Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 1989–1993, March 1982
Immunology

Dissociation of two signals required for activation of resting B cells

(T-cell–B-cell collaboration/H-2 restriction/anti-immunoglobulin/B-cell activation)

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Communicated by Niels K. Jerne, December 2, 1981

ABSTRACT **Cellular interactions involved in the T cell-dependent activation of B cells were analyzed by using lines and clones of helper T cells specific for determinants expressed on the B cell surface. Activation of male antigen-, *M* locus-, and H-2-specific T cells was shown to support polyclonal Ig production by a population of B cells that did not require T-cell–B-cell interaction for induction/amplification. However, these T cells alone did not activate gradient-purified small (resting) B cells. The activation of small B cells was shown to require not only a signal derived through an antigen-specific T-helper cell–B cell interaction but in addition a second signal that could be provided by anti-Ig antibodies.**

The role of a direct major histocompatibility complex (MHC)-restricted interaction between thymus-derived helper cells (T_H) and B cells in the latter's activation to immunoglobulin secretion has been widely investigated (1–8). Recently, it has been established that the requirement for a MHC-restricted T_H–B interaction correlates with the B cell's state of activation: the induction of resting (small) B cells is limited by this interaction whereas the induction/amplification of activated (blasted) B cells is not (7, 9).

In addition, in a hapten-carrier response, the activation of resting hapten-specific B cells requires that hapten be covalently linked to carrier (linked recognition) (10). Thus, through surface immunoglobulin (sIg) receptors, hapten-specific B cells indirectly acquire carrier determinants, creating an antigen bridge through which *H-2*-restricted T_H activity can be mediated.

The question arises as to whether the role of sIg in mediating B-cell activation is solely to focus T_H-derived activation signals (11) or whether involvement of sIg receptors in binding antigen is critical and in itself integrally related to the process of B-cell activation (12). To distinguish between these two alternatives, focusing of the T_H-dependent signal must be dissociated from sIg receptors. This was accomplished by using T cells specific for B-cell surface determinants distinct from sIg. This experimental design was modeled after previous studies demonstrating that such T cells can function as polyspecific B-cell activators (13).

MATERIALS AND METHODS

Animals. BALB/c, C3H/HeJ, CBA, CWB, C57BL/6, and B10.A(4R) mice were obtained from the Institute for Biomedical Research (Füllinsdorf, Switzerland). BALB/b and C3H-H-2° mice were obtained from OLAC (Bicester, Oxon, England). BALB/k mice, derived from a breeding pair obtained as a kind gift of F. Lilly, were maintained in the animal colony of the Basel Institute for Immunology (Basel). Male and female mice were used as indicated.

T Cell Preparation. Anti-H-2^k-specific T cells were obtained by immunizing CWB mice with C3H spleen cell suspensions injected at the base of the tail (13, 14). Draining lymph nodes from immunized CWB mice were harvested at day 7, and T cells were isolated by using nylon wool fractionation (15). These cells were restimulated at 5- to 7-day intervals with x-irradiated C3H spleen cells. The anti-H-2^k clone $D^k/1$ was obtained by limiting dilution of CWB responders after the third restimulation, in the presence of C3H fillers and T-cell growth factor (16).

Other T-cell lines and clones were obtained as described (16). Both the line 5R/L and clone 5R/33 were male-specific and restricted to IA^b (16). In addition, they both were stimulated by non-H-2 antigens present in CBA/J ($H\text{-}2^k$), DBA/2J ($H\text{-}2^d$), and C3H/Tif ($H\text{-}2^k$) but not in B10.BR ($H\text{-}2^k$), C3H/He ($H\text{-}2^k$), and B10.D2 ($H\text{-}2^d$) mice. This strain distribution of mixed lymphocyte reactivity mirrors that of the *M* locus (17), but recent experiments indicate that determinants other than M1s may be involved (18).

B Cell Preparation. Splenic B cells were prepared by depleting erythrocytes and T cells as described (19, 20). The resulting cell suspensions were used not only as responding B-cell populations but also, subsequent to 2000 rads (200 grays) of x-irradiation, as a source of antigen-presenting cells (APC). Small B cells were prepared by Percoll (Pharmacia) density gradient separation of splenic B cells. Small B cell populations contained 0.5–3% of the level of Ig-secreting cells detected in unfractionated splenic B cell populations as assessed by the protein A plaque assay (see below). All of the small B cell populations used responded to lipopolysaccharide with the production of 6–10 $\times 10^4$ Ig-secreting cells per 10^6 input B cells after 5 days of culture.

Antibodies. Sera were collected from rabbits hyperimmunized either with purified TEPC-183 myeloma protein (IgM, κ) or with purified papain-solubilized H-2^d molecules. (The latter serum was a kind gift of O. Henriksen.) IgG fractions were isolated on a column of protein-A-Sepharose CL-4B. The anti-Ig antibody (anti-TEPC-183) reacted with Ig molecules of all classes and subclasses, by Ouchterlony and immunoprecipitation analyses, and stained between 35% and 55% of adult mouse spleen cells in indirect immunofluorescence tests. The anti-H-2 antibody stained 90–100% of adult mouse spleen cells in indirect immunofluorescence and precipitated several minor species but mostly molecules of 46 and 11 kilodaltons from membrane-radioiodinated spleen cells. Bivalent $F(ab')_2$ fragments were prepared by pepsin digestion of intact IgG molecules, and monovalent Fab' fragments were prepared by mild reduction and alkylation of the $F(ab')_2$ fragments. All of the antibody fragments retained the indirect immunofluorescence staining properties of the parent antibodies. For specificity controls, a por-

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Abbreviations: T_H, thymus-derived helper cell; APC, antigen-presenting cell; MHC, major histocompatibility complex; sIg, surface immunoglobulin; pfc, plaque-forming cells.

Table 1. Specificity of an alloreactive T-cell clone in polyspecific B-cell activation

Anti-Thy 1.2-treated spleen cells*	Haplotype			Response, pfc per 10^6 input B cells	
	K	*IA*	*D*	No addition	With $D^k/1$†
C3H	*k*	*k*	*k*	400	36,800
BALB/k	*k*	*k*	*k*	800	32,600
BALB/b	*b*	*b*	*b*	1200	4,200
BALB/c	*d*	*d*	*d*	1100	2,100
B10.A(4R)	*k*	*k*	*b*	1300	900
B10.MBR	*b*	*k*	*q*	1100	1,100
C3H-H-2°	*d*	*d*	*k*	2300	33,100

* Triplicate cultures, each containing 5×10^4 of the indicated B cells.
† B cells were cultured in absence of T cells or in the presence of 2×10^3 $D^k/1$ cells per culture.

tion of $F(ab')_2$ anti-Ig antibody was circulated over a series of columns consisting of purified myeloma proteins coupled to Affi-Gel 10 beads. The resulting antibody preparation was termed "negatively adsorbed." The $F(ab')_2$ anti-Ig molecules that bound to the Ig columns were eluted with low pH, dialyzed, and concentrated. This antibody fraction was termed "positively adsorbed."

DNA Synthesis. Cells (2×10^5) were cultured in flat-bottomed microtiter wells containing 0.2 ml of fluid. The medium was RPMI-1640 containing 10 mM Hepes (pH 7.5), 100 units of penicillin and 100 μg of streptomycin per ml, 2 mM L-glutamine, 5% fetal calf serum, and 50 μM 2-mercaptoethanol plus antibodies at the indicated final concentrations. After 2 days of culture at 37°C in a 5% CO_2/95% air atmosphere, 0.5 μCi of [^{3}H]thymidine (5 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was added per well. The radioactivity incorporated into DNA was measured 24 hr later. The values expressed are means ± SEM from triplicate determinations.

***In Vitro* Helper Assays and Enumeration of Ig-Secreting Cells.** A modification of the Mishell–Dutton culture technique (21) was used as described (19). Mixtures of T_H, APC, and B cells were cultured in 0.2-ml volumes. Anti-Ig was included in some cultures at concentrations indicated. After 5 days of incubation, replicate cultures were harvested, pooled, and assayed for Ig-secreting cells. Total Ig-secreting cells were enumerated by using protein A-conjugated sheep erythrocytes as indicator cells (22) and developed with a polyspecific rabbit anti-mouse Ig isotype antiserum. Plaque-forming cells (pfc) were assessed by using the Cunningham modification (23) of the Jerne plaque technique (24); the results are expressed as pfc per 10^6 input B cells. Haplotypes of plaque-forming cells were determined by incubating pools of 13–18 replicate cultures in 0.5 ml of Eagle's minimal essential medium containing no antiserum, anti-H-$2K^b$, anti-H-$2K^k$, or a combination of anti-K^b and anti-K^k. These antisera were obtained from the Research Resources Branch (National Institute of Allergy and Infectious Diseases) and all were used at a final dilution of 1:40. After a 30-min incubation on ice in the presence of anti-H-2 antiserum, guinea pig serum was added as a source of complement and the mixtures were further incubated at 37°C for 30 min. Recovered cells were washed once and assayed for total Ig pfc.

RESULTS

A clone of T cells ($D^k/1$) derived from an H-2^b anti-H-2^k mixed lymphocyte reaction was determined to be specific for D^k as assessed by its ability to induce polyclonal B-cell responses only

Table 2. T-cell–B-cell interaction does not limit the activation of all B cells

Anti-Thy 1.2-treated spleen cells*	X-irradiated anti-Thy 1.2-treated spleen cells†	Response, pfc per 10^6 input B cells			
		No addition	With 5R/L‡	With 5R/33§	With $D^k/1$¶
B6, male	—	800	9,900	10,400	
	B6 male	700	22,500	22,100	
	B6 female	800	8,900	9,500	
B6, female	—	600	1,600	700	
	B6 male	300	12,600	15,600	
	B6 female	400	2,000	1,200	
B10.A(4R), male	—	400		800	
	B6 male	300		10,100	
	B6 female	100		1,300	
CBA, female	—	2600	10,800	14,300	
	CBA female	2200	20,900	18,100	
C3H-H-2°	—	400			19,400
	C3H-H-2°	300			30,300
B10.MBR	—	400			1,700
	C3H-H-2°	700			25,900
B10.A(4R)	—	200			1,300
	C3H-H-2°	200			22,100

* See footnote * in Table 1.
† Splenic B cells were x-irradiated with 2000 rads and 1.25×10^4 were added per culture where indicated.
‡ Male-specific T cells derived from bulk cultures after the third restimulation (5R/L) were added at 1×10^4 per culture.
§ The male-specific T cell clone 5R/33 was added at 5×10^3 per culture.
¶ See footnote † in Table 1.

in cell populations bearing the appropriate determinants. Mixture of C3H/HeJ (the original stimulator) B cells and the alloreactive clone resulted in a polyspecific B-cell response of almost 100-fold over background (Table 1). Of the BALB H-2 congeneic cells tested, only those from BALB/k responded. The specificity within $H\text{-}2^k$ was defined as D^k by the same criteria with B10.A(4R), B10.MBR, and C3H-H-2° used as B-cell sources.

The results in Table 2 demonstrate that the polyspecific pfc responses mediated by male-specific and allospecific T_H originate from B cells which only require soluble products derived from T_H–APC interactions for activation or further amplification. Whereas the polyspecific responses induced by 5R/L and 5R/33 were limited to male B6 splenic B cells, the addition of male B6 but not female B6 APC resulted in the induction of polyspecific responses by both female B6 and male B10.A(4R) splenic B cells. Similarly, the $D^k/1$ clone induced polyspecific responses by inappropriate splenic B cells [B10.MBR and B10.A(4R)] in the presence of APC bearing D^k determinants. These results demonstrate that this polyspecific induction of B cells is not limited by a direct T-cell–B-cell interaction.

Resting B cells are not susceptible to this mode of T-cell dependent B-cell activation in the absence of a signal provided by anti-Ig. When 5R/L, 5R/33, or $D^k/1$ T_H were added to small B cells, levels of pfc were not increased over background (Table 3). The lack of response was not due to insufficient APC for T_H activation because the addition of exogenous sources of APC had no effect. However, polyspecific responses by male B6, CBA, and C3H-H-2° small B cells were induced by T_H when both an appropriate source of exogenous APC and anti-Ig were present simultaneously.

The lack of response without a source of exogenous APC or with inappropriate APC demonstrates that anti-Ig is unable to obviate the requirement for a T_H–APC interaction (Table 3). In

Table 3. Activation of small B cells requires two signals

Small B cells*	X-irradiated anti-Thy 1.2-treated spleen cells†	Rabbit anti-mouse Ig‡	Response, pfc per 10^6 input small B cells: No addition	With 5R/L§	With 5R/33¶	With $D^k/1$‖
			Experiment I			
B6, male	—	−	<100	<100		
	—	+	<100	<100		
	B6, male	+	<100	**34,600**		
			Experiment II			
B6, male	B6, male	−	200	400	100	
		+	<100	**141,000**	**12,400**	
B6, female		−	100	200	900	
		+	<100	600	500	
CBA	CBA	−	400	200	100	
		+	<100	**66,900**	**16,500**	
			Experiment III			
B6, male	—	−	500		600	
	B6, male	−	600		700	
		+	700		**11,800**	
	B6, female	−	700		900	
		+	600		1,400	
B6, female	—	−	700		800	
	B6, male	−	900		300	
		+	1000		1,200	
	B6, female	−	500		1,300	
		+	600		600	
4R, male	—	−	1100		800	
	B6, male	−	1100		900	
		+	700		1,200	
	B6, female	−	800		1,100	
		+	800		1,100	
			Experiment IV			
C3H-H-2°	—	−	<100			200
	—	+	<100			<100
	C3H-H-2°	−	<100			300
		+	<100			**10,100**

* Percoll gradient-fractionated small B cells were cultured at 5×10^4 per well.
† See footnote † in Table 2.
‡ Where indicated, cultures were supplemented with $F(ab')_2$ preparations of rabbit anti-mouse Ig at a final concentration of 25 μg/ml.
§ See footnote ‡ in Table 2.
¶ See footnote § in Table 2.
‖ See footnote † in Table 1.

addition, the presence of anti-Ig and conditions satisfying T_H activation were not sufficient to induce polyspecific responses by small B cells not bearing determinants recognized by T_H. Thus, 5R/L and 5R/33 were unable to induce female B6 or male B10.A(4R) small B cells in the presence of male B6 APC and anti-Ig. This result suggests that, in addition to the signal provided by anti-Ig, induction of small B cells requires a direct interaction with activated T_H.

This was tested by determining which B cells responded in cultures containing both male B6 and male B10.A(4R) small B cells, supplemented with 5R/L, male B6 APC, and anti-Ig. Treatment of the polyspecific pfc from such cultures with anti-H-2 antisera specific for K^b determinants resulted in the loss of all pfc, whereas treatment of pfc with anti-K^k antiserum did not affect the number of pfc recovered (Table 4). Thus, two distinct signals are obligatory for the induction of small (resting) B cells.

To demonstrate that the relevance of anti-Ig was beyond that of nonspecific perturbation of B-cell membranes, its activating potential was compared with that of anti-H-2. Although both of these reagents were found to interact with the B-cell membrane, only anti-Ig was able to supplement the T_H-derived activation signal (Fig. 1*A*). Moreover, the inability of anti-H-2 to mediate the appropriate signal was not due to its inhibition of the male-specific, *I-A*-restricted T_H–B-cell interaction because the polyspecific response obtained in the combined presence of anti-Ig and anti-H-2 was identical to that obtained in the presence of anti-Ig alone. The activity of $F(ab')_2$ preparations of anti-Ig in supplementing the T_H-derived signal could be adsorbed by and eluted from Ig-coated beads (Fig. 1*B*). Furthermore, Fab' monomers, which were still reactive with B-cell sIg, were ineffective. It is likely, therefore, that the anti-Ig signal is mediated through B-cell sIg receptors and that crosslinking is critical.

Although $F(ab')_2$ anti-Ig alone did not mediate pfc production (Table 3), it stimulated the incorporation of [^{3}H]thymidine by both unfractionated and small spleen cells (Fig. 1*C*). Anti-H-2 antibodies had no effect. The ability to induce [^{3}H]thymidine incorporation correlated with activity against sIg, and crosslinking remained essential (Fig. 1*D*). If the incorporation of [^{3}H]thymidine is considered an accurate reflection of cell growth, it follows that B-cell proliferation can occur in the absence of differentiation.

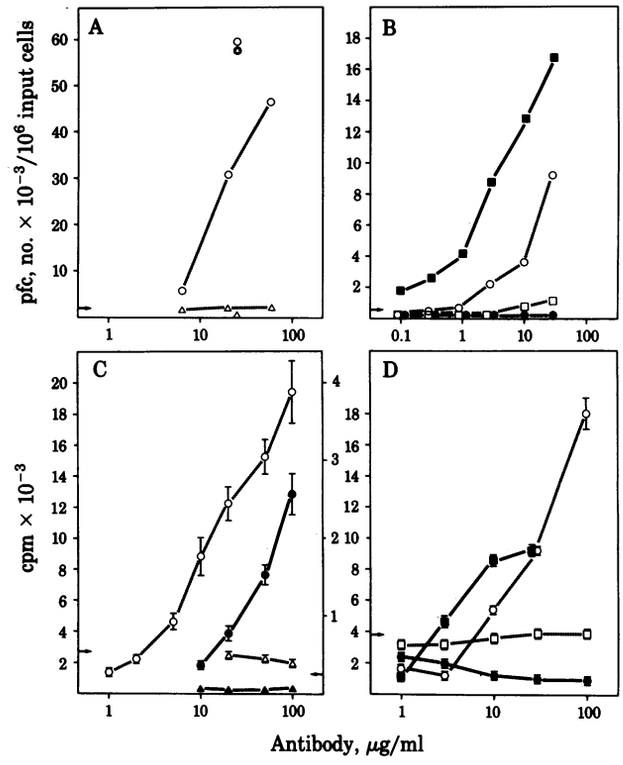

FIG. 1. Anti-Ig-mediated B-cell activation. (*A*) Mixtures of Percoll gradient-fractionated small B cells derived from spleens of C57BL/6 males, T cells from 5R/L, and x-irradiated male C57BL/6 splenic B cells were cocultured at 5 × 10^4, 1 × 10^4, and 1.25 × 10^4 cells per well, respectively. Cultures were supplemented with various quantities of $F(ab')_2$ anti-Ig (○) or anti-H-2 Ig (△) or both (◎). At day 5, Ig-secreting cells were counted. (*B*) Small B cells derived from spleens of C3H-H-2°, D^k/1 T cells, and x-irradiated C3H-H-2° splenic B cells were cocultured at 5 × 10^4, 2 × 10^3, and 1.2 × 10^4 cells per well, respectively. Cultures were supplemented with various quantities of positively adsorbed (■), negatively adsorbed (□), or unadsorbed (○) preparations of $F(ab')_2$ anti-Ig. In addition, the activity of Fab' monomers (●) prepared from unadsorbed $F(ab')_2$ preparations was assessed. At day 5, Ig-secreting cells were counted. (*C*) Cultures containing either 2 × 10^5 unfractionated male C57BL/6 whole spleen cells (○, △) or 2 × 10^5 Percoll gradient-fractionated male C57BL/6 small spleen cells (●, ▲) were supplemented with various quantities of anti-Ig (○, ●) or anti-H-2 (△, ▲) $F(ab')_2$. Cultures were pulsed at day 2 with [^{3}H]thymidine and cells were harvested on day 3. Right-hand ordinate scale is for ● and ▲. (*D*) See *C*. Cultures containing 2 × 10^5 unfractionated C57BL/6 spleen cells were supplemented with various quantities of $F(ab')_2$ anti-Ig preparations as in *B*.

DISCUSSION

The experiments described identify two signals required for the activation of small (resting) B cells. An antigen-specific T_H–B-cell interaction, as previously demonstrated in more conventional systems analyzing T cell-dependent B-cell induction (5–9), was found to be obligatory. Because in systems of antigen-specific B-cell activation the delivery of the T_H-derived signal involves B-cell sIg receptors, the latter's involvement in B-cell activation, beyond the obvious role of focusing, is unclear. In

Table 4. T-cell–B-cell interaction limits the activation of small B cells

Small B cells*	X-irradiated anti-Thy 1.2-treated spleen cells†	Response, pfc per 10^6 input small B cells in presence of 5R/L and anti-Ig‡ after treatment with: —	Anti-H-2K^b	Anti-H-2K^k	Anti-H-2K^b + anti-H-2K^k
B6, male plus 4R, male	B6, male	23,300	100	21,300	100

* See footnote * in Table 3. Mixtures contained 2.5 × 10^4 small B cells from both male C57BL/6 and male B10.A(4R).

† See footnote † in Table 2.

‡ Cultures contained 1 × 10^4 5R/L and 25 μg of $F(ab')_2$ anti-Ig per ml. Haplotype of plaque-forming cells was determined by anti-H-2-mediated cytotoxicity.

the present study, T_H–B-cell interactions were dissociated from sIg receptors by using T cells specific for B-cell surface determinants distinct from sIg. This procedure revealed that two signals are required for T cell-dependent activation of resting B cells.

Mixtures of male-specific or H-2-specific T cells with small B cells bearing the appropriate determinants did not result in polyspecific B-cell induction. In addition to a direct T_H interaction, activation of small B cells required a second signal which could be delivered by anti-Ig (Table 3). The likelihood that the anti-Ig-derived signal is mediated through B-cell sIg receptors is supported by adsorption studies (Fig. 1*B*) (25). As suggested by previous studies (26), crosslinking of B-cell receptors by anti-Ig is critical (Fig. 1*B*). One might speculate that anti-Ig in these studies mimicks the role of antigen. Therefore, antigen would normally play two roles in B-cell activation, focusing T_H interactions and delivering a signal via sIg.

The present study demonstrates that the T_H-derived signal need not be focused through sIg and that the Ig perturbation can be independent of the T_H interaction. Previous studies have demonstrated synergy between anti-Ig and soluble T-cell products in the induction of polyspecific B-cell responses from unfractionated B cells (27).

The experimental design used was modeled after previous studies demonstrating that T cells specific for B-cell surface determinants function as polyspecific B-cell activators (13). Those authors did not observe bystander responses and concluded that all B cells are directly activated by T_H interactions without the need for sIg-mediated signals. Although the reasons for the discrepancy between their results and our experiments regarding bystander responses are not clear, it is possible that the lack of observed bystander response in those studies is due to the use of unfractionated spleen cells as APC. Also, different cell clones were used. However, the positive demonstration of bystander responses derived from unfractionated B cells (this work, ref. 5, and unpublished data) show that some B cells are stimulated without direct T-cell–B-cell interaction. We demonstrate here that small B cells are not responsive to either direct or indirect T-cell influences alone but become responsive with the addition of an anti-Ig-delivered signal.

The induction of small B cells to Ig secretion required not only a T_H–B-cell interaction (Table 4) but also a T_H–APC interaction (Table 3): the combined presence of anti-Ig and small B cells expressing the appropriate determinants did not, in the absence of appropriate APC, satisfy the requirements for T_H activation for the support of polyspecific B-cell responses (Table 3). Perhaps the requirement for T_H–APC interaction reflects more stringent requirements for T_H function in B-cell activation, as opposed to T-cell proliferation, because B cell-presented antigen may be sufficient for the latter (28).

The activation and effector functions of T_H have been classically defined as limited by the recognition of antigen in association with *I-A* region-encoded products of the MHC. This phenomenon is seen at the level of both T_H–APC (29–32) and T_H–B-cell (28, 33) interactions. It leads to the question of whether products of the *I-A* region are unique in providing, with antigen, the appropriate channels for the donation and acceptance of T_H-derived helper signals. Two T_H specificities analyzed in this study suggest that neither *I-A*-region products specifically nor MHC-encoded products in general need be involved in delivery of help to small B cells. Both D^k- and *M*-locus-specific T cells provided the necessary supplements to induce resting B cells in a polyspecific fashion, suggesting that multiple B-cell determinants are able to serve this function.

As shown in the activation of antigen-specific B cells (8), the polyclonal activation of B cells described herein can be accomplished by using cloned T_H populations. Thus, one need not postulate a requirement for multiple T_H subsets in the activation of resting B cells (34).

The authors thank Brigitte Gross, Katrin Turton, and Brigitte Geschke for excellent technical assistance and Sharon Buser-Boyd for preparation of the manuscript. The Basel Institute for Immunology was founded and is supported by Hoffmann–La Roche, Basel, Switzerland. M.H.J. is a recipient of a Leukemia Society of America Scholarship.

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