MAJOR HISTOCOMPATIBILITY COMPLEX-RESTRICTED, POLYCLONAL B CELL RESPONSES RESULTING FROM HELPER T CELL RECOGNITION OF ANTIIMMUNOGLOBULIN PRESENTED BY SMALL B LYMPHOCYTES

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The simplest model for the function of helper T cells in the antibody response is that they act by recognizing antigen on the surface of antigen-specific B cells. This model accommodates the classic experiments of Mitchison (1), showing that T cells and B cells must recognize portions of the same antigen molecule in order to collaborate (linked recognition). Also, since helper T cells recognize antigen in the context of class II major histocompatibility complex (MHC)[1] antigens, this model predicts the finding that T cell–B cell collaboration is often MHC-restricted (2–5), and that *Ir* genes, which influence T cell recognition of antigen, are expressed in B cells (6).

Since T-B collaboration occurs at antigen concentrations in the nanogram per milliliter range, the model also requires that the B cell serve as a particularly efficient antigen-presenting cell for those antigens which can bind its antigen receptor. A direct test of this prediction, however, is severely limited by the clonal distribution of antigen receptors. Even with primed cell populations in vitro, the frequency of antigen-specific, MHC-restricted T-B collaboration is exceedingly low, and the collaboration is not visible until the B cells begin to make antibody.

The advent of long-term T cell lines has solved half of this problem. Polyclonal, MHC-restricted B cell responses can be induced in unprimed B cells using alloreactive T cell lines or clones (7, 8), or using protein antigen–specific T cell clones and very high antigen concentrations, which enable B cells to take up antigen nonspecifically (9, 10). These experiments show directly that B cells can induce and receive help by acting as antigen-presenting cells. Since most, but not all (8, 11) of these experiments do not explicitly involve membrane Ig (mIg), they suggest that a signal delivered via mIg is not absolutely necessary for B cell activation when activation is a consequence of helper T cell recognition of

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[1] *Abbreviations used in this paper:* Anti-Ars, antiphenylarsonate; C, complement; Con A, concanavalin A; FBS, fetal bovine serum; IL-2, interleukin 2; mAb, monoclonal antibody; MHC, major histocompatibility complex; mIg, membrane Ig; NRG, normal rabbit globulin; PAS, protein A–Sepharose; RF(ab')$_2$, rabbit F(ab')$_2$.

antigen on the B cell surface; a conclusion reached earlier by Cammisuli et al. (12) for primed B cells. However, since these experiments circumvent mIg, they cannot approach the question of how the antigen receptor on the B cell might function in antigen-specific responses to enhance T-B collaboration. Possible roles include passive antigen binding or "focusing" (13), as well as more active roles in antigen processing and presentation (14), or transmembrane signalling to enable the B cell to induce and receive a helper signal (15).

In our previous studies on the role of mIg in B cell responses, we used anti-Ig as a polyclonal model for antigen, and T cell–derived, stable, antigen-nonspecific, soluble helper factors in place of T cells. We, and others (15–20), have shown that high concentrations (>1 μg/ml) of anti-Ig activate B lymphocytes, as measured by an increase in RNA content per cell (16), cell size (17, 18), or DNA synthesis (15), and induce responsiveness to T cell–derived helper factors that enable the cells to proliferate and secrete Ig (15, 19, 20). This model is based on the classical experiments of Dutton et al. (21), and Schimpl and Wecker (22), which showed that helper T cell function in the in vitro response to heterologous erythrocytes can be replaced by soluble, antigen-nonspecific factors produced by activated T cells. However, the response to erythrocytes and high concentrations of anti-Ig may differ from the response to low concentrations of protein antigens. Helper T cell function for low concentrations of protein antigens cannot be replaced by the same factors (4, 5, 23), nor are hapten-protein conjugates as effective as anti-Ig in activating isolated, hapten-specific B cells (24, 25). Moreover, B cells from the CBA/N mouse, which fails to respond to anti-Ig, or to antigen and helper factors under certain conditions, can still be induced to respond by MHC-restricted helper T cells (5, 26). Consequently, helper T cell recognition of antigen on the B cell surface appears to induce helper signals that are not provided by stable helper factors.

To investigate the role of mIg in a polyclonal model of MHC-restricted T-B collaboration that involves these other signals, we have taken advantage of the recent finding (14, 27) that B cell blasts present rabbit anti-Ig very efficiently to mouse lymph node T cells primed with normal rabbit Ig, as measured by T cell proliferation in vitro. We have extended the system by preparing mouse T cell lines and T cell hybridomas that are specific for rabbit Ig, and by looking at the responses of B cells as well as T cells in the presence of rabbit anti-IgM and anti-IgD. We show here that small, resting B cells can efficiently present either anti-IgM or anti-IgD to our T cell lines. Efficient presentation depends upon mIg interaction and results in a B cell as well as T cell response. T cell proliferation or lymphokine release, and polyclonal B cell responses occur with concentrations of anti-Ig that are several orders of magnitude lower (1–10 ng/ml) than those required to activate B cells in the absence of T cells, or to induce B cell responsiveness to stable, antigen-nonspecific helper factors (>5 μg/ml) (28). Consequently, this polyclonal model system also requires helper T cell functions that cannot be replaced by such factors, and so more closely resembles the antibody response to low concentrations of a protein antigen. Also, like the antibody response to low concentrations of protein antigens, the polyclonal responses described here require linked recognition, and are largely MHC-restricted at the T-B cell level.

Materials and Methods

Mice. Mice were obtained from The Jackson Laboratory, Bar Harbor, ME, and used at 8–32 wk of age.

Antibodies and Cell Lines. Class-specific, affinity-purified rabbit antibodies to mouse IgM and IgD were prepared as described previously, and their specificity was checked by immunoprecipitation of radiolabeled mouse spleen cell plasma membranes (18). $F(ab')_2$ rabbit antiphenylarsonate antibody (anti-Ars) was prepared similarly. Affinity-purified goat anti-IgM was a gift of Fred Finkelman (Uniformed Services University of the Health Sciences, Bethesda, MD), and was lightly arsanilated on amino groups with an imidoester analog of tyrosine azophenylarsonate, as described (29). Normal rabbit globulin (NRG) was prepared from pooled normal or preimmune serum on protein A–Sepharose (PAS), or was purchased from Sigma Chemical Company (St. Louis, MO). $F(ab')_2$ fragments of NRG or specific antibodies were prepared by digestion with pepsin, and residual, intact IgG was removed on PAS (Sigma Chemical Co.).

The M12.4.1 B cell lymphoma of BALB/c origin (30) was a gift of Laurie Glimcher (Harvard School of Public Health, Boston, MA). The 8-azaguanine–resistant BW5147 AKR thymoma cell line, also from Glimcher, was a gift of Paula Hochman (Tufts University Medical School, Boston, MA).

T Cell Hybridomas. Antigen-specific T cell hybridomas were established by the method of Kappler et al. (31), by fusion of BW5147 with rabbit globulin–specific T cells. The T cells were either from an established cell line or were T cell blasts obtained from draining lymph nodes of (C3H/HeJ × DBA/2J)F_1 (C3D2) mice that had been primed with 100 μg of $F(ab')_2$ of normal rabbit IgG in the tail and footpads, and boosted in vitro with $F(ab')_2$ NRG (100 μg/ml) and irradiated spleen cells. 2.5×10^7 T cell blasts or 10^7 cells of a T cell line were fused to equal numbers of BW5147 cells, and distributed into 400 microculture wells in 0.1 ml HAT (hypoxanthine, aminopterin, thymidine) medium, which was changed every 5 d. Hybrid growth was detectable at 2–4 wk. Growing wells were expanded to 1-ml cultures, then tested for antigen-induced interleukin 2 (IL-2) release, and the ability to induce B cell proliferation and Ig secretion in the presence of $F(ab')_2$ rabbit anti-Ig. Positive cultures were cloned and subcloned as soon as possible at 0.1 input cells/well. The hybridomas occasionally lost activity, and so required periodic recloning or recovery from liquid nitrogen storage.

T Cell Lines. Antigen-specific T cell lines were established from lymph node cells of C3D2 mice that had been primed with $F(ab')_2$ NRG in vivo and boosted in vitro as described above. After the second stimulation with antigen and irradiated spleen cells, limiting dilution lines, at 100 input cells/well, were established in vitro according to Kimoto and Fathman (32), using an ammonium sulfate fraction (40–80% saturation) of supernatant from concanavalin A (Con A)–treated rat spleen cells as a source of IL-2. Growing microwells were transferred to 1-ml cultures and tested for ability to support $F(ab')_2$ anti-Ig–dependent B cell responses. Active lines were transferred to upright culture flasks, and were maintained by restimulation of 4×10^5 cells every 2–3 wk with 2×10^7 irradiated spleen cells (done in 8 ml of medium containing 100 μg/ml $F(ab')_2$ NRG, and IL-2 from rat spleen Con A supernatant). Although most of the limiting dilution lines have the properties of clones (stable phenotype, restriction to one of the two maternal MHC haplotypes), they have not been subcloned at high plating efficiency, and are not certain to be clones. Unlike the bulk cultures, which become overgrown with antigen-independent, autoreactive T cells in ~8 wk, the limiting dilution lines have remained antigen-dependent for 1 yr in culture. They have been frozen and recovered from liquid nitrogen storage. Most of the lines have remained dependent on IL-2 in addition to antigen and irradiated spleen cells, but the line shown in Table II was weaned from IL-2, after which it showed a much better proliferative response to antigen.

Cell Preparation. Spleen cell suspensions were treated with an anti-Thy-1.2 (HO-13-14) monoclonal antibody (mAb) plus agarose-absorbed guinea pig complement (C). Small B cells were then isolated by counterflow centrifugal elutriation (model J-6B; Beckman Instruments, Palo Alto, CA) (33). Briefly, $1–5 \times 10^8$ cells in 8 ml culture medium or balanced salt solution with 1.5% fetal bovine serum (FBS) were treated with deoxyribo-

nuclease, loaded into the elutriation chamber with a starting countercurrent flow rate of 13.5 ml/min, and spun at 4°C at a constant speed of 3,200 rpm. A small-cell fraction with very few contaminating large cells was eluted, typically at 14–17 ml/min, although the exact flow rate depended on the medium in which the cells were suspended. The fractions for each experiment were size-analyzed using a Coulter Channelyzer (Coulter Electronics, Inc., Hialeah, FL). The small-cell fraction, 5–20% of the input cell number, gave a nearly symmetrical peak, with a mean cell volume of 120 μm^3. The small-cell fraction was also depleted of accessory cells, as shown by the ablation of Con A response in the small-cell fraction of untreated spleen cells. When indicated, cells were irradiated with a cesium source.

Cell Cultures. Unless otherwise noted, 10^5 B cells and 3×10^4 T cells were cultured in flat-bottomed 96-well plates in 0.2 ml RPMI 1640 (Gibco Laboratories, Grand Island, NY) containing 5×10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 10% heat-inactivated FBS (2G034; M.A. Bioproducts, Walkersville, MD). Some cultures received mouse spleen Con A supernatant (50% solution) (34) as a source of T cell–derived helper factors, instead of T cells.

Assays. DNA synthesis was estimated by incorporation of [^{3}H]thymidine (1 μCi/well, 2 Ci/mmole; New England Nuclear, Boston, MA) during a 4 h pulse on day 3. The polyclonal assay for Ig-secreting cells as direct reverse plaques, using sheep red cells coated with anti-mouse Fab, has been described (35). The H-2 type of the secreting cells was determined by incubating cultured cells with anti-H-2K^k mAb (clone 11-4.1; American Type Culture Collection, Rockville, MD) for 30 min at 4°C, and then with absorbed rabbit C at 37°C for 45 min. IL-2 production by the T cell hybridomas was measured by proliferation of an IL-2-dependent T cell line as described (19). Our unit of IL-2 has half-maximal activity at a dilution of 1:8–1:16 in the IL-2 assay.

Results

Rabbit Ig-specific T Cell Hybridomas and T Cell Lines. T cell lines were derived from lymph node cells of C3D2 mice primed with the F(ab')$_2$ fragment of NRG, and stimulated in vitro with antigen and irradiated syngeneic spleen cells. We obtained limiting dilution lines using IL-2, and selected them for their ability to stimulate B cell proliferation and Ig secretion, dependent on the presence of rabbit anti-Ig. We maintained them by periodic restimulation with antigen, spleen cells, and IL-2. T cell hybridomas were generated by fusing primed C3D2 lymph node cells, enriched in vitro for specific T helper cells, or long-term specific T cell lines to BW 5147. The T cell hybridomas were screened for (*a*) IL-2 production, (*b*) ability to induce B cell proliferation, and (*c*) ability to induce B cell Ig secretion. We considered it necessary to screen in all three assays, because it was not clear whether all activities were related to each other. We found that, from four separate fusions, 124 out of 1,000 wells positive for cell growth were active in at least one of the assays. They were cloned as soon as possible, and selected clones with helper activity were subcloned. We obtained ~20 stable T cell hybridoma clones. All clones with helper activity for B cell proliferation were also IL-2 producers and vice versa, but only ~40% of these also induced Ig secretion. So far, we have found no hybridoma clones that have completely lost their ability to secrete IL-2 but are still able to deliver help for B cell proliferation.

Functional Characteristics of the Rabbit Ig-specific T Cell Hybridomas and T Cell Lines. The following characteristics of the T cell hybridomas and T cell lines are representative of the 12 independently established T cell hybridomas and five T cell lines that we have studied more carefully. Table I shows the requirements for IL-2 release by the T cell hybridoma 2R50.20. 2R50.20 produces no

TABLE I

Functional Characteristics of a Rabbit Globulin–specific T Cell Hybridoma

T-depleted spleen cells	Additions	IL-2 release*		B cell proliferation‡		2R50.20 stimulation of:§	
		2R50.20	BW 5147	2R50.20	BW 5147	B cell Ig secretion (secreting cells/culture)	B cell recovery ($\times 10^4$ cells/culture)
	μg/ml	*cpm*		*cpm*			
C3D2 (H-2^K × H-2^d)	Nil	303	380	2,890	1,450	540	5.2
	F(ab′)$_2\alpha\mu$ 1	ND‖	ND	30,285	6,308	ND	ND
	F(ab′)$_2\alpha\mu$ 5	4,531	303	243,395	16,191	3,700	38.6
	F(ab′)$_2\alpha\delta$ 10	2,371	412	119,558	8,064	21,700	24.5
	LPS					58,700¶	23.3¶
B10.D2 (H-2^d)	Nil	328	302	1,881	1,472	230	5.8
	F(ab′)$_2\alpha\mu$ 1	ND	ND	92,368	3,414	58,400	31.6
	F(ab′)$_2\alpha\mu$ 5	8,345	292	158,412	8,431	ND	ND
	F(ab′)$_2\alpha\delta$ 2	6,981	358	198,666	1,138	55,200	25.5
	F(ab′)$_2\alpha\delta$ 10	7,204	328	108,431	1,699	45,400	29.5
	LPS			96,168¶			
B10.BR (H-2^k)	Nil	287	345	1,002	ND	30	4.3
	F(ab′)$_2\alpha\mu$ 1	292	361	2,992	ND	0	3.1
	F(ab′)$_2\alpha\mu$ 5	332	392	9,321	ND	ND	ND
	F(ab′)$_2\alpha\delta$ 2	392	299	989	ND	ND	ND
	F(ab′)$_2\alpha\delta$ 10	301	336	1,298	ND	0	4.5
	LPS			89,499¶			

2R50.20, the T cell hybridoma, is compared with BW 5147, the fusion partner. 3×10^4 T hybridoma cells (2R50.20), or fusion partner tumor cells (BW 5147), were given 3,000 rad and incubated with 10^5 T-depleted spleen cells and various additions.

* After 24 h, culture supernatants were tested for IL-2 activity.

‡ Cultures were pulsed with [^{3}H]thymidine at 68–72 h of culture.

§ Cell recoveries and numbers of Ig-secreting cells were determined on day 4.

‖ ND, not done.

¶ 10^5 T-depleted spleen cells were cultured with 50 μg/ml LPS without T cells.

IL-2 constitutively and has no autoreactivity. It secretes IL-2 when it is stimulated with rabbit F(ab′)$_2$ anti-Ig [RF(ab′)$_2$ anti-IgM or RF(ab′)$_2$ anti-IgD] on the appropriate antigen-presenting cell. 2R50.20, derived from H-2^k × H-2^d lymph node cells, recognizes only cells bearing the H-2^d haplotype. The fusion partner, BW 5147, releases no IL-2 under these conditions. The T cell hybridomas can also deliver help to B cells, measured by >10-fold enhancement of DNA synthesis in syngeneic B cells (B cell proliferation). The response with the fusion partner, BW 5147, shows the intrinsic stimulatory activity of the anti-Ig preparations used (Table I). In addition, 2R50.20 induces actual B cell proliferation, as measured by cell recovery after 4 d of culture, and, finally, induces high rate Ig secretion in syngeneic B cells even with less than mitogenic doses of anti-Ig (Table I).

Table II shows the antigen-presenting cell requirements of a limiting dilution

TABLE II

T Cell Line Is Specific for Rabbit Globulin Rather Than Activation Antigens on B Cells

Additions to culture*	T cell proliferation	
	μg/ml	*cpm*
None		110
Rabbit F(ab')$_2$ anti-IgM	0.001	880
	0.01	9,930
	0.1	29,180
	1.0	18,360
	10.0	14,110
Goat anti-IgM	0.001	80
	0.01	90
	0.1	170
	1.0	200
	10.0	300
Rabbit F(ab')$_2$ NRG	190	12,007

* Cell cultures contained 10^5 irradiated (1,000 rad) syngeneic T-depleted spleen cells and 3×10^4 CDC35 T cells.

time (CDC 35), after being "weaned" of IL-2, for proliferation in response to antigen. This line requires only ng/ml concentrations of anti-Ig for activation. The specificity for rabbit Ig determinants is shown by the failure of goat anti-IgM antibodies, at submitogenic as well as mitogenic concentrations, to stimulate the T cell. This rules out the possibility that the T cell line is reactive to an activation antigen expressed on B cells in response to cross-linking mIg with anti-IgM antibodies. The same is true for the T cell hybridoma 2R50.20, as shown by assaying T cell help induction for B cell proliferation (Fig. 1). Moreover, Fig. 1 *a* shows that a rabbit antibody with a specificity not directed to mIg [RF(ab')$_2$ anti-Ars] is, even at concentrations 100 times higher than optimal for anti-Ig, unable to activate the T cell hybridoma. However, by using arsanilated goat anti-Ig plus RF(ab')$_2$ anti-Ars, the rabbit anti-Ars is bound to cross-linked mIg in a hapten sandwich. Under these conditions, rabbit Ig determinants can be presented, and the T cell hybridoma is able to deliver help to the B lymphocytes. It is important to note that the response does not require mitogenic concentrations of goat anti-Ig antibodies. Mixing unarsanilated goat anti-Ig and RF(ab')$_2$ anti-Ars does not induce a response (Fig. 1 *b*). This indicates that B cell proliferation in response to the T cell hybridoma depends on recognition of rabbit Ig determinants on the B cell.

Antigen Presentation by Small, Resting B Lymphocytes. To determine whether these responses required either conventional accessory cells or activated B lymphocytes in the starting T-depleted spleen cell population, we subjected the cells to counterflow elutriation centrifugation. This procedure not only gives us small, resting B lymphocytes, but also depletes the B cells of accessory cells. We found the accessory cell depletion to be as good as fractionation by plastic adherence and two passes over Sephadex G-10, as it abrogates the Con A response of the fractionated spleen cells before treatment with anti-Thy-1. Fig. 2 shows

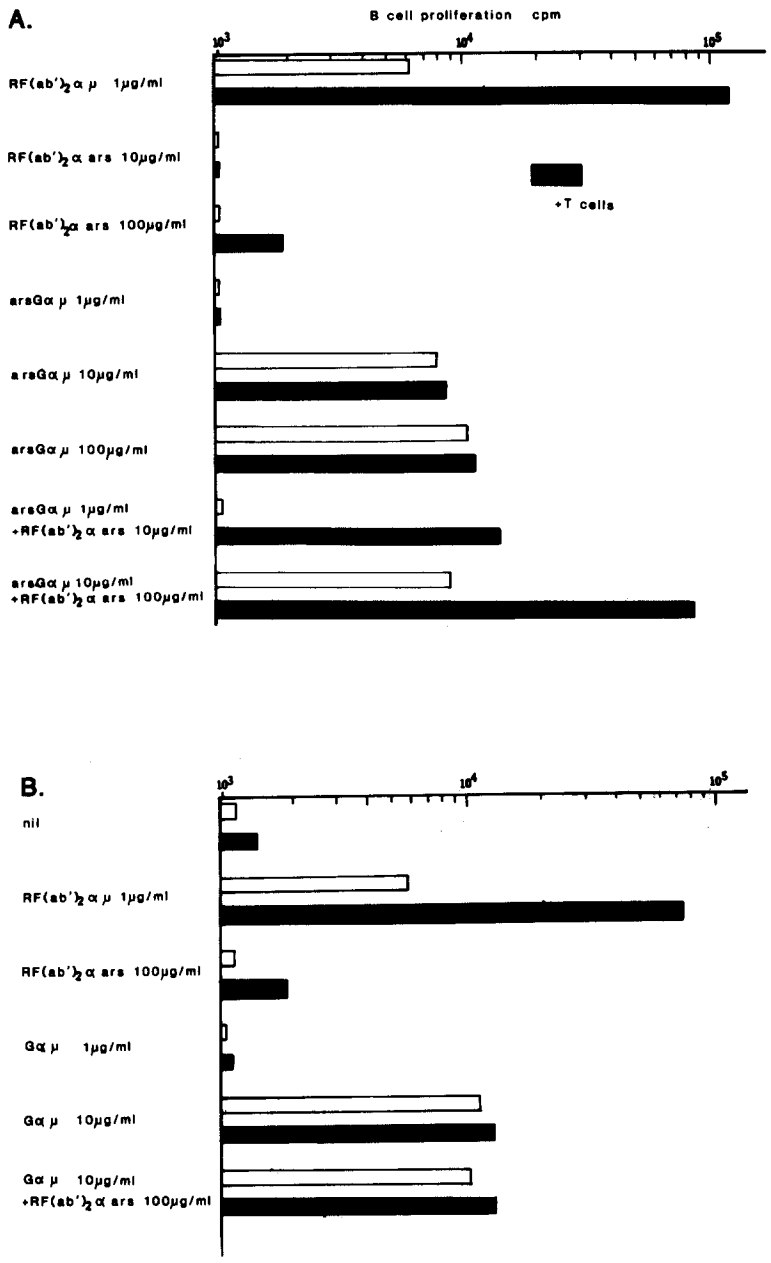

FIGURE 1. Induction of help for B cell proliferation depends upon attachment of rabbit globulin to receptor Ig on the B cell. 10^5 T-depleted spleen cells were cultured with (solid bars) or without (open bars) 3×10^4 irradiated T hybridoma cells (2R50.20), and [^{3}H]-thymidine incorporation was measured at 72 h. T cells cause a large enhancement of the proliferative response to rabbit F(ab')$_2$ anti-IgM [*RF(ab')$_2$αμ*], but not to goat anti-IgM (*Gαμ*) or arsanilated Gαμ (*ars Gαμ*). Rabbit antiphenylarsonate antibody [RF(ab')$_2$αars], which does not bind to the B cell, does not stimulate a response unless added together with *ars Gαμ*, which allows it to attach to the B cell in a hapten sandwich. The experiment in *B* shows that the *Gαμ* must be arsanilated in order to allow T cell–dependent stimulation with *RF(ab')$_2$αars*.

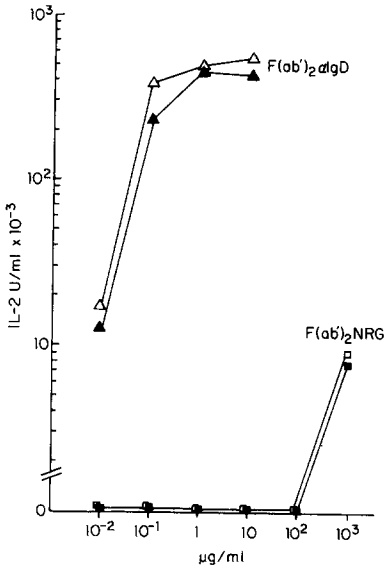

FIGURE 2. Rabbit anti-Ig is presented at least 10,000-fold more efficiently than NRG by T-depleted spleen cells or small B cells. 10^5 small DBA/2 B cells (open symbols) or 10^5 unfractionated, T-depleted spleen cells (closed symbols) were cultured with 3×10^4 2R50.20 hybridoma cells and indicated amounts of F(ab')$_2$ anti-IgD (△, ▲) or F(ab')$_2$ NRG (□, ■). Supernatants were assayed for IL-2 at 16 h.

that small B lymphocytes depleted of accessory cells are fully capable of presenting anti-Ig to an NRG-specific T cell hybridoma, as measured by IL-2 release. The small cells are as effective as unfractionated anti-Thy-1 plus C–treated spleen cells, which includes accessory cells as well as large B cells (Fig. 2).

Previous studies have shown that accessory cell–depleted B cell blasts (14, 27), or B tumor cells (36) present RF(ab')$_2$ anti-Ig at much lower concentrations than RF(ab')$_2$ NRG. Our experiments confirm the remarkable efficiency with which anti-Ig is presented by B cells (Fig. 2). In addition, we show that a mIg$^-$ B cell tumor line, M12.4.1, which cannot engage in mIg-mediated events, shows the same high concentration requirement for F(ab')$_2$ anti-Ig as for F(ab')$_2$ NRG (Fig. 3). This important control shows that the T cell hybridoma cannot distinguish our anti-Ig from our NRG preparation (by allotype or variable region differences) when presented on a surface Ig$^-$ cell. However, unlike others (14), who found that antigen-presenting function was limited to B cell blasts, we find that small B cells present anti-Ig very efficiently, even at concentrations far below that required to induce an increase in cell size in the absence of T cells (18). This discrepancy can now be explained by the finding that the antigen-presenting function of small, resting B cells is highly radiation-sensitive (37) for antigens the B cell takes up, presumably, via nonspecific binding. Using low concentrations of anti-Ig, which can bind and cross-link mIg of small B cells, we find the same result: the antigen-presenting capability of small B cells is lost rapidly at doses >1,000 rad (Fig. 4). This radiation sensitivity is in contrast to all classical antigen-presenting cells, whose antigen-presenting function is radioresistant. In the experiment shown in Fig. 4, the antigen-presenting function of the unfraction-

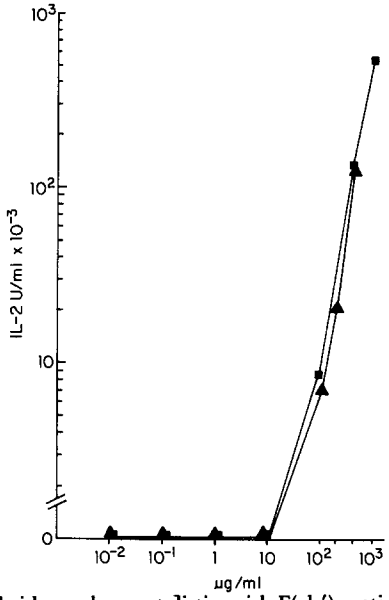

FIGURE 3. The T cell hybridoma does not distinguish F(ab')$_2$ anti-IgD (▲) from F(ab')$_2$ NRG (■) when presented on surface IgD$^-$ B lymphoma cells. 5 × 10^4 M12.4.1 cells were cultured with 3 × 10^4 2R50.20 cells, and IL-2 was measured after 16 h.

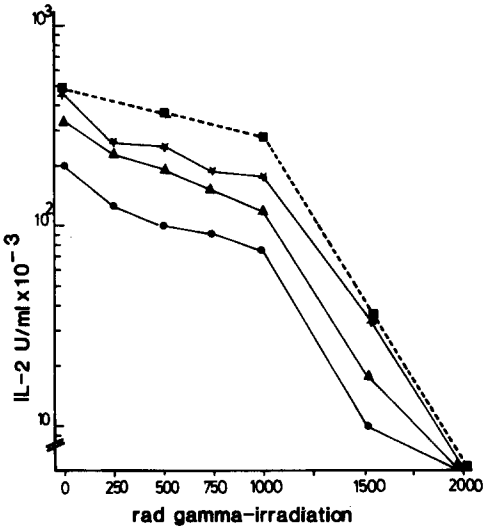

FIGURE 4. Radiosensitivity of antigen presentation. 10^5 small B cells (solid lines) or 10^5 T-depleted spleen cells (broken line) were irradiated with the indicated doses and cultured with 3 × 10^4 2R50.20 cells and F(ab')$_2$ anti-IgD (*, ■, 10 μg/ml; ▲, 1 μg/ml; ●, 0.1 μg/ml). IL-2 accumulation was measured at 16 h.

ated, T-depleted spleen cells is, likewise, shown to be radiosensitive. In other experiments (not shown), we have found a radioresistant cell in unfractionated spleen which presents anti-Ig efficiently and may be a B cell blast which was

activated in vivo before the spleen was removed. Activated B cells are radioresistant and are present in variable numbers among normal spleen cells (14).

Polyclonal Activation of Small, Resting B Lymphocytes. Small, resting B lymphocytes differ from larger, in vivo preactivated B lymphocytes in their requirement for activation (38, 39). Therefore, we wanted to know whether accessory cell–depleted, small B cells (which, as shown above, can activate the T cell hybridoma) are also able to receive T cell help. Fig. 5 shows that 2R50.20 can also deliver help to small, resting B cells over a range of concentrations of anti-IgM as well as anti-IgD. The response is proportional to the B cell concentration per culture and does not disappear at low cell concentrations, as does the response to anti-Ig and helper factors. In addition, there is no significant difference between unfractionated B cells, which include large B cells and accessory cells, and accessory cell–depleted, small, resting B lymphocytes, even at very low cell concentrations.

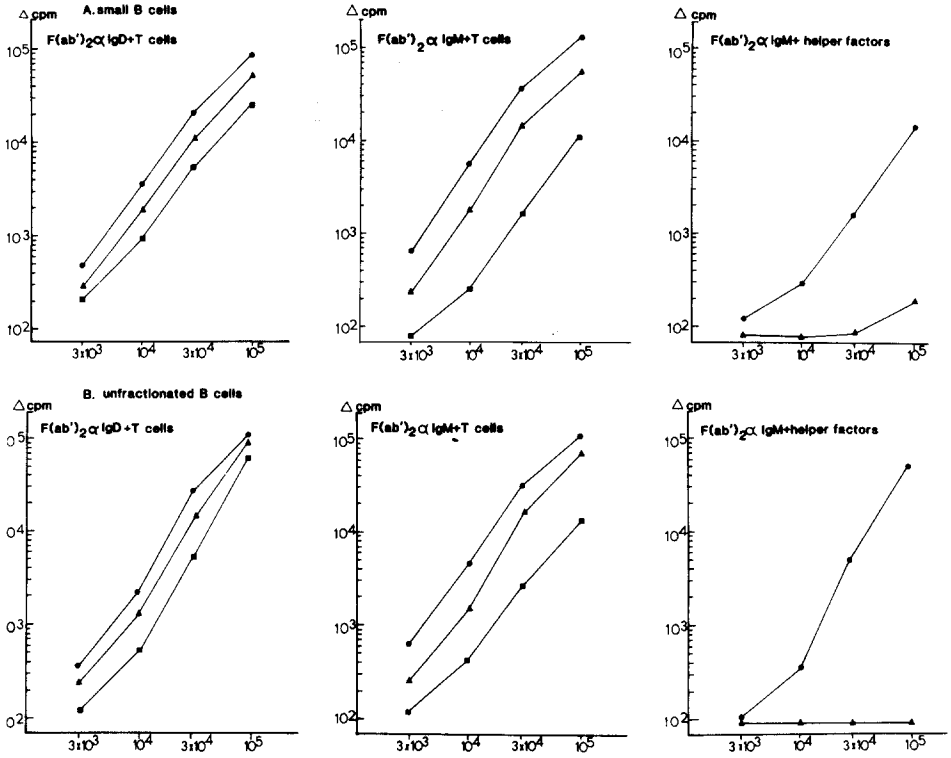

FIGURE 5. Small B cells respond as well as unfractionated T-depleted spleen cells to anti-Ig and a rabbit globulin–specific T cell hybridoma, even at low cell concentrations. Cultures contained either 5×10^4 irradiated 2R50.20 cells or helper factors (50% mouse splenic Con A supernatant) and $F(ab')_2$ anti-IgD (●, 10 μg/ml; ▲, 2 μg/ml; ■, 0.4 μg/ml) or $F(ab')_2$ anti-IgM (●, 5 μg/ml; ▲, 1 μg/ml; ■, 0.2 μg/ml). [^{3}H]Thymidine incorporation was measured at 72 h. Cultures which received $F(ab')_2$ anti-IgM (0.2 μg/ml) or $F(ab')_2$ anti-IgD (10 μg/ml, 2 μg/ml, 0.4 μg/ml) with helper factors showed only background responses. Subtracted background responses without anti-Ig were for small B cells (101, 162, 238, 774 cpm) and unfractionated, T-depleted spleen cells (104, 189, 396, 1541 cpm) for 3×10^3, 10^4, 3×10^4, and 10^5 cells/well, respectively.

Nature of T Cell Help. As shown above, the help mediated by T cells in these cultures is MHC-restricted, since T cell recognition of antigen on the B cell surface is MHC-restricted. However, the question remained whether this MHC restriction applies only to the requirements for T cell activation, or if T cells, even after activation by an appropriate antigen-presenting cell, would help only B cells bearing appropriate MHC antigens. We tested this possibility in experiments where small B lymphocytes of appropriate, and different MHC haplotype were mixed and cultured together with anti-Ig and a T cell line. At the end of the culture, we measured the T cell help received by small B cells of the inappropriate MHC haplotype by treating the culture with anti-H-2 antibodies (to the appropriate H-2 haplotype) and C, immediately before the assay for Ig-secreting cells.

Table III shows the result of such an experiment. The T cell line, CDB41, which is specific for rabbit Ig on H-2^k B lymphocytes, cannot be activated by anti-Ig on small H-2^b B cells, but these B cells can be activated by high concentrations of anti-IgM and lymphokines. As expected, H-2^b Ig-secreting cells cannot be destroyed by treatment with anti-H-2^k and C before assay. On the other hand, this T cell line, derived from a C3D2F_1 (H-2^k × H-2^d) mouse, can be activated, and can deliver help to small C3D2 B cells. The Ig-secreting cells from this response, as well as the response to lymphokines plus high concentrations of anti-IgM, can be abolished by treatment with anti-H-2^k plus C. When the two B cell populations were mixed and activated with T cells and low concentrations of anti-IgM or anti-IgD, the Ig-secreting cells present at the end of culture were all derived from C3D2 B cells, as shown by their sensitivity to anti-H-2^k plus C. That is, the response is strictly MHC-restricted in the effector phase, even when B cells with the inappropriate MHC outnumber the responding B cells by 7 to 1 (Table III, Exp. 2).

However, at the higher concentrations of anti-IgM or anti-IgD, we see a small but significant deviation from MHC-restriction in the response. This deviation implies that MHC-restricted recognition of antigen on the B cell surface may not be an essential part of the machinery of delivery of help, although the presenting B cells are preferential recipients of help, particularly at low antigen concentration.

In any event, it is clear that the helper T cells do more to the antigen-presenting B cells than produce, locally, the same set of stable, MHC-unrestricted factors that have been shown to support the proliferative and secretory responses that isolated B cells show to mitogenic concentrations of anti-Ig, since the T cells produce B cell secretory responses under conditions of anti-Ig activation that are not sufficient to induce a response with exogenous helper factors alone. These conditions include low concentrations of $F(ab')_2$ anti-IgM (Fig. 5), $F(ab')_2$ anti-IgD in soluble form at any concentration (Fig. 5) (15, 18), and intact anti-Ig (Table III, Exp. 1), which we have shown blocks B cell activation even in the presence of helper factors by cross-linking Ig to the Fc receptor for IgG (18, 34).

Role of mIg. This leads to the question of what role mIg plays in B cell activation, where these different types of T cell help are required. Fig. 6*a* directly compares the amounts of anti-Ig required to induce a B cell response when T cell help is provided by MHC-restricted T cells, versus soluble lympho-

TABLE III
MHC Restriction of T Cell Help

Additions to culture				Ig-secreting cells after treatment with:		
Responding, small, T-depleted spleen cells	T cell help	Anti-Ig		Nil	C only	Anti-H2^k plus C
			μg/ml			
Experiment 1						
5 × 10^4 B10 (H-2^b)	Helper factors	F(ab′)$_2$ anti-IgM	10.0	15,900	22,800	11,800
	T cells	F(ab′)$_2$ anti-IgM	0.1	40	—	—
5 × 10^4 C3D2 (H-2^k × H-2^d)	Helper factors	F(ab′)$_2$ anti-IgM	10.0	24,400	23,000	10
	T cells	F(ab′)$_2$ anti-IgM	0.1	25,900	25,900	<10
5 × 10^4 B10 plus 5 × 10^4 C3D2	Helper factors	F(ab′)$_2$ anti-IgM	10.0	27,300	44,900	18,400
	T cells	None		700	—	—
	T cells	F(ab′)$_2$ anti-IgM	0.01	3,200	970	<10
			0.1	19,300	18,900	<10
			1.0	37,700	32,700	3,500
			10.0	7,800	7,700	1,600
	T cells	F(ab′)$_2$ anti-IgD	0.01	3,800	1,100	10
			0.1	14,300	23,100	180
			1.0	29,200	30,200	2,900
			10.0	32,400	48,400	6,460
	T cells	Intact anti-IgM	0.01	11,800	—	—
			10.0	7,520	—	—
		Intact anti-IgD	0.01	3,100	—	—
			10.0	22,380	—	—
Experiment 2						
8 × 10^4 C3D2 plus 8 × 10^4 B10	Helper factors	F(ab′)$_2$ anti-IgM	10.0	59,400	48,400	23,200
	T cells	F(ab′)$_2$ anti-IgD	0.1	17,600	13,300	50
4 × 10^4 C3D2 plus 3 × 10^5 B10	T cells	F(ab′)$_2$ anti-IgD	0.1	17,300	16,100	90

Small B cells were cultured 4 d with 3 × 10^4 T cells (line CDB 41) or helper factors (50% mouse splenic Con A supernatant). After 4 d, the H-2 type of the Ig-secreting cells was determined by treatment with anti-H-2K^k plus C before the reverse plaque assay.

kines. The anti-Ig dose response for B cell differentiation is reported to follow the ability of anti-IgM to induce G_0–G_1 transition (>1 μg/ml) (16–18), if T cell help is provided in the form of lymphokines. However, when T cell help is provided by MHC-restricted T helper cells, the anti-Ig dose response seems to be independent of direct anti-Ig effects, and resembles only the dose response for T cell activation (Table II). For this cell line (CDC35), even concentrations of ~1 ng/ml anti-Ig induce a B cell response. Consequently, if an mIg-mediated signal is required for the response that is also dependent on MHC-restricted T helper cells, the strength of the signal appears to be relatively minimal; concen-

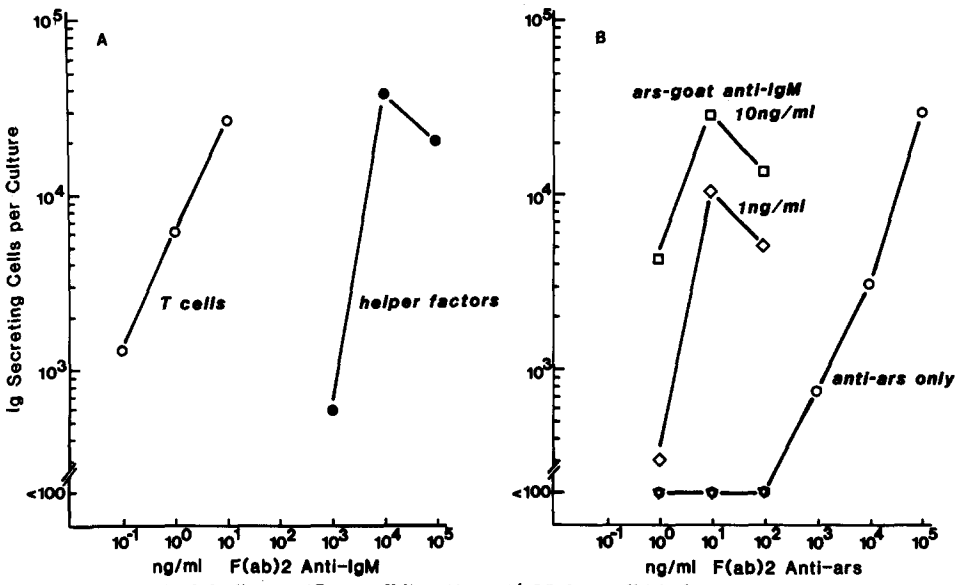

FIGURE 6. A globulin-specific T cell line (3×10^4 CDC35 cells) induces a secretory response from 10^5 small B cells at 10,000-fold lower concentrations of anti-Ig than that required with helper factors. (*B*) Rabbit $F(ab')_2$ anti-Ars, which does not bind specifically to the B cell, will also induce a response with T cells present, but at much higher concentrations (○). However, ng/ml quantities of $F(ab')_2$ anti-Ars are adequate if linked to the B cell surface by a hapten sandwich with arsanilated goat anti-IgM at 1 ng/ml (◇) or 10 ng/ml (□). 10 ng/ml goat anti-IgM which was not arsanilated (▽) failed to enhance the response to $F(ab')_2$ anti-Ars.

trations 10,000-fold lower than those inducing G_0–G_1 transition seem to be sufficient (Fig. 6*a*). Therefore, we wanted to know if a rabbit antibody that does not bind specifically to mIg can induce a response in small B cells as well. Fig. 6*b* shows that such an antibody (i.e., $RF(ab')_2$ anti-Ars) can indeed induce a T cell–dependent B cell response. Because $F(ab')_2$ anti-Ars cannot bind mIg, it is not, as shown above, presented by the B cells as efficiently as anti-Ig; response requires about four orders of magnitude more antibody (Fig. 6*b*). Giving a putative mIg-mediated signal with very low concentrations of goat anti-Ig antibodies does not alter the response to rabbit anti-Ars. However, if the goat anti-Ig antibody is arsanilated, then rabbit anti-Ars can bind to it, and thus, indirectly, to mIg. The dose response then shifts to concentrations of rabbit anti-Ars comparable to the necessary concentrations of rabbit anti-Ig. This experiment, like the experiment shown in Fig. 1, is the polyclonal analog of linked recognition. It demonstrates the important role of mIg for antigen presentation by B cells. However, a mIg-transduced B cell signal, comparable to the one crucial for the response of small B cells to stable lymphokines, is not required if T cell help is provided by MHC-restricted helper cells. It remains to be determined whether mIg plays more than a passive, antigen-focusing role in enhancing antigen presentation in T-B collaboration.

Discussion

Based on the experiments described above, we conclude that small B cells are extremely efficient at presenting rabbit anti-Ig to our rabbit Ig-specific T cell

lines and hybridomas. MHC-restricted recognition of rabbit globulin on the small B cell surface results both in T cell activation and a vigorous polyclonal B cell response. Antigen presentation and the ensuing B cell response does not require the well-characterized blastogenic response to anti-Ig, since the concentrations of anti-Ig required with some of the T cell lines (Fig. 6) are 1,000-fold lower than the concentrations required to induce blast transformation (18) or increases in membrane Ia expression (40), DNA synthesis (41), or responsiveness to stable, antigen-nonspecific helper factors (28).

Efficient presentation of rabbit anti-Ig by small B cells is mediated by the endogenous mIg on the B cell surface (Figs. 1–3, and 6). If one makes the reasonable assumption that antigens would be presented with comparable efficiency by antigen-specific B cells (42), then the experiments reported here provide a new and useful polyclonal model for mIg-mediated, MHC-restricted T cell help in the antibody response to low concentrations of protein antigens.

According to the clonal selection theory, the interaction of antigen with mIg on the B cell surface enables the B cell to respond, and so accounts for the specificity of the antibody response. The role of mIg in directly producing activating signals, or in acting as a passive, antigen-specific address for other B cell–activating signals, has been a subject of much debate. In the model presented here, the primary role of mIg is to enable the B cell to bind antigen and present it to the helper T cell, especially when antigen is present at very low concentration. A signalling role for cross-linked mIg does not appear to be essential in this system, since an unrelated rabbit antibody that does not bind mIg (anti-Ars) will nevertheless induce T and B cell activation if present at much higher concentration (Fig. 6). This result confirms other experiments, with antigen-specific T cells at high antigen concentration (9, 10, 39), alloreactive T cells (7, 8), or apparently autoreactive T cells (26, 43, and our unpublished results) that polyclonally activate B cells in the absence of apparent mIg involvement. This is not to deny the wealth of evidence for positive and negative signals that are delivered through mIg in other experimental systems, some of which are likely to be physiologically relevant. Even in systems where B cells can be driven without mIg involvement, antigen or anti-Ig can enhance or suppress responses (8, 11, 24, 43, 44).

Although mIg signalling appears to be unnecessary for T cell–dependent B cell activation, it may play more than a passive (antigen "focusing") role in binding antigen to the B cell surface. Antigen presentation requires a time- and energy-dependent process of antigen processing. Two laboratories (45, 46) recently showed that antigen processing for protein antigens requires, and may be limited to, denaturation or partial proteolysis, since prefixed cells could present partially hydrolyzed or denatured protein antigens, but not native globular proteins, to T cell hybridomas like ours. Consequently, it is likely that our globulin-reactive T cells also see processed antigen rather than native antigen bound to mIg. Thus, mIg may be functionally specialized to enable the B cell to efficiently process and present antigen to the helper T cell, perhaps by aiding in antigen internalization (47). This question can be approached by determining requirements for cross-linking, using monovalent antibodies, and by comparing

mIg with other B cell surface molecules for the efficiency of presentation of attached rabbit antibodies. These experiments are in progress.

In contrast to our findings, some experiments in other laboratories suggest that small, resting B cells are deficient in their ability to present alloantigens or protein antigens to T cells, even when their radiosensitivity is taken into account (8, 37, 48). It should be noted that the experiments reported here were performed with T cell lines and hybridomas, which may have less stringent requirements for activation than normal or in vivo primed T cells. For instance, a requirement for MHC-appropriate accessory cells may reflect a requirement for an accessory cell function, e.g., production of IL-1 or some other factor, for activation of helper T cells to a stage where they can interact directly with an antigen-presenting small B cell. Also, our limiting dilution lines and cloned hybridomas, though induced and maintained by conventional procedures, were selected for their ability to induce B cell responses. If T cell lines are heterogeneous in their requirements for accessory cells, or in their preferences for recognizing antigen on different kinds of antigen-presenting cells, then we may have selected those that are particularly adept at recognizing antigen presented on small B cells.

Although much remains to be learned, our experiments also tell us something about the mechanisms by which T cells help B cells when they recognize antigen on the B cell surface. At the very low concentrations of anti-Ig able to induce a B cell response in the presence of T cells, the B cells are not responsive to stable, antigen-nonspecific helper factors of the kind found in the culture supernatants of Con A–activated spleen cells (Fig. 6). Consequently, as stated above, the T cells must be providing helper functions in addition to the secretion of this kind of helper factor. One possibility is that this kind of T cell help requires close cell contact mediated by specific recognition, by analogy with target cell lysis by cytolytic T cells. In a mixture of target cells and "innocent bystander" cells bearing inappropriate MHC alleles, cytolytic T cells will kill their MHC-appropriate targets and spare the bystanders. When we performed similar experiments with our helper T cells, we found that the MHC-appropriate, antigen-presenting B cell accounted for the majority of the Ig secretory response. That is, T-B collaboration appeared to be MHC-restricted in the effector phase, since, under some conditions, bystander B cells bearing inappropriate MHC alleles did not become secreting cells even when mixed in the same culture vessel with MHC-appropriate B cells that were responding optimally to activated helper T cells (Table III). However, the MHC restriction was not absolute; the degree of the bystander response depended on the particular T cell line and the antigen concentration. When we looked earlier in the sequence of B cell activation, at DNA synthesis in MHC-inappropriate bystanders (unpublished experiments), we found less MHC restriction, in agreement with others (10), who detected DNA synthesis or blast transformation of MHC-inappropriate bystanders in polyclonal B cell responses induced by antigen-specific T cells at high antigen concentrations. We conclude that a limiting helper T cell function acts at short range, since the MHC-appropriate, antigen-presenting B cell is helped preferentially, particularly when one measures high-rate Ig secretion, a late event in the B cell activation sequence. Local help for the antigen-presenting B cell would explain

the finding of MHC-restriction of T-B cell collaboration in vivo (2, 3). However, since MHC-inappropriate B cells, which cannot serve as antigen-presenting cells (as determined by T cell activation), can receive some help, it seems likely that the molecular machinery of help is distinct from the machinery of specific recognition; i.e., that help does not require binding of B cell Ia plus antigen to the T cell receptor. Whether help is mediated by a labile, short-range factor or by cell contact remains to be determined.

Summary

Anti-Ig has been widely used as a model for antigen receptor–mediated B cell activation. B cells activated with mitogenic concentrations of anti-Ig (~10 μg/ml) become responsive to a set of T cell–derived, antigen-nonspecific helper factors that enable the B cells to proliferate, and, in some cases, mature to Ig secretion. In the present experiments, we show that anti-Ig can also be used as a model for major histocompatibility complex (MHC)-restricted, antigen-specific T-B cell collaboration. We used murine helper T cell lines and T cell hybridomas specific for a protein antigen, the $F(ab')_2$ fragment of normal rabbit IgG. Small B cells are very efficient at presenting rabbit anti-IgM or rabbit anti-IgD to these rabbit Ig–specific T cell lines and hybridomas, and the responding (initially) small B cells, appear to be the only antigen-presenting cells required. Efficient presentation depends upon binding of rabbit antibody to mIg on the B cell surface. MHC-restricted recognition of rabbit Ig determinants on the B cell surface results in a polyclonal B cell response. This response is qualitatively different from the well-studied response to blastogenic concentrations of anti-Ig plus stable, T cell–derived helper factors, since it (*a*) requires 1,000-fold lower concentrations of anti-Ig, (*b*) involves helper T cell functions other than, or in addition to, the local production of the same stable helper factors, and (*c*) is largely MHC-restricted at the T-B cell level.

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