SPECIFIC T HELPER CELLS THAT ACTIVATE B CELLS POLYCLONALLY

In Vitro Enrichment and Cooperative Function*

By ANDREI A. AUGUSTIN and ANTÓNIO COUTINHO

From the Basel Institute for Immunology; and the Department of Microbiology, Columbia University College of Physicians and Surgeons, New York 10032; and the Department of Immunology, Umeå University, Umeå, Sweden

The analysis of B-cell triggering mechanisms and clonal regulation has been successfully approached with ligands that are competent to activate a large fraction of all cells (1–3). Polyclonal activation of B cells has also proved to be a useful tool in studying Ig synthesis and secretion (4), in the analysis of functional B-cell subpopulations (5) and their development (6), and in the description of the antibody repertoire (7, 8) by allowing the determination of relative frequencies of clonotypes in the absence of antigen selection.

Each B-cell mitogen known to date activates only a fraction of all bone marrow-derived lymphocytes (9, 10). Although some discrepancies in quantitative aspects of the problem still exist, it is now clear that partially distinct subsets of B cells respond to the various mitogens (5, 11, 12). In particular, much attention has been focused on the existence of B-cell subpopulations responding to either thymus-independent or thymus-dependent forms of the same antigenic determinants (13–16). As was pointed out before (10), this cell population that secretes antibodies subsequently to T cell-macrophage help has thus far escaped all analyses using direct B-cell mitogens. The study of this cell subset would provide new information on both its antibody repertoire and the mechanism of cooperative B-cell triggering which, to date, remains controversial (17).

We attempted to develop a system for polyclonal B-cell induction mediated by T cells, in the absence of extraneously added ligands, such as antigen or mitogen. Previous experiments by Cammisuli et al. (18) had already indicated that cooperative B-cell induction could take place in the absence of specific interactions involving Ig receptors on the responding B cell, by using helper cell recognition of determinants artificially attached to B-cell surfaces. We have now improved that approach with three major modifications. To ensure that the antigens would remain on the B-cell surface throughout the cooperative culture, we activated T cells directly against minor antigens expressed on B-cell surfaces. Furthermore, we used an in vitro system by which a manifold enrichment of specific helper cells can be achieved. Finally, we measured the responses of all B cells, regardless of their antibody specificity. In this way, a very high frequency of specific T-B cell interactions was obtained in the helper assay, and this might make the system an optimal tool for the study of T-B cell collaboration.

* Supported in part by the Swedish Medical Research Council.

Materials and Methods

Mice. Mice of both sexes, between 6 and 24 wk of age were used. C3H/HeJ were obtained from the Institute for Biomedical Research, Füllinsdorf, Switzerland, whereas C3H/Tif and the athymic mutants derived from this strain were purchased from Bomholtgaardt, Ry, Denmark.

In Vivo Immunizations. Splenic lymphocytes from C3H/Tif female mice were used for immunization. Erythrocytes were removed from spleen cell suspensions by hypoosmotic lysis and subsequent passage of the whole suspension through cotton wool. The remaining cells were suspended in complete Freund's adjuvant (CFA)[1] at a final concentration of 10^9 cells/ml. C3H/HeJ mice were injected with 50 μl of suspension at the base of the tail.

Long-Term Selective Cultures for Helper T Cells. 4 d after immunization, the inguinal and paraaortic lymph nodes of the immunized mice were removed and teased through an 80-gauge steel mesh. Stimulator cells were always splenic lymphocytes prepared identically as for the in vivo immunization and irradiated with 3,300 rad. Long-term cultures were set up in RPMI-1640 medium (Microbiological Associates, Walkersville, Md.) supplemented with a 2.5×10^{-2} M final concentration of Hepes (pH corrected with 1 N NaOH to a value of 7.2), fresh glutamine, asparagine, penicillin/streptomycin, and 5% human serum previously tested for growth supporting properties. (A detailed description of the long-term culture conditions has been reported elsewhere [19].) Long-term cultures were incubated in Falcon culture flasks (No. 3033; Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.) in a standing position. Each culture contained 15×10^6 responder cells and 30×10^6 irradiated stimulator cells, all suspended in a total vol of 5 ml culture medium. Every 7 d, cultures were fed with 2 ml of fresh medium containing 10^7 irradiated stimulator spleen cells.

T-Cell Proliferation Assays. Lymph node cell suspensions taken from immunized or normal C3H/HeJ mice as well as cells recovered from long-term proliferation cultures were used as responders in these assays. Stimulator cells were either total splenic lymphocyte suspensions or purified splenic T-cells (nylon-wool passed) and splenic B-cells (from suspensions treated with anti-Thy-1.2 serum and complement).

Cell suspensions were distributed in Falcon microtiter plates (No. 3040). Each culture contained 2×10^5 responder cells and 6×10^5 stimulator cells irradiated with 3300 rad in a total vol of 0.2 ml of the culture medium described above. Lymphocyte proliferation was assessed by incubating the cultures for 16 h in 1 μl of methyl-[^{3}H]thymidine ([^{3}H]Tdr; Amersham Corp., Arlington Heights, Ill.; sp act = 2 Ci/mmol). Cultures were harvested on filter pads and radioactivity measurements were made in scintillation fluid in a Packard Tri-Carb liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill.).

Polyclonal Helper Activity of T Cells. The polyclonal helper activity of T cells was assayed by simply mixing them in the same culture with spleen cells from either normal or nude mice, and by monitoring the appearance of Ig-secreting plaque-forming cells (PFC) in the cultures, using the protein A plaque assay (20). In most experiments, we have used a 1:1 ratio of T cells and spleen cells, which proved to be optimal in a number of preliminary titrations. The cells were cultured at a total concentration of 2×10^5/ml in 0.2-ml aliquots in microtiter plates (Falcon No. 3040) in RPMI-1640 medium supplemented with 5×10^{-5} M 2-mercaptoethanol, 10^{-2} M Hepes, and 10% fetal calf serum (Gibco Biocult Ltd., Irvine, Scotland; batch No. K88). In all experiments, each cell population was also cultured alone and in the presence of various B-cell mitogens at optimal concentration, in particular lipopolysaccharide (LPS) from *Brucella abortus equi* (a kind gift from Dr. C. Galanos and Dr. O. Lüderitz, Max-Planck-Institut für Immunobiologie, Freiburg, Federal Republic of Germany) or liprotein (LP) from *Escherichia coli* (a gift from Dr. J. Andersson, Biomedicum, Uppsala, Sweden).

Developing antisera to mouse IgM, IgG1, IgG2a and IgG2b were obtained by immunizing rabbits with MOPC 104E, MOPC 21, a mixture of UPC 10 and RPC5, and MOPC 195 purified myeloma proteins, respectively, whereas rabbit anti-IgG3 was purchased from Litton-Bionetics. Each rabbit was primed with 1 mg of protein in CFA, boosted with 100 μg of protein

[1] *Abbreviations used in this paper:* CFA, complete Freund's adjuvant; [^{3}H]Tdr, [^{3}H]thymidine; LP, liprotein from *Escherichia coli*; LPS, lipopolysaccharide from *Brucella abortus equii*; MHC, major histocompatibility complex; PFC, plaque-forming cells; SpA, protein A from *Staphylococcus aureus*.

in CFA 1 wk later, and regularly, every month thereafter, with 100 μg of alumina-precipitated protein. Pooled bleeds from individual rabbits, before and after purification of antibodies that bind protein A from *Staphylococcus aureus* (SpA) on a protein A-Sepharose (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) column (21) were tested in developing PFC in the protein A plaque assay on a number of myeloma or hybridoma cells secreting Ig of the various subclasses. Probably, as a result of the high dilution of antibodies used in the plaque assay (in the range of 1:1,000 to 1:5,000 of the original antisera) these antisera proved in many cases specific for PFC development of the corresponding heavy H-chains subclasses even before any specific absorption, at those dilutions which were optimal developing PFC of the corresponding subclass. Anti-δ alloantiserum (a courtesy of L. Forni at the Basel Institute for Immunology, Basel, Switzerland) was obtained by immunizing CWB mice with C3H.SW spleen cells and by absorbing the resulting antiserum with donor thymocytes and Sepharose-coupled IgM. The anti-μ antiserum previously tested for inhibition of the LPS-induced PFC responses was a gift from F. Melchers (Basel Institute for Immunology) and it has been prepared by immunizing rabbits with MOPC 104E myeloma protein as described (22).

Results

Priming and Enrichment of B Cell-specific T-helper Cells. One of us has recently developed an *in vitro* system for the functional enrichment of antigen-specific T-helper cells (19). We attempted to apply the same priming and in vitro conditions to select for helper cells recognizing non-major histocompatibility complex (MHC) antigenic differences expressed on B-cell surfaces. We chose two H-2-compatible C3H substrains (C3H/HeJ and C3H/Tif) (23) which differ at several loci, two of which, at least, result in distinct antigenic patterns of B-cell surfaces. These are the *lps* locus on chromosome 4 (24, 25), which encodes an antigenic surface receptor for LPS expressed in roughly 30% of all splenic B cells, and another locus, unlinked to the former, which controls primary mixed leukocyte reactions between these two substrains and has been arbitrarily classified as *Mls* locus (23). C3H/HeJ mice were immunized with C3H/Tif spleen cells and lymph nodes from the immunized mice were cultured in vitro with irradiated stimulator cells in selective helper cell cultures (19). 2–6 wk after the initiation of the cultures, the cells were harvested and tested for responsiveness and specificity for the immunizing antigens. Over 90% of the living cells recovered expressed surface antigens recognized by a rabbit anti-mouse brain antiserum, whereas only a minority expressed Ig. Roughly 20 and 4% of the initial input of lymph node cells could be recovered from these cultures at 2 and 6 wk, respectively, in the absence of repeated restimulation. With weekly feeding and restimulation with irradiated C3H/Tif spleen cells, however, the recoveries were consistently higher, ranging between 20 and 40% even after 4–5 wk of culture.

As shown in Table I, C3H/HeJ T cells recovered from the selective cultures exhibited marked proliferative responses to the immunizing C3H/Tif spleen cells. Interestingly, the T cell-proliferative response cannot be evoked by a B cell-depleted suspension of stimulator spleen cells, whereas T-cell depletion has no effect on the stimulatory capacity of C3H/Tif cells. We conclude that C3H/HeJ T cells selected by our procedures are most likely specific for surface determinants expressed by B and not by T cells from C3H/Tif mice. It is also apparent from the results in Table I that, although there is a detectable primary stimulation of C3H/HeJ lymph node cells by C3H/Tif spleen cells (23), such a reaction can be augmented by in vivo priming. It appears, however, that the increase in reactivity is primarily achieved by enrichment in selective long-term cultures—less than threefold effect by in vivo priming, compared

TABLE I

Proliferative Responses of C3H/HeJ Lymph Node Cells to C3H/Tif Spleen B Cells Before and After Priming and In Vitro Enrichment

	Responder lymph node cells	Stimulator cells	CPM/culture
			$\times 10^{-3}$
A	Normal	—	0.10
	Normal	C3H/HeJ spleen	0.21
	Normal	C3H/Tif spleen	1.96
B	In vivo primed*	—	0.36
	In vivo primed	C3H/HeJ spleen	0.64
	In vivo primed	C3H/Tif spleen	5.49
C	In vivo primed‡ and in vitro enriched	—	2.97
	In vivo primed and in vitro enriched	C3H/HeJ spleen	16.28
	In vivo primed and in vitro enriched	C3H/Tif spleen	239.41
	In vivo primed and in vitro enriched	C3H/Tif$_{(B\ cells)}$§	8.49
	In vivo primed and in vitro enriched	C3H/Tif$_{(T\ cells)}$‖	218.52
	In vivo primed and in vitro enriched	A/J spleen	10.82

Uptake of [^{3}H]Tdr in triplicate cultures was measured on day 4 of culture in A and B with 5×10^5 responder and 10^6 3,300-rad-irradiated stimulator cells, and on day 3 in C with 2×10^5 responder and 6×10^5 irradiated stimulator cells.

* Mice primed 4 d previously with one intra-tail injection of C3H/Tif spleen cells.

‡ The same cells 22 d after in vitro restimulation with irradiated C3H/Tif spleen cells.

§ Nylon-wool-column-passed spleen cells.

‖ Anti-Thy-1.2-and-complement-treated spleen cells.

to >100-fold enrichment after in vitro selection. As previously described (19) and shown in Table I, in parallel with the positive selection for specific cells we observed negative selection and disappearance of other T-cell specificities, such as alloreactive, H-2-specific responses.

Helper Activity of Enriched T Cells. After we demonstrated that a large number of T cells specific for surface determinants on B cells from C3H/Tif had developed in these cultures which select for helper cells, we moved on to directly test the helper activity displayed by these cells. The experiments consisted in mixing various proportions of C3H/HeJ anti-C3H/Tif-enriched T cells with normal C3H/Tif spleen cells, or with spleen cells from C3H nude mice derived from the C3H/Tif substrain. From these cultures, we monitored B-cell induction and clonal expansion by measuring numbers of high-rate Ig-secreting cells in the SpA plaque assay (20). As shown in Table II, we observed the appearance of large numbers of IgM PFC in these cultures, at the same level of magnitude, as in C3H/Tif spleen cell cultures stimulated by B-cell mitogens, such as LP and LPS. Longer periods of in vitro selection resulted both in a progressive loss of background reactivity as detected by PFC induction in C3H/HeJ cells (compare experiments 1 A and B) as well as in increased functional reactivity to C3H/Tif cells (compare experiments 2 A and B). C3H/HeJ anti-C3H/Tif T cells also stimulated a vigorous IgG PFC response in culture of C3H/Tif spleen cells. One experiment is presented in Fig. 1 showing the kinetics of IgM PFC development as well. In most experiments where the same initial cell density (2×10^5 spleen cells/ml) was used the peak T cell-dependent IgM PFC responses preceeded by 1 d that detected in mitogen-stimulated cultures. This is probably a result of the deterioration

TABLE II

IgM PFC Responses Induced in C3H/Tif Spleen Cells by C3H/HeJ Anti-C3H/Tif Helper T Cells or by LPS and LP

Experiment number	Responding spleen cells	Stimulation by			
		Macrophage	LPS	LP	T cells
1A	C3H/HeJ	62	22	2,440	873
	C3H/Tif	84	5,373	10,173	3,653
	C3H/Tif nu/nu	1,100	16,267	19,947	7,893
1B	C3H/HeJ	15	<80	4,547	84
	C3H/Tif	63	6,760	11,093	2,847
2A	C3H/HeJ	<10	<10	890	246
	C3H/Tif	18	8,338	10,200	7,900
2B	C3H/Tif	165	20,400	19,600	19,850

In 0.2-ml cultures, 2×10^4 spleen cells from either C3H/HeJ or C3H/Tif were cultured alone, in the presence of 50 μg/ml of LPS or LP, or together with 2×10^4 C3H/HeJ anti-C3H/Tif T cells. These were collected from the in vitro selective cultures at day 9 and 16 in Exp. 1 (A and B, respectively) and at days 14 and 21 in Exp. 2. The IgM PFC responses in the test cultures were measured on day 4 and the results are the mean value observed in triplicate cultures. No PFC were detected in cultures of T cells stimulated by either LP or LPS.

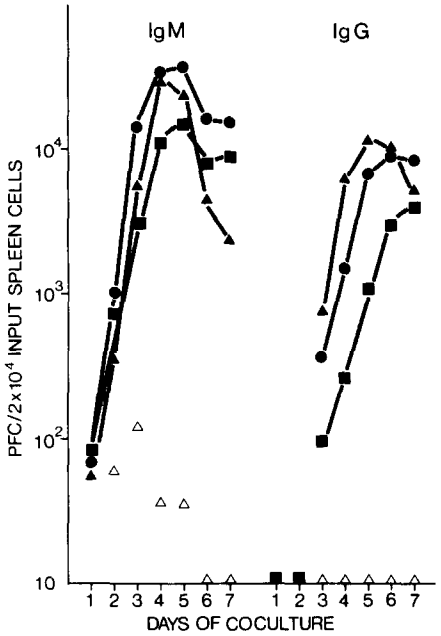

FIG. 1. Kinetics of IgM- and IgG-secreting PFC response in cultures of 2×10^4 C3H/Tif spleen cells stimulated by LPS, LP, or C3H/HeJ anti-C3H/Tif T cells, which were enriched for 28 d in vitro. No PFC could be detected in cultures of T cells with either LPS or LP. Control cocultures of C3H/HeJ spleen with T cells did not develop IgG PFC, and the numbers of IgM PFC were 35, 36, 304, 360, 625, 715, and 205, from days 1–7, respectively. Addition to cultures of C3H/Tif cells: △, none; ■, 50 μg/ml LPS; ●, 50 μg/ml LP; ▲, T cells, at a 1:1 ratio to the spleen cells.

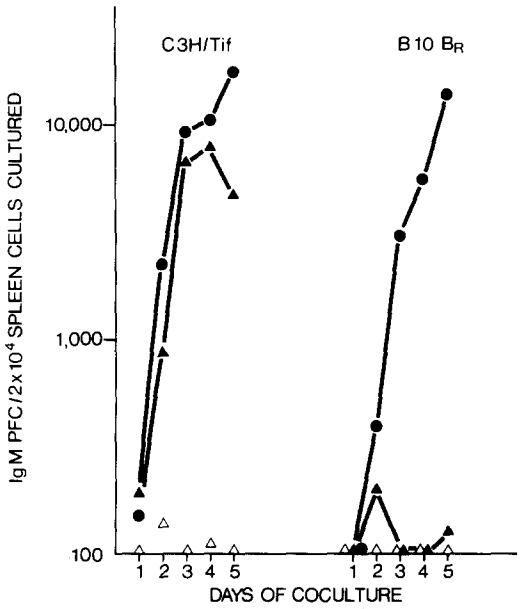

FIG. 2. Kinetics of the IgM PFC responses developed in cultures of either C3H/Tif or B10.BR spleen cells (2×10^4 cells/culture), stimulated by 50 μg/ml LP (●) or by C3H/HeJ anti-C3H/Tif T cells (2×10^4 cells/culture) enriched for 2 wk *in vitro* (▲). Numbers of PFC detected in unstimulated cultures are also shown (△).

of culture conditions in the cooperative responses, because twice as many cells are proliferating in these cultures. The peak of IgG PFC followed by 1 or 2 d that of IgM PFC. In no experiments were IgG PFC detected in the first 2 d of culture and we repeatedly observed a clear shift in the ratios of IgM:IgG PFC by day 3, suggesting that most IgG-secreting PFC arise from clones previously secreting IgM, as shown for mitogen-activated B cells (26). Although the T cell-induced IgM PFC responses were, in some experiments, lower than LPS- or LP-induced responses, the IgG PFC responses were invariably higher in cultures stimulated by T cells. Fig. 1 provides an example of this.

The helper activity of C3H/HeJ anti-C3H/Tif T cells was, as the proliferative responses shown in Table I, specific for the immunizing agents. Not only did these cells induce very low numbers of PFC in self C3H/HeJ spleens (Table II, Fig. 1) but also they failed to induce a PFC response in spleen cells from H-2-compatible B10.BR mice (Fig. 2).

Polyclonality of the T Cell-dependent B-Cell Response. The magnitude of the helper cell-induced PFC responses would indicate that a large fraction of all splenic B cells were activated, because it was comparable to that induced by LPS or LP which stimulate every third B cell in the spleens of C3H/Tif mice (10). The high number of helper cell-reactive B cells suggested that responding B cells were activated irrespectively of the antibody receptor specificities. Direct evidence for this assumption was obtained by assaying, in parallel, spleen cultures from C3H/Tif mice activated by either LPS or T-helper cells against a panel of different antigens in the plaque assay. The additional measurement of total numbers of IgM-secreting PFC in the same cell suspensions provided a quantitative estimate of the frequency of each antibody

TABLE III

Frequencies of Specific Antibody-secreting Cells among LPS or T Cell-induced PFC

Target antigen on PFC assay	PFC induction by	
	LPS	T cells
Sheep erythrocytes	1/576	1/1,139
Horse erythrocytes	1/505	1/735
Goat erythrocytes	1/1,093	1/1,898
Donkey erythrocytes	1/7,785	1/7,595
Dx α-1-3*	1/13,388	1/3,255
Phosphorycholine	1/3,825	1/1,266
$Trinitrophenyl_{30}$	1/196	1/138
$Hydroxydinitrophenyl_2$	1/49	1/105
$Nitroiodophenyl_2$	1/172	1/217

C3H/Tif spleen cells were stimulated in mass cultures with either 50 μg/ml LPS or C3H/HeJ anti-C3H/Tif T cells (enriched for 3 wk in vitro) at a 1:1 ratio. By day 5 of culture, roughly one-third of all cells in LPS-stimulated and one-ninth in T cell-stimulated cultures were detected as IgM-secreting PFC. Each cell suspension was also plaqued at appropriate dilutions with the indicated target erythrocytes and complement. The results show the ratio between the number of direct PFC detected for each antigen and the numbers of IgM-secreting PFC counted in the same cell suspension.

* Dx α-1-3, dextran B1355 used as a target antigen in a plaque assay in the presence of excess dextran B 512 and, consequently, detecting only α-1-3 specificities.

TABLE IV

Subclasses of Ig Secreted by LP- or T Cell-induced PFC

Cultures stimulated by	Classes of Ig-secreting PFC			
	IgM	IgG1	IgG2 (a + b)	IgG3
Macrophages	194	<10	<10	<10
LP	33,480	167	1,240	3,853
T cells	8,080	8,640	6,320	<20

Spleen cells from C3H/Tif mice (2×10^4/culture) were stimulated with either 50 μg/ml LP or 2×10^4 T cells collected after 5 wk of in vitro enrichment. Triplicate cultures were harvested on day 6 and assayed for PFC using subclass-specific developing antisera in the protein-A plaque assay. No plaques could be detected in control cultures of T-cells stimulated by LP. The results shown are mean values of PFC/culture observed.

specificity among all activated and IgM-secreting B cells. The results shown in Table III demonstrate that B cells directly activated by T helper cells can in fact secrete antibodies of all the specificities we examined. Furthermore, the frequencies of specific PFC to heterologous erythrocytes or to nitrophenyl haptens were very similar to those measured in LPS cultures, whereas large differences were observed for the frequencies of PFC to bacterial antigens phosphorylcholine and Dx α-1-3 (dextran B1355 used as a target antigen in a plaque assay in the presence of excess dextran B 512 and, consequently, detecting only α-1-3 specificities) between the two cell populations. Assuming that there is no preferential clonal expansion or maturation determined by combining site specificities of the dividing B cells, these measurements reflect the approximate frequencies of specificities among the initially responding B cells. The

TABLE V

Inhibition of LPS- or Helper Cell-induced PFC Responses by Anti-μ Antibodies

Experiment number	Stimulation by	IgM PFC/culture: additions to cultures		
		None	Anti-μ	Normal rabbit serum
1	(Day 3) LPS	1,110	<10	ND*
	T cells	1,390	310	ND
2	(Day 4) LPS	9,560	<10	10,720
	T cells	17,440	3,420	12,160
3	(Day 6) LPS	8,724	47	9,480
	T cells	9,030	5,500	4,680

C3H/Tif spleen cells (2×10^4/culture) were stimulated by either 50 μg/ml LPS or 2×10^4 C3H/HeJ anti-C3H/Tif T cells. These have been enriched in vitro for 3, 4, and 5 wk, for Exps. 1, 2, and 3, respectively. A rabbit anti-μ antiserum or normal rabbit serum at a final dilution of 1:100 were added as indicated, for the whole culture period. The day of assay of the test cultures is indicated in parentheses for each experiment. Untreated cultures and those receiving anti-μ or normal rabbit sera contained <50 PFC in all experiments.

* ND, not done.

assumption appears reasonable because the frequencies of LPS-induced PFC are in fact identical to the frequencies of LPS-reactive B cells of the same specificities, when determined in limiting-dilution conditions (7). These observations confirm previous reports that T cell-dependent B-cell activation could take place in the absence of specific interactions with Ig receptors of the responding cells (18), when memory cells were used, and they establish that this is also the case for normal, unprimed B cells.

Distinctive Characteristics of T-helper Cell- and Mitogen-dependent B-Cell Activation. The availability of an assay for polyclonal T-helper cell dependent B-cell activation offered the possibility of investigating several questions directly concerning B-cell triggering and regulation of differentiated function in the clonal progeny. A few surprising observations will be reported here.

The first concerns the isotypes of Ig secreted by the activated B cells. As shown above, the IgG PFC response in cultures activated by T helper cells is consistently higher than that induced by LPS or LP. The analysis of IgG subclasses in these cultures has further revealed a sharp difference between direct and cooperative B-cell responses. As shown in Table IV, there is a nearly complete dependence of T helper cells for the development of an IgG1 response and a complete dependence on thymus-independent mitogen for the generation of IgG3 PFC. On the other hand, both types of activation result in a substantial IgG2 response, although clearly higher responses can be observed with T-helper cells. These observations confirm previous reports on the high IgG3:IgG1 ratio in LPS-stimulated B-cell responses ([27] and J. F. Kearney. Personal communication.) and they agree with current views on the high degree of T-cell dependence of IgG1 production. Moreover, they demonstrate that helper cell-dependent IgG production can take place in the absence of antigen, and that the regulation of the Ig class depends on the quality of the stimulus received by B cells.

The other sharp distinction between thymus-dependent and -independent B-cell

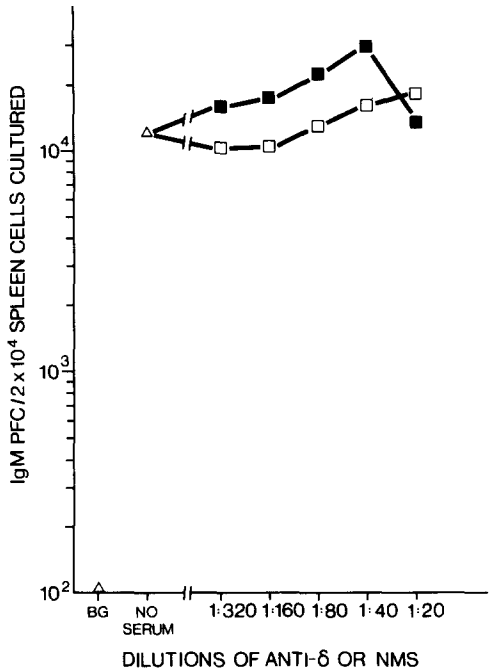

FIG. 3. Influence of CWB anti-C3H.SW anti-δ or normal CWB Ig in the C3H/Tif PFC response to C3H/HeJ anti-C3H/Tif helper cells. Normal or anti-δ Ig were added for the whole culture period, at the indicated final dilutions, from 3.5 mg/ml solutions. The helper cells had been enriched for 5 wk *in vitro* and they were used at a 1:1 ratio to spleen cells (2×10^4/culture). The IgM PFC responses were measured on day 4 of culture, but identical results were obtained from day 2–6.

activation concerns the inhibitory effects of anti-μ antibodies in the PFC responses. As first reported by Andersson et al. (22) some batches of anti-μ antisera inhibit the development of PFC in LPS-stimulated cultures, without affecting B-cell proliferation. Various interpretations have been offered to this finding: conformational changes induced on mitogen receptors by antibodies binding to IgM receptors (22), intracellular turning off of Ig synthesis by internalization of anti-μ antibodies (27), or even Fc receptor-mediated suppressive signals (28). Previous experiments by Cammisuli et al. (18) suggested that helper cell-dependent induction of B cells might not be susceptible to this type of inhibition. The direct test of this possibility was performed by titrating anti-μ antiserum in cultures of C3H/Tif spleen cells activated by either LPS or T helper cells. As shown in Table V, although this particular anti-μ antiserum results in complete suppression of LPS-induced PFC (29), it inhibits only partially the cooperative B-cell response. These findings exclude that the inhibitory effects of anti-μ antibodies can be totally attributed to intracellular blockade of Ig synthesis (2, 27), and they also make the available alternative explanations appear unlikely (22, 28).

On the basis of experiments where antigen-specific responses could be inhibited by anti-δ antibodies which do not affect LPS-induced PFC, it has been claimed that membrane δ on small B lymphocytes plays a fundamental role in T cell-dependent triggering (30).

Because inhibition of antigen-specific responses might be a result of hindrance of antigen-binding and, consequently, cannot be used as indication for a role of membrane δ other than binding, we investigated the influence of a conventional anti-δ

TABLE VI

B-Cell Activation by T Cells Requires Recognition of Antigens on the B-Lymphocyte Surface

Responding spleen cells	Exp. 1		Exp. 2		Exp. 3A		Exp. 3B	
	No additions	T cells	No additions	T cells	No additions	T cells	No additions	T cells
C3H/HeH	15	84	<10	605	<10	360	53	2,788
C3H/Tif	63	2,847	105	23,120	36	29,120	129	18,240
C3H/HeJ + x-irradiated C3H/Tif	30	267	<10	165	<10	256	<10	3,477

C3H/HeJ anti-C3H/Tif cells, cultured in vitro for 2 or 4 wk (Exps. 1, 2, and 3, respectively) were cocultured with spleen cells from either C3H/HeJ, C3H/Tif, or a 1:1 mixture of 3,000-rad-irradiated C3H/Tif and untreated C3H/HeJ cells. In Exps. 1, 2, and 3A, 2×10^4 T cells and 2×10^4 spleen cells were used, whereas in Exp. 3B, 6×10^4 T cells were cocultured with 6×10^4 spleen cells. The IgM PFC responses were measured on day 4 on triplicate cultures, and the results corrected to a fixed input of untreated spleen cells.

antiserum on the T-helper cell-dependent PFC responses. In this system, because no antigen-binding is required by the responding B cells, any effect of anti-δ could in fact be interpreted as indicating particular physiological properties of this surface molecule. As shown in Fig. 3, however, we could not detect any activity of the anti-δ antibodies, significantly different from that of normal mouse serum, although control experiments demonstrated their binding to the majority of splenic B cells. It is possible, therefore, that in those experiments referred to above (30, 31), the inhibitory activity of anti-δ was exclusively a result of inhibition of antigen binding. Alternatively, less efficient culture systems might be more sensitive for detecting inhibitory activities. PFC responses in those experiments were 100- to 1,000-fold lower than those we observed for the same antibody specificity (see Table III), in the absence of antigen-specific selection.

T Helper Cell-dependent B-Cell Activation Requires T-Cell Recognition of Antigens on the Responding B-Cell Surface. A considerable controversy is currently maintained over the question of the mechanisms of cooperative B-cell responses. Although it is clear that soluble factors can augment or reveal an ongoing response, the initiation of the B-cell responses has been shown to require Ia (and possibly antigen) recognition on the responding B cell, by the specific helper cell (32, 33). The only exception to this rule (the in vitro responses to erythrocyte antigens) may have trivial explanations because alloreactive cell lines function as helper cells in such systems (34) and the in vivo anti-erythrocyte PFC response shows the same stringent requirements for direct B-cell recognition by the helper cell (32). We have investigated this problem in our system of polyclonal helper cell-dependent activation by measuring the PFC responses developed by bystander C3H/HeJ spleen cells cocultured with irradiated spleen cells from C3H/Tif mice and C3H/HeJ anti-C3H/Tif T-helper cells. As shown in Table VI, the results demonstrate that activation of B cells requires that T helper cells directly recognize the immunizing antigen on the responding cell. Even at very high cell densities, when the background self activation is quite high, we found no evidence for bystander activation. We conclude, therefore, that T cell-dependent B-cell activation to growth and maturation is not initiated by soluble products generated by an independent T-cell activation.

Discussion

For studying the physiology of cooperative B-lymphocyte activation, and determining the sequence of events leading to clonal growth and antibody secretion, an optimal system should allow the turning on by helper T cells of a large proportion of B cells

from a normal population. Such cooperative B-cell responses, therefore, should not depend on the antigenic specificity of individual B lymphocytes and could be defined as polyclonal.

If, on the other hand, the polyclonal activator T-helper cells were highly enriched, specific T-B cell interactions would be very frequent and they would take place between large populations of cells. T cell-dependent B-cell induction and clonal regulation could then be the object of quantitative analysis.

The experimental system presented here meets these two requirements and might, therefore, constitute a useful tool for studying help. We have achieved T-B cell collaboration on the basis of specific antigen recognition by highly enriched T cells when the antigen is a non-Ig constituent of the membrane of large majority (or even all) B cells. Previous reports have described T-B cell interactions which are not mediated by the classical hapten-carrier tandem. Some, however, dealt with T-cell recognition of Ig idiotypes on responding B cells and are, therefore, equivalent to oligoclonal, antigen-dependent cooperation (35, 36). Others, although bypassing B-cell Ig receptors, did not use enriched T cells and studied only the responses of antigen-specific B cells (18).

Our results qualify the collaborative B-cell responses as polyclonal, because all antigen specificities tested among the resulting PFC were detectable at frequencies comparable to those induced by polyclonal mitogens. All these B cells were activated in the absence of any additional soluble or particulate antigen strongly indicating that, in this case, T-B lymphocyte collaboration can occur in the absence of antigen recognition by B cells. Moreover, the direct involvement of the B-cell Ig receptors in T-B cell interaction and B-cell activation is very unlikely, because no Ig allotype or idiotype differences have been recorded between C3H/HeJ and C3H/Tif.

The C3H/HeJ T cells recovered from long-term cultures appear to be progressively enriched for reactivity to C3H/Tif as measured by their proliferative responses to irradiated stimulator cells. This parallels the progressive increase in the PFC responses induced by these cells inC3H/Tif spleen. In a large number of independent experiments we have invariably observed this correlation between the T cell proliferative responses and their helper capacity. The progressive enrichment of T cells specific for C3H/Tif B cells in long-term cultures also parallels an increase in the average antigen specificity exhibited by the entire T-cell population, as assessed by the ratios of PFC induced in C3H/Tif B cells versus C3H/HeJ B cells.

The enriched T cells are specific for an antigen, or a group of antigens, present on B but not on T cells of C3H/Tif mice, and also absent from C3H/HeJ, A/J, and B10.BR spleen cells. The antigen(s) recognized by these C3H/HeJ anti-C3H/Tif helper cells is not yet identified. That it is expressed on B but not on T cells is in agreement with both products of the *lps* and *Mls* loci (11, 37), which are known to differ between these two strains. Several attempts to enrich for helper T cells of C3H/Tif origin which would specifically activate C3H/HeJ B cells, in the same type of experiments as those reported here, were not successful, suggesting asymmetry in the reactivity of T cells to B-cell determinants differing between these two strains of mice.

From all experiments presented, we would like to underline here those where C3H/HeJ B cells mixed with irradiated C3H/Tif cells could not be induced by the enriched helper cells. This suggests that help occurs after T-cell recognition of antigen presented on B-cell surfaces and that only the B cell presenting that antigen can be triggered.

These conclusions are substantiated by the findings that irradiated C3H/Tif cells display full competence to activate enriched helper cells as shown in Table I. Furthermore, MHC restriction cannot be invoked to explain the unique direction of help toward C3H/Tif B cells, because both substrains have the H-2k haplotype (23) and antigen-specific help in carrier-hapten systems occurs indiscriminately between cells from both strains (unpublished observations). Finally, although T-cell populations after long-term in vitro enrichment in our conditions are depleted of macrophages (19), both spleen cell populations mixed in the test cultures possess macrophages. It appears, however, that macrophages do not deviate the direction of help, and therefore we suggest that they do not represent the channel of T-B collaboration. We conclude, therefore, that recognition of antigens on the B-cell surface by specific T cells or T-cell products is a *sine qua non* requirement for the initiation of cooperative B-cell activation. These conclusions are in agreement with results obtained for T-B cell cooperation in specific antibody responses (32, 33) and with the reported inability of nonspecific soluble factors to substitute for helper cells in the antibody responses of normal B cells to soluble antigens (17). On the other hand, several independent observations substantiate the ability of T cell-released soluble factors to induce B cells of unrelated specificities to Ig secretion (4, 38–40). Experiments in progress have in fact demonstrated that soluble helper factors are generated in the same cultures where we fail to detect bystander B cell responses. These discrepancies might result from different cell populations and culture conditions used in ours and in those experiments, and the observations may not be contradictory. Thus, in our system, which employs very low numbers of B cells, the detection of responses requires extensive clonal growth. Soluble factors, on the other hand, may well be able to induce terminal differentiation in given subpopulations of B cells without proliferation (38), and we would have missed these responses. Furthermore, whereas soluble helper factors appear to be able to maintain growth of already activated B cells (C. Martinez-A., A. A. Augustin, and A. Coutinho. Manuscript in preparation; and F. Melchers. Personal communication.), the bystander B-cell population used in our experiments is practically devoid of blast cells. These considerations indicate that much more extensive work needs to be done in these systems.

The observation on the Ig classes secreted upon helper cell- or mitogen-dependent B-cell activation is also worth discussing. Assuming that we are measuring the responses of the same set of initially reactive B cells (as experiments already performed with purified, mitogen-derived blasts would suggest) two major alternatives could explain the selectivity in class expression by cells responding to qualitatively distinct stimuli. An instructive point of view would argue that the switch in expression of C_H genes is not only fixed to a certain direction but also that the number of consecutive translocations would depend on the quality and quantity of external stimuli. In other words, switching on of IgG1 gene would require more help, whereas any help would be in excess for the chain of translocations to stop at IgG3. The selective alternative would have to argue that although the regulation of C_H gene expression is independent of external stimuli, it is, on the other hand, somehow correlated with the functional expression of growth receptors. In this case, cells turning on IgG1 genes would selectively express growth receptors for help but not for LPS or LP, whereas the opposite would be true for cells switching to IgG3 expression. Experiments in progress aim at deciding between these alternatives.

Summary

C3H/HeJ T cells which specifically recognize B cell-surface antigens of the related, major histocompatibility complex-compatible C3H/Tif strain, can be substantially enriched in vitro by long-term exposure (2–6 wk) of primed lymph node cells to the relevant cellular antigens. These enriched T cells contain functional helper cells as demonstrated by their capacity to induce large numbers of Ig-secreting plaque-forming cells (PFC) in cultures of antigenic B cells. The cooperative interaction results in activation of a large fraction of all splenic B cells, with consequent exponential growth and maturation to high rate secretion of IgM, IgG1, and IgG2, but not IgG3. The IgM PFC response includes antibody specificities to a number of different antigens and can be considered, therefore, as polyclonal. The T helper cell-dependent B-cell response is insensitive to inhibition by anti-δ antibodies, and in contrast with lipopolysaccharide-induced PFC responses, is only partially sensitive to the inhibitory effects of anti-μ antibodies. Finally, B-cell activation to growth and maturation by helper T cells strictly required direct T-cell recognition of antigens on the surface of responding B cells, leading us to the conclusions that if any soluble factors are generated in the collaborative process, they are either antigen specific or incompetent to initiate B-cell growth.

We thank Carina Olsson and Margaretha Tuneskog for very good technical assistance and Luciana Forni for immunofluorescence determinations.

Received for publication 13 August 1979 and in revised form 13 October 1979.

References

1. Andersson, J., O. Sjöberg, and G. Möller. 1972. Mitogens as probes for immunocyte activation and cell cooperation. *Transplant. Rev.* **11**:131.
2. Coutinho, A., and G. Möller. 1975. Thymus-independent B-cell induction and paralysis. *Adv. Immunol.* **21**:113.
3. Andersson, J., A. Coutinho, F. Melchers, and T. Watanabe. 1977. Growth and maturation of single clones of normal murine T- and B-lymphocytes *in vitro*. *Cold Spring Harbor Symp. Quant. Biol.* **41**:227.
4. Melchers, F., and J. Andersson. 1973. Synthesis, surface deposition and secretion of immunoglobulin M in bone marrow-derived lymphocytes before and after mitogenic stimulation. *Transplant. Rev.* **14**:76.
5. Gronowicz, E., and A. Coutinho. 1975. Functional analysis of B-cell heterogeneity. *Transplant. Rev.* **24**:3.
6. Melchers, F., J. Andersson, and R. A. Phillips. 1977. Ontogeny of murine B-lymphocytes development of Ig synthesis and of reactivities to mitogens and to anti-Ig antibodies. *Cold Spring Harbor Symp. Quant. Biol.* **41**:147.
7. Andersson, J., A. Coutinho, and F. Melchers. 1977. Frequencies of mitogen-reactive B-cells in the mouse. II. Frequencies of B-cells producing antibodies which lyse sheep or horse erythrocytes and trinitrophenylated or nitroiodophenylated sheep erythrocytes. *J. Exp. Med.* **145**:1520.
8. Eichman, K., A. Coutinho, and F. Melchers. 1977. Absolute frequencies of lipopolysaccharide-reactive B cells producing A5A idiotype in unprimed streptococcal A carbohydrate-primed, anti-A5A idiotype sensitized and anti-A5A idiotype suppressed A/J mice. *J. Exp. Med.* **146**:1436.
9. Andersson, J., A. Coutinho, W. Lernhardt, and F. Melchers. 1977. Clonal growth and

maturation to immunoglobulin secretion *in vitro* of every growth-inducible B-lymphocyte. *Cell.* **18:**27.

10. Andersson, J., A. Coutinho, and F. Melchers. 1977. Frequencies of mitogen-reactive B cells in the mouse. I. Distribution in different lymphoid organs from different inbred strains of mice at different ages. *J. Exp. Med.* **145:**1511.
11. Gronowicz, E., and A. Coutinho. 1974. Selective triggering of B cell subpopulations by mitogens *Eur. J. Immunol.* **4:**471.
12. Forni, L., and A. Coutinho. 1978. An antiserum which recognizes lipopolysaccharide-reactive B cells in the mouse. *Eur. J. Immunol.* **8:**56.
13. Quintans, J., and H. Cosenza. 1976. Antibody response to phosphorylcholine *in vitro*. II. Analysis of T-dependent and T-independent responses. *Eur. J. Immunol.* **6:**399.
14. Gorczynski, R. M., and M. Feldman. 1975. B-cell heterogeneity. Difference in the size of B lymphocytes responding to T dependent and T independent antigens. *Cell. Immunol.* **18:**88.
15. Andersson, B., and H. Blomgren. 1975. T cell response to polyvinyl pyrrolidone is linked to maturity of B cells. *Nature (Lond.).* **253:**476.
16. Möller, G., and C. Fernandez. 1978. Immunological tolerance to the thymus-independent antigen dextran can be abrogated by thymus-dependent dextran conjugates: evidence against clonal deletion as the mechanism of tolerance induction. *Scand. J. Immunol.* **8:**29.
17. Marrack, P., L. Harwell, J. W. Kappler, D. Kawahara, D. Keller, and E. Swierkosz. Helper T cell interactions with B cells and macrophages. *In* Recent Developments in Immunological Tolerance and Macrophage Function. P. Baram, C. W. Pierce, and J. R. Battisto, editors. Elsevier/North Holland, Amsterdam. In press.
18. Cammisuli, S., C. Henry, and L. Wofsy. 1978. Role of membrane receptors in the induction on *in vitro* secondary anti-hapten response. I. Differentiation of B memory cells to plasma cells is independent of antigen-immunoglobulin receptor interaction. *Eur. J. Immunol.* **8:**656.
19. Augustin, A., M. Julius, and H. Cosenza. 1979. Antigen-specific stimulation and trans-stimulation of T cells in long-term culture. *Eur. J. Immunol.* **9:**665.
20. Gronowicz, E., A. Coutinho, and F. Melchers. 1976. A plaque assay for all cells secreting Ig of a given type or class. *Eur. J. Immunol.* **6:**588.
21. Goding, J. W. 1976. Conjugation of antibodies to fluorochromes: modifications to the standard methods. *J. Immunol. Methods.* **13:**215.
22. Andersson, J., W. W. Bullock, and F. Melchers. 1974. Inhibition of mitogenic stimulation of mouse lymphocytes by anti-mouse immunoglobulin antibodies. I. Mode of action. *Eur. J. Immunol.* **4:**715.
23. Coutinho, A., T. Meo, and T. Watanabe. 1977. Independent segregation of two functional markers expressed on the same B cell subset in the mouse: the Mls determinants and LPS receptors. *Scand. J. Immunol.* **120:**422.
24. Watson, I., K. Kelly, M. Largen, and B. A. Taylor. 1978. The genetic mapping of a defective LPS response gene in C3H/HeJ mice. *J. Immunol.* **120:**422.
25. Coutinho, A., L. Forni, and T. Watanabe. 1978. Genetic and functional characterization of an antiserum to the lipid-A-specific triggering receptor on murine B cells. *Eur. J. Immunol.* **8:**63.
26. Kearney, J. F., and A. R. Lawton. 1975. B lymphocyte differentiation induced by lipolysaccharide. I. Generation of cells synthesizing four major immunoglobulin classes. *J. Immunol.* **115:**671.
27. Kearney, J. F., J. Klein, D. E. Bockman, M. D. Cooper, and A. R. Lawton. 1978. B cell differentiation induced by lipopolysaccharide. V. Suppression of plasma cell maturation by anti-μ: mode of action and characteristics of suppressed cells. *J. Immunol.* **120:**158.
28. Sidman, C. L., and E. R. Unanue. 1976. Control of B lymphocyte function. I. Inactivation of mitogenesis by interaction with surface immunoglobulin and Fc receptor molecules. *J. Exp. Med.* **144:**382.

29. Andersson, J., A. Coutinho, and F. Melchers. 1979. The role of immunoglobulin in the induction of B-lymphocytes. *In* Cells of Immunoglobulin Synthesis. B. Pernis and H. J. Vogel, editors. Academic Press, Inc., New York. 209.
30. Cambier, J. C., F. S. Ligler, J. W. Uhr, J. R. Kettman, and E. S. Vitetta. 1978. Blocking of primary *in vitro* antibody responses to thymus-independent and thymus-dependent antigens with antiserum specific for IgM or IgD. *Proc. Natl. Acad. Sci. U. S. A.* **75:**432.
31. Zitron, I. M., D. E. Mosier, and W. E. Paul. 1977. The role of surface IgD in the response to thymus-independent antigens. *J. Exp. Med.* **146:**1707.
32. Sprent, J. 1978. Restricted helper functions of F_1 hybrid T cells positively selected to heterologous erythrocytes in irradiated parental strain mice. II. Evidence for restrictions affecting helper cell induction and T-B cell collaboration, both mapping to κ-end of the H-2 complex. *J. Exp. Med.* **147:**1159.
33. Marrack, P., and J. Kappler. 1978. The role of H-2 linked genes in helper T cell function. III. Expression of immune response genes for trinitrophenyl conjugates of TG-A-L in B cells and macrophages. *J. Exp. Med.* **147:**1596.
34. Waterfield, J. D., G. Dennert, S. L. Swain, and R. W. Dutton. 1979. Continuously proliferating allospecific T cells. I. Specificity of cooperation with allogeneic B cells in the humoral antibody response to sheep erythrocytes. *J. Exp. Med.* **149:**808.
35. Eichmann, K., I. Falk, and K. Rajewsky. 1978. II. Antigen independent cooperation between T and B lymphocytes that possess similar and complementary idiotypes. *Eur. J. Immunol.* **8:**853.
36. Woodland, R., and H. Cantor. 1978. Idiotype-specific T helper cells are required to induce idiotype-positive B memory cells to secrete antibody. *Eur. J. Immunol.* **8:**600.
37. von Boehmer, H., and J. Sprent. 1974. Expression of M locus differences by B cells but not T cells. *Nature (Lond.).* **249:**363.
38. Coutinho, A., G. Möller, W. W. Bullock, and J. Andersson. 1973. B cell precursors differentiate into antibody-secreting cells by nonspecific T cell factors. *Scand. J. Immunol.* **2:** 83.
39. Geha, R. S., F. Mudawwar, and E. Schneeberger. 1977. The specificity of T helper factor in man. *J. Exp. Med.* **145:**1436.
40. Chiorazzi, N., S. M. Fu, and H. G. Kunkel. 1979. Induction of polyclonal antibody synthesis by human allogeneic and autologous helper factors. *J. Exp. Med.* **149:**1543.