradiated B lymphocytes plus  $1 \times 10^5$  T lymphocytes to GAT and to PPD were 7,269 and 10,514 cpm, respectively.

TABLE IV

*T Lymphocytes, but Not B Lymphocytes, Must Be Obtained from Donors Primed to the Stimulating Antigen*

Priming of B cell donor	Priming of T cell donor	Proliferative response			
		Medium	GAT	DNP-OVA	PPD
			<i>cpm</i>		
GAT (irradiated)	GAT	332	1,166	NT*	1,926
GAT	No T cells	1,078	2,158	5,466	8,705
DNP-OVA	No T cells	2,022	1,299	2,387	7,879
GAT	GAT	2,097	85,707	6,919	84,138
DNP-OVA	GAT	2,050	74,554	3,486	74,036
GAT	DNP-OVA	1,873	5,256	85,273	68,444
DNP-OVA	DNP-OVA	2,346	2,686	86,678	80,572

\* Not tested.

indicated that in the added B lymphocyte population only one cell type was limiting (Fig. 1).

*Proliferating B Lymphocytes Do Not Have to be Obtained from Specifically Primed Donors.* The experiments presented so far do not determine whether the proliferating B lymphocytes are themselves specific for the antigen used to stimulate the response. To

TABLE V  
*B Lymphocytes from Primed and Unprimed Donors Are Comparable in the Proliferative Response*

Experiment	B cells			T cells	Proliferative response		
	Priming of donors	Source of cells	Treatment of cells		Medium	GAT	PPD
						<i>cpm</i>	
1	GAT	LN	2,000 rad	+	73	3,394	4,246
	GAT	LN	—	—	1,237	2,240	9,096
	GAT	LN	—	+	2,209	29,646	56,699
	Unprimed	spleen	—	—	1,008	1,382	4,429
	Unprimed	spleen	—	+	2,849	56,092	57,052
2	GAT	LN	2,000 rad	+	106	7,460	20,274
	GAT	LN	—	—	173	193	843
	GAT	LN	—	+	497	58,297	125,445
	Unprimed	spleen	—	—	409	583	1,915
	Unprimed	spleen	—	+	1,596	99,304	77,802
	Unprimed	LN	—	—	218	181	1,180
	Unprimed	LN	—	+	931	103,614	149,601

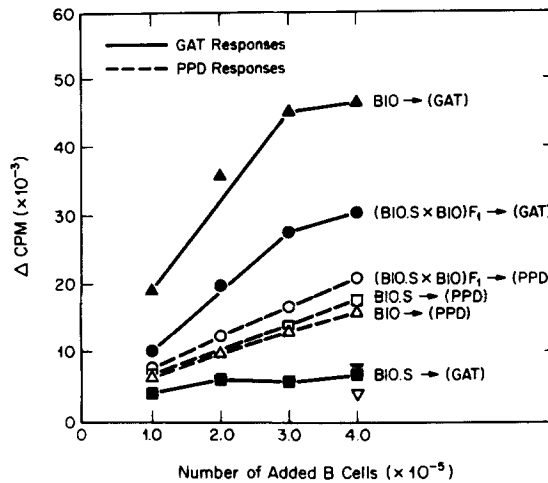


FIG. 2. Genetic restriction of B lymphocyte proliferative response.  $(B10.S \times B10)F_1$  mice were primed to GAT in CFA. 10 d later, lymph node T lymphocytes were prepared and a constant number ( $0.85 \times 10^6$ ) of these  $F_1$  lymphocytes was added  $1 \times 10^5$  irradiated  $F_1$  spleen cells and to various numbers of B10, B10.S, or  $F_1$  B lymphocytes. The responses of these cell mixtures to GAT and to PPD were determined. B10 B lymphocytes ( $\blacktriangle$ ),  $F_1$  B lymphocytes ( $\bullet$ ), and B10.S B lymphocytes ( $\blacksquare$ ) responding to GAT; B10 B lymphocytes ( $\Delta$ ),  $F_1$  B lymphocytes ( $\circ$ ), and B10.S B lymphocytes ( $\square$ ) responding to PPD.  $\blacktriangledown$  denotes the response of  $4 \times 10^6$  irradiated B10 B lymphocytes and  $0.85 \times 10^6$   $F_1$  T lymphocytes responding to GAT and  $\blacktriangledown$  to PPD.

address this question, we immunized mice to either GAT or DNP-OVA and prepared T and B lymphocytes from donors of both types. B lymphocytes from GAT-primed animals were then mixed with DNP-OVA-primed lymphocytes and challenged with either GAT, DNP-OVA, or PPD. Similarly, DNP-OVA-primed B lymphocytes were tested with GAT-primed T lymphocytes (Table IV). It is clear from this experiment

that only the T lymphocytes had to be obtained from a donor primed to the antigen used for stimulation. The immune status of the B lymphocyte donor was not related to the magnitude of the response. This conclusion was strongly supported by the observation that normal B lymphocytes from unprimed donors could be induced to proliferate by primed T lymphocytes (Table V). Similar responses were obtained with B lymphocytes taken from primed or unprimed donors. Furthermore, both mesenteric lymph node and splenic B lymphocytes from unprimed donors were effective.

*Genetic Restriction of T-B Interaction.* To examine the role of MHC-encoded genes in this interaction of primed T lymphocytes and unprimed B lymphocytes, we took advantage of the fact that the responses to certain antigens are under the control of specific immune response (*Ir*) genes that have been mapped to the I region of the MHC. Two strains of mice, B10 and B10.S, have been characterized as responders and nonresponders, respectively, to the antigen GAT, both by the T cell proliferative assay and by specific anti-GAT antibody production (15, 16). Both strains are responders to PPD.

Primed lymph node T lymphocytes from (B10.S × B10)<sub>F1</sub> animals were used to stimulate normal B lymphocytes from B10, B10.S, or F<sub>1</sub> mice (Fig. 2). To ensure that the antigen-presenting function was not limiting, we added irradiated F<sub>1</sub> spleen cells to all cultures. F<sub>1</sub> T lymphocytes cultured with irradiated B lymphocytes (as well as irradiated spleen) gave a background uptake of tritiated thymidine to GAT of about 6,000 cpm. When nonirradiated B10 or F<sub>1</sub> B lymphocytes were used, significant responses were observed with GAT or PPD as antigens. B10.S B lymphocytes failed to respond when GAT was used but responded normally to PPD. Thus, the ability of T lymphocytes to cause proliferation by B lymphocytes correlated with the *Ir* genetic type of the B lymphocyte donor. It seems most likely that the failure of the B10.S B lymphocytes to proliferate in cultures containing F<sub>1</sub> T lymphocytes, irradiated F<sub>1</sub> spleen cells, and GAT represents an inability of the B10.S B lymphocyte to be stimulated by GAT-activated F<sub>1</sub> T lymphocytes. It might be argued, however, that the B10.S B lymphocyte population failed to respond because of the failure of the F<sub>1</sub>

TABLE VI  
*Genetic Restriction of B Lymphocyte Proliferation*

	Me- dium	GAT			PPD		
	cpm remain- ing	cpm remain- ing	Δ cpm	Percent- age of comple- ment control	cpm remain- ing	Δ cpm	Percent- age of comple- ment control
Complement control	18,322	110,735	92,413	100	168,379	150,057	100
Anti-H-2 <sup>b</sup> + complement treated	13,288	22,120	8,832	9.5	50,362	37,074	24.7
Anti-H-2 <sup>a</sup> + complement treated	15,171	82,779	67,608	73.2	53,362	38,191	25.4

T lymphocytes from (B10.S × B10)<sub>F1</sub> mice primed to GAT in CFA were mixed with B lymphocytes from nonprimed B10 and B10.S donors and cultured with medium alone or with GAT or PPD. 3 d later, cells were labeled with tritiated thymidine. 16 h later, cells were treated with either complement alone, or anti-H-2<sup>b</sup> or anti-H-2<sup>a</sup> antisera, in the presence of rabbit complement. Remaining viable cells were isolated with Ficoll-Paque and were assayed for radioactivity.

T lymphocytes to be stimulated by GAT. This would be anticipated if the B lymphocyte population were the only source of antigen-presenting cells, because B10.S antigen-presenting cells have been shown to be deficient in their ability to stimulate responses to GAT by F<sub>1</sub> T lymphocytes (15). We regard this possibility as quite unlikely because all cultures contained an excess of irradiated F<sub>1</sub> spleen cells, which should supply a rich source of competent antigen-presenting cells. To demonstrate directly that under identical conditions, GAT-stimulated F<sub>1</sub> T lymphocytes preferentially cause proliferation of B10 B lymphocytes, we mixed both B10.S and B10 B lymphocytes in the same culture together with primed F<sub>1</sub> T lymphocytes and antigen. Cultures were incubated for 3.5 d and then labeled with tritiated thymidine. Aliquots of the labeled cells were treated with anti-H-2<sup>b</sup> or anti-H-2<sup>a</sup> antisera in the presence of rabbit complement. Dead cells were then removed by Ficoll-Hypaque purification. Any diminution in the total radioactivity after treatment should reflect the contribution of that population of cells that was eliminated by the antisera. In mixed cultures of F<sub>1</sub> T, B10 B, and B10.S B lymphocytes responding to GAT, anti-H-2<sup>b</sup> plus complement treatment eliminated 90% of the proliferative response to GAT, whereas anti-H-2<sup>a</sup> plus complement treatment eliminated only 27% of the response (Table VI). Because the F<sub>1</sub> T lymphocytes should be eliminated by either anti-H-2<sup>a</sup> or anti-H-2<sup>b</sup> treatment, these results could be interpreted to indicate that 73% of the proliferative response to GAT was contributed by B10 B lymphocytes, 17% by F<sub>1</sub> T lymphocytes, and 10% by B10.S B lymphocytes. Furthermore, because the killing by antisera and complement may not have been complete, the estimate of the contribution of the B10.S B lymphocytes should be regarded as a maximum estimate. When PPD, to which both strains could respond, was used as antigen, the two antisera eliminated approximately equal proportions (75%) of responding cells, suggesting that 50% of the response was due to F<sub>1</sub> T lymphocytes, and 25% was due to each of the B lymphocyte types present. These results indicate that in a mixture of "responder" and "nonresponder" B lymphocytes, the nonresponder B lymphocytes exhibit little or no proliferation and do not inhibit the proliferation of the responder B lymphocytes. We conclude from these experiments that I-region genes regulate the interaction between T and B lymphocytes that leads to the induction of B lymphocyte proliferation, even when the response appears not to be specific for the antigen used to stimulate the T lymphocytes.

### Discussion

The results presented here indicate that proliferative responses of B lymphocytes depend upon the stimulation of antigen-primed T lymphocytes and that the B lymphocyte response, although *Ir*-gene restricted, is apparently antigen nonspecific. That it was, in fact, B lymphocyte proliferation that was being studied is indicated by the following considerations. T lymphocytes cultured alone at densities of  $0.5 \times 10^5$ – $1 \times 10^5$  per ml or in the presence of irradiated spleen cells as a source of antigen-presenting cells displayed only limited thymidine uptake in response to antigen. The addition of a source of B lymphocytes to these cultures resulted in a marked increase in the proliferative response, which was directly related to the number of added B lymphocytes. This effect of B lymphocytes was ablated if they had been irradiated before their addition to the T cells. Direct examination of blast cells from a culture containing both T and B lymphocytes indicated that a majority of the cells bore



membrane Ig; furthermore, the Thy-1 marker was expressed on only a relatively small fraction of the cells, which suggests that the Ig<sup>+</sup> cells were not Thy-1<sup>+</sup> cells that had acquired Ig passively. Finally, when parental B lymphocytes were cultured with GAT-primed F<sub>1</sub> T lymphocytes, the majority of the cells proliferating in response to GAT were of parental histocompatibility type. In the mixture of (B10 × B10.S)F<sub>1</sub> T lymphocytes primed to GAT and B10 and B10.S B lymphocytes, antiserum directed at H-2<sup>b</sup> determinants killed cells that had taken up ~90% of the cell-associated thymidine, whereas anti-H-2<sup>s</sup> antibody led to only 27% killing. This indicates that ~73% of the proliferation can be ascribed to B lymphocytes from the B10 donor.

A second interesting feature of this B lymphocyte proliferative response is that B lymphocytes from primed and unprimed donors are essentially equivalent in their capacity to proliferate in the presence of antigen and primed T lymphocytes. This strongly suggests but does not unequivocally demonstrate that the B lymphocytes that respond are not simply those that bear receptors for the antigens used to stimulate responses. Rather, it appears that the responding B lymphocytes may be of a wide range of antigenic specificities. Preliminary characterization of the antibody-producing cells that develop in these cultures indicates that both anti-sheep cell antibodies and anti-trinitrophenyl (TNP) antibodies are produced even when an antigen such as GAT is used for stimulation. This supports the idea that the B lymphocyte activation is polyclonal in nature.

Our results indicate that this antigen-stimulated T lymphocyte-dependent activation of B lymphocyte proliferation is regulated by genes in the MHC. We initially observed that antigen-dependent B lymphocyte proliferation was not obtained if the B and T lymphocytes were drawn from histoincompatible donors (data not shown). This suggested a role for MHC genes, but because substantial mixed lymphocyte responses were observed in these cultures, we could not interpret our results unambiguously. Consequently, we chose a system in which we could investigate histocompatibility restriction without concern for allogeneic effects. The opportunity to do so was presented by an *Ir* gene-controlled system. Thus, we used primed T lymphocytes from F<sub>1</sub> hybrids prepared by mating B10 and B10.S mice. The former possesses a responder allele at the *Ir* locus that controls responsiveness to GAT, whereas the latter lacks this gene. Such F<sub>1</sub> T cells, together with irradiated F<sub>1</sub> spleen cells as a source of antigen-presenting cells, stimulated proliferation in response to GAT by B10 but not B10.S B lymphocytes. If an antigen to which both strains were responsive was used (i.e., PPD), both B10 and B10.S B lymphocytes could be stimulated to proliferate. Perhaps the most decisive evidence that this *Ir* gene-controlled B lymphocyte proliferative response required "responder" B lymphocytes and not simply responder antigen-presenting cells was provided by the experiment in which F<sub>1</sub> T lymphocytes, B10 B lymphocytes, and B10.S B lymphocytes were cultured in the same well. Under such circumstances, the B10 B lymphocytes proliferated well and the B10.S B lymphocytes proliferated poorly.

The surprising feature of these results is that this *Ir* gene-restricted T-B collaboration is apparently antigen nonspecific. This was unanticipated because MHC-restricted cellular interactions have generally been interpreted to involve the recognition by T lymphocytes of both antigen and an MHC gene product, such as an Ia antigen, on the surface of antigen-presenting cells and B lymphocytes (17-20). Thus, GAT-primed F<sub>1</sub> T lymphocytes would be expected to interact when they encounter GAT and B10

Ia antigens on the surface of either an antigen-presenting cell or a B lymphocyte. Our observation that B10 B lymphocytes that lack specific receptors for GAT are nonetheless recognized by the GAT-Ia<sup>b</sup>-specific T lymphocytes could be interpreted to indicate that the T lymphocytes have a partial specificity for Ia<sup>b</sup> that is sufficiently great to allow them to interact with B10 B cells even though the latter have not bound GAT. If so, this might provide a peripheral analogue to the intrathymic events that are thought to determine the "restriction phenotype" (21). However, two other ideas must be considered. The first is that the B lymphocytes being activated are really specific for GAT and thus have bound GAT to their membrane receptors. We regard this as very unlikely for the reasons outlined above. However, additional studies aimed at testing this possibility are in progress. A second possibility is that the B lymphocytes, although they do not bear receptors specific for GAT, nonetheless have bound GAT to their membranes nonspecifically. This GAT, together with Ia<sup>b</sup>, might then be recognized by the T lymphocytes and allow a collaborative interaction. This would indicate that antigens not bound by the Ig receptors might nonetheless be a suitable target for antigen-specific helper cells. Previous work by Cammisuli, Henry, and Wofsy (22, 23) has indicated that collaborative interactions through membrane sites other than membrane Ig can, in fact, lead to antibody synthesis. Studies aimed at distinguishing between these three possibilities are now in progress.

Marrack and Kappler (24) have recently reported a phenomenon with several similarities to that which we have described here. They have shown that "the bystander" activation of anti-sheep erythrocyte (SRBC) antibody-secreting cells in the presence of T lymphocytes specific for the branched chain polymer poly(Tyr,Glu)-poly, D,L-Ala--poly Lys [(T,G)-A--L] expresses histocompatibility and *Ir* restriction. In their experiments, this restricted activation of precursors of anti-SRBC antibody-producing cells depended upon the presence of (T,G)-A--L. Because they included SRBC in each of their cultures, it seems possible that the role of SRBC in their system was to activate B lymphocytes to an extent that those cells were now susceptible to the action of T cells specific for Ia and (T,G)-A--L. In our system, which measures cellular proliferation, those cells that proliferate in a polyclonal fashion may be the cells that have already been partially activated in the animal.

### Summary

For the purpose of examining more closely the interaction between T and B lymphocytes, we have developed an *in vitro* T lymphocyte-dependent B lymphocyte proliferation assay. Proliferation of B lymphocytes in response to antigen was found to depend on the presence of primed T lymphocytes; the B lymphocytes could be derived from nonprimed animals. It appears that these B cells were nonspecifically recruited to proliferate. This nonspecific recruitment, however, was found to be *Ir*-gene restricted in that B lymphocytes from B10.S mice, which are genetic nonresponders to the polymer Glu<sup>60</sup>-Ala<sup>30</sup>-Tyr<sup>10</sup> (GAT), could not be stimulated by GAT-primed (responder × nonresponder)F<sub>1</sub> T cells. The apparent lack of antigen specificity in the face of *Ir* gene-restricted T-B interaction may have important implications in our understanding of the recognition unit(s) on T lymphocytes.

The authors thank Dr. Ronald Schwartz for helpful discussions during the course of this work.

*Received for publication 9 December 1980.*

## References

1. Schimpl, A. T. Hunig, and E. Wecker. 1974. Separate induction of proliferation and maturation of B cells. *In Progress in Immunology*. L. Brent and J. Holbrow, editors. Elsevier North-Holland, Inc., New York. **2**:135.
2. Gorczynski, R. M., and A. J. Cunningham. 1978. Requirement for matching T cell and B cell subsets in secondary anti-hapten antibody responses. *Eur. J. Immunol.* **8**:753.
3. Marrack, P., L. Harwell, J. W. Kappler, D. Kawahara, D. Kelles, and J. E. Swierkosz. 1979. Helper T cell interactions with B cells and macrophages. *In Immunologic Tolerance and Macrophage Function*. P. Barnum, J. R. Battisto, and C. W. Pierce, editors. Elsevier North-Holland, Inc., New York. 31.
4. Andersson, J., M. H. Schreier, and F. Melchers. 1980. T-cell dependent B-cell stimulation is H-2 restricted and antigen dependent only at the resting B-cell level. *Proc. Natl. Acad. Sci. U. S. A.* **77**:1612.
5. Augustin, A. A., and A. Coutinho. 1980. Specific T helper cells that activate B cells polyclonally. In vitro enrichment and cooperative function. *J. Exp. Med.* **151**:587.
6. Elfenbein, G. J., E. M. Shevach, and I. Green. 1972. Proliferation by bone marrow derived lymphocytes in response to antigenic stimulation in vitro. *J. Immunol.* **109**:870.
7. Dutton, R. W. 1975. Separate signals for the initiation of proliferation and differentiation in the B cell response to antigen. *Transplant. Rev.* **23**:66.
8. Lipsky, P. E., and A. S. Rosenthal. 1976. The induction and regulation of guinea pig B-lymphocyte proliferation in vitro. *J. Immunol.* **117**:1594.
9. Yano, A., R. H. Schwartz, and W. E. Paul. 1977. Antigen presentation in the murine T-lymphocyte proliferative response. I. Requirement for genetic identity at the major histocompatibility complex. *J. Exp. Med.* **146**:828.
10. Tse, H. Y., R. H. Schwartz, and W. E. Paul. 1980. Cell-cell interaction in the T cell proliferative response. I. Analysis of the cell types involved and evidence for nonspecific cell recruitment. *J. Immunol.* **125**:491.
11. Schwartz, R. H., C. L. Horton, and W. E. Paul. 1977. T-lymphocyte-enriched murine peritoneal exudate cells. IV. Genetic control of cross-stimulation at the T-cell level. *J. Exp. Med.* **145**:327.
12. Mage, M. G., L. L. McHugh, and T. L. Rothstein. 1977. Mouse lymphocytes with and without surface immunoglobulin: preparative scale separation in polystyrene tissue culture dishes coated with specifically purified anti-immunoglobulin. *J. Immunol. Methods.* **15**:47.
13. Mishell, R. I., and R. W. Dutton. 1967. Immunization of dissociated spleen cell cultures from normal mice. *J. Exp. Med.* **126**:423.
14. Tse, H., and R. W. Dutton. 1976. Separation of helper and suppressor T lymphocytes on a Ficoll velocity sedimentation gradient. *J. Exp. Med.* **143**:1199.
15. Yano, A., R. H. Schwartz, and W. E. Paul. 1978. Antigen presentation in the murine T lymphocyte proliferative response. II. Ir-GAT-controlled T lymphocyte responses require antigen-presenting cells from a high responder donor. *Eur. J. Immunol.* **8**:344.
16. Kapp, J. A., C. W. Pierce, and B. Benacerraf. 1973. Genetic control of immune responses in vitro. I. Development of primary and secondary plaque-forming cell responses to the random terpolymer L-Glutamic acid<sup>60</sup>-L-Alanine<sup>30</sup>-L-Tyrosine<sup>10</sup> (GAT) by mouse spleen cells in vitro. *J. Exp. Med.* **138**:1107.
17. Rosenthal, A. S., and E. M. Shevach. 1973. Function of macrophages in antigen recognition by guinea pig T lymphocytes. I. Requirement for histocompatible macrophages and lymphocytes. *J. Exp. Med.* **138**:1194.
18. Katz, D. H., M. Graves, M. E. Dorf, H. DiMuzio, and B. Benacerraf. 1975. Cell interactions between histoincompatible T and B lymphocytes. VII. Cooperative responses between lymphocytes are controlled by genes in the I-region of the H-2 complex. *J. Exp. Med.* **141**: 263.

19. Paul, W. E., E. M. Shevach, S. Pickeral, D. W. Thomas, and A. S. Rosenthal. 1977. Independent populations of primed F<sub>1</sub> guinea pig T lymphocytes respond to antigen-pulsed parental peritoneal exudate cells. *J. Exp. Med.* **145**:618.
20. Yano, A., R. H. Schwartz, and W. E. Paul. 1977. Antigen presentation in the murine T lymphocyte proliferative response. I. Requirement for genetic identity at the major histocompatibility complex. *J. Exp. Med.* **146**:828.
21. Zinkernagel, R. M. 1978. Thymus and lymphohemopoietic cells: their role in T cell maturation, in T cells' H-2-restriction-specificity and in H-2 linked Ir gene control. *Immunol. Rev.* **42**:224.
22. Cammisuli, S., C. Henry, and L. Wofsy. 1978. Role of membrane receptors in the induction of an in vitro secondary anti-hapten response. I. Differentiation of B memory cells to plasma cells is independent of anti-immunoglobulin receptor interaction. *Eur. J. Immunol.* **8**:656.
23. Cammisuli, S., and C. Henry. 1978. Role of membrane receptors in the induction of an in vitro secondary anti-hapten response. II. Antigen immunoglobulin receptor interaction is not required for B memory cell proliferation. *Eur. J. Immunol.* **8**:6.
24. Marrack, P., and J. W. Kappler. 1980. The role of H-2-linked genes in helper T cell function. VII. Expression of I region and immune response genes by B cells in bystander help assays. *J. Exp. Med.* **152**:1274.