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Antigen-specific helper T-cell clone supernatant is sufficient to induce both polyclonal proliferation and differentiation of small resting B lymphocytes

(T-cell lymphokines/B-cell stimulation/major histocompatibility complex restriction)

L. LECLERCQ, G. BISMUTH, AND J. THÈZE

Département d'Immunologie, Institut Pasteur, 25, rue du Docteur Roux, 75015 Paris, France

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ABSTRACT **Gradient-purified resting B lymphocytes can be polyclonally stimulated by antigen-specific major histocompatibility complex (MHC)-restricted helper T lymphocytes as well as by antigen-activated helper T-cell supernatant. In contrast to what has been described so far, we show that helper T-cell supernatant (in the absence of any other added stimulus, such as that provided by anti-μ antibodies) is sufficient to induce both proliferation of resting B cells and their differentiation into IgM-secreting cells. The stimulation induced by the helper T-cell supernatant takes place in serum-free medium and is not MHC-restricted. Our findings strongly support the existence of a B-cell activating factor acting on the resting B cell and causing it to enter the G_1 phase of the cell cycle in a MHC-unrestricted manner.**

The different steps of the stimulation of resting B lymphocytes resulting in proliferation and clonal expansion followed by maturation to the immunoglobulin-secretion stage are not yet fully understood (1–6). B cells can be stimulated by antigen-specific major histocompatibility complex (MHC)-restricted helper T lymphocytes, which can help antigen-specific B lymphocytes and, in some circumstances, induce a polyclonal stimulation of other B cells (1–3, 5–11). The requirements for MHC-restricted helper T–B cell interactions and linked recognition have been related to the state of B-cell activation: resting B cells can be activated via a pathway that requires covalent carrier–hapten linkage and MHC-restricted T-cell recognition of B cells and accessory cells, whereas activated or blasted B cells can be activated via a different pathway that requires MHC-restricted helper T-cell recognition only of accessory cells but not of B cells (2, 6). A single helper T-cell clone is able to activate B cells via both pathways (6).

B lymphocytes can also be polyclonally activated from the resting stage to a proliferating stage by mitogens such as lipopolysaccharide or by the combined and sequential use of low doses of anti-μ antibodies and T-cell-derived growth factor, called B-cell growth factor (12–16). After exposure to anti-μ antibodies, B cells become sensitive to B-cell growth factor (12, 15). Differentiation into immunoglobulin-secreting cells seems to be brought about by yet other T-cell-derived factors, called B-cell differentiation factor or B-cell maturation factor (17–20).

Indeed, upon recognition of their specific antigen on antigen-presenting cells expressing appropriate Ia molecules, helper T cells release a variety of antigen nonspecific lymphokines, including interleukin 2, B-cell growth factor, B-cell differentiation factor, interferon-γ (IFN-γ), and hemopoietic growth factor such as colony-stimulating factor and interleukin 3 (IL-3) (for a review, see ref. 16). However, among these lymphokines, none has been shown to provide the signal delivered by the helper T cell upon contact with the resting B cell, or by anti-μ antibodies. We report here on the polyclonal stimulation of gradient-purified resting B lymphocytes induced by antigen-activated helper T-cell supernatant. Our findings strongly support the existence of a B-cell-activating factor acting on the resting B cell and causing it to enter G_1 phase in a MHC-unrestricted manner, as recently suggested by De Franco and co-workers (21, 22).

MATERIALS AND METHODS

Mice. DBA/2, C57BL/6, A/J, BALB/c, C3H/HeJ, C3H/HePas, CBA/J, CBA/N, B6.C H-2^{bm12} mice were bred in the animal facilities of the Pasteur Institute. CBA/N mice were a kind gift of P. Truffa-Bachi. Two-month-old female mice were used throughout the experiments.

Antigens, Mitogens, and Culture Medium. The synthetic random terpolymer poly(Glu^{60}, Ala^{30}, Tyr^{10}) (GAT) (lot no. 13), the synthetic random copolymer poly(Glu^{60}, Ala^{40}) (GA), (lot no. 2), and concanavalin A were purchased from Miles (Paris). Lipopolysaccharide from *Salmonella typhimurium* was purchased from Difco. Complete culture medium consisted of RPMI 1640 (Flow Laboratories, Irving, Scotland) containing 5% fetal calf serum/penicillin/streptomycin/fungizone/1 mM glutamine/50 μM 2-mercaptoethanol/10 mM Hepes. In one experiment, cells were cultured in Iscove's modified Dulbecco's medium supplemented with albumin, transferrin, and soybean lipid prepared as described (23) and kindly given to us by the laboratory of F. Melchers (Basel Institute of Immunology). Fetal calf serum devoid of mitogenic activity as tested by the limulus assay was kindly given to us by the laboratory of L. Chedid (Pasteur Institute).

B-Cell Preparations. B cells were prepared by treatment of spleen cell suspensions with a combination of rat monoclonal anti-mouse T-cell antigen antibodies, and complement. Rat anti-Thy-1, anti-Lyt-1, and anti-Lyt-2 hybridomas were a generous gift of A. Coutinho (Pasteur Institute) and of M. Pierrès (Marseille-Luminy, France). After treatment, the remaining cells no longer responded to concanavalin A. In some experiments, after T-cell depletion, resting B cells were prepared on a Percoll density gradient (Pharmacia, Paris) according to Ratcliffe *et al.* (11).

GAT-Specific T-Cell Clone and Preparation of Supernatant. Helper T-cell clone 52-3 is derived from a GAT-specific BDF_1 T-cell line, is restricted by the *H-2^d* haplotype, and does not crossreact with the synthetic copolymer GA (24). Clone 52-3 was propagated in complete medium containing DBA/2-irradiated (3300 rads; 1 rad = 1.0×10^{-2} gray) spleen cells (2×10^6 per ml) and 100 μg of GAT per ml. 52.3 T-cell supernatants were obtained by first coculturing T cells

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Abbreviations: GA, copolymer Glu^{60}, Ala^{40}; GAT, terpolymer Glu^{60}, Ala^{30}, Tyr^{10}; MHC, major histocompatibility complex; IFN-γ, interferon-γ; IL-3, interleukin 3.

(5×10^5 cells per ml) and DBA/2-irradiated spleen cells (2.5×10^6 cells per ml) in complete medium in the presence of GAT (100 μg/ml) for 36 hr. Control supernatants were obtained by coculture in the presence of GA. Irradiated spleen cells were then removed on Lymphoprep (Nyegaard, Oslo) and T cells (10^6 per ml) were allowed to secrete in antigen-free medium for 24 hr. Supernatants from the second culture were collected, filtered on a 0.22 μm Millipore membrane, and stored at −20°C. 52.3 T-cell supernatants did not show any growth-promoting activity for granulomonocyte precursors or an IL-3-dependent cell line (AD3) or IFN-γ, as determined by G. Milon and A. Hovanessian (personal communication).

B-Cell Proliferation and Immunoglobulin Secretion Assay. Splenic B cells were cultured at the indicated concentrations with various dilutions of 52.3 T-cell supernatant in 96-well flat bottom plates (Falcon 3040) and their proliferation was measured on day 3 after a 16-hr pulse with 0.5 μCi of [^{3}H]thymidine (1 Ci/mmol; 1 Ci = 37 GBq) (Commissariat à l'Energie Atomique, Saclay, France) per well. Results were expressed as the arithmetic mean of triplicate cultures. Standard errors of the mean never exceeded 10%.

For the immunoglobulin secretion assay, 5×10^4 B cells were cultured in complete medium for 4 days in the presence of various dilutions of T-cell supernatants. The culture supernatants were then assayed for IgM secretion in an ELISA as described (24).

RESULTS

Antigen-Stimulated Helper T-Cell Clone Supernatant Is Sufficient to Induce the Proliferation of Unprimed B Cells. In the presence of GAT, the GAT-specific T cells induced a very intense proliferation of DBA/2 B cells. Compared to the background B-cell proliferation (2500 cpm), the polyclonal B cell proliferation was significant at a B-cell/T-cell ratio of 50:1 (15,500 cpm) and at a 1:1 ratio was comparable to that induced by 2 μg of lipopolysaccharide per ml (72,000 cpm). This prompted us to try to induce this polyclonal proliferation with antigen-free activated T-cell supernatant prepared as described.

T-cell supernatants were titrated into B-cell cultures. As shown in Table 1, in the absence of any other stimulus, unprimed B cells were driven to proliferate when activated T-cell supernatant was added to the culture. This effect is dose dependent: in the presence of GAT-activated 52.3 supernatant, the proliferation reaches a plateau at a concentration of 25% (vol/vol), then it decreases and is hardly detectable at 1%. Furthermore, the biological activity leading to this proliferation is inducible by the antigen, because the control GA-"activated" T-cell supernatant (although also seeded at 10^6 cells per ml in the second step of the supernatant preparation) is 1/30th as active as the GAT-induced T-cell supernatant.

Similar results were obtained with a different batch of fetal calf serum known to contain no mitogenic activity, such as endotoxins and peptidoglycans (data not shown). B cells were also cultured in Iscove's serum-free medium supplemented with transferrin, albumin, and soybean lipids. As shown in Table 1 (Exps. 2 and 3), B cells proliferate with the same intensity in fetal calf serum-containing medium and in serum-free medium when T-cell supernatant is added to the cultures, whether the T-cell supernatant was prepared in fetal calf serum-containing (batch 1) or -lacking (batch 2) medium.

GAT-activated 52.3 T-cell supernatant (at a final concentration of 10%) is active even at very low B-cell concentrations. Detectable [^{3}H]thymidine incorporation was observed with as few as 10^4 DBA/2 unprimed B cells (Table 1; Fig. 1). If the specific proliferation in the presence of T-cell supernatant is plotted versus the B-cell concentration, we clearly see that the slope of the log–log plot is close to 1, showing that only one limiting cell type in the B-cell population is being titered over this range of B-cell concentration. The stimulation index in these cultures shows a sharp decrease when the B-cell concentration increases. Thus, most of the following experiments were done at 10^4 or 5×10^4 B cells per well, because both concentrations showed a significant specific proliferation and a good stimulation index.

Table 1. 52.3 helper T-cell clone supernatant in the absence of any other stimulus induces unprimed B-cell proliferation

Addition to culture	[^{3}H]Thymidine incorporation of DBA/2 B cells					
	Exp. 1		Exp. 2		Exp. 3	
	cpm	SI	cpm	SI	cpm	SI
None	2,986		551		612	
Lipopolysaccharide	53,145	17.8	9806	17.8	9282	15.2
GAT-activated 52.3 supernatant						
Batch 1						
25%	28,635	9.6	—	—	—	—
10%	23,545	7.9	5573	10.1	5097	8.3
1%	8,011	2.7	—	—	—	—
0.1%	3,881	1.3	—	—	—	—
Batch 2						
10%	—	—	5485	9.9	4843	7.9
Control 52.3 supernatant						
25%	5,569	1.9	—	—	—	—
10%	4,733	1.6	—	—	—	—
1%	3,278	1.1	—	—	—	—

Splenic B cells (Exp. 1, 5×10^4; Exps. 2 and 3, 10^4) were cultured in the presence of lipopolysaccharide (2 μg/ml), activated (batch 1), or control 52.3 supernatant. Exp. 3 was carried out in Iscove's serum-free medium. Batch 2 supernatant was prepared by allowing the activated 52.3 T cells to secrete in fetal calf serum-free medium. Proliferation was measured on day 3 after a 16-hr pulse with 0.5 μCi of [^{3}H]thymidine. SI, stimulation index (cpm in stimulated cultures/background cpm in unstimulated cultures); —, not done.

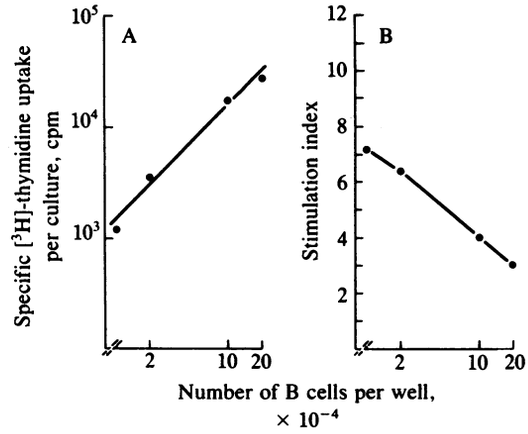

FIG. 1. GAT-activated 52.3 T-cell supernatant is active over a wide range of B-cell concentration. Various numbers of DBA/2 splenic B cells were cultured with or without 10% (vol/vol) GAT-activated 52.3 T-cell supernatant. Proliferation was measured on day 3 after a 16-hr pulse with 0.5 μCi of [^{3}H]thymidine. (*A*) Specific [^{3}H]thymidine uptake per culture = cpm in stimulated cultures minus background cpm in unstimulated cultures (262 cpm for 10^4 B cells and 13.985 cpm for 2×10^5 B cells). (*B*) Stimulation index = cpm in stimulated cultures divided by background cpm in unstimulated cultures.

Table 2. Proliferation of unprimed B cells in the presence of helper T-cell supernatant is not MHC-restricted

Strain	H-2 haplotype	[^{3}H]Thymidine incorporation, cpm per culture			
		None	LPS	52.3	SI
DBA/2	*H-2^d*	981	20,842	9,114	9.3
BALB/c	*H-2^d*	1443	14,494	7,196	5
C57BL/6	*H-2^b*	1620	36,054	11,466	7.1
B6.C H-2^{bm12}	*H-2^b*	2421	41,965	17,182	7.1
A/J	*H-2^a*	2426	18,917	10,408	4.3
C3H/HE Pas	*H-2^k*	2848	38,410	14,566	5.1
C3H/He J	*H-2^k*	933	1,113	7,402	7.9
CBA/J	*H-2^k*	4586	54,888	39,023	8.5
CBA/N	*H-2^k*	1059	20,562	7,020	6.6
DBA/1	*H-2^q*	2851	46,754	16,690	5.8

Splenic B cells (5×10^4) of various genetic origins were cultured without additions (None) or in the presence of lipopolysaccharide at 20 μg/ml (LPS) or 10% (vol/vol) GAT-activated 52.3 T-cell supernatant (52.3). Proliferation was measured on day 3 after a 16 hr-pulse with 0.5 μCi of [^{3}H]thymidine. SI, stimulation index (cpm in 52.3-stimulated cultures divided by background cpm in unstimulated cultures).

Proliferation of Unprimed B Cells in the Presence of 52.3 Helper T-Cell Supernatant Is Not MHC Restricted. GAT-activated 52.3 T-cell supernatant induced a significant proliferation of B cells of 10 strains of mice that belonged to 5 different H-2 haplotypes (Table 2). Although the absolute [^{3}H]thymidine incorporation varied among the different strains, as did the background stimulation, the stimulation index did not show more than a 2-fold variation, ranging from 4.3 to 9.3.

The fact that a significant proliferation occurred with C3H/HeJ B cells in the presence of T-cell supernatant, whereas the same B cells did not respond to lypopolysaccharide strongly argues against the possibility that the proliferation is mediated by some bacterial component in the T-cell supernatant, acting in a lipopolysaccharide-like manner. CBA/N B cells also demonstrated a significant proliferation. This indicates that in our assay the B cells responding to the T-cell factors do not exclusively consist of the Lyb5$^+$ B-cell population, which is missing in CBA/N mice and can be stimulated in a MHC-unrestricted way (6).

Table 3. 52.3 T-cell supernatant alone drives small resting B cells to proliferate

DBA/2 cells	Addition to culture	[^{3}H]Thymidine uptake in culture			
		Exp. 1		Exp. 2	
		cpm	SI	cpm	SI
Unseparated	None	981		427	
	LPS	20,842		3133	
	52.3	9,114	9.2	3317	7.8
Resting B cells (1.09–1.08)	None	668		211	
	LPS	17,000		1306	
	52.3	6,754	10.1	2942	13.9
Large B cells (1.065–1.055)	None	1,680		792	
	LPS	46,318		7350	
	52.3	15,838	9.4	5720	7.2

Small and large B-cell populations were prepared from unseparated DBA/2 splenic B cells by Percoll density separation. Lipopolysaccharide (LPS) was added at 2 μg/ml and GAT-activated T-cell supernatant (52.3) was added at a final concentration of 10%. Proliferation was measured on day 3 after a 16 hr-pulse with 0.5 μCi of [^{3}H]thymidine. B cells were seeded at 5×10^4 per well in Exp. 1 and at 10^4 per well in Exp. 2. SI, stimulation index (cpm in stimulated culture/background cpm in unstimulated cultures).

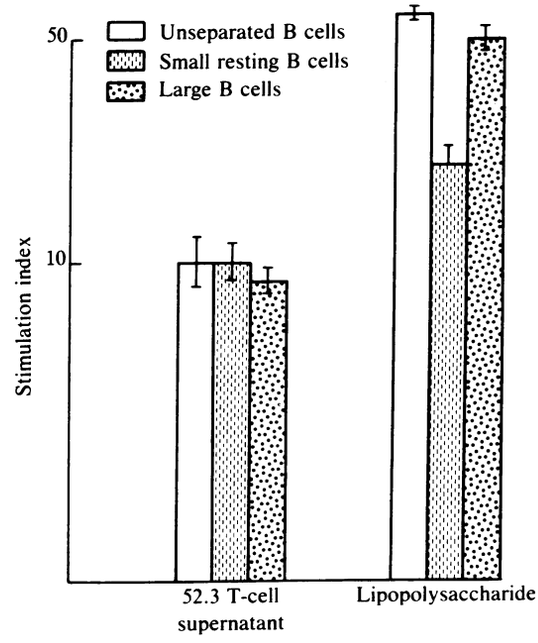

FIG. 2. GAT-activated 52.3 T-cell supernatant can induce resting B cells to differentiate to IgM secretion. Resting and large B-cell populations were prepared from unseparated DBA/2 splenic B cells by Percoll density separation. B cells (10^4) were cultured in fetal calf serum containing medium alone, or in the presence of lipopolysaccharide (2 μg/ml) or 10% (vol/vol) GAT-activated 52.3 T-cell supernatant. Cell-free supernatants of a 4-day culture were assayed for the presence of IgM by ELISA. Stimulation index = IgM secretion in stimulated cultures divided by IgM secretion in unstimulated cultures. In the experiment reported, background secretion of IgM on day 4 was 20 ng/ml, 5 ng/ml, and 100 ng/ml for unseparated B cells, small resting B cells, and large B cells, respectively.

52-3 Helper T-Cell Supernatant Is Sufficient to Induce Polyclonal Proliferation and Differentiation of Resting B Cells. To further understand the mechanism of action of the T-cell supernatant, we wished to determine if it affected resting B cells. We separated B cells into resting B cells and less dense cells collected at the 1.09–1.08 and at the 1.065–1.055 interface of the Percoll gradient, respectively. This last fraction is enriched in large B cells and presumably contains cells that have recently been activated *in vivo*. We first studied whether T cells themselves could activate resting B cells in the absence of anti-Ig antibodies. In the presence of GAT and various numbers of B cells, irradiated 52-3 T cells induced the same [^{3}H]thymidine uptake by both unseparated B cells and resting B cells (data not shown).

Furthermore, as shown in Table 3, 52-3 T-cell supernatant induces the proliferation of resting B cells and that of unseparated B cells to the same extent. In the large B-cell fraction, the background stimulation was higher than in the control unseparated B cells, but the stimulation index in the presence of T-cell supernatant was identical in both cultures.

As shown in Fig. 2, helper T-cell supernatant is also able to drive the differentiation of resting B cells to Ig secretion. Even though resting B cells secrete less IgM than unseparated B cells (see legend to Fig. 2), the IgM stimulation index is the same in a 4-day culture of unseparated B cells, resting B cells, or large B cells in the presence of helper T-cell supernatant. This stimulation index is of the same order of magnitude as that obtained in the presence of 2 μg of lipopolysaccharide per ml.

DISCUSSION

The experiments reported here show that upon activation, an antigen-specific MHC-restricted helper T-cell clone releases

in its supernatant factors that are sufficient to induce the polyclonal proliferation of resting B cells and their differentiation into Ig-secreting cells. For simplicity, we limited our report to the findings obtained with a single GAT-specific helper clone, which was selected because its supernatant did not contain any IFN-γ, colony-stimulating factor, or IL-3, thus allowing easier interpretations of the results. In addition, this supernatant was able to induce the proliferation of unprimed lymph node B cells, which are virtually deprived of hematopoietic cells (data not shown); this argues against the contribution of hematopoietic precursors to the cellular proliferation measured. The features of the 52.3 T-cell supernatant are not unique to this clone: we were able to prepare active supernatant from other GAT-specific cell lines and clones, propagated in complete medium in the presence of syngeneic irradiated spleen cells, rat concanavalin A supernatant and antigen, or adapted to grow in complete medium supplemented only with 20% rat concanavalin A supernatant.

Cell titration experiments (Fig. 1) showed that only one cell type is being affected by the T-cell supernatant, and thus suggest that the B cells are the target of the biological activity of the supernatant. In addition, a density separation was used to isolate B cells shown by others (5, 11, 21) to consist of resting cells. Such a resting B-cell population proliferated with the same intensity as unseparated B cells (Table 3), thereby showing that most of the responding cells in unseparated B cells consist of resting B cells. However, we cannot rule out the participation of adherent cells, given the findings of Corbel and Melchers (25) who have shown that such cells (or factors derived from them) are necessary to lipopolysaccharide-induced B-cell activation. Even if the complete removal of adherent cells (which is extremely hard to accomplish), was to abolish the stimulation of resting B cells by the T-cell supernatant, this would still not disprove that B cells are the target of the helper T-cell supernatant activity. Adherent cells and their factors may be essential to B cells for mere "nutritional" purposes (26).

The factor(s) contained in the T-cell supernatant acts across the MHC barrier. The possibility that, in the cultures of MHC-incompatible B cells, the responding cells exclusively belonged to a subpopulation of *in vivo* activated B-cell blasts (known to be sensitive to MHC-unrestricted T-cell-derived factors) (2), is ruled out by the proliferation intensity. Indeed MHC-incompatible B cells proliferated to the same extent (Table 2) as syngeneic B cells, for which we have shown that the responding population consists of resting B cells. The response of CBA/N B cells (Table 2) (only consisting of the $Lyb5^-$ subpopulation) indicates that in other strains the T-cell factors are not affecting exclusively the $Lyb5^+$ B-cell subpopulation, which does not require a MHC-restricted helper T–B cell interaction (6). The lack of MHC restriction in the activity of the antigen-free helper T-cell supernatant suggests that, under our culture conditions, the double occupancy on resting B cells of immunoglobulin by antigen and Ia by helper T cells as postulated by Anderson *et al.* (2) is not an absolute requirement for the excitation of the resting B cell from the G_0 to G_1 phase to take place.

The response of C3H/HeJ B cells (Table 2) (which are nonresponders to lipopolysaccharide) strongly argues against the possibility that the activity of the helper T-cell supernatant is mediated by lipopolysaccharide contamination of the culture medium, which is in agreement with the fact that helper T-cell supernatant induced the proliferation of DBA/2 B cells in serum-free medium (Table 1). This point is also supported by the fact that in the presence of the helper T-cell supernatant, B cells do not only proliferate and differentiate to IgM secretion, they also start to produce IgG with a typical thymodependent pattern distinctly different from that induced by lipopolysaccharide (ref. 27; unpublished observations).

In our hands, helper T-cell supernatant is sufficient to induce a polyclonal proliferation and differentiation of resting B cells. These results are in contrast with the current consensus that resting B cells become sensitive to B-cell replication factors only after they have been "excited" by a signal transmitted through the membrane immunoglobulin that induces the expression of the receptors for such factors. Such a signal may be delivered by anti-Ig antibodies (12, 14, 15) or by helper T-cell recognition of antigen and Ia on B cells (5). There are two possible ways in which to account for our results, both of which involve the existence of a lymphokine participating in the early steps of the resting B-cell activation: (*i*) the proliferation of resting B cells in the presence of our T-cell supernatant operates through a pathway other than the sequential one summarized earlier and is mediated by a lymphokine(s) different from B-cell growth factor; (*ii*) alternatively, our T-cell supernatant contains a B-cell activating factor acting on a resting B cell and causing it to enter the G_1 phase in a MHC-unrestricted manner, and to become sensitive to B-cell growth factor also contained in the T-cell supernatant. Indeed, the existence of such a factor was recently postulated by De Franco *et al.* (21) on the basis of the lack of MHC restriction in the activation of resting B cells by antigen-specific T-cell clones.

The discrepancy between our results and those of other investigators is not yet totally clear: it may be a matter of concentration of T-cell-derived factors, or of the culture conditions used to propagate the T cells. For instance, work done with sheep erythrocyte-specific or ovalbumin-specific T-cell clones, used T cells that were grown in serum-free medium and the supernatants were derived from the activation of 5×10^4 T cells per ml, whereas we used a 20-fold higher T-cell concentration to prepare our supernatants. In fact, the supernatant of a sheep erythrocyte-specific T-cell clone prepared as described above did exhibit a slight activity on resting B cells, as recently reported by Sidman *et al.* (20).

Whatever the mechanism accounting for the results presented here, it is important to consider the *in vivo* consequences of such an activity and its relevance to the specificity of an immune response. Even though the membrane immunoglobulin does not seem to play a role in the proliferation of resting B cells *in vitro* in the presence of helper T-cell supernatant, it may play a crucial role *in vivo* by a postulated antigen-bridging process, which would focus T cell help to B cells, having bound the antigen for which the T cell is specific (28). Antigen focusing by the B cells would locally yield antigen concentrations equivalent to the high concentrations used to elicit the production of factors by the helper T cells *in vitro*. Furthermore, if upon antigen recognition on the B cells (22), helper T cells produce a 30-fold greater amount of signal than nonactivated helper T cells, the specificity of the immune response can be maintained, assuming that the B-cell-activating factor is only acting at a short range, thus exposing antigen-specific B cells to much more factor than other B cells. It should be mentioned that the low densities at which B cells were cultured may have yielded local concentrations of factors on each B cell that differ from the *in vivo* situation in which B cells are always found at much higher densities. The need for a close contact between helper T cells and B cells (3–5, 7, 12) to drive proliferation of resting B cells may simply reflect conditions under which the production of the B-cell-activating factor is limiting, thus emphasizing the advantage of the antigen-specific B cells over the other B cells. Under such conditions, the concentration of the factor on by-stander B cells will not reach the threshold of stimulation. However, it also seems probable that the production of nonspecific immuno-

globulin that accompanies the development of an antigen-specific response *in vivo* (29) reflects the action of molecule(s) such as those discussed in this paper.

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