ACTIVATION OF B LYMPHOCYTES BY MONOVALENT ANTI-Lyb-2 ANTIBODIES

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Small resting B lymphocytes are activated when their cell surface immunoglobulin (Ig) receptors interact with specific antigen or anti-Ig antibodies. Such a receptor-ligand interaction leads to enlargement and proliferation of B lymphocytes. These activated B cells can subsequently mature into Ig-secreting cells in the presence of appropriate accessory cell signals (1–3). However, monovalent anti-Ig antibodies cannot induce B cell activation even though they can bind to the surface Ig (4–7). It has thus been concluded that membrane Ig has to be cross-linked to induce early steps in B cell activation (membrane depolarization; 8) as well as later steps (proliferation). There are several B cell mitogens as well as antibodies to B cell membrane components that stimulate B cell proliferation but probably interact with B cell surface moieties other than Ig (9–11). It is not clear if cross-linking of demonstrated or postulated receptor structures is also essential for these agents to transform resting B cells into activated cells.

Recently we have described a model system in which a monoclonal antibody (MAb) to a murine B lymphocyte differentiation antigen (Lyb-2) triggers B cell activation (12). We have shown that under serum-free culture conditions, MAb to the Lyb-2 molecule transform small B cells into blast cells and induce B cell proliferation. Here we investigate the possibility that this B cell activation process requires cross-linking in addition to the binding of antibody to the Lyb-2 molecules. We have prepared divalent F(ab')2 fragments and monovalent Fab' fragments and tested their ability to induce B cell-specific blast transformation and proliferation. Our results demonstrate that monovalent anti-Lyb-2 is sufficient to induce B cell enlargement and proliferation so we conclude that cross-linking of Lyb-2 molecules is unnecessary for B cell activation.

Materials and Methods

Animals. DBA/2J and C3H/HeJ mice were obtained from The Jackson Laboratory, Bar Harbor, ME. BALB/c 1cr mice were obtained from the Laboratory Animal Facility of the Institute for Cancer Research, Philadelphia, PA.

Preparation of Antibodies. Anti-Lyb-2 (γ5κ) MAb was purified from ascites from mice bearing the hybridoma clone 10.1.D2 as described earlier (12). Anti-κ antibodies were purified from the serum of goats immunized with the mouse Igκ myeloma protein.

This work was supported by grants AI-15879 and CA-06927 from the National Institutes of Health and by the Commonwealth of Pennsylvania. Current address of B. Subbarao is the Department of Microbiology and Immunology, University of Kentucky, Lexington, KY 40536.
PC8916. Immune goat sera were extensively absorbed with IgG1 and IgG2a mouse myeloma proteins to remove anti-light chain antibodies. Anti-μ antibody was affinity purified from the negatively absorbed antiserum on an HPCM27 IgMx column. Affinity-purified goat anti-μ antibodies were passed over a protein A-Sepharose column at pH 8.5 and the protein A-bound fraction was eluted with acid and concentrated.

Preparation of F(ab')2 and Fab' fragments. Anti-Lyb-2 MAb and the protein A-binding fraction of goat anti-μ antibodies were digested with pepsin at an enzyme to protein ratio of 1:50 at 37°C for different time periods (13). The pepsin digest was analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to monitor the appearance of species with the molecular weight of F(ab')2 fragments. Digests containing appreciable amounts of such fragments were passed through a protein A-Sepharose column at pH 8.5 to remove the intact antibody from the F(ab')2 fragments (14). Material that did not bind the column was concentrated and used as F(ab')2 fragments. This preparation had no undigested intact antibodies as shown by SDS-PAGE analysis. Fab' fragments were prepared by reduction of F(ab')2 fragments with 0.02 M dithiothreitol at room temperature for 1.0 h and then alkylated with 0.024 M iodoacetamide at 37°C for 15 min (15). All fragments were dialyzed against saline, 0.22-μm filter-sterilized, and stored at 4°C until further use.

 Cultures and Media. Spleen cells or splenic B cell preparations (see below) were cultured in serum-free Iscove's/Ham's F12 medium (16) at 37°C either at 2.5 × 10^6 cells/ml in 24-well Costar 3424 plates (Data Packaging, Cambridge, MA) or at 5 × 10^6 cells/0.2 ml in 96-well Costar 3596 plates in a 5% CO2 atmosphere for 2–4 d. Supernatant from concanavalin A-activated rat spleen cells (Con A SN) was prepared by culturing 5.0 × 10^6 spleen cells in 1.0 ml serum-free Iscove's/Ham's F12 medium with 2.0 μg/ml Con A for 24 h and collecting the SN. Con A SN was filtered and stored frozen at -20°C. Free Con A was blocked by the addition of 20 mM α-methylmannoside.

 Cell Separations. Adherent cells were depleted by incubating spleen cells in 60-mm plastic dishes for 1.0 h at 37°C in RPMI 1640 medium with 5% fetal calf serum and then passing the nonadherent cells through prewarmed Sephadex G-10 columns once or twice according to the procedure of Ly and Mishell (17). Depletion of adherent cells was assessed by staining for nonspecific esterase. Adherent cell-depleted populations contained <0.5% esterase-positive cells. T cells were depleted by treatment with monoclonal anti-Thy-1.2 from the clone 13-4 (18) and monoclonal anti-Lyt-2 from the clone 3.168 (19) plus absorbed rabbit complement (Low-Tox M; Accurate Chemical & Scientific Corp., Westbury, NY).

 Assays for B Cell Activation and Maturation. Two assays were used to measure early steps of B cell activation. First, the increase in the size of B cells was determined by flow cytometry. Spleen cells cultured with various antibody preparations for 2 d were harvested, washed, and stained with an F(ab')2 fragment of a fluoresceinated goat anti-mouse Ig (Cappel Laboratories, Cochranville, PA) to detect B cells and with ethidium bromide to detect dead cells. The forward light scatter of 25,000 viable B cells was determined. Small lymphocytes gave a unimodal light scatter distribution centered at channel 400 with the amplifier gain set at four and a 400 mW argon laser output. Cells with light scatter greater than channel 650 were defined as blast cells and their percentage calculated. A second assay for B cell activation was for proliferation, which was determined by measuring the incorporation of [3H]thymidine (6.0 Ci/mM; New England Nuclear, Boston, MA) over a 4-h period after 48 h of culture. Finally, maturation to antibody secretion was determined after the addition of Con A SN by using the protein A plaque-forming cell (PFC) assay (20) with rabbit anti-μ antibodies added to detect IgM-secreting cells.

 Results and Discussion

 Comparison of the Ability of Fab' Fragments of Anti-μ and Anti-Lyb-2.1 MAb to Induce Blastogenesis in Small B Cells. To assess the importance of receptor cross-linking in an early step of B cell activation, we measured the increase in B lymphocyte volume after exposure to monovalent or divalent fragments of either anti-μ (21) or anti-Lyb-2 antibodies. Spleen cells were cultured with either F(ab')2...
Monovalent Fragments of Anti-Lyb-2.1 MAb but not of Goat Anti-μ Antibodies Induce Blast Transformation of B Lymphocytes

| Addition to culture* | DBA/2 B cells (Lyb-2.1) | BALB/c B cells (Lyb-2.2) |
|---------------------|--------------------------|--------------------------|
|                     | Percent blast cells% | [3H]Thymidine incorporation | [3H]Thymidine incorporation |
| Control Ig          | 15.7 ± 2.3              | 4.5 (1.09)                | 2.3 (1.25)                 |
| Anti-Lyb-2.1 MAb    | 35.7 ± 3.1†            | 55.7 (1.01)               | 2.5 (1.20)                 |
| Fab′ anti-Lyb-2.1 MAb | 29.0 ± 4.0†          | 27.1 (1.03)               | 3.8 (1.03)                 |
| Fab′ anti-Lyb-2.1 MAb | 30.5 ± 2.1†          | 33.0 (1.00)               | 4.8 (1.19)                 |
| Fab′ goat anti-μ    | 43.0 ± 4.6†            | 50.3 (1.17)               | 29.3 (1.09)                |
| Fab′ goat anti-μ    | 17.3 ± 3.1              | 6.2 (1.11)                | 2.8 (1.03)                 |

* The antibodies and the fragments were used at a concentration of 50 μg/ml.
† 2.5 x 10⁶ spleen cells were cultured in 1.0 ml of Iscove’s/F12 serum-free medium for 48 h at 37°C in a 5% CO₂ atmosphere. Blast cells were enumerated by flow cytometry as described in Materials and Methods.
‡ Spleen cells were cultured at the concentration of 5 x 10⁶/0.2 ml culture in serum-free Icsove’s/F12 medium in Costar 3596 plates. After 48 h, cultures were pulsed for 4 h with 1.0 of [3H]-thymidine, harvested onto filter discs, and counted. Results are expressed as the geometric mean (×10⁻³) of values from triplicate cultures with SE in parentheses.
† A two-tailed Student’s t test was used to evaluate the statistical significance of the percent blast cells in the experimental groups vs. the control Ig group. Values indicated are significantly different from control Ig with P < 0.05.

or Fab′ fragments in serum-free Icsove’s/Ham’s F12 medium for 48 h and the number of B cell blasts was determined as described in Materials and Methods. As can be seen from Table I, both F(ab′)₂ and Fab′ fragments of anti-Lyb-2 MAb were able to induce a substantial fraction of B cells to increase in size and the antibody fragments were nearly as effective as the intact antibody. Only the surface Ig⁺ lymphocytes were stimulated to blast transformation in these cultures (data not shown) and the response was restricted to Lyb-2.1⁺ strains. In contrast to these results, monovalent Fab′ fragments of goat anti-μ antibodies were unable to increase the percent of B cell blasts in these serum-free cultures even though the Fab′ anti-μ was very effective in inducing the blast cell transformation of B cells. The viability of the cells after 2 d of culture in serum-free medium was similar in all groups and ranged from 70 to 80%. Further, the recovery of cells did not vary significantly in the different groups, suggesting that these effects of antibody fragments are due to induction of blastogenesis rather than the selective survival of blast cells in the critical groups.

B Cell Proliferation Is Induced by the Fab′ Fragment of Anti-Lyb-2 MAb. We have shown previously (12) that intact anti-Lyb-2.1 MAb induces B cell proliferation. Since the monovalent antibody fragments were able to activate B cells into blast cells, we investigated if they would induce proliferation and if the Fab′ fragments retained the specificity for the Lyb-2.1 allele. Results in Table I show that this indeed is the case; that is, Fab′ anti-Lyb-2 induced DBA/2 B cells to proliferate to at least the same extent as the intact antibody. As expected (4–6), the F(ab′)₂ anti-μ but not the monovalent Fab anti-μ was able to induce proliferation. Since the proliferation induced by Fab′ anti-Lyb-2.1 MAb in the BALB/c spleen cells (Lyb-2.1⁺) was slightly above background, it was formally possible that the fragment contained very low levels of contamination with lipopolysaccharide (LPS). This explanation seems to be unlikely for two reasons. First, the extent of proliferation induced by the Fab′ or anti-Lyb-2.1 MAb in the LPS-
unresponsive C3H/HeJ (Lyb-2.1) mice was of almost the same magnitude as observed with BALB/c mice (data not shown). Second, the dose-response relationships for intact antibody and Fab' fragments are similar (data not shown). We think it is unlikely that the biologic effects of Fab' fragments of anti-Lyb-2.1 MAb can be attributed to redimerization of the reduced and alkylated fragments. Gel electrophoresis of Fab' fragments of anti-Lyb-2.1 under nondenaturing conditions showed no evidence of any higher molecular weight species. The lack of activity of Fab' anti-μ demonstrates that goat Fab' fragments do not dimerize under our serum-free culture conditions, and we assume that mouse Fab' fragments behave similarly. Finally, Dower, Ozato, and Segal (22) have shown functional monovalency of mouse Fab' fragments prepared by methods identical to ours.

The nature of B cell activation achieved by the Fab' anti-Lyb-2.1 MAb or by the whole antibody appear to be similar, since in both cases the activated B cells mature into Ig-secreting cells in the presence of factors from Con A SN (Table II). Once again, monovalent fragments of anti-μ did not synergize with Con A SN to induce polyclonal activation of B cells. Fab' anti-μ antibody did have a negative influence in that the background response in the presence of Con A SN alone was diminished substantially. The small response to the fragments of anti-Lyb-2 antibody alone (i.e., in the absence of Con A SN) may be due to direct activation of a small number of preactivated cells in the cultures.

**Cellular Requirements for the Proliferation Induced by Fab' of anti-Lyb-2.1 MAb.** Even though the Fab' fragments of anti-Lyb-2.1 MAb cannot cross-link the Lyb-2 molecules by themselves, it is possible that they may do so with the assistance of accessory cells such as adherent cells or T cells. This possibility was tested by evaluating the ability of Fab' anti-Lyb-2.1 MAb antibody to induce proliferation in spleen cells that were depleted of T cells or adherent cells. The proliferation induced by the Fab' fragment of the anti-B cell MAb was not at all diminished by the removal of the adherent cells or T cells, suggesting that the B cells are being stimulated directly by the monovalent antibody fragments (data not shown). By contrast the same T-depleted or adherent cell-depleted cells were substantially reduced in their proliferative response to concanavalin A.

Our results demonstrate that monovalent Fab' fragments of anti-Lyb-2.1 are at least as effective as the intact antibodies in stimulating a fraction of normal B cells to become blast cells and another or the same fraction to proliferate. It appears unlikely that monomeric anti-Lyb-2 fragments are indirectly aggregated at the surface of an accessory cell, since the Fab' fragments appear to be effective in the apparent absence of T cells and adherent cells. In addition, we have direct evidence to show that apparently normal numbers of Lyb-2 molecules are still detectable on spleen cells after in vitro culture with intact anti-Lyb-2.1 MAb for 24 h (our unpublished observations), suggesting that the antibody does not induce Lyb-2 aggregation and endocytosis or shedding. It appears that in this system, receptor occupancy is sufficient to provide an activation signal to B lymphocytes and that cross-linking of receptors or receptor modulation is not required. It is possible that monovalent or divalent antibody binding to Lyb-2 activates local aggregation via an allosteric effect on the membrane-bound domain of Lyb-2. We thus can be confident that cross-linking of Lyb-2 is not a requirement for B cell activation, but local aggregation of Lyb-2 molecules by a
mechanism not dependent on multivalent ligands cannot be excluded.

Do Lyb-2 molecules have a functional role in the physiology of B lymphocytes? Based on the analogy that anti-receptor antibodies may mimic the action of the ligand that binds the receptor, we suggested (12) that Lyb-2 is a receptor for a B cell growth factor (12). It seems unlikely that small molecular weight ligands can induce receptor cross-linking unless binding takes place in the context of a large, multivalent carrier molecule. We thus assert that functional activity as a monovalent ligand might be expected of a small, polypeptide growth factor, and that Lyb-2 should be viewed tentatively as a receptor in search of its ligand.

Summary

Although activation of B lymphocytes by antigen or anti-Ig antibody has been shown to require cross-linking of surface Ig molecules, cross-linking is not necessary for B cell activation by anti-Lyb-2 monoclonal antibody (MAb). Monovalent Fab' fragments of anti-Lyb-2 MAb are as effective as the intact antibody in inducing blast cell transformation of small B cells and B cell proliferation in the apparent absence of T cells and adherent cells. In the presence of factors from T cells, B cells activated by Fab' fragments of anti-Lyb-2 MAb were induced to mature into Ig-secreting cells. Since monovalent Fab' fragments probably cannot induce receptor aggregation, it appears that receptor occupancy is sufficient to induce B cell activation with anti-Lyb-2 MAb.

We thank Kathy Feinstein, Susan Swetavage, and Eileen Walsh for their technical assistance, Peter Lopez for the fluorescence-activated cell sorter analysis, and Donna Platz for preparing the manuscript.

Received for publication 23 December 1983 and in revised form 22 March 1984.

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