HAPTEN REACTIVE INDUCER T CELLS

II. Evidence That a Secreted Form of the T Cell Receptor Induces Antibody Production*

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There is good evidence that B cells can be activated by several distinct pathways. The intensity and isotype of the antibody response of a subset of B cells to certain antigens (Type 2 T-independent antigens) is determined by nonspecific factors produced by T cells and macrophages (1–5). A different activation pathway is required for antibody responses to low doses of soluble antigen (6). This pathway has two cardinal features. First, "classical" inducer T cells must be focused onto target B cells by a foreign protein containing a portion that covalently links the determinant seen by the T inducer and B cells, forming a so-called "antigen bridge." Second, I-A region products on B cells must be the same as those expressed on antigen-presenting cells (APC)[1] that first initiated clonal expansion of the inducer T cell. These observations have given rise to the theory that activated inducer cells bearing receptors for a particular peptide associated with I-A gene products activate B cells that display I-A as well as the peptide antigen bound to the B cell surface immunoglobulin (Ig) receptors. However, there is little direct evidence to support this hypothesis, and no evidence that recognition and activation may be mediated by a secreted form of the inducer cell surface receptor.

In a previous report we described the derivation and the specificity of activation of a panel of hapten-reactive inducer T cell clones. One group of clones, termed conjugate specific, is activated by hapten conjugated to the carrier protein used for in vitro selection and expansion. The second group, termed hapten specific, is activated by a particular hapten coupled to virtually all foreign and autologous proteins. Both types of clones corecognize conventional antigen in association with products of the I-A locus (7).

In this report, we examine the biologic activity of molecules synthesized and secreted by these hapten-reactive inducer clones. After stimulation by antigen-

[1] Abbreviations used in this paper: ABM, antigen-binding molecules; APC, antigen-presenting cell; B.A., Brucella abortus; BGG, bovine gamma globulin; BSA, bovine serum albumin; Con A, concanavalin A; DME, Dulbecco's modified Eagles; FITC, fluoresceinisothiocyanate; GLA, L-glutamic acid-L-lysine-L-alanine; GLT, L-glutamic acid-L-lysine-L-tyrosine; Ig, immunoglobulin; IL-2, Interleukin 2; KLH, keyhole limpet hemocyanin; LPS, lipopolysaccharide; MHC, major histocompatibility complex; NP, (4-hydroxy-3-nitrophenyl) acetyl; OVA, ovalbumin; PAA, polyacrylamide; PBS, phosphate-buffered saline; PFC, plaque-forming cells; SRBC, sheep erythrocytes; TNBS, trinitrobenzene sulfonic acid; TNP, 2,4,6-trinitrophenyl.

pulsed adherent cells or the T cell mitogen concanavalin A (Con A), a single inducer clone secretes both antigen-specific inducer peptides as well as nonspecific factors that activate B cells. The latter amplify the response of B cells to Type 2 T-independent antigens and the in vitro response to sheep erythrocytes (SRBC). The former consist of antigen-binding molecules (ABM). ABM induce plaque-forming cell (PFC) responses specific for a determinant recognized by immune B cells, provided that cultures containing ABM and B cells are stimulated with antigen that links the epitope recognized by ABM with the B cell epitope. Induction of B cells by ABM is limited to B cells that express the same I-A allele as the source of the ABM and this H-2–restricted interaction between ABM and B cells reflects binding by ABM to I-A products on B lymphocytes. Finally, analysis of the fine specificity of B cell activation by inducer molecules suggests that inducer T cells can activate B cells by secreting a modified form of their cell-bound receptor.

Materials and Methods

Animals. BALB/c, C57BL/6, C57BL/10, CB6F$_1$, and B10.A(5R) mice were obtained from The Jackson Laboratory, Bar Harbor, ME. B10.A(3R) and B10.A(4R) mice were bred in the Michael Redstone Animal Facility of the Dana-Farber Cancer Institute from breeding pairs donated by Dr. D. Murphy, Yale University School of Medicine, and from Dr. D. Sachs, the National Institutes of Health.

Antisera and Complement. Monoclonal (mc) anti–Thy-1.2 was kindly donated by Dr. Ed Clark, University of Washington, Seattle, WA. Monoclonal antisera to Ly 1.2 and 2.2 were obtained from Dr. F. W. Shen, Memorial Sloan-Kettering Cancer Center. Rabbit complement was obtained from Pel-Freeze (Rogers, AR). Guinea pig complement was purchased from Gibco Laboratories (Grand Island, NY).

Antigens and Immunizations. Fluoresceinisothiocyanate (FITC), nitrophenyl (NP), and trinitrophenyl (TNP) conjugates were prepared as described (7). The double conjugates FITC-KLH-TNP or FITC-BGG-TNP were prepared by incubating 50 mg FITC (Sigma Chemical Co., St. Louis, MO) and 40 mg trinitrobenzenesulfonic acid (TNBS) (ICN Nutritional Biochemicals, Cleveland, OH) with 100 mg keyhole limpet hemocyanin (KLH) (Calbiochem-Behring Corp., San Diego, CA) or 100 mg bovine gamma globulin (BGG) in 10 ml of 2% K$_2$CO$_3$ at room temperature for 2 h, followed by rotation overnight at 4°C and extensive dialysis. Ficoll (mol wt 400,000 Pharmacia, Inc., Piscataway, NJ) and lipopolysaccharide (LPS) (*Escherichia coli* 0127:B8; Sigma Chemical Co.) were conjugated with E-TNP-L-lysine (ICN Nutritional Biochemicals) as described (8). TNP conjugated *Brucella abortus* (B.A.) was prepared as described by Mosier (9). TNP-conjugated polyacrylamide (PAA) was prepared as previously described (10). The synthetic polypeptides L-glutamic acid-L-lysine-L-alanine (GLA) and L-glutamic acid-L-lysine-L-tyrosine (GLT) were a generous gift of Dr. M. Dorf, Harvard Medical School.

Mice were primed to TNP-KLH, NP-KLH, or TNP-Bovine serum albumin (BSA) by i.p. immunization with 200 µg antigen in complete Freund's adjuvant. In some cases mice were boosted with 100 µg antigen in saline 6–10 wk later. Mice were used 4–8 wk after the last immunization.

Lyt$^+$ T Cell Clones. The derivation and maintenance of the hapten-specific T cell clones was described in the preceding paper (7).

Media. Cells were cultured in Dulbecco's modified Eagle's Medium (DME) (Gibco Laboratories) supplemented with essential and nonessential amino acids, essential vitamins, 10% fetal calf serum, 1 mM sodium pyruvate, 2 mM L-glutamine, and 5 × 10^{-5} M 2-mercaptoethanol (complete medium).

Preparation of Supernatants. Pulsed adherent cells (PAC) were prepared by incubating 5 × 10^6 irradiated (2,000R) spleen cells syngeneic with the clone with 100 µg/ml antigen in 24-well tissue culture plates (Linbro, # 76-033-05, Flow Laboratories, McLean, VA).

After 2 h, the wells were washed with phosphate-buffered saline (PBS) to remove nonadherent cells (>98% of the cell population) and excess antigen. 10^6 Cl.Ly1-T1 or Cl.Ly1-N5 were then added. After 24 h, the supernatants were removed, spun at 1,200 rpm and stored at $-20°C$. In some cases, supernatants were incubated with TNP-BGG Sepharose, FITC-BGG Sepharose, or NP-OVA Sepharose (0.5 ml Sepharose/ml supernatant) for 2 h at 4°C. After washing, columns were eluted with 0.1 M ammonium hydroxide and neutralized with 0.5 M sodium phosphate. The eluate was then applied to a PD 10 column (Pharmacia) and eluted with complete medium. In some cases, supernatants were prepared by incubating 10^6 Cl.Lyt-T1 or Cl.Ly1-N5 with 1 μg Con A in 1 ml complete medium. Supernatants were harvested after 24 h and 10 mg/ml α-methylmannoside was added. Supernatants were fractionated as described above.

B Cell Culture. B plus accessory cells were prepared by sequential positive and negative selection. After elution from rabbit anti-mouse Fab-coated plates (11), cells were incubated with monoclonal (mc) anti–Thy-1.2, anti-Ly1.2, and anti-Ly2.2 plus rabbit complement for 45 min at 37°C and washed with PBS. Lack of residual T cells was verified by immunofluorescence using anti–Thy-1 antibody, as well as by the loss of proliferative response to Con A and the anti-SRBC plaque-forming cells (PFC) response in vitro. No attempts were made to deplete macrophages from these populations. Accessory cells are still present as judged by the reconstitution of a primary anti-SRBC response with positively selected $Ly1^+$ T cells.

3×10^6 (B plus accessory cells) were incubated in complete medium (1 ml volumes in 24-well (Linbro) plates) at 37°C in 10% CO_2, and supplemented daily with 0.1 ml medium. PFC were enumerated on day 4. Antigen was added at the following concentration: 0.1 μg/ml hapten-protein antigens, 0.05 ml of a 10% TNP-PAA solution, 0.01 μg/ml TNP-ficoll or 5×10^6 SRBC/ml. In some cases, cultures were supplemented with adherent cells (macrophages) as described above.

Incubation of Inducer Molecules with Activated B Cells. Activated B cells were prepared by incubating 7×10^7 spleen cells with 0.4% hapten-PAA conjugate in 10 ml complete medium in 25-cm² tissue culture flasks (Corning, #25100). After 3 d the viable cells were separated on a gradient of Lymphocyte Separation Medium (Litton Bionetics, Kensington, MD). These cells were >70% large "blasts." Cells were incubated with the eluate from a hapten-Sepharose column (10^7 B cells/ml eluate) for 1 h at 4°C, centrifuged at 1,200 rpm for 10 min and the supernatant was stored at $-20°C$.

PFC Assay. PFC were determined by the Jerne plaque technique as modified by Dresser and Greaves (12). TNP-SRBC were prepared as described by Rittenberg and Pratt (13). NP-SRBC were prepared as described by Sherr et al (14). FITC-SRBC were prepared as described by Ramos and Moller (15). Anti-SRBC PFC were assayed on SRBC. In some cases rabbit anti-mouse Ig (Cappel Laboratories, Cochranville, PA) was used to develop IgG PFC. All PFC results represent the mean of duplicate or triplicate cultures; in all cases the standard error was <10%.

The distribution of affinity of the antibody response was determined by the PFC inhibition procedure of Andersson (16). E-TNP-L-lysine (ICN Pharmaceuticals, Cleveland, OH) or NP-caproate (Biosearch, San Rafael, CA) was used to inhibit anti-TNP and anti-NP plaques, respectively and was added to both the agarose and the complement where indicated. Unless otherwise indicated the number of PFC per culture represent only those plaques that were inhibited with 10^{-5}M TNP-lysine or NP-caproate.

Results

Demonstration of Antigen-specific Inducer Activity by a TNP-specific T Cell Clone

We have generated a series of hapten-specific, $Thy1^+$, $Ly1^+2^-3^-$ T cell clones. Cl.Ly1-N5 is activated only by NP-OVA associated with I-A^b gene products; Cl.Ly1-T1 is activated by TNP coupled to virtually any protein associated with I-A^d APC (7).

We asked whether the TNP-specific clone Cl.Ly1-T1 might induce purified (B plus accessory cells) from mice immune to FITC to secrete anti-FITC antibody. Cultures containing Cl.Ly1-T1, B cells from donors immune to FITC-BGG, and the double conjugate FITC-KLH-TNP produced strong anti-FITC PFC responses (Fig. 1). Cultures containing a mixture of FITC-KLH and TNP-KLH did not produce significant responses even when the concentration of each conjugate was 10–100-fold higher than FITC-KLH-TNP (not shown). Thus, Cl.Ly1-T1 induces B cells to secrete anti-hapten antibody to hapten-protein antigens only if the determinant recognized by the clone (i.e., TNP) is covalently coupled to the determinant recognized by immune B cells.

Cl.Ly1-T1 and Cl.Ly1-N5 Synthesize Molecules that Mediate Specific Inducer Function

To obtain molecules that mediate the activity of the intact inducer clone, supernatants were prepared by incubating Cl.Ly1-T1 with either (a) syngeneic antigen-pulsed APC or (b) Con A in the absence of other cells. These supernatants were applied to hapten-coupled Sepharose 4B columns. The eluate from a TNP-Sepharose column was tested for induction of anti-FITC PFC responses using the assay described above. The eluate induced anti-FITC PFC responses in cultures containing the double conjugate FITC-BGG-TNP but not in cultures containing FITC-BGG + TNP-BGG (Fig. 1). The effluent contained no activity. Thus, both the inducer clone as well as products synthesized by the clone activate B cells via an antigen bridge.

We next examined the ability of hapten-reactive inducer cells or their products to activate (B plus accessory cells) to produce antibody to the same hapten.
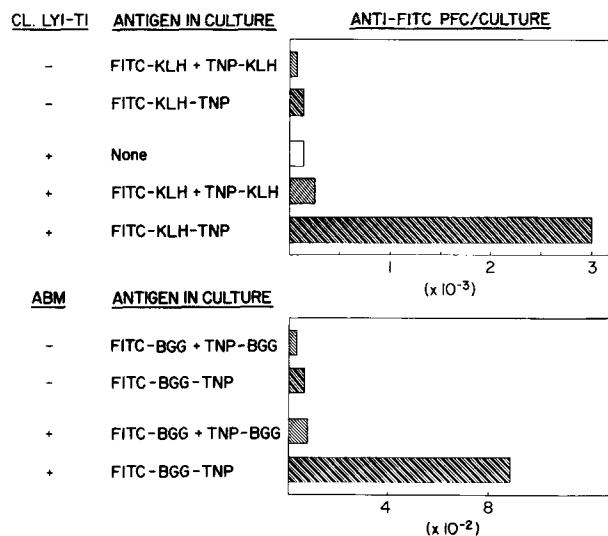

FIGURE 1. Cl.Ly1-T1 and ABM activate B cells via an antigen bridge. Purified B cells were prepared from Balb/c mice primed with FITC-BGG (top panel) or FITC-KLH (bottom panel). 3×10^6 B cells were incubated with 0.1 μg/ml antigen and 3×10^4 Cl.Ly1-T1 cells or 0.1 ml ABM derived from Cl.Ly1-T1 in a final volume of 1 ml. PFC shown are the average of duplicate cultures, in the absence of hapten inhibitors.
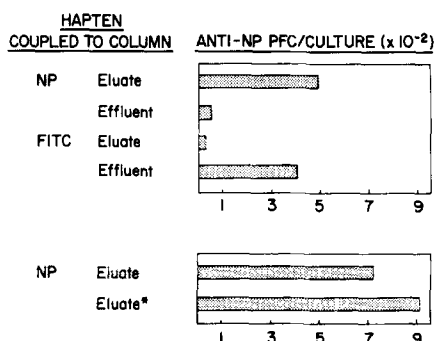
FIGURE 2. Secreted ABM binds specifically to hapten. 3×10^6 purified B cells from NP-KLH–primed C57BL/6 mice were incubated with 0.1 μg/ml NP-OVA. Supernatant from Cl.Ly1-N5 was fractionated on the indicated column and 0.1 ml of the eluate or effluent was added to the culture. PFC inhibitable by 10^{-5} M NP-caproate are shown and represent the average of duplicate cultures. *The eluate was centrifuged for 60 min at 100,000 g before addition to cultures.

Supernatants of the NP-specific clone Cl.Ly1-N5 prepared as described above were applied to Sepharose columns coupled with NP or FITC. The eluate and effluent fractions were tested for induction of antibody production by B cells primed to NP-KLH. The eluate from the NP-Sepharose column induced anti-NP PFC responses (Fig. 2); the effluent (representing ~95–98% of internally labeled protein (data not shown)) did not. The eluate from the NP-coupled column contained molecules specifically bound to NP but not to another hapten: the effluent but not the eluate from a FITC-coupled column induced an anti-NP PFC response[2] (Fig. 2). These findings demonstrate that (a) hapten- or antigen-binding molecules (ABM) can induce B cells to produce antibody to the same hapten, and (b) since ABM bind to Sepharose coupled with the appropriate hapten, NP, but not an inappropriate hapten (FITC), ABM mediate at least part of the B cell induction. Additionally, a dose-response curve of inducer activity in supernatants of both clones showed that (a) substantial inducer activity was demonstrable even at dilutions of 1:1,000 (the equivalent of 10^4 cloned cells) and (b) the activity present in the unfractionated supernatant was completely recovered in the eluate (Table I).

Although only IgM PFC were detected in these assays, in all cases 75–80% of the anti-TNP PFC were inhibitable with 10^{-5} M TNP-lysine. In contrast, the small numbers of anti-TNP or NP PFC produced in cultures in the absence of inducer cells or ABM were not inhibited with 10^{-5} M TNP-lysine or NP-caproic acid.

To determine whether ABM are shed or secreted by the clone, the supernatant from Cl.Ly1-N5 was applied to and eluted from an NP-coupled column and was centrifuged for 60 min at 100,000 g. This procedure did not decrease inducer

[2] 10-fold more TCA-precipitable CPM of internally labeled peptides bound to the NP-Sepharose than to the FITC-Sepharose column (6.25 × 10^5 cpm bound to NP Sepharose (~5% of input cpm) vs. 5 × 10^4 cpm to FITC-Sepharose (<1% of the TCA-precipitable cpm) applied)). The [^35S]-methionine labeled peptides eluted from NP-Sepharose columns resolved as 4–5 bands ranging from 25–60 kdaltons after sodium dodecyl sulfate–polyacrylamide (12%) gel electrophoresis under reducing conditions.

TABLE I

Titration of Inducer Activity Produced by Cl.Ly1-T1 and Cl.Ly1-N5*

Addition to culture		PFC/Culture	
		Expt. 1	Expt. 2
Unfractionated supernatant	1:10	2,160	1,050
	1:100	945	690
	1:1,000	675	180
Eluate (ABM)	1:10	2,790	950
	1:100	990	620
	1:1,000	720	210
Effluent	1:10	205	45
	1:100	135	35
	1:1,000	135	27

* 3×10^6 purified B cells from NP-KLH primed C57BL/6 (Expt. 1) or TNP-KLH primed BALB/c (Expt. 2) mice were incubated with 0.1 µg/ml NP-OVA or TNP-BGG, respectively. Supernatant from Cl.Ly1-N5 (Expt. 1) or Cl.Ly1-T1 (Expt. 2) was fractionated on NP-Sepharose or TNP-Sepharose, and added to cultures at the indicated concentration. Anti-NP (Expt. 1) or anti-TNP (Expt. 2) PFC were assayed after 4 d. PFC inhibitable by 10^{-5} M NP-caproate or TNP-lysine, respectively, are shown and represent the average of duplicate cultures.

activity in the eluate (Fig. 2), suggesting that inducer activity is mediated by secreted ABM rather than mediated by fragments of cell membranes or liposomes.

Evidence that ABM Represent a Secreted Form of the Cell Surface Receptor

H-2 restriction of specific inducer activity by ABM. To determine whether inducer activity mediated by ABM is restricted to B cells/macrophages sharing the same H-2 genes, the eluate fraction of supernatant from clone Cl.Ly1-T1 ($H-2^d$) was added to cultures containing purified (B plus accessory cells) from either TNP-KLH–primed BALB/c or C57BL/6 mice and irradiated CB6F₁ adherent cells from unprimed mice. The eluate induced BALB/c but not C57BL/6 B cells to produce strong anti-TNP PFC responses (Fig. 3).

To map this restriction more precisely, the eluate prepared from clone Cl.Ly1-N5($H-2^b$) was added to purified (B plus accessory cells) from NP-KLH–primed B10 ($H-2^b$) or B10.A (4R) ($H-2^{h4}$) mice, which differ only at the H-2K and I-A loci. A substantial anti-NP response was induced in cultures containing B10 but not B10.A (4R) (B plus accessory) cells.

The above experiments show that anti-hapten responses to hapten-protein conjugates were obtained only when inducer ABM were incubated with B cells (and possibly macrophages) identical at the I-A locus (Fig. 3). One explanation for this observation is that the secreted ABM represents a modified form of the cell-bound receptor and, like the inducer cell, recognizes I-A gene products in association with antigen. To directly test this hypothesis, ABM were incubated with syngeneic or allogeneic B cells and then assayed for antigen-specific inducer activity. Incubation with activated B cells from allogeneic strains had no effect on subsequent inducer activity; in contrast, incubation with strains identical at the I-A region (e.g., B6 vs. B10A.4R) resulted in the complete loss of inducer activity (Table II). In experiments not shown we found that ABM inducer activity was also depleted after incubation with unactivated syngeneic, but not allogeneic

PFC/Culture
$(\times 10^{-3})$

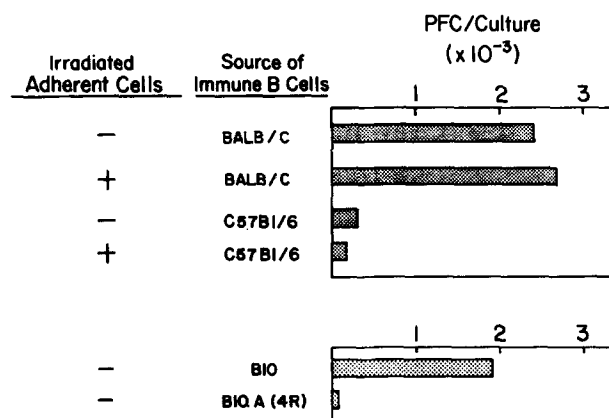| Irradiated Adherent Cells	Source of Immune B Cells	

FIGURE 3. Specific inducer activity of ABM is H-2 restricted. Upper panel: 3×10^6 purified B cells from TNP-KLH primed mice were incubated with 0.1 μg/ml TNP-BGG. Supernatant from Cl.Ly1-T1 was fractionated on TNP-Sepharose and 0.1 ml eluate added to the cultures. Where indicated, cultures were supplemented with irradiated adherent cells from unprimed CB6F1 mice. After 4 d cultures were assayed for anti-TNP PFC. Lower panel: 3×10^6 purified B cells from NP-KLH–primed mice were incubated with 0.1 μg/ml NP-OVA. Supernatant from Cl.Ly1-N5 was fractionated on NP-Sepharose and 0.1 ml eluate added to the cultures. After 4 d cultures were assayed for anti-NP PFC. PFC inhibitable by 10^{-5} M TNP-lysine or NP-caproate, respectively, are shown and represent the average of duplicate cultures.

TABLE II

Loss of Inducer Activity Following Preincubation of ABM with Syngeneic Activated B Cells

	Source of ABM*	Source of activated B cells used for preincubation[‡]	PFC/Culture[§]
Expt. 1	Cl.Ly1-T1 (BALB/c origin)	a, None	1,350
		b, C57BL/6	1,420
		c, A/J	1,310
		d, BALB/c	190
		e, b + d[∥]	1,375
Expt. 2	Cl.Ly1-N5 (C57BL/6 origin)	None	860
		C57BL/6	0
		B10.A (4R)	840

* Supernatants prepared from Cl.Ly1-T1 (Expt. 1) or Cl.Ly1-N5 (Expt. 2) were fractionated on NP-Sepharose or TNP-Sepharose to obtain ABM.

‡ Where indicated, the ABM was preincubated with activated B cells (see Materials and Methods) before addition to cultures.

§ 3×10^6 purified B cells from TNP-KLH primed BALB/c (Expt. 1) or NP-KLH primed C57BL/6 (Expt. 2) mice were incubated with 0.1 μg/ml TNP-BGG or NP-OVA respectively, and the indicated ABM. After 4 d, cultures were assayed for anti-TNP (Expt. 1) or anti-NP (Expt. 2) PFC.

∥ A mixture of supernatants incubated with activated B cells from group b (C57BL/6) and group d (BALB/c) were tested for inducer activity to rule out the possibility that after incubation with syngeneic B cells (group d) supernatants did not acquire suppressive activity.

B cells. However, removal of inducer activity was incomplete, implying that activated B cells express more surface I-A than do resting B cells. This result does not bear on whether the secreted ABM is composed of two separate proteins that bind to antigen and to I-A, respectively, or one that binds both. However, collectively these experiments show that activation of B cells is mediated by

secreted inducer molecules that recognize both antigen and I-A products on B cells.

Fine specificity of B cell activation by ABM.   Cl.Ly1-T1 is activated by autologous or foreign proteins such as the synthetic polymer TNP-GLA that are restricted by I-A^d gene products (e.g. TNP-GLA). In contrast, TNP coupled to I-E controlled polymers (such as GLT) (25) does not activate Cl.Ly1-T1 (7). We reasoned that if ABM secreted by Cl.Ly1-T1 represents a secreted form of the receptor, then ABM might display the same fine specificity as the receptor. Failure of ABM to recognize and bind to TNP-GLT/I-A complexes on B cells might prevent delivery of the inducer signal to the target B cell. This was the case (Fig. 4): incubation of TNP-primed B cells with ABM and TNP-GLA but not TNP-GLT elicited strong anti-TNP responses.

These findings show that secreted inducer molecule(s) that activate PFC responses co-recognize I-A and antigen on B cells with the same fine specificity as the cell-bound receptor. The most parsimonious interpretation of these observations is that ABM represents a secreted product of the same gene(s) that encode the T cell receptor.

Separation of Antigen-specific from Nonspecific Helper Factors

Although antigen-specific B cell activation by Cl.Ly1-T1 and Cl.Ly1-N5 is mediated by molecules that co-recognize antigen and I-A displayed on B cells (Figs. 2–4), these clones also produce factors that induce B cell responses independent of an antigen bridge (nonspecific help). We attempted to separate the inducer cell products responsible for the two functions. Supernatants from antigen-stimulated Cl.Ly1-T1 were applied to TNP-coupled Sepharose columns. The eluate, effluent, and unfractionated supernatant were tested for nonspecific and specific inducer activity (Fig. 5). Unfractionated supernatant as well as the eluate from the TNP-Sepharose column stimulated the production of anti-TNP PFC by B cells from mice immune to TNP-KLH while the effluent did not induce a significant anti-TNP PFC response at any concentration tested. Moreover, neither the whole supernatant nor the TNP-column eluate induced B cells from mice primed to FITC-KLH to respond to FITC-BGG, confirming and extending earlier results indicating the specificity of B cell activation by ABM (Figs. 2 and 3).

These data also show that the unfractionated supernatant induces virgin B cells to generate anti-SRBC PFC. However, in contrast to induction of hapten-
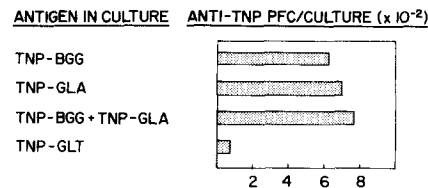
ANTIGEN IN CULTURE   ANTI-TNP PFC/CULTURE (x 10^{-2})

TNP-BGG

TNP-GLA

TNP-BGG + TNP-GLA

TNP-GLT

2   4   6   8

FIGURE 4.   Fine specificity of B cell activation by ABM. 3×10^6 purified B cells from TNP-KLH–primed BALB/c mice were incubated with 0.1 μg/ml antigen and 0.1 ml of ABM derived from Cl.Ly1-T1. In the absence of eluate, there were 50 PFC/culture. PFC inhibitable by 10^{-5} M TNP-lysine are shown and represent the average of duplicate cultures. The results from one of two similar experiments is shown.
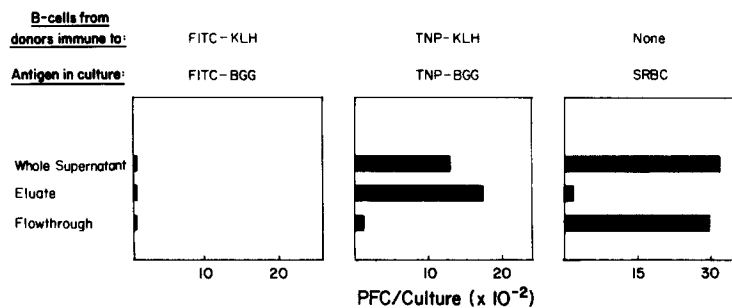
FIGURE 5. A single helper cell produces antigen-specific and nonspecific molecules. Purified B cells were prepared from BALB/c mice which had been primed as indicated. 3×10^6 cells were incubated with 0.1 μg/ml FITC-BGG, TNP-BGG or 5×10^6 SRBC/ml, and 0.1 ml of the supernatant, eluate or effluent from Cl.Ly1-T1 as indicated. The results from one of three similar experiments are shown.

specific PFC responses, the inducer activity resided in the column effluent. Thus, a single helper cell produces both antigen-specific and nonspecific inducer molecules; the two can be separated on Sepharose columns coupled with the appropriate antigen.

Clone Supernatants Enhance B Cell Responses to Type 2 T-independent Antigens

Responses to the Type 1 T-independent antigens TNP-lipopolysaccharide (TNP-LPS) and TNP-*Brucella abortus* (TNP-BA) appear to be independent of T cells. However, recent studies have shown that responses to the Type 2 T-independent antigens TNP-polyacrylamide (TNP-PAA) and TNP-ficoll are in fact dependent on T cells or their products (1, 2). We therefore asked whether Cl.Ly1-T1 or Cl.Ly1-N5 inducer cell products might induce B cells to respond to Type 1 or 2 T-independent antigens. Purified B cells (and macrophages) from unprimed mice were incubated with different T-independent antigens (Table III). Addition of unfractionated clone supernatant greatly increased the response to TNP-PAA and TNP-ficoll (Type 2 antigens), but had no effect on the response to TNP-LPS or TNP-BA (Type I antigens). Induction was due to nonspecific factors rather than to TNP-specific ABM because (*a*) whole supernatants enhanced the response to FITC-PAA (Table III), (*b*) the effluent rather than the eluate fraction from TNP Sepharose columns contained inducer activity, and (*c*) supernatants of Cl.Ly1-N5 (H-2^b) and Cl.Ly1-T1 (H-2^d) were equally effective in augmenting an anti-TNP-PAA response of H-2^d B cells, indicating lack of MHC restriction.

Discussion

These studies show that hapten-specific inducer clones produce soluble factors that activate B cells to secrete antibody. Cells of a single inducer clone produce two types of inducer molecules: antigen-specific molecules (ABM) that induce responses to hapten-protein conjugates and nonspecific molecules. The latter do not require Ia homology between the strain of origin of the inducer cell and B cells to induce antibodies to SRBC and Type 2 T-independent antigens. These

TABLE III

Nonspecific Factors Produced by Activated Clones Facilitate a B Cell Response to Type 2
T-independent Antigens

Antigen in culture*	Supernatant[‡]	PFC/culture[§]
TNP-LPS	−	4,020
	+	3,920
TNP-B.A.	−	3,260
	+	3,240
TNP-PAA	−	520
	+	9,120
TNP-Ficoll	−	185
	+	1,720
FITC-PAA	Unfractionated	5,126
	Eluate	480
	Effluent	4,880

* 3×10^6 purified B cells were prepared from virgin BALB/c mice and incubated with 0.01 μg/ml
TNP-LPS, 10^9 TNP-B.A. organisms, 0.5% hapten-PAA, or 0.01 μg/ml TNP-ficoll.

‡ Unfractionated supernatant derived from Cl.Ly1-T1 was added to the cultures, with the exception
of cultures containing FITC-PAA in which case the supernatant was fractionated on a TNP-
Sepharose column. In all cases, supernatant or fractions were added at a final concentration of
10%.

§ After 4 d of culture, anti-TNP or anti-FITC PFC were assayed in the absence of inhibitors. One
of six similar experiments is shown.

clones also synthesize and secrete factors that bind to antigen (ABM) and activate
only those B cells that are (a) linked to the antigen via a bridge and (b) syngeneic
with the inducer clone at I-A (H-2 restriction). We show that this phenomenon,
"antigen-specific, H-2–restricted" help, reflects binding of ABM to both I-A and
antigen on the B cell surface.

These studies explain, in part, the failure despite extensive effort to identify
inducer molecules that mediate the activity of the intact cell. There are several
reasons for this. First, considerably higher concentrations of antigen-specific
peptides may be synthesized by homogeneous populations (clones) of T cells than
by heterogeneous populations or by T cells that have been fused with tumors.
Second, both antigen-specific and nonspecific factors are synthesized solely by
the T cell clones because both are produced after activation either by antigen-
pulsed APC or by Con A in the absence of other cell types. It is essential to
separate nonspecific helper factors from ABM on antigen-coupled columns
before assay because antigen-specific inducer activity is normally masked by the
nonspecific factors produced after clonal activation.

Previous studies demonstrated that supernatants from T cell hybridomas can
augment in vitro PFC responses of cultures containing both T and B cells (17,
18). This augmenting activity is lost after passage of supernatants through
columns conjugated with (a) anti-I-A (17, 18), (b) antiidiotype (17), (c) anti-V_H
(17, 18), and (d) antigen (17, 18). In all cases biologic activity was measured by
PFC generated from spleen cells rather than purified B cells: the augmenting
activity of these factors may reflect an effect on T cells rather than a direct effect
on B cells. There is a single report suggesting that factors from T cell clones
specific for horse erythrocytes bind specifically to erythrocytes and "prepare"
small B cells to make antibody after a second encounter with B cell replication

and maturation factors (19). However, this study and others show that activated Ly1$^+$ T cells produce nonspecific lymphokines that are sufficient to stimulate division and production of anti-SRBC PFC by cultures containing purified B cells and macrophages (3–5).

ABM present in the eluate from hapten-coupled Sepharose columns induce anti-hapten PFC responses to hapten-protein conjugates but do not activate responses to SRBC or Type 2 antigens. B cell growth factors have been shown to be required for the latter responses (1–5) and may well be involved in the former. Perhaps enough of this material is complexed with the ABM and is efficiently targeted to the appropriate B cell. Preliminary results show that some BCGF activity is present in column eluates but at much lower concentrations than in the effluent (data not shown).

The concept of a hapten-specific helper T cell is relatively recent. The majority of T cells recognize protein carriers, whereas B cells can produce antibody to either hapten or protein determinants. The findings reported here emphasize that there is no functional division of labor based on specificity for protein and haptenic determinants. This is most directly shown by our demonstration that a hapten can "help" itself. Previous failures to demonstrate hapten-specific help probably reflect the predominance of inducer clones that recognize hapten only in association with a unique amino acid sequence on the carrier protein (7).

Hapten-specific helper activity mediated by ABM is H-2 restricted since (a) help is restricted to B cells that share I-A identity and (b) help can be depleted by incubation of inducer ABM on syngeneic but not allogeneic activated B cells. It is therefore of interest that ABM activity is retained on hapten-coupled Sepharose columns that lack I-A. Possibly, multiple epitopes stabilized on a solid surface provide a sufficient matrix for low affinity binding in the absence of MHC products. Alternatively, it is possible that MHC products are carried over from the cell culture.

We used the double hapten conjugate FITC-KLH-TNP to show that significant antibody responses require targeting of the cloned cells (Cl.Ly1-T1) or its product(s) to the B cell by an antigen bridge, despite the fact that IgM and not IgG PFC were induced. Roehm et al. (20), who measured only IgM PFC, also demonstrated a requirement for linked recognition in cultures containing low doses of antigen and an antigen-specific T cell hybridoma. This is in contrast to the findings of Asano et al. (6). Their results suggest that Lyb5$^-$ B cells require linked recognition as well as H-2 identity between B cells, T cells, and macrophages to produce IgG. In contrast, Lyb5$^+$ B cells produce IgM in the absence of an antigen bridge, although high doses of antigen are required.

The above data may be reconciled if B cells (e.g. Lyb5$^-$) that require linked recognition and MHC identity fail to switch to IgG production in the absence of factors provided by a second T cell. Limiting dilution studies of Rosenberg and Asofsky (21) suggest that while one type of helper cell is sufficient for an IgM PFC response in vitro, two are needed for an IgG response (21). Isakson et al. (22) have shown that some T cell lines or hybridomas produce factors that are capable of mediating an IgM to IgG transition in vitro (22). We have isolated a clone that is reactive to autologous I-A on activated B cells (Clayberger, C., R. H. DeKruyff, and H. Cantor, manuscript submitted). The cell, or its products,

stimulate an IgM to IgG switch in the in vitro system described here as well as in other in vivo and in vitro systems. For example, addition of this clone or its products to cultures containing ABM and hapten-primed B cells results in a strong IgG response. This type of cell may be particularly important in the response to antigens containing repeating epitopes (e.g. highly conjugated TNP-BGG) to which an IgG response is not as readily obtained (23).

After activation, Cl.Ly1-T1 and Cl.Ly1-N5 also produce antigen nonspecific, H-2–restricted factors that promote B cell responses to SRBC or Type 2 T-independent antigens. Responses to both antigens depend on factors from activated T cells and/or macrophages, which may include interleukin 1, interleukin 2, and B cell growth factors (5). Addition of factors from activated macrophages alone had no effect on the anti-SRBC or Type 2 responses studied here (data not shown), presumably because preparations contained sufficient numbers of macrophages; all nonmacrophage factors necessary for activation of these PFC responses were provided by T cell clones.

The finding that ABM inducer activity in the supernatant is not diminished after ultracentrifugation (100,000 g for 1 h) suggests that ABM are secreted products of inducer cells. In principle cytoplasmic proteins released from dead cells may remain in the supernatant after centrifugation. However, ABM activitry is highest early after activation of the clones (24), when cell viability is highest (>95%). Experiments to demonstrate that ABM are secreted, rather than shed or released, are currently in progress.

The data reported here thus strongly support the view that ABM represent a modified, secreted form of the cell surface receptor. If the fine specificity of activation of Cl.Ly1-T1 is determined by a cell-bound receptor, then this recognition structure has the following three properties: (a) it distinguishes completely between TNP and FITC, (b) it recognizes a portion of the I-A gene product, and (c) it does not recognize TNP conjugated to I-E–controlled polymers such as GLT (25), but responds well to TNP coupled to I-A–controlled polymers such as GLA. The specificity of ABM activation of B cells displays these features: (a) ABM binds to TNP-Sepharose and not FITC-Sepharose columns. (b) inducer activity is lost after incubation with B cells expressing the I-A genotype identical to that of the clone, and (c) ABM from Cl.Ly1-T1 activates B cells cultured with TNP-GLA but fails to stimulate B cells cultured with TNP-GLT. Thus, ABM are characterized by properties analogous to those displayed by the cell-bound receptor and most likely represent a modified form of this structure.

Taken together, these findings indicate that at least some antibody responses to proteins may not depend on cognate B cell–T cell interaction. Instead, a secreted form of the inducer T cell receptor induces antibody secretion by antigen-specific, I-A identical B cells. The fine specificity of secreted inducer molecules, as defined here, ensures that B cell clones activated by secreted ABM belong to the same pool as those triggered by the intact inducer cell.

Summary

The biologic activity of molecules synthesized and secreted by hapten-specific inducer T cells was examined. After activation, a single inducer clone secretes both antigen-specific inducer peptides as well as nonspecific factors. The nonspe-

cific factors augment the in vitro response of B cells to sheep erythrocytes (SRBC) and Type 2 T-independent antigens. The antigen-specific molecules (ABM) induce plaque-forming cell (PFC) responses in cultures containing ABM, B cells, and antigen that links the epitope recognized by ABM with the B cell epitope. Induction of B cells by ABM is limited to B cells expressing the same I-A allele as the source of the ABM and this reflects binding by ABM to I-A products on B lymphocytes. The data reported here strongly support the view that inducer cells can activate at least some B cells by secretion of a modified form of the T cell surface receptor.

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