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Role of the Bp35 cell surface polypeptide in human B-cell activation

(B lymphocytes/proliferation/monoclonal antibodies)

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ABSTRACT **A 35-kDa polypeptide, Bp35, expressed on the surface of all B cells, plays a role in B-cell activation. Monoclonal antibodies to Bp35 stimulate human tonsillar B cells to proliferate. The activation induced by anti-Bp35 is similar to anti-Ig-mediated in several ways: the activation does not require T cells but is augmented by T-cell-derived allogeneic factors; monovalent Fab fragments to Bp35 do not trigger proliferation but instead block activation by whole antibody, indicating that cross-linking is required; and induction by anti-Bp35, like the induction by anti-Ig, is inhibited by monoclonal anti-IgM via an Fc domain-dependent mechanism. However, several features of anti-Bp35-mediated proliferation are clearly different from activation by anti-Ig: anti-Bp35 monoclonal antibodies do not require attachment to beads to function, the proliferation induced by anti-Bp35 and anti-Ig is additive, and Fab fragments of anti-Bp35 augment proliferation induced by anti-Ig. Models for the possible function of the Bp35 polypeptide as either a "bridge" or a "second signal" with surface Ig in B-cell activation are discussed.**

B lymphocytes can be stimulated to proliferate by relatively high concentrations of antisera specific for surface immunoglobulin (Ig) receptors (1–4). Low doses of anti-Ig, which alone do not stimulate B cells to divide, can, in the presence of T-cell-derived B-cell stimulatory factors (BSF), induce substantial proliferation (5–7). A current model is that, after an initial activation signal (e.g., by low doses of anti-μ heavy chain antisera), receptors are expressed for accessory cell-derived growth and differentiation factors such as BSF and interleukin 1 (IL-1); in the presence of these factors, B cells divide and differentiate (6–9). Although specific receptors for the T-cell growth factor interleukin 2 (IL-2) have been characterized (10), analogous structures on B cells have yet to be fully elucidated.

Recently, a number of new polypeptides on B cells have been identified with monoclonal antibodies (mAb) (refs. 11–14, 35, 36). One antigen, Bp35, a 35-kDa polypeptide first defined by the mAb B1 (12), is only expressed on cells of the B-cell lineage (12, 14, 35). Bp35 is a phosphoprotein expressed at high density on germinal-center B cells (15, 16). Recently, we have found that Bp35 is expressed at higher levels on B cells able to proliferate in response to T-cell factors and at lower levels on resting B cells not able to respond to growth factors without other signals (36). Here we show that the Bp35 molecule plays some role in B-cell activation: anti-Bp35 antibodies alone trigger resting tonsillar B cells to proliferate. This proliferation is enhanced by T-cell factors and is distinct from proliferation triggered through surface IgM.

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MATERIALS AND METHODS

Antibodies. The monoclonal antibodies used in this study have been described (14) or will be described elsewhere. They include 2H7 (IgG2b) and 1F5 (IgG2a) mAbs specific for Bp35 (CD20), which block the binding of each other and thus are specific for the same epitope or a closely related epitope; HB10a (IgG2b) mAb specific for HLA-DR; 2C3 (IgG1) and 4B8 (IgG2a) specific for IgM; 9BA5 (IgG2b) specific for the p55 55-kDa B-cell associated antigen; and H616 (IgG2a) specific for the p76 76-kDa B-cell surface antigen. Purification of mAbs and conjugation with fluorescein, using fluorescein-5-isothiocyanate (17), or with R-phycoerythrin, using 3-(2-pyridylthio)propionic acid *N*-hydroxysuccinimide ester (18), for one- and two-color cytofluorographic analysis as detailed elsewhere (37). mAbs were conjugated to CNBr-activated Sepharose 4B beads (Pharmacia) at a ratio of 10 mg/ml of Sepharose. Fab fragments of the 1F5 and 2C3 antibodies were prepared by papain digestion followed by separation on a protein A-Sepharose or Sephacryl S-200 (Pharmacia) to remove undigested antibody and Fc fragments. Antibodies or Fab fragments were dialyzed extensively against phosphate-buffered saline prior to use in cell-culture experiments.

Cell Preparations. Tonsillar cell suspensions were prepared by teasing tissue gently in R15 (RPMI 1640 medium with penicillin, streptomycin, glutamine, pyruvate, and 15% fetal bovine serum). Lymphocytes in tonsillar cell suspensions or lymphocytes in heparinized blood diluted 1:2 in R15 were isolated using Lymphocyte Separation Medium (Litton Bionetics). Cells were washed twice, resuspended in 30% Percoll at 10^8 cells per ml and layered over Percoll (Pharmacia) step gradients (19) in 15-ml conical tubes with 2.5 ml of 60%, 55%, 50%, 45%, and 40% Percoll. After centrifugation at 3000 × *g* for 5 min, cells from the 45/50 (Fx1), 50/55 (Fx2), and 55/60 (Fx3) interfaces and the pellet were isolated and tested.

Cell Cultures. Cells were cultured in quadruplicate in 96-well Microtiter plates in 200 μl of R15 at 10^6 cells per ml. After 1–7 days, cells were incubated with 0.5 μCi of [^{3}H]thymidine per well (New England Nuclear, 6.7 Ci/mmol; 1 Ci = 37 GBq). After 18–24 hours, cells were harvested onto glass-fiber filters with a cell harvester, and radioactivity was measured in a liquid scintillation counter. For a source of T-cell factors, T cells were cultured for 2 days with mitomycin C-treated allogeneic cells. These mixed lymphocyte reaction-derived T-cell factors (MLR-TF) were used at a concentration of 12.5%–50% (19, 20).

Flow Cytometry. Flow cytometry with a modified FACS IV cell sorter (Becton Dickinson) and quantitative two-color

Abbreviations: BSF, B-cell-stimulating factor(s); mAb, monoclonal antibody; MLR-TF, mixed lymphocyte reaction-derived T-cell factors (i.e., allogeneic T-cell supernatant).

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analyses are described elsewhere (35–37). In brief, 5×10^5 cells were incubated for 30 min on ice with fluorescein- and/or phycoerythrin-conjugated mAb, washed twice, and passed through fine gauze just before analysis. A 488-nm laser line was used, and a 560-nm dichroic mirror (Becton Dickinson) split the emission wavelengths. Additional 580-nm long-pass and 540-nm short-pass filters (Ditric Optics, Hudson, MA) were placed in front of the red (phycoerythrin) and green (fluorescein) photomultiplier tubes, respectively. A compensator built by T. Nozaki (Stanford University) was used to correct any residual spillover of green and red signals.

RESULTS

A Subpopulation of B Cells Is Stimulated by Anti-Bp35. Peripheral blood or tonsillar B cells separated by Percoll step gradients differ in their ability to proliferate in response to MLR-TF alone or Sepharose-conjugated anti-μ (19, 36). As illustrated in Fig. 1*A*, low-density tonsillar B cells proliferated in response to MLR-TF but not in response to Sepharose-anti-μ. In contrast, higher density B cells (Fig. 1*B*) did not respond to T-cell factors but did respond weakly to anti-μ beads. High- and low-density tonsillar B cells also differ in their ability to be triggered by antibodies to Bp35: although the lower density B cells did not respond to anti-Bp35 antibodies, the higher density B cells did respond. Although there was little or no response to either anti-Bp35 or T-cell factors alone in this experiment, together anti-Bp35 and MLR-TF induced a strong proliferative response. In subsequent experiments, we found that the peak of the response to anti-Bp35 plus T-cell factors occurred on day 3 and then waned (Fig. 2); furthermore, we found that anti-Bp35 mAb alone could induce cells in the higher density fractions to proliferate significantly above background levels (>10-fold). Changes in forward-angle scatter profile were evident on day 3, when 15–20% of anti-Bp35-treated cells were the size of blasts while only 0.5–2% of MLR-TF-treated cells were (21).

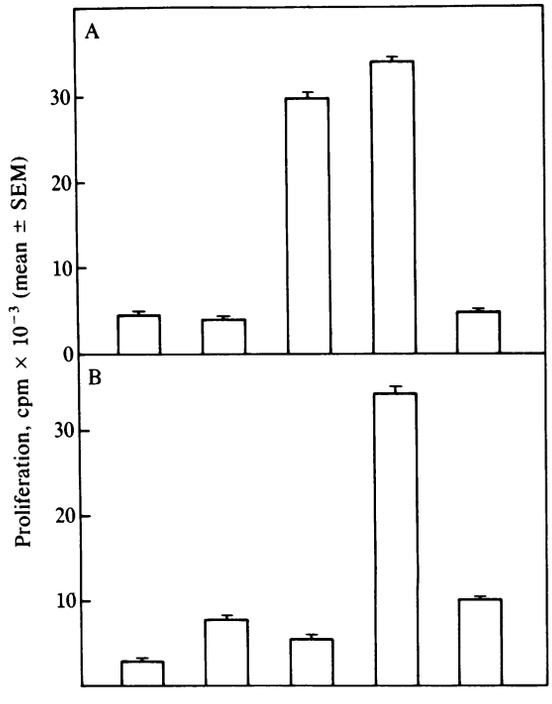

FIG. 1. Proliferation of high- and low-density tonsillar B-cell fractions in response to anti-Bp35 antibodies and/or allogeneic T-cell factors. Proliferation (day 3), measured by incorporation of [^{3}H]thymidine, of Percoll gradient Fx2 cells (2.8% erythrocytes + T cells) (*A*) and Percoll gradient pellet cells (29.6% erythrocytes + T cells) (*B*) in response to medium (from left to right) without additions, with 1F5 anti-Bp35 mAb at 5 μg/ml, with 33% MLR-TF, with 1F5 and MLR-TF, or with Sepharose-anti-μ at 50 μg/ml.

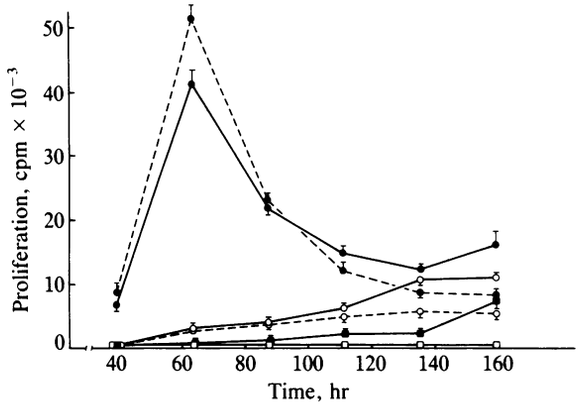

FIG. 2. Kinetics of anti-Bp35-antibody induced proliferation of dense tonsillar B lymphocytes. Unfractionated (67% B cells, —) or T-cell-depleted (99% B cells, -----) cells were cultured with anti-Bp35 (5 μg/ml) alone (○) or together with 33% MLR-TF (●). Mean proliferation ± standard error of quadruplicate samples was measured at various times. MLR-TF alone (■) and medium (□) controls were assayed concurrently.

Anti-Bp35-Induced Proliferation Does Not Depend on T Cells. The responsive lymphocyte fractions invariably contained 60–85% surface IgM$^+$ B cells, few or no detectable monocytes, and 15–40% T lymphocytes. Therefore, it was important to ascertain whether T cells were required for the anti-Bp35-induced proliferation. To test this possibility, we compared the responsiveness of unfractionated and T-cell-depleted B-cell fractions to anti-Bp35 antibody. Tonsillar lymphocytes contain three major populations of cells: Bp35^{dull}IgM^{bright} (IgM^{bri}) B cells, Bp35^{bri}IgM^{dull} B cells, and IgM$^-$Bp35$^-$ non-B cells (35, 36). After separation on Percoll gradients, the dense lower fractions are depleted of the Bp35^{bri} B cells reactive to MLR-TF (Fig. 1). After further depletion of T cells from the dense pellet fraction, a highly purified population IgM^{bri}Bp35^{dull} B cells of >98% purity was obtained.

As shown in Table 1, removal of T cells did not reduce the ability of anti-Bp35 antibodies to trigger B-cell proliferation and, if anything, enhanced B-cell proliferation. T-cell supernatants still augmented the effect of anti-Bp35, and the kinetics of the proliferative response was the same whether or not T cells were present (Fig. 2). These results suggest

Table 1. Augmentation and inhibition of anti-Bp35-induced B-cell proliferation

	Anti-Bp35*	Mean proliferation of B-cell fraction	
		With T cells	Without T cells
Medium	−	660 ± 16	5,162 ± 527
	+	10,546 ± 898	25,404 ± 2235
MLR-TF (50%)	−	1,977 ± 148	3,012 ± 427
	+	29,177 ± 2028	107,221 ± 3780
Anti-μ*	−	698 ± 30	1,377 ± 388
	+	947 ± 86	1,878 ± 53

Dense tonsillar fractions were either untreated (62% sIgM$^+$Bp35$^+$ cells) or depleted of T cells by E-rosetting (98.6% sIgM$^+$Bp35$^+$ cells). Proliferation is expressed as cpm (mean ± SEM, n = 4) recovered in cells that were cultured for 72 hr and then incubated with [^{3}H]thymidine for 18 hr.

*Anti-Bp35 (1F5) and anti-μ (2C3) antibodies used at 5 μg/ml.

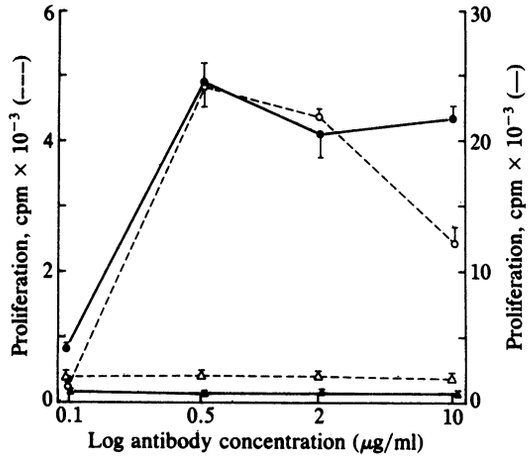

FIG. 3. Dose–response curves for induction of proliferation of dense tonsillar B cells by anti-Bp35 (○, ●) or control anti-p76 (△, ▲) in the presence (——) or absence (-----) of 33% MLR-TF. Mean proliferation ± standard error of quadruplicate samples was measured on day 3.

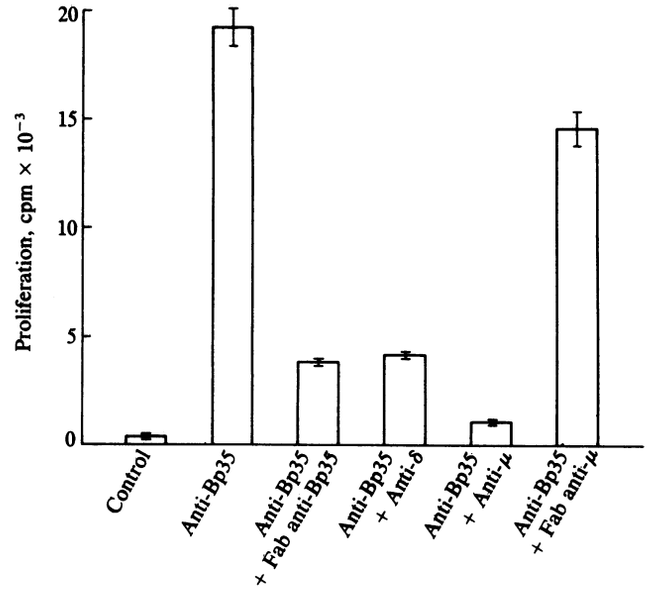

FIG. 4. Inhibition of the proliferation of dense tonsillar B cells induced by anti-Bp35 (5 μg/ml) and 25% allogeneic T-cell supernatant (MLR-TF). Mean proliferation ± standard error was measured on day 3. Similar results were obtained in experiments without MLR-TF. Inhibiting antibodies: Fab anti-Bp35 fragments (5 μg/ml), anti-δ (5 μg/ml), anti-μ (5 μg/ml), and Fab anti-μ fragments (10 μg/ml).

that anti-Bp35 mAb acts directly on Bp35$^+$ B cells and does not require T cells for B-cell activation.

Cross-Linking of Bp35 Is Required to Stimulate B Cells. For comparison in antibody titration experiments using the same number of B cells, 0.2–1.0 μg of anti-Bp35 mAb per ml was needed to induce proliferation. Maximal staining of 10^6 B cells with fluorescein-conjugated-anti-Bp35 was obtained at approximately 0.2 μg/ml. As shown in Fig. 3, anti-Bp35 mAb at 0.5 μg/ml induced B-cell proliferation whereas a 100-fold greater concentration of an IgG2a mAb to the p76 B-cell surface antigen or an IgG2b mAb to the p55 B-cell antigen (data not shown) had no effect. Similar dose–response curves were obtained with or without the presence of T-cell factors.

The fact that saturating amounts of mAb were required for stimulation suggested that cross-linking might be necessary to transmit the activation signal. To test this possibility, the stimulating activity of Fab fragments of anti-Bp35 mAb were compared to whole antibody. Fab fragments at 0.5–20 μg/ml alone or with MLR-TF did not induce proliferation (e.g., Table 2, experiments 2 and 4). Fab fragments, however, did consistently block the ability of whole mAb to induce proliferation with or without MLR-TF (Fig. 4). Therefore, it appears that cross-linking is necessary for anti-Bp35 mAb to exert its effect. In the presence of whole mAb at 5 μg/ml, Fab at ≥5 μg/ml was necessary to obtain ≥50% inhibition.

Monoclonal antibodies to surface IgM or surface IgD also blocked proliferation induced by anti-Bp35 (Fig. 4). This effect was specific for surface Ig, since a similar dose of a control mAb to p76 antigen did not inhibit proliferation. The Fc portion of anti-μ appears to play some role in the inhibition, because even saturating amounts (1–10 μg/ml) of Fab fragments of anti-μ did not inhibit efficiently, whereas the intact antibody at one-tenth these concentrations was inhibitory.

To assess the possible role of the Fc domain in anti-Bp35-induced proliferation, we compared the ability of two anti-Bp35 mAbs of different IgG subclasses to trigger B-cell proliferation (Table 3). While the 1F5 IgG2a mAb induced B-cell proliferation, the 2H7 IgG2b mAb did not, even though it is specific for the same epitope or a closely associated epitope. This isotype difference suggests that

Table 2. Anti-Bp35 and anti-Ig together augment B-cell proliferation

Experiment	Anti-Bp35 (μg/ml)	25% MLR-TF	Proliferation, cpm* Alone	With S-anti-μ
1	—	−	384 ± 163	1,811 ± 91
	Whole (1)	−	1,154 ± 208	5,067 ± 269
2	—	−	246 ± 4	1,488 ± 55
	Whole (5)	−	2,153 ± 184	6,928 ± 98
	Fab (5)	−	161 ± 4	3,758 ± 233
3	—	−	162 ± 5	1,567 ± 114
	Whole (10)	−	322 ± 31	1,907 ± 96
	—	+	422 ± 22	5,560 ± 279
	Whole (10)	+	4,128 ± 144	13,007 ± 128
4	—	−	126 ± 7	4,117 ± 158
	Whole (5)	−	1,118 ± 22	11,701 ± 117
	Fab (5)	−	331 ± 19	18,430 ± 1234
	—	+	366 ± 22	22,355 ± 4611
	Whole (5)	+	18,015 ± 524	67,873 ± 223

Dense tonsillar B cells were cultured at 10^6 cells per ml, either alone or with 2C3 anti-μ heavy chains coupled to Sepharose (S-anti-μ; ≈10 μg/ml). After 72 hr, [^{3}H]thymidine was added and the cultures were incubated for an additional 18 hr.
*Mean ± SEM, n = 4.

Table 3. IgG2a and IgG2b anti-Bp35 antibodies differ in their ability to induce B-cell proliferation

Anti-Bp35*	25% MLR-TF	Proliferation, cpm
None	−	250 ± 42
	+	864 ± 44
1F5 (IgG2a)	−	1,624 ± 39
	+	31,659 ± 1770
2H7 (IgG2b)	−	236 ± 20
	+	924 ± 36

Dense tonsillar lymphocytes were cultured and proliferation was measured as described in *Materials and Methods* and the legend to Table 1.
*Used at 2 μg/ml. Similar results were obtained at concentrations of 0.5–10 μg/ml.

accessory cells may be involved in the anti-Bp35-induced proliferation, since a similar isotype-dependence of activation by mAb to CD3 on T cells is caused by differences in monocyte recognition of the mAb isotype (22).

Proliferation Triggered by Anti-Bp35 and Proliferation Triggered by Anti-Ig Are Distinct. B-cell proliferation induced by either anti-Bp35 or by certain anti-Ig reagents are similar in several ways: both appear to require cross-linking, both are augmented by allogeneic T-cell factors, and both are inhibited by free anti-μ via an Fc domain-dependent mechanism (refs. 2, 5–7, 23–25; Table 1 and Fig. 4). Thus it was of interest to examine whether they induce proliferation via a common pathway. First, we tested the effect of treating B cells with anti-Bp35 and Sepharose-anti-μ together or with each separately. As illustrated in Table 2 (experiments 1 and 2), significantly more proliferation was induced with the two antibodies together than with either alone. This was true even when optimal doses of anti-μ were added to cultures or when T-cell factors were added to cultures as well (e.g., experiments 3 and 4). These results suggested that the signals induced by anti-μ and anti-Bp35 are distinct yet additive. Furthermore, Fab fragments of anti-Bp35, although they were not stimulatory themselves (see above), could augment the induction by Sepharose-anti-μ. The converse was not true; Fab fragments of anti-μ did not augment anti-Bp35-induced proliferation. The fact that Bp35- and surface Ig-mediated proliferation were additive suggested that Bp35 and Ig may be associated on the membrane. However, in experiments in which surface Ig was capped or modulated and shed from cells by using goat anti-human Ig antisera, no effect on or association with Bp35 was discernible with phycoerythrin-coupled anti-Bp35 antibody. Thus, Bp35 did not co-cap or modulate with surface Ig.

DISCUSSION

Human B cells can be induced to proliferate via a surface polypeptide distinct from surface immunoglobulin: mAb to Bp35, alone or with T-cell-derived factors, induces B cells to divide. Strong proliferation is induced in the absence of T cells, so the antibody apparently acts directly on B cells. The relatively high concentrations of mAb needed to activate the B cells and the ability of monovalent Fab fragments to block the activity of the whole antibody suggest that cross-linking of Bp35 is necessary to trigger proliferation.

Although anti-Bp35 alone in some experiments induces B-cell proliferation, the antibody also acts synergistically with T-cell-derived factors (Fig. 1). A similar dose–response curve for anti-Bp35 antibody is obtained with or without T-cell factors (Fig. 3) suggesting that anti-Bp35 may first have to activate B cells before MLR-TF can augment proliferation. Within 24 hr after exposure to anti-Bp35 antibodies, B cells are stimulated to increase surface levels of HLA-DR, p76, and C3dr receptors (21). It is possible that anti-Bp35 also induces an increase in expression of receptors for factors such as BSF present in T-cell supernatants (9). More detailed studies will be necessary to test this possibility.

Anti-Bp35-induced proliferation is similar to anti-Ig-mediated triggering in several ways: both appear to require cross-linking, both are augmented by T-cell factors, and both are inhibited by monoclonal anti-μ via an Fc domain-dependent mechanism (2, 6, 23–25). Yet there are also features that clearly distinguish these triggering mechanisms. First, anti-Bp35 mAb do not require attachment to beads to function and, unlike free anti-μ mAb, do not inhibit proliferation even at high doses. This difference cannot be attributed to differences in the IgG subclass of the antibodies used since a mAb to μ chain (4B8) of the same IgG2a isotype as the 1F5 anti-Bp35 antibody inhibits proliferation, whereas 1F5 does not (data not shown). Second, the proliferation induced by anti-Bp35 and anti-μ on beads is additive (Table 2). This is true in the presence of MLR-TF and even when optimal doses of anti-μ are used. Third, Fab fragments of anti-Bp35, although they cannot trigger B cells to divide by themselves, can augment anti-μ-induced proliferation. The converse is not true: Fab anti-μ does not affect anti-Bp35 triggering. Finally—a point worth reemphasizing—the anti-Bp35 and anti-μ mAbs do not block the binding of each other and precipitate distinct polypeptides (ref. 14 and unpublished data). Thus, anti-Bp35-triggering resembles and augments anti-Ig-induced proliferation yet is distinct.

Like proliferation induced by anti-Ig on beads, anti-Bp35-mediated proliferation is inhibited by anti-μ or anti-δ antibody via an Fc domain-dependent mechanism. Aggregated Ig or immune complexes, unlike anti-Ig, do not inhibit proliferation triggered by either bacterial lipopolysaccharide (24) or by stimulatory anti-μ (2, 26). This suggests that inhibition requires cross-linking of membrane Ig with Fc receptors on B cells. Recent studies of Phillips and Parker (27) support this conclusion: when Fc receptors on B cells were blocked with a specific mAb, anti-μ or anti-γ antibodies stimulated rather than inhibited proliferation. In our studies, a mAb to B-cell antigen p76, which is distinct from surface IgM (35), did not inhibit proliferation even though an anti-μ mAb of the same subclass and at the same concentration did inhibit. This result suggests that the inhibitory effect may be mediated specifically via surface Ig and Fc receptors cross-linking and not via the cross-linking of Fc receptors to other antigens such as p76.

The fact that either whole or Fab anti-Bp35 augment the proliferation induced by anti-Ig on beads suggests that Bp35 normally may function in some way as a positive signal for B-cell proliferation. Surface Ig is an unusual receptor since not only can it bind its ligand but, in another form, it is secreted as a functional antibody (28). Membrane Ig differs from secreted Ig in its COOH-terminal region where it has a hydrophobic tail which anchors it to the membrane (29). Apparently, only three hydrophilic amino acids of membrane Ig are intracytoplasmic, and these are not phosphorylated (29). How surface Ig after it binds antigen can transmit a signal for proliferation with such a small cytoplasmic tail is not known. Since anti-Ig on large beads induces proliferation, internalization of the receptor is most likely not involved in the signaling mechanism (2). It has been suggested that capping of surface Ig may be necessary for triggering (e.g., see ref. 25). During this process, surface Ig may need to interact with other membrane structures capable of serving as "bridges" into the cytoplasm to signal proliferation. In other words, the evolutionary constraints of being both a receptor and a secreted molecule may have required surface Ig to interact with a bridge molecule rather than have its own intracytoplasmic signal. Although not formally demonstrated to be such a bridge, the Bp35 molecule does meet a number of the requirements that would be necessary for one: it is on the surface of all B cells at high density; when it is cross-linked directly, B-cell proliferation is induced; and it is a phosphoprotein (15), which would be necessary if, as has been suggested, a phosphokinase-dependent pathway is utilized (2, 30). Furthermore, the studies of Parker and Phillips (2, 26, 27) provide a precedent for bridging between surface Ig and membrane structures (i.e., Fc receptors), although in their studies a negative or inhibitory signal was transmitted.

On the other hand, no close association between Bp35 and Ig on the membrane was discernible, and the two molecules are not always coordinately expressed. Furthermore, the anti-Bp35 and anti-Ig signals are additive. In an alternative model, a "second-signal" model, the Bp35 molecule may serve as a receptor for a distinct signal normally necessary for B-cell proliferation. Several models for B-cell induction

have postulated a need for "second signals" mediated by receptors other than Ig (31, 32, 34). B cells can be triggered to proliferate by stimulating structures distinct from the Ig receptor, such as the receptor for lipopolysaccharide. Bp35 and the lipopolysaccharide receptor are similar in that triggering via both is inhibited by free anti-μ (24). Macrophages have been implicated as essential for B-cell proliferation (32). The Bp35 structure normally may serve as a receptor for an early signal from macrophages or other accessory cells. The bridge and second-signal models for Bp35 function cannot yet be clearly distinguished, but both have the advantage of being testable.

Proliferation induced through Bp35 cell surface structures resembles the T-cell proliferation induced via Tp19-29 CD3 structures. First, Bp35 and CD3 are, respectively, the prototype pan B- and pan T-cell markers distinct from antigen-specific receptors. Second, IgG antibodies to either Bp35 or CD3 induce proliferation but Fab fragments are not effective (Table 2 and ref. 33). Third, IgG2a mAbs to either Bp35 (1F5) or CD3 (OKT3) consistently induce proliferation, whereas IgG2b (Bp35) or IgG1 (CD3) subclass mAbs either do not induce proliferation or only do so in certain individuals (Table 3 and ref. 22). van Wauwe *et al.* (22) have suggested that specific monocytic Fc receptors that interact with only certain murine isotypes are necessary for inducing T-cell activation. What role Fc receptors on accessory cells play in Bp35-mediated activation is not known.

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