

## Ia-MEDIATED SIGNAL TRANSDUCTION LEADS TO PROLIFERATION OF PRIMED B LYMPHOCYTES

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Considerable controversy exists regarding the nature and multiplicity of signals required for T cell-dependent activation of quiescent B lymphocytes. While some evidence suggests the antigen and T cell-derived lymphokines are sufficient to drive this process (1), recent studies indicate that proliferative signals are provided by isolated helper T (Th) cell membranes (2) or intact Th cells made incapable of secreting lymphokines by cyclosporin A treatment (3). Finally, a number of very recent reports indicate that although requirements for Th recognition of antigenic peptide during in vitro thymus-dependent immune responses can be obviated by stimulation of Th cells with anti-CD3 antibodies, T cell-B cell contact is still required (4-6). These data, considered in view of the fact that Th cell antigen receptors and CD4 molecules (7) engage Ia and our biochemical evidence that Ia molecules can act as signal transducers (8), led us to explore the possibility that ligand binding to membrane Ia (mIa) molecules leads to transduction of signals that promote B cell proliferation and/or differentiation.

Previous studies that bear on the possibility of mIa-mediated signaling have demonstrated that soluble anti-Ia antibodies antagonize LPS-induced B cell proliferation (9, 10) and promote differentiation of CH12 lymphoma cells to secrete IgM (11). These effects are surprising in view of the fact that Ia ligation by Th cells occurs early in the immune response and appears to be necessary, in the case of small B cells, for initiation of proliferation (12). Thus, we hypothesized that a properly timed signal through Ia may lead to proliferation of B cells. To test this hypothesis, we developed a model system in which quiescent B cells were first primed by exposure to immunoregulatory molecules they are thought to encounter preceding direct T-B cell interaction and were subsequently exposed to anti-Ia antibodies before various biologic parameters were measured. Results demonstrate that Ia binding ligands provide proliferative signals to appropriately primed normal B cells.

### Materials and Methods

*Mice.* Akr and (C578L/6 × DBA2)F<sub>1</sub> (BDF<sub>1</sub>) mice were produced in the National Jewish Center animal facility and used in the experiments described at 6-12 wk of age.

*Reagents.* Recombinant IL-4 and IL-5 were derived from constitutive supernatants of IL-4

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and IL-5 structural gene transfectants mIL4X63Ag 8.653 and mIL5X63Ag 8.653 (13) provided by Dr. Fritz Melchers (Basel Institute for Immunology, Basel, Switzerland). These cells were shown to be mycoplasma free using the GenProbe test (GenProbe, San Diego, CA). Units of IL-4 and IL-5 were based on induction of Ia expression in normal B cells (14) and the BCL1 assay kindly performed by Dr. Sue Tonkonogy, respectively. Mixed lymphocyte culture supernatants were prepared as previously described (15). LPS (refined standard endotoxin, *S. typhimurium*, G30/C21) was purchased from RIBI (Hamilton, MT). Antibodies used in these studies include monoclonal anti- $\mu$  (Bet-2; reference 16), anti-H-2K (M1/42.398; 17) and anti-I-A<sup>db</sup> (D3.137.5.7; 14), anti-I-E (14.4.4S), anti-I-A $\beta^k$  (10.3.6, 10.2.16, 40M) (18), 39J (18) anti-I-A $\alpha^k$  (2A2; 19), anti-Lyb8 (Cy34.1.2), anti-Lyb2 (10-1.D.1), anti-B220 (RA3-3A1/69), anti-IgD (JA12.5; 20), and anti-IgG-Fc $\gamma$  (2.4G2; 21). Antibodies not referenced are described and provided by American Type Culture Collection (Rockville, MD). All antibodies were purified by protein A or protein G chromatography. Polystyrene plates were coated with antibodies by overnight incubation at concentrations noted. Plates were washed extensively before use.

**B Cell Isolation and Culture.** Quiescent B cells ( $\rho > 1.080$ ) were isolated from spleens of normal adult mice as previously described (20). Cell in this population were  $>90\%$  mIg<sup>+</sup> and  $>95\%$  in G<sub>0</sub> based on flow cytometric immunofluorescence and acridine orange cell cycle analyses. Cells were cultured ( $10^6$ /ml) in complete Iscove's modified Dulbecco's medium containing 10% FCS (HyClone, Logan, UT) and 2-ME ( $5 \times 10^{-5}$  M).

**Assays of Activation.** Cells were stained with acridine orange and analyzed by flow cytometry as previously described (22) using an Ortho cytofluorograf 50H with 2150 computer. For analysis of thymidine uptake, cultures ( $10^5$ /cells/200  $\mu$ l in 96-well tissue culture plates) were labeled for 3 h with 1  $\mu$ Ci [<sup>3</sup>H]thymidine before being harvested and counted. Ig (IgM and total IgG) production was assessed using an ELISA (23). Lymphocyte activation was also assessed upon the MTT assay of mitochondrial metabolic activity as previously described (24).

## Results and Discussion

Based on observations that IL-4 primes B cells for T-B conjugate formation (25) and proliferation (26, 27), it has been proposed that IL-4, produced during Th cell/antigen-presenting macrophage interactions, may provide an important B cell signal before T-B interactions. Clearly antigen binding to B cells leads to signal transduction (28-31) and is essential for subsequent antigen processing and presentation leading to direct T-B interactions. Therefore, we employed IL4 and mIg binding ligands, in this case low doses of the monoclonal anti-IgM antibody Bet-2, for B cell priming. Like thymus dependent antigens, soluble Bet-2 binds to antigen receptors and induces Ca<sup>2+</sup> mobilization, phosphoinositide hydrolysis, Ia expression and proto-oncogene expression, but is not mitogenic, and thus is an appropriate thymus-dependent antigen surrogate (28-38; Klemsz and Cambier, unpublished observation). It should be noted that for all responses described below, priming with *both* ligands was required. In the optimal situation, quiescent B cells were primed 12-16 h with IL-4 and Bet-2 before being transferred to plastic plates that had been precoated with anti-Ia mAbs used as a surrogate for Th cells. As shown in Fig. 1 C, quiescent B cells that had been primed as described above, underwent an immediate morphological transformation upon transfer to anti-Ia coated plates. This response, which was detectable within 10-20 min of cell transfer, was characterized by formation of extended fibrillar pseudopods. Most B cells ( $>80\%$ ) underwent this transformation within 6 h of initiation of culture (with anti-Ia) and retained this morphology for 36-48 h. Primed cells transferred to uncoated plates or plates coated with anti-MHC class I (M1.42.398, anti-H-2K) antibodies did not undergo this response (Fig.

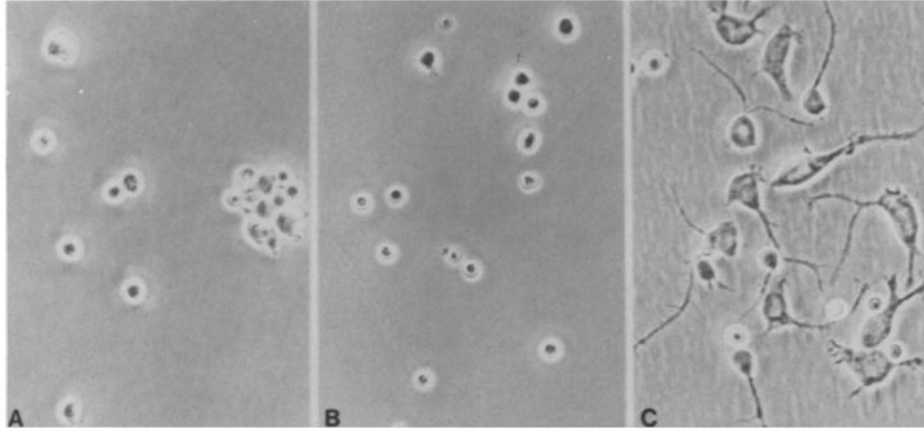


FIGURE 1. Immobilized anti-I-A antibodies induce rapid morphologic transformation by IL-4- and anti- $\mu$ -primed B cells. Quiescent ( $\rho > 1.080$ ) BDF1 B cells ( $10^6/\text{ml}$ ) were primed for 16 h with IL-4 (100 U/ml) and anti- $\mu$  mAb (Bet-2, 10  $\mu\text{g}/\text{ml}$ ) before being transferred (with primary stimuli) to uncoated plates (A), anti-H-2K, (M1.42.398, 10  $\mu\text{g}/\text{ml}$ ) coated plates (B), or anti-I-A (D3.137, 10  $\mu\text{g}/\text{ml}$ ) coated plates (C). After  $\sim 12$  h incubation at 37°C, photomicrographs ( $\times 400$ ) were taken of representative fields. Results are representative of approximately 50 experiments.

1 A and B) even when plates were coated with fourfold more anti-H-2K than anti-Ia (data not shown).

We next determined whether primed B cells enter cell cycle, proliferate, and differentiate to secrete Ig in response to anti-Ia binding. Cells were primed as before and transferred to antibody-coated or uncoated plates, and cultured for various times before cell cycle analysis was performed by flow cytometric analysis following acridine orange staining. Hydroxyurea was included in some cultures to block daughter cell generation (22), thus allowing determination of the absolute frequency of cells that entered cell cycle. As can be seen in Fig. 2 A in these cultures,  $\sim 95\%$  of primed cells exhibited significantly increased RNA content, indicative of entry into  $G_1$ , 48 h after transfer to anti-Ia-coated plates. Some increase in  $G_1$  phase cells was seen in populations cultured with IL-4 plus anti- $\mu$  on either uncoated or anti-H-2K-coated plates compared with cells primed with only IL-4. These findings suggest that while the combination of IL-4 and the monoclonal anti- $\mu$  provide all necessary signals to stimulate entry of a small percentage ( $\sim 15\%$ ) of B cells into  $G_1$ , the further addition of an Ia-mediated signal stimulates most members of the population to enter cell cycle. It should be noted that inclusion of higher doses of Bet-2 anti- $\mu$  ( $\geq 50 \mu\text{g}/\text{ml}$ ) in cultures did not result in a significant increase in the percentage of cells that entered cycle on anti-H-2K-coated plates.

Cell cycle analysis of primed cells cultured for 48 h on immobilized anti-Ia without hydroxyurea revealed that anti-Ia stimulated progression of many cells through cell cycle. Shown in Fig. 3 is an isometric display of data generated by flow cytometric analysis of acridine orange stained cells. Integration of cells containing hyperdiploid DNA (cells in channels  $> 30$  on the DNA axis) revealed that  $\sim 32\%$  of cells were in

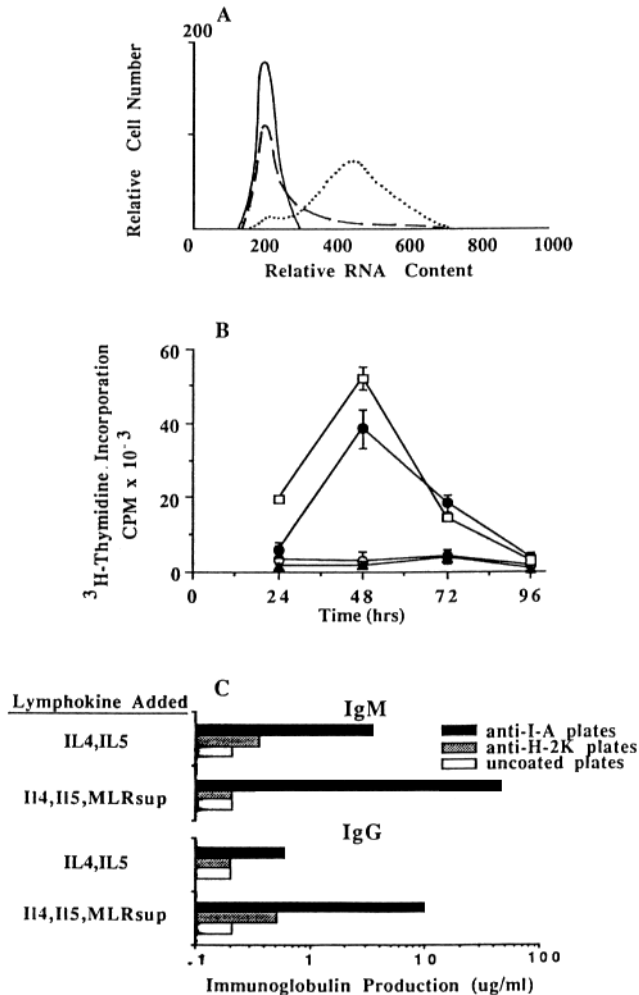


FIGURE 2. IL-4 and anti- $\mu$ -primed B cells respond to immobilized Ia binding ligands by entry into cell cycle, proliferation, and differentiation to secrete IgM and IgG. Quiescent BDF1 B cells ( $\rho < 1.080$ )  $10^6$ /ml were primed for 16 h and stimulated as described in Fig. 1 before analysis of entry into cell cycle (A) after an additional 48 h, and [ $^3$ H]thymidine uptake (B) after an additional 24–96 h of culture. Alternatively, cells were washed after 48 h incubation on antibody-coated plates, and recultured for 72 h (on antibody-coated plates) in the lymphokine mixtures (100 U IL-4/ml, 200 U IL-5/ml, 20% vol/vol MLR supernatant) indicated before Ig production was assessed (C). Shown in A are RNA histograms generated by flow cytometric analysis of acridine orange-stained cells that had been primed as in Fig. 1 and cultured with 20 mM hydroxyurea (Hu) and IL-4 on uncoated plates (—) with Hu, anti- $\mu$  and IL4 on anti-H-2K coated plates (---) with Hu, anti- $\mu$  and IL4 on anti-I-A coated plates (.....). Data are representative of three separate experiments. Shown in B are time course analyses of thymidine uptake by cells stimulated with optimal concentration of LPS (20  $\mu$ g/ml) under culture conditions described in Fig. 1 except that no IL-4 or anti- $\mu$

was present, and cells were cultured on uncoated plates (□). Alternatively, anti- $\mu$ - and IL-4-primed cells were cultured on uncoated (▲), anti-H-2K-coated (○), or anti-I-A-coated (●) plates. Shown are mean and SD of assays of triplicate cultures. Data are representative of six separate experiments. Shown in C are IgM and IgG levels in cultures of quiescent B cells that had been primed with IL-4 and anti- $\mu$ , transferred (with stimuli) to anti-I-A, anti-H-2K, or uncoated plates for 48 h, and then washed three times and recultured an additional 72 h with the lymphokines indicated, before IgM and IgG levels were assayed using an ELISA. Control response to LPS were 168  $\mu$ g/ml IgM and 96  $\mu$ g/ml IgG. Data are representative of three separate experiments.

S, G<sub>2</sub>, or M at the time of analysis. By extrapolation from cell cycle progression rates seen in lymphomas, these findings predict that a large proportion (>70%) of cells were proliferating. As an alternative assay of proliferation, we analyzed [ $^3$ H]thymidine incorporation uptake in companion cultures. As shown in Fig. 2 B, IL-4 plus anti- $\mu$ -primed, anti-Ia-stimulated cells exhibited [ $^3$ H]thymidine incorporation comparable to LPS-stimulated cells. Further, IL-4 plus anti- $\mu$ -primed cells did not incorporate significant [ $^3$ H]thymidine if cultured on anti-H-2K-coated or

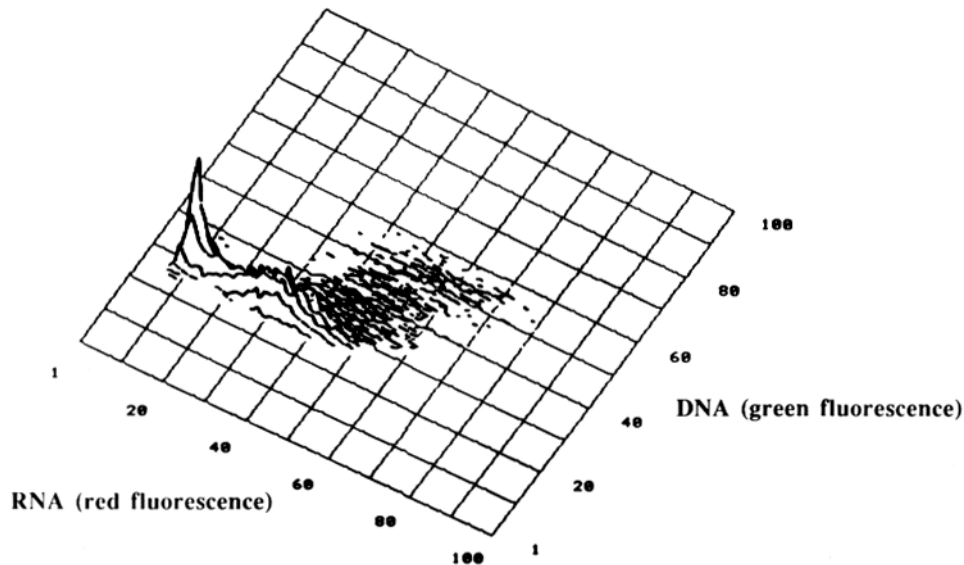


FIGURE 3. Anti-Ia antibodies stimulate progression of primed B cells through cell cycle. Quiescent BDF1 cells were primed for 16 h before being transferred to anti-Ia-coated plates (D3.137) as described in Fig. 1. Cells were harvested and stained with acridine orange 48 h later, and then analyzed by flow cytometry. Shown is an isometric display constructed based on analysis of 20,000 cells. The display illustrates the relative number of cells at each coordinate of RNA (red fluorescence) and DNA (green fluorescence) content. These data are representative of five replicate experiments.

uncoated plates. Thus, although IL-4 and monoclonal anti- $\mu$  (Bet-2) promote entry of some B cells into  $G_1$  (Fig. 2 A), these cells apparently do not proceed into S phase without an additional signal(s), i.e., that provided by immobilized anti-Ia.

Next we assessed the ability of immobilized anti-Ia to stimulate Ig production by primed B cells. In these experiments, quiescent B cells were primed for 16 h as before, and cultured for 48 h (growth phase) on anti-Ia-coated plates. Cells were then washed three times and recultured with IL-4 and IL-5 but without anti- $\mu$ . 72 h later, supernatants were harvested and assayed for Ig content. As shown in Fig. 2 C, anti-Ia but not anti-H-2K, when used in combination with IL-4 and IL-5, provided all signals necessary to support differentiation of primed cells to become Ig secretors. However, this response was much less than that induced by LPS (see Fig. 2 C, legend). Further, significant Ig production was not seen in the absence of lymphokines (data not shown). Supplementation of cultures during the final 72 h with a mixed lymphocyte culture (15) supernatant known to contain additional lymphokines, resulted in an approximate 10-fold increase in production of IgM and IgG. In preliminary experiments, we have attempted to reconstitute this response with various combinations of purified and/or recombinant mouse IL-1, IL-2, IL-4, IFN $\gamma$ , IL-5, and human IL-6 but have thus far failed (data not shown). These findings are consistent with the possibilities that an additional lymphokine is necessary for induction of differen-

tiation or that the lymphokines used work optimally only when provided in a certain sequence or a certain ratio.

To assure that the effects of anti-Ia in our experiments were a function of ligand binding to Ia and not a result of some peculiar crossreactivity of the D3.137 anti-I-A<sup>db</sup> mAb with some other cell surface marker, we compared the ability of a number of anti-Ia antibodies to induce primed B cells to proliferate. As shown in Fig. 3, immobilized anti-Ia specific for I-E, I-A $\beta$  chain, and I-A $\alpha$  chain exhibited a comparable ability to induce proliferation. Thus, ligand binding to either I-E or I-A ( $\alpha$  or  $\beta$  chains) results in signaling. It should be noted that D3.137 anti-Ia<sup>db</sup> did not stimulate Ia<sup>k</sup> B cells (data not shown).

Finally, we assessed the specificity of the response to anti-Ia by comparing the ability of immobilized mAbs specific for a variety of B cell surface markers to activate primed B cells. In this experiment, activation was assessed based on the MTT assay of mitochondrial metabolic activity (24) since this assay has proven to be the most sensitive indicator of Ia mediated signaling. As shown in Fig. 4, antibodies specific for Fc receptor (a rat IgG), IgD (a rat IgG), H-2K (a rat IgG), Lyb8 (a mouse IgG1), and Lyb2 (a mouse IgG2b) did not stimulate primed B significantly compared to anti-Ia. Surprisingly, anti-B220 (a rat IgM) exhibited significant stimulatory activity. We have subsequently observed that antibodies against a variety of other B cell markers including J11D (a rat IgM), anti-Qa2 (a mouse IgM) and anti-CD23 (a rat IgG) do not induce this response (data not shown). Companion experiments demonstrated that anti-B220 stimulates 60–80% of primed cells to undergo morphologic transformation indistinguishable from that described in Fig. 1, however, this ligand does not stimulate significant thymidine incorporation (data not shown). These findings are particularly interesting in view of recent evidence that CD45 is associated with a tyrosine phosphatase activity (37) consistent with its (CD45) functioning as a signal transducer.

The data presented demonstrate that immobilized Ia binding ligands stimulate proliferation of small B cells primed by contact with IL-4 and membrane Ig binding

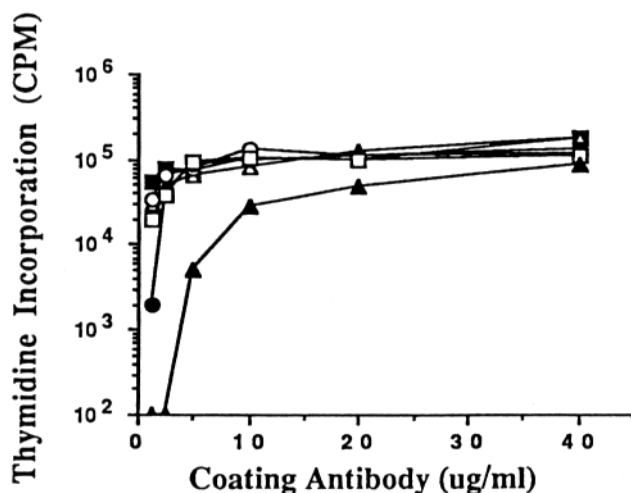


FIGURE 4. Multiple anti-I-A antibodies stimulated proliferation of primed B cells. Quiescent B cells were isolated from AKR mice and primed for 16 h with IL-4 and anti- $\mu$  as described in Fig. 1. Cells were then transferred to plates that had been coated with varied concentrations of anti-I-E (■, 14.4.4S), anti-I-A  $\beta$  ( $\Delta$ , 10.3.6; O, 10.2.16; ●, 40M; and □, 39F), or anti-I-A $\alpha$  ( $\blacktriangle$ , 2A2) antibodies and cultured for 48 h before [<sup>3</sup>H]thymidine uptake was assessed. Data are representative of three separate experiments.

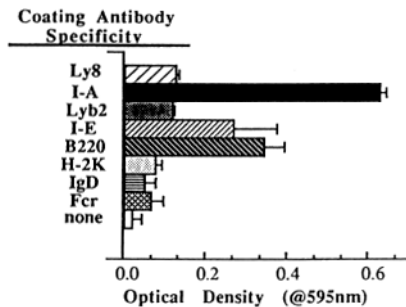


FIGURE 5. Specificity of anti-I-A activation of primed B cells. Quiescent B cells were prepared from BDF1 mice and primed for 16 h with IL4 and anti- $\mu$  as described in Fig. 1 before being transferred to 96-well plates that had been precoated with 10  $\mu$ g/ml monoclonal anti-Ly8 (Cy34.1.2), anti-I-A (D3.137), anti-Lyb2 (10-1.D.2), anti-I-E (14.4.4S), anti-B220 (RA3-3A1/69), anti-H-2K (M1.42.398), anti-IgD (JA12.5), or anti-Fcr (2.4G2), or were not precoated. After an additional 48 h incubation, activation was assessed using the MTT assay. Shown are the mean  $\pm$  standard deviation of triplicate cultures. Data are representative of four separate experiments.

ligands. Based on these data, we hypothesize that IL-4, produced as a result of antigen-presenting macrophage-Th cell interaction, and antigen provide the first B cell signals leading to "thymus-dependent" humoral immune responses. This combination of signals induces quiescent B cells to become competent to interact directly with the Th cell. This competence is probably a function, at least in part, of increased Ia expression induced by IL-4 and antigen (20). The physical interaction of Th cells and B cells results in turn in T cell signaling via  $\alpha\beta$ /CD3 binding to antigen/Ia, and B cell signaling via CD4 (7) binding to Ia. The primary involvement of CD4 binding to monomorphic Ia determinants in mediating this signal would explain the ability of Th cells to activate histoincompatible B cells, provided antigen-presenting histocompatible B cells are also present (22, 39). B cell signaling mediated through Ia molecules results in entry of most cells into cycle and proliferation, and prepares the cells to differentiate to secrete Ig provided appropriate lymphokines are present. We believe that it is the lack of the Ia-mediated signal that has resulted in the previous failure to achieve comparable activation of quiescent B cells in the presence of antigen and lymphokines versus antigen and Th cells (12, 22, 35, 36, 39).

### Summary

One of the most controversial questions in immunology is the molecular basis by which Th lymphocytes deliver activating signals to quiescent B lymphocytes during T cell-dependent immune responses. Recent studies suggest that T cell-dependent activation of quiescent B lymphocytes may involve signaling mediated by direct T helper cell-B cell contact. Since B cell membrane-associated MHC-encoded class II molecules (Ia) must be recognized by Th lymphocytes for generation of T cell-dependent humoral immune responses, they are obvious candidates for receptors of this signal. Here we report that stimulation of quiescent murine B cells with IL-4 and antibodies against the B cell antigen receptor for 12-16 h primes cells to proliferate in response to immobilized mIa binding ligands. In the presence of additional lymphokines, these B cells differentiate to secrete Ig of IgM and IgG classes. These results suggest that Ia molecules are receptors for direct, T helper cell-B cell contact mediated signaling that results in B cell proliferation.

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