ROLE OF MEMBRANE IMMUNOGLOBULIN (Ig) CROSSLINKING IN MEMBRANE Ig–MEDIATED, MAJOR HISTOCOMPATIBILITY–RESTRICTED T CELL–B CELL COOPERATION

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Recent evidence from a number of laboratories (1–5) supports a simple and unifying model of major histocompatibility complex (MHC)[1]-restricted T cell help in the antibody response, in which the B cell acts as a very efficient antigen-presenting cell by means of its specific antigen receptor, membrane immuno-globulin (mIg). In our own recent experiments (4) we analyzed a polyclonal model of MHC-restricted T cell–dependent B cell activation in which we use rabbit anti-Ig as a soluble protein antigen, and provide help with MHC-restricted helper T cell lines or T cell hybridomas that are specific for rabbit Ig. These studies have shown that resting B cells are very efficient in antigen presentation if antigen binds to either mIgM or mIgD. Presentation results in induction of T cell help, which preferentially activates antigen-presenting B cells, and causes them to proliferate and differentiate to Ig secretion. Moreover, we found that B cell activation strictly parallels the amount of T cell activation, even at concentrations of anti-Ig that are too low to directly activate B cells in the absence of T cells, i.e., <10 ng/ml. Therefore, we wanted to test whether mIg-transduced signals, even at very low antigen concentrations, are important for efficient antigen presentation and responsiveness to the MHC-restricted T helper cells. Also, we wanted to address the question of whether mIg is a specialized receptor for antigen processing and presentation, or merely functions passively to bind antigen to the B cell surface (6).

There is now abundant evidence that mIg, when crosslinked by antigen or anti-Ig, can transduce an activating signal to the B cell. mIg crosslinking can lead to an increase in intracellular Ca^{++} concentration (7), decreased binding of cyanine dyes (8, 9), induction of MHC class II molecule expression (10), increase in cell size (11–13), induction of RNA (8) and DNA (13, 14) synthesis, and induction of responsiveness to soluble lymphokines for proliferation and Ig secretion (15–17). Moreover, membrane Ig crosslinking results in conversion of

mIg to a detergent-insoluble state (18), followed by capping and enhanced endocytosis of mIg with its bound ligand (19, 20). These specialized functions of mIg can easily be imagined to be directly involved in antibody responses to soluble protein antigens requiring MHC-restricted, T cell–B cell interactions. It is important to note that all these functions have been shown to be strictly dependent on mIg crosslinking, since they cannot be induced by haptens or by monovalent Fab' fragments of anti-Ig antibodies (7–9, 21, 22).

Consequently, we investigated the requirement for mIg crosslinking on resting B lymphocytes for efficient antigen presentation, and for responsiveness to MHC-restricted T cell help. Surprisingly, we find that mIg crosslinking is required for neither. Moreover, rabbit antibodies bound to MHC class I molecules are also presented very efficiently, although three to fivefold less efficiently than rabbit antibodies bound to IgM or mIgD.

Materials and Methods

Mice. (C3H/HeJ × DBA/2J)F$_1$ mice (C3D2) were obtained from The Jackson Laboratory (Bar Harbor, ME), and used at 8–20 wk of age. C3H/St mice were purchased from West Seneca Laboratories (West Seneca, NY).

Antibodies. Class-specific, affinity-purified rabbit antibodies to mouse IgM and IgD were prepared as described previously (12). Normal rabbit globulin was purchased from Sigma Chemical Co. (St. Louis, MO). F(ab')$_2$ normal rabbit globulin (NRG) was adsorbed on a mouse serum–Sepharose column. For the preparation of the anti-H-2 serum, rabbits were immunized with affinity-purified H-2K^k, which was a gift from M. Mescher, Harvard University Medical School (Boston, MA), and showed only class I heavy chain and β_2 microglobulin on reducing sodium dodecyl sulfate (SDS) gels. Immune serum was adsorbed with agarose, and the IgG fraction was isolated on protein A–Sepharose. F(ab')$_2$ fragments were prepared and adsorbed on a mouse serum column. This antibody preparation did not induce DNA synthesis in B cells in the absence of T cells.

The specificity of all antibodies was checked by immunoprecipitation of radiolabeled mouse spleen cell plasma membranes. Briefly, mouse spleen cells were isolated on Ficoll-Hypaque ($\sigma = 1.09$) and labeled with ^{125}I using lactoperoxidase and glucose oxidase. Cells were lysed in 20 mM phosphate buffer containing 2 mM phenylmethylsulfonylfluoride, 2 U/ml aprotinin, 0.5% Nonidet P-40 (NP-40), and 0.02% NaN$_3$, and precleared with 50 μl/ml of protein A–Sepharose. Antibodies plus a fivefold capacity excess protein A–Sepharose were added to 0.2 ml of lysate, and incubated at 4°C for 1 h. The beads were then washed extensively with 0.5% NP-40, 50 mM Tris, 400 mM NaCl, 0.02% NaN$_3$, pH 8.5, boiled in reducing sample buffer, and loaded onto a 16 cm × 18 cm × 1.5 mm SDS-polyacrylamide gel. Anti-μ and anti-δ antibodies were judged to be specific using 20 μg of antibody. Anti-H-2K was judged to be specific using 100 μg of IgG (Fig. 1). Fig. 1 also shows that both rabbit anti-μ and rat anti-μ monoclonal antibody (mAb) (b-7-6) precipitate two bands from C3H spleen cells, with molecular masses of 75 and 82 kilodaltons (kD) in this gel system. The same result can be obtained using DBA/2 cells (not shown). Both of these appear to be surface proteins, since further isolation of viable cells after iodination does not change the amount of either band. In addition, both bands are also isolated from iodinated preparations of small B cells (not shown), indicating that the faster-migrating band is probably not being secreted from the cells (23).

F(ab')$_2$ fragments of specific antibodies or NRG were prepared by digestion with pepsin as described (12), and residual, intact IgG was removed on protein A–Sepharose CL-4 B (Sigma Chemical Co.).

Fab' fragments were prepared by reduction and alkylation of the F(ab')$_2$ fragments (21). Fab' fragments were isolated by gel permeation chromatography on an Ultrogel AcA 44 column (LKB Instruments, Inc., Gaithersburg, MD). They eluted in a single peak. The absence of divalent F(ab')$_2$ fragments was confirmed by SDS–polyacrylamide
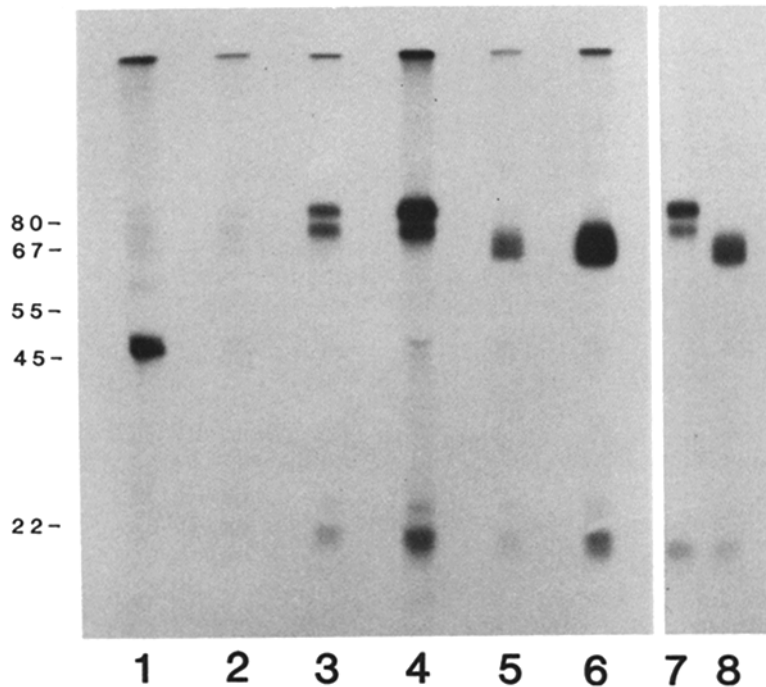
FIGURE 1. Immunoprecipitation of ^{125}I-labeled C3H/St spleen cells. The following amounts of antibodies were added to 0.2 ml of lysate containing 5 × 10^6 cells: lane *1*, 100 μg anti-H-2K (IgG fraction); lane *2*, 100 μg anti-H-2K preimmune IgG fraction; lane *3*, 100 μg anti-H-2K preimmune IgG plus 0.04 μg rabbit anti-μ; lane *4*, 100 μg anti-H-2K preimmune IgG plus 0.4 μg anti-μ; lane *5*, 100 μg anti-H-2K preimmune IgG plus 0.04 μg rabbit anti-δ; lane *6*, 100 μg anti-H-2K preimmune IgG plus 0.4 μg anti-δ; lane *7*, 2 μg rat anti-μ mAb (b-7-6); lane *8*, 2 μg mouse anti-δ mAb (10-4.22). Molecular mass (kD) is given at the left.

gel electrophoresis under nonreducing conditions. Fab' anti-IgM and Fab' anti-IgD failed to induce Ia expression or DNA synthesis in B cells. The rat anti-IgM mAb (b-7-6) (24) was a gift from L. Schmidt (University of Massachusetts Medical School). All the antibody preparations were spun in an Airfuge (Beckman Instruments, Inc., Fullerton, CA) at 100,000 g for 20 min before addition to cultures, except for the experiment shown in Fig. 2.

T Cell Lines. The establishment of the T cell lines specific for F(ab')$_2$ NRG has been described (4). They were maintained by restimulation of 5 × 10^4 T cells/ml with 2 × 10^6 irradiated 3,000 rad (Cs source) C3D2 spleen cell/ml, 100 μg/ml F(ab')$_2$ NRG, and 1% interleukin 2 (IL-2) (40–80% ammonium sulfate cut of culture supernatant from rat spleen cells activated with concanavalin A [Con A]). On the basis of stimulation by B cells of different mouse strains, and inhibition of stimulation by anti–class II mAb, the T cell line CDC 35 is restricted to I-A^d. CDC 25 appears to be restricted to the hybrid molecule I-A$^{d/k}$, since it responds to C3D2F1 B cells, but not to cells from either parent, and it is inhibited by an anti-I-A^k mAb (10.2.16; American Type Culture Collection, Bethesda, MD) but not by anti-I-E^k mAb (14.4.4s and M5/114.15.2; American Type Culture Collection).

Cell Preparation. Spleen cell suspensions were treated with anti-Thy-1.2 (HO-13-4, American Type Culture Collection; and J1j.10 [25]) mAb plus agarose-absorbed guinea pig complement. Cells in Figs. 2, 3, and 6 were also treated with anti-L3T4 mAb (GK 1.5 [26]), followed by anti–rat κ mAb (MAR 18.5 [27]) for enhancement of cytotoxicity. Small B cells were then isolated by counterflow elutriation centrifugation as described (4). The

small cell fraction was size-analyzed using a Coulter Channelyzer (Coulter Electronics, Inc., Hialeah, FL). The size distribution of the small cell fraction, 5–15% of the input cell number, was nearly symmetrical, with a mean cell volume of 120 μm^3. The T cell lines were used 2 wk after their last restimulation. They were separated from dead cells and debris before culture by centrifugation over Ficoll-Hypaque ($\sigma = 1.09$).

Cell Cultures. Typically, 10^5 B cells and 3×10^4 T cells (2,000 rad) were cultured in flat-bottomed, 96-well plates (3598; Costar, Cambridge, MA) in 0.2 ml RPMI 1640 (Gibco Laboratories, Grand Island, NY) containing 5×10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin, 100 $\mu g/ml$ streptomycin, 2 mM glutamine, and 10% heat-inactivated fetal bovine serum (FBS) (2G034; M. A. Bioproducts, Walkersville, MD). Some cultures contained 50% mouse spleen Con A supernatant, and 10 mg/ml α-methyl-D-mannoside (Sigma Chemical Co.) as a source of T cell–derived helper factors, instead of T cells.

Assays. IL-2 production by the T cell lines was measured by proliferation of an IL-2-dependent T cell line in 96-half-well plates (25870; Corning Glass Works, Corning, NY), as described (16). DNA synthesis in B cells was estimated by incorporation of [^{3}H]-thymidine (1 $\mu Ci/well$, 2 Ci/mmole; New England Nuclear, Boston, MA) during a 6-h pulse on day 3 of cultures, which were set up with irradiated (2,000 rad) T cells. The assay for polyclonal Ig-secreting cells as direct reverse plaques, using sheep red cells coated with anti–mouse Fab, has been described (22).

Cell Pulsing and Fluorescence Staining. Selected small B cells were washed twice with Hepes-buffered RPMI 1640 medium, which contained 1% FBS and 0.1% NaN_3. The cells were then incubated at a concentration of 10^6 cells/ml with different amounts of rabbit antibodies for 2 h at 4°C. Thereafter, the cells were washed in the presence of 0.1% NaN_3 through an FBS cushion, and then with a phosphate-buffered salt solution (PBS) containing 1% FBS. Cells that were used for cell culture were washed in parallel, but no NaN_3 was present after the incubation period with rabbit antibodies throughout the washing protocol. For cell surface staining, cells were incubated at 4°C for 20 min with fluorescein isothiocyanate (FITC)-conjugated F(ab')$_2$ goat anti–rabbit IgG [F(ab')$_2$ fragment–specific, 1312-0111; Cappel Laboratories, Cochranville, PA). The cells were then washed and fixed. All antibodies used were spun at 100,000 g for 20 min in an airfuge before use. Flow cytometry was performed on a fluorescence-activated cell sorter (FACS) 440 (Becton-Dickinson Immunocytometry Systems, Mountain View, CA) equipped with a logarithmic amplifier. The mean fluorescence intensity, in arbitrary units, was derived by taking the antilogarithm of the mean channel number.

Results

Influence of Crosslinking mIgM on Antigen Presentation Via mIgD and Subsequent T Cell–dependent B Cell Activation. In a previous report (4), we showed that efficient antigen presentation by small B cells is mediated by the endogenous mIg on the B cell surface. This seemed not to require a strong activating signal via the mIg receptor, since small B cells presented concentrations of anti-Ig that do not activate B cells in the absence of T cells. The use of anti-Ig allows us to look at presenting and activating functions of the two mIg isotypes present on a resting B cell independently. We wondered whether crosslinking of mIgM with activating doses of anti-Ig influences mIgD-mediated antigen presentation and subsequent B cell activation. We titrated rat anti–mouse IgM mAb into cultures of small resting B cells containing different amounts of rabbit F(ab')$_2$ anti-IgD, and T cells from a line specific for F(ab')$_2$ NRG, and restricted to the I-A^d molecule. The amounts of F(ab')$_2$ anti-IgD used do not activate resting B cells, as judged by their inability to induce increased Ia expression, blast transformation, or DNA synthesis independent of T cells (not shown).

Fig. 2A shows that crosslinking mIgM with submitogenic as well as mitogenic
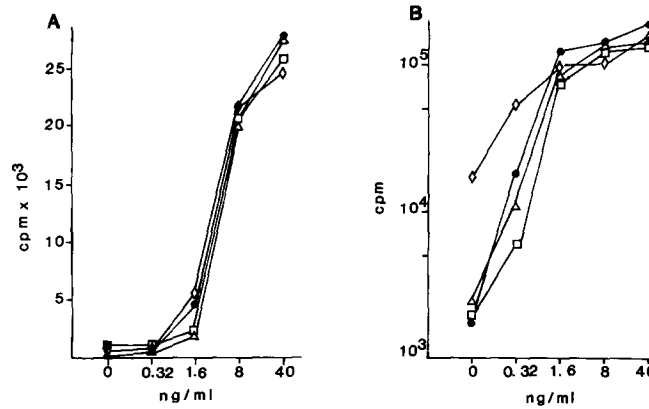
FIGURE 2.   Influence of crosslinking mIgM receptors on antigen presentation via mIgD receptors and subsequent B cell activation. 10^5 small C3D2 B cells were cultured with 3×10^4 irradiated T cells (CDC35) and indicated amounts of F(ab')$_2$ anti-IgD (●). Parallel cultures received additionally rat anti-IgM mAb (b-7-6, open symbols; △, 0.01 µg/ml; □, 0.1 µg/ml; ◇, 1 µg/ml). (A) Supernatants were assayed for IL-2 at 24 h. (B) [³H]Thymidine incorporation by B cells was measured at 66 h. 1 µg/ml rat anti-IgM induced 18,500 cpm [³H]thymidine incorporation in the absence of T cells.

doses of rat anti-IgM does not affect the efficiency of presentation of rabbit F(ab')$_2$ anti-IgD antibodies to a NRG-specific T cell line. Moreover, as shown in Fig. 2B, crosslinking mIgM does not induce enhanced susceptibility in small B cells to respond to the MHC-restricted T cell helper signal, even at concentrations of rat anti-IgM that can induce DNA synthesis in small B cells in the absence of T cells (24).

Crosslinking of mIg Is Not Required for Antigen Presentation by Small B Cells or for Subsequent B Cell Activation.   Since crosslinking of mIg induces rapid mIg internalization (20), which might aid in a possible antigen processing step, we still considered it likely that mIg crosslinking is involved in efficient antigen presentation via mIg. Also, it was possible that a minimal, otherwise undetectable, crosslinking signal transduced through mIg even at very low anti-Ig concentrations is required to receive the helper signal from the T cell. Therefore, we prepared monovalent Fab' fragments from our rabbit F(ab')$_2$ anti-Ig that were devoid of divalent antibodies, as judged by SDS gels under nonreducing conditions, and by their inability to induce DNA synthesis, lymphokine responsiveness, or Ia on small B cells even at concentrations up to 10 µg/ml (data not shown).

Nevertheless, small B cells present monovalent anti-IgM as efficiently as the divalent antibody. Both forms of antigen show exactly the same dose response and require only nanogram per milliliter amounts of antibody for the T cell response, whereas F(ab')$_2$ NRG is required at much higher concentrations (Fig. 3A). Even at the earliest timepoints of the response, monovalent anti-Ig is as efficient as the divalent antibody. This indicates that signaling by mIg crosslinking is not directly involved in efficient antigen presentation by small B lymphocytes. In addition, as Fig. 3B illustrates, monovalent and divalent anti-Ig antibodies show the same dose response for MHC-restricted, T cell–dependent B cell activation, indicating that crosslinking of mIg is not a requirement for the response of a resting B cell to MHC-restricted help.
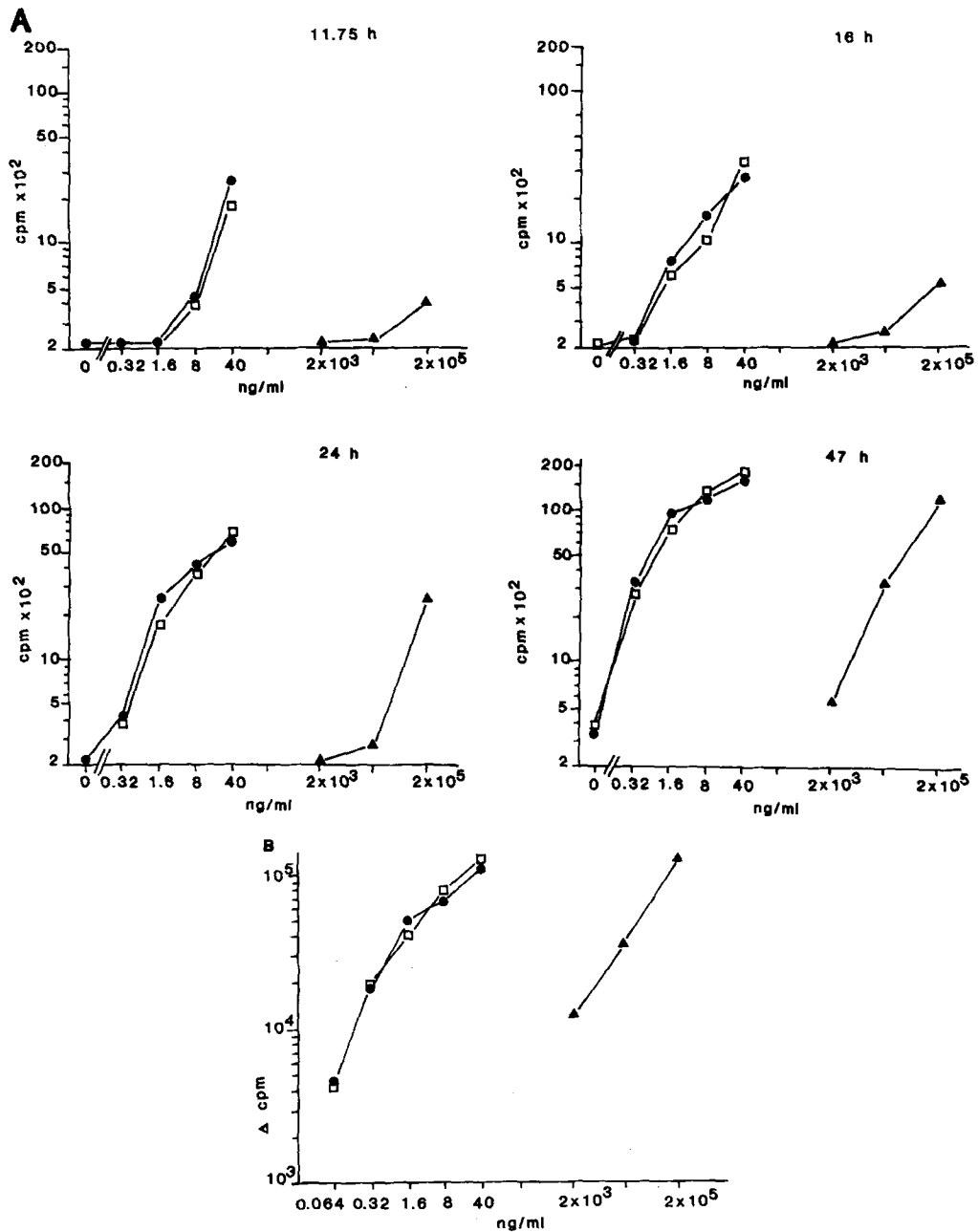
FIGURE 3. Crosslinking of mIg is not required for either antigen presentation by small B cells or for B cell activation. 10^5 small C3D2 B cells were cultured with 3×10^4 T cells (CDC35). Cultures received the indicated amount of F(ab')₂ anti-IgM (●), Fab' anti-IgM (□), or absorbed F(ab')₂ NRG (▲). (A) IL-2 accumulation was measured at 11.75 h, 16 h, 24 h and 47 h. (B) [³H]Thymidine incorporation by B cells was measured at 68 h.

This B cell activation results in actual cell division, as measured by cell recovery after 4 d of culture, and finally leads to high-rate Ig secretion (Table I). Monovalent Fab' anti-IgM, in contrast to divalent F(ab')₂ anti-IgM at 10 μg/ml, fails to induce responsiveness to conventional lymphokines present in Con A supernatant (Table I). In similar experiments using T cell hybridomas, which consistently required more anti-Ig for activation and were also less effective in inducing an MHC-restricted B cell response, we found different reactivity patterns among the hybridomas in their responses to anti-IgM, anti-IgD (monovalent and divalent), anti-H-2, and NRG. We think this may be due to the high anti-Ig concentrations required by the hybridomas, which saturate mIg on the B cells. However, the two NRG-specific T cell lines we studied (CDC25 and CDC35), which use different restriction molecules, gave consistently comparable results in experiments like those shown in Figs. 3, 4, and 6.

mIg Is Specialized as a Receptor for Efficient Antigen Presentation. All specialized functions of mIg demonstrated so far depend strictly on crosslinking of the receptor. Having shown the absence of a requirement for crosslinking mIg for efficient antigen presentation as well as responsiveness to the MHC-restricted T helper signal, we wanted to address the question of whether mIg is nevertheless specialized for antigen presentation in some way other than, and in addition to, its ability to bind antigen, or if other integral membrane proteins like class I MHC molecules can also serve as receptors for efficient antigen presentation by small B lymphocytes. We compared the T and B cell responses using rabbit anti-H-2 class I antibodies with those using rabbit anti-Ig. The titer of the F(ab')₂ rabbit anti-H-2 globulin fraction was compared to affinity-purified F(ab')₂ anti-IgD by the capacity of serial dilutions of each antibody to stain the surface of small B cells (as in Fig. 5, below). The comparison showed that, based on the protein concentration, similar staining required ~50-fold more of the anti-H-2 globulin than affinity-purified anti-Ig. Therefore, for these experiments, we added 50 μg F(ab')₂ rabbit IgG of the preimmune anti-H-2 serum to each

TABLE I

Crosslinking of mIg Is Not Required for High-rate Ig Secretion

T cell help	Antigen (μg/ml)		Ig-secreting B cells/culture*	B cell recovery (cells × 10⁻⁴/culture)
Helper factors	F(ab')₂ αμ	10	22,000	21
	Fab' αμ	10	<10	0.8
T cells (CDC25)	F(ab')₂ αμ	0.003	37,000	32.5
		0.001	17,000	13.7
		0.0003	566	3.1
		0.0001	<10	1.5
	Fab' αμ	0.003	32,000	31.5
		0.001	17,400	15.6
		0.0003	1,742	3.7
		0.0001	<10	1.5
	Nil		<10	1.5

* 10⁵ small B cells were cultured with 3 × 10⁴ T cells (line CDC25), or helper factors (50% mouse splenic Con A supernatant). Ig-secreting cells were determined at day 4 in a reverse plaque assay.

microgram of the affinity-purified F(ab')₂ anti-IgD in order to have comparable rabbit globulin concentrations.

Fig. 4 compares the amounts of antibodies required to induce IL-2 release in an F(ab')₂ NRG-specific T cell line. The amounts of F(ab')₂ anti-IgD and F(ab')₂ anti-H-2 are given as the amounts of specific antibodies present, estimated for the anti-H-2 as described above. The experiment shows that F(ab')₂ anti-IgD and F(ab')₂ anti-H-2 are both ~10,000 times more efficient than nonspecific rabbit Ig over the entire dose response. However, F(ab')₂ anti-Ig is ~5 times better than F(ab')₂ anti-H-2. It is unlikely that the biological response we obtain with the rabbit anti-H-2K antibody is due to a contaminating anti-Ig reactivity, since the anti-H-2K serum precipitates no detectable Ig heavy chain band from radiolabeled spleen cells (Fig. 1, lane 1), while anti-IgD, added in a 1:2,500 ratio to the preimmune anti-H-2 serum, precipitates, under the same conditions, an easily detectable band (Fig. 1, lane 5). At the concentrations of anti-H-2 used, even a 1:2,500 contaminant would not explain the observed response (Fig. 4). Importantly, Fig. 4 shows that the T cell line does not distinguish the preimmune rabbit Ig of the anti-IgD from the anti-H-2 serum. Therefore, the result seen comparing F(ab')₂ anti-IgD and F(ab')₂ anti-H-2 is not likely to be due to the fine specificity of the T cell line used. Also, similar results were obtained with the other T cell line studied, CDC35 (our unpublished results). To consolidate this result and control for possible differences in binding of the two antibodies under the staining and culture conditions, we pulsed small B cells for 2 h at 4°C in the presence of 0.1% azide with different amounts of divalent as well as monovalent anti-IgM and anti-H-2 class I antibodies, respectively. These pulsed B cells were split, and one part was washed and stained for bound rabbit Ig (Fig. 5). The other part was washed and put into culture with an NRG-specific T cell line, without further addition of antigen. Fig. 6A shows T and B cell responses as a function of the amount of rabbit Ig bound to the B cell at the beginning of culture. The level of T cell activation depends on the amount of rabbit Ig bound,
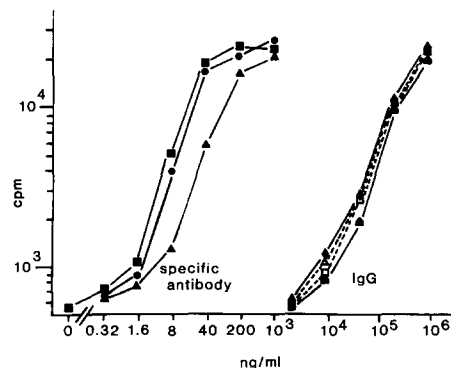

FIGURE 4. The difference in immunogenicity of F(ab')₂ anti-H-2K and F(ab')₂ anti-Ig is not due to fine specificity. 8 × 10⁴ small C3D2 B cells were cultured with 3 × 10⁴ T cells (CDC25). Cultures contained Fab' anti-IgD (●); F(ab')₂ anti-IgD plus 50 µg F(ab')₂ rabbit IgG from anti-H-2 preimmune serum per microgram of F(ab')₂ anti-IgD (■); F(ab')₂ anti-H-2K (▲); IgG from anti-IgD preimmune serum (△); IgG from anti-H-2 preimmune serum (□); F(ab')₂ fragments of anti-H-2 preimmune IgG (▲); F(ab')₂ NRG (◆). IL-2 accumulation was measured at 22 h.
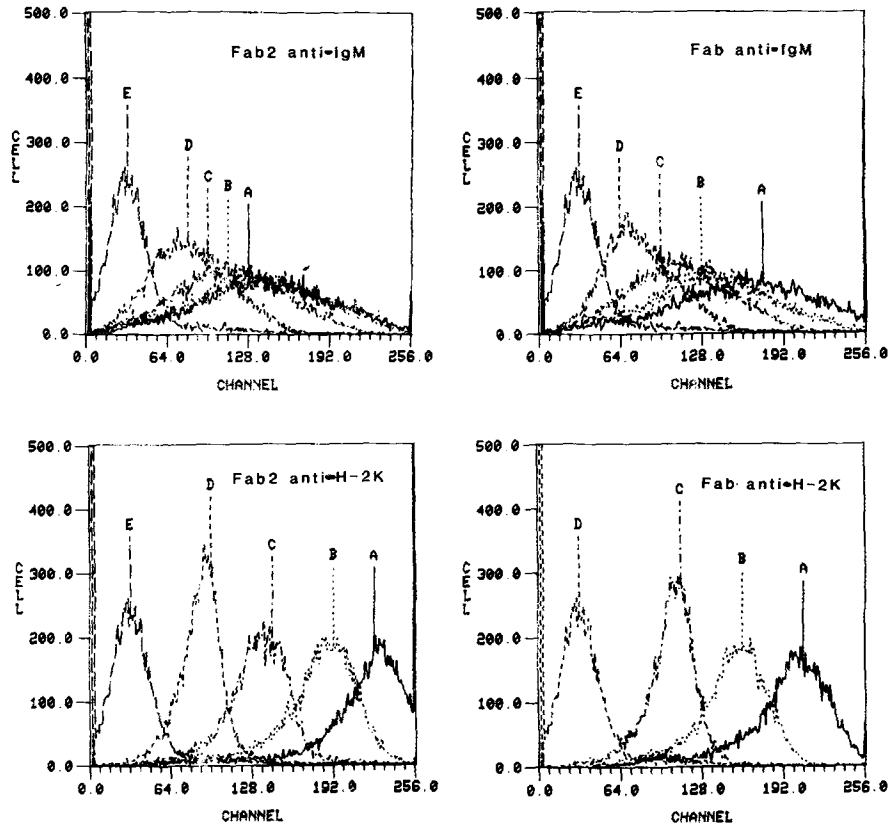
FIGURE 5. Small B cells (10^6 cells/ml) were incubated at 4°C in culture medium containing 0.1% NaN_3 with the following amounts of purified antibodies or IgG fractions (μg/ml). F(ab')$_2$ anti-IgM: A = 1, B = 0.2, C = 0.04, D = 0.008, E = 0; Fab' anti-IgM: A = 1, B = 0.2, C = 0.04, D = 0.008, E = 0; F(ab')$_2$ anti-H-2: A = 50, B = 10, C = 2, D = 0.4, E = 0; Fab' anti-H-2: A = 50, B = 10, C = 2, D = 0. Anti-IgM is affinity purified; anti-H-2 is an IgG fraction. After 2 h, the cells were washed and stained with fluorescein isothiocyanate-labeled F(ab')$_2$ goat anti–rabbit antibodies.

and is the same whether F(ab')$_2$ anti-Ig or monovalent Fab' anti-Ig is used. However, about threefold more divalent rabbit anti-H-2 Ig must be bound to induce a comparable response. Monovalent anti-H-2 is considerably less effective. Fig. 6B shows that the B cell proliferative response is strictly parallel to T cell activation. Monovalent and divalent anti-Ig are equally effective, whereas anti-H-2 is about threefold less effective. Although this threefold difference seems very small compared to the ~10,000-fold difference using absorbed F(ab')$_2$ NRG, it is reproducible, and suggests a specialized role for mIg in presentation beyond antigen binding.

Discussion

The experiments presented here were designed to answer the question of whether mIg plays more than a passive role in merely binding antigen onto the B cell surface, that is, either by enabling the B cell to carry out subsequent steps
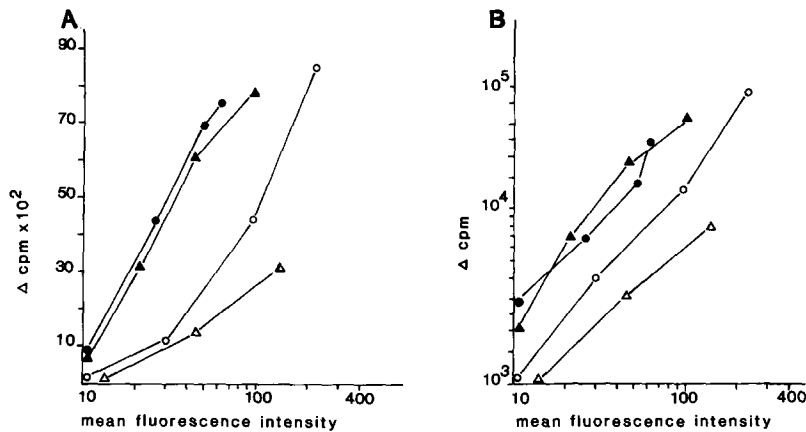
FIGURE 6.   Comparison of antibodies directed to mIg vs. class I MHC molecules for their efficiency in antigen presentation and B cell activation. Small B cells were pulsed, and a portion was stained as described in the legend to Fig. 5. Another portion of the cells was washed and placed into culture. Cultures contained 10^5 pulsed small C3D2 B cells and 3×10^4 irradiated T cells of the line CDC25. IL-2 (A) was measured after 24 h, B cell proliferation (B) after 68 h. The response is plotted against the mean fluorescence intensity of the B cells after staining with florescein isothiocyanate-labeled F(ab')₂ goat anti–rabbit IgG. F(ab')₂ anti-IgM (●), Fab' anti-IgM (▲), F(ab')₂ anti-H-2K (○), Fab' anti-H-2K (△).

of antigen presentation or by enabling the B cell to receive help from the T cell once presentation has occurred. Since antigen presentation has been shown to be a function of the product of the concentrations of antigen and class II restriction molecules (28), the finding that crosslinking mIg leads to enhanced expression of class II molecules on B cells (10), as well as the demonstration of mIg as an activating receptor, imply that a signal transduced through mIg may induce or enhance the capability of a small B cell for antigen presentation or responsiveness to the MHC-restricted T helper event (29–34). We have previously shown (4) that nanogram per milliliter concentrations of anti-Ig, which by themselves are not sufficient to induce measurable activation events, are nevertheless enough for a good T cell–dependent B cell response. However, this finding did not rule out an important potential signal transduced through mIg in these responses, especially if the signal were not manifest in the absence of helper T cells. The mechanisms of signal transduction by membrane Ig appears to depend on mIg crosslinking, since, without exception, all the direct effects of antigen or anti-Ig on B cells require multivalent antigen or divalent antibody (7–21), and can be inhibited by hapten or monovalent Fab' fragments, respectively.

By adding anti-Ig either as divalent F(ab')₂ fragments or as monovalent Fab' fragments, we could dissociate the amount of antigen in culture from the potential to crosslink mIg. We were surprised to find monovalent Fab' anti-Ig to be as efficient as divalent F(ab')₂ anti-Ig for antigen presentation, as well as for B cell activation. We think it is unlikely that the T cell itself crosslinks mIg when it recognizes anti-Ig on the B cell surface, since, as argued below, we believe the T cell recognizes anti-Ig after it is processed and no longer associated with mIg. The excellent responses we obtained with anti-H-2K antibodies also argue against any necessary involvement of mIg signalling in MHC-restricted T

cell help. Similar conclusions have been drawn from studies (35) with alloreactive T cells, which stimulate polyclonal B cell responses in the absence of overt mIg involvement.

Even in the absence of a requirement for crosslinking, we thought it was still possible that mIg might be functionally specialized for presentation of protein antigens. This appears to be true, since presentation of $F(ab')_2$ anti-IgM or $F(ab')_2$ anti-IgD is about three- to fivefold more efficient than presentation of $F(ab')_2$ anti-H-2K. This reproducible difference is the same, whether based on comparing similar concentrations of each antibody added to culture, or similar amounts bound to the surface of small B cells at the beginning of culture. The difference is not due to a preference of the T cells for certain rabbit globulin sequences, which might differ among the various antibodies used. It is not likely to be related to affinity differences, because the avidity of divalent $F(ab')_2$ anti-H-2 should be at least as high as the monovalent Fab' anti-Ig (36), and subsaturating concentrations of monovalent as well as divalent anti–class I antibodies remain on the B cell surface even after 2 h at 37°C (our unpublished observation).

The use of anti-Ig also allowed us to assess the roles of the two antigen receptor isotypes, IgM and IgD, which are coexpressed on small B cells. Both antigen receptors are independently able to present antigen and enable efficient T cell– B cell interactions (4). Neither requires crosslinking (Figs. 3 and 4). Moreover, independently crosslinking mIgM, even with doses of rat anti-IgM that induce DNA synthesis in small B cells, does not enhance presentation of anti-IgD or responsiveness to MHC-restricted T cell help (Fig. 2). This result stresses the independence of antigen presentation from mIg signalling. Moreover, the MHC-restricted T helper cell seems to be able to provide all important signals for activation of a small B lymphocyte, since mIg signalling does not, in this case, enhance MHC-restricted T cell–dependent B cell activation. This is not to deny the importance of positive signals through mIg, as shown in other T cell–dependent systems (29, 30, 32). Our T cells were selected for efficient cooperation with B cells; it could well be that B cell activation by T cells that are not as efficient in T cell–B cell collaboration can be influenced by or is dependent upon signals transduced through mIg.

Antigen presentation of protein antigens by B cell lymphomas (37) and macrophages (38) appears to require a time- and energy-dependent step called antigen processing, which involves internalization, denaturation, and partial hydrolysis in an acid compartment, and reexpression of antigen fragments on the cell surface in a form that can be recognized together with class II molecules by the antigen receptor of the T cell. This raises the question of whether B cells must process antigen bound to mIg before it can be recognized by helper T cells. This question was addressed directly by Lanzavecchia (5) in a model system involving mIg-mediated antigen presentation by antigen-specific, Epstein-Barr virus–transformed B cell lines. He found that anti-Ig inhibited presentation if added before but not after incubation of the B cell lines with antigen, implying that antigen was no longer bound to mIg at the time of presentation to the T cell. Similar results have been obtained by Abbas and coworkers (39) in a murine system involving presentation by isolated, antigen-specific normal B cells. Exper-

iments in progress in our laboratory suggest that selected small B cells also need to process anti-Ig before it can be recognized by our NRG-specific lines.

Summary

Resting murine B lymphocytes can present rabbit anti-Ig to T cell lines specific for normal rabbit globulin. The T cell–B cell interaction is major histocompatibility complex (MHC)-restricted, and leads to activation, proliferation, and differentiation of the resting B cell into an antibody-secreting cell. Efficient antigen presentation and B cell activation depends upon binding of rabbit globulin to (membrane) mIg. To investigate the role of mIg in this polyclonal model for a T cell–dependent primary antibody response, we determined whether crosslinking of mIg is required either for efficient antigen presentation, as measured by helper T cell activation, or for the B cell response to T cell help, since all the direct effects of anti-Ig on B cells require crosslinking of mIg. We found that monovalent Fab' fragments of anti-IgM or anti-IgD work as efficiently as their divalent counterparts. Therefore, a signal transduced through the antigen receptor seems not to be required when T cell help is provided by an MHC-restricted T helper cell recognizing antigen on the B cell surface. Moreover, rabbit globulin bound to class I MHC molecules in the form of anti-H-2K also results in efficient antigen presentation and T cell–dependent B cell activation. However, mIg still appears to be specialized for antigen presentation, since anti-Ig is presented about three- to fivefold more efficiently than anti-H-2K.

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