B CELL ACTIVATION

I. Anti-Immunoglobulin-induced Receptor Cross-linking Results in a Decrease in the Plasma Membrane Potential of Murine B Lymphocytes*

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Although the nature of the antigen receptors on B lymphocytes was proposed by Ehrlich (1) as early as 1900 and demonstrated to be immunoglobulin (Ig) by Moller (2) over 20 years ago, very little is known about its precise function in B cell activation. Speculation regarding the role of membrane-associated Ig (mIg)[1] in antigen-specific B cell activation ranges from transmission of the first signal for proliferation and differentiation (3) to serving merely as a bridge to focus antigen-specific T cell help or mitogenic moieties of thymus-independent antigens to secondary receptors which in turn transduce activating signals (4–6).

An active role for mIg in signal generation is supported by the ability of anti-Ig reagents to polyclonally stimulate B cells to divide, as first demonstrated by Sell and Gell (7) and later extended by others (8–12). These results suggested that cross-linking of surface Ig generates a signal sufficient to stimulate proliferation of B cells. Differentiation into antibody-secreting cells, however, appeared to require a second signal(s) supplied by T cells or by supernatants from activated T cells (13, 14).

With the recent observations by Howard et al. (15) demonstrating that, in the absence of T cells or T cell-derived factors, B cells do not undergo proliferation in response to anti-Ig reagents, the function of mIg in B cell activation is again unclear. In view of these observations, we undertook this study to evaluate the role of mIg in the mediation of a very early event in B lymphocyte activation, decreased plasma membrane potential (depolarization).

Plasma membrane depolarization has been shown to be an initial and pivotal event in activation and entry into the mitotic cycle in various somatic cell systems (16–18) and is believed to be an early event in mitogen-induced T and B lymphocyte activation as well (19, 20). In this study, we demonstrate that very soon (detectable within 5 min) after exposure to anti-Fab, B lymphocytes undergo a measurable

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[1] *Abbreviations used in this paper:* AO, acridine orange; Con A, concanavalin A; $DiOC_5[3]$, 3,3′-dipentyloxacarbocyanine iodide; $DxSO_4$, dextran sulfate; FCS, fetal calf serum; LPS, lipopolysaccharide; mIg, membrane-associated immunoglobulin; PHA, phytohemagglutinin.

decrease in membrane potential. Membrane depolarization occurs in >80% of splenic B cells in response to anti-Fab. Finally, the ineffectiveness of monovalent anti-Fab to induce detectable changes in membrane potential suggests that receptor Ig cross-linking is necessary to induce this response.

Materials and Methods

Mice. BALB.k mice were obtained from Dr. Frank Lilly of the Albert Einstein School of Medicine, New York, and bred in our facility. (C57BL/6 × DBA/2)F_1 mice ($B6D2F_1$) were purchased from The Jackson Laboratory, Bar Harbor, ME. Mice used for all experiments were 6–8 wk old.

Antibodies. Supernatants from hybridoma T24 anti-Thy-1 (a gift from Dr. Ian Trowbridge) (21) and hybridoma HO 13.4.9. anti-Thy-1.2 (Salk Institute, San Diego, CA) (22) were titered and used throughout this study for depletion of T cells. Monoclonal anti-Qa2, the secreted product from hybridoma D3-262 (S. Tonkonogy, personal communication) was a gift from Dr. Sue Tonkonogy, Duke University Medical Center, Durham, NC). Alloantisera with specificity against mouse $H\text{-}2D^d$ was the kind gift of Dr. Alan Whitmore, Duke University Medical Center.

Culture Conditions. Spleens were removed aseptically from animals killed by cervical dislocation. They were then teased apart and forced through a fine-mesh steel screen. Erythrocytes were removed by treatment with Gey's solution. Resulting cells were washed three times in balanced salt solution. T cells were removed by treatment with anti-Thy-1.2 and anti-Thy-1 plus complement. The resulting population, henceforth referred to as B cells, was washed three times in balanced salt solution and cultured at indicated concentrations in RPMI 1640 containing 10% fetal calf serum (FCS) (lot 100273; Sterile Systems, Logan, UT), glutamine, streptomycin, penicillin, 5×10^{-5} M 2-mercaptoethanol, nonessential amino acids, sodium pyruvate, insulin, and oxaloacetic acid.

Mitogens. Lipopolysaccharide (LPS) (lot 3123-25; Difco Laboratories Inc., Detroit, MI) was used at 50 μg/ml in all experiments. Dextran sulfate ($DxSO_4$) (500,000 mol wt; Gibco Laboratories, Grand Island, NY) was used at 20 μg/ml in all experiments.

Preparation of Anti-Fab. Rabbit anti-mouse Fab was raised by subcutaneous injection of 100 μg of Fab fragments of normal mouse Ig in complete Freund's adjuvant. Anti-Fab antibodies were affinity purified using normal mouse IgG-Sepharose with elution using 3.5 M $MgCl_2$. The resulting antibody was passed over a staphylococcal protein A-Sepharose column. The bound protein was eluted with 0.1 M sodium acetate, pH 4.3. Pepsin digestion of the resulting anti-Fab antibody was accomplished using standard methods (23). Purified $F(Ab')_2$ fragments were isolated by passing the neutralized digest over the staphylococcal protein A-Sepharose column, which retained the undigested Ig.

Preparation of Fab Fragments of Rabbit Anti-Mouse Fab. The affinity- and protein A-purified rabbit anti-mouse Fab was digested with papain using standard procedures. Purified Fab fragments were isolated by passing the digest over staphylococcal protein A-Sepharose.

[^{3}H]Thymidine-incorporation Assay. Triplicate cultures of 2×10^5 viable B cells were incubated in 200 μl media in 96-well, flat-bottomed microtiter plates (3595; Costar, Data Packaging, Cambridge, MA) at 37°C, 7% CO_2. Cultures were pulsed with 1 μCi tritiated thymidine and 18 h later harvested onto filter paper disks with a MASH harvester (Flow Laboratories, Inc., McLean, VA). The incorporated radioactivity was counted by liquid scintillation spectrophotometry.

Acridine Orange (AO) Cell Cycle Analysis. The straining procedure of Darzynkiewicz et al. (24, 25) was used for determination of cell entry into cycle (G_0–G_1 transition). Briefly, this involves incubation of cells after mild EDTA-detergent treatment at low pH (0.1% Triton X-100, 1 mM EDTA, 0.1 N HCl, pH 3.8) in an aqueous solution of AO (13 μM). Because of the differential binding characteristics of AO to single- and double-stranded nucleic acids, DNA fluoresces green, whereas RNA fluoresces red upon excitation by 488-nm laser light. Relative integrated red and green fluorescence was determined using the Cytofluorograf System 50-H (Ortho Diagnostic Systems Inc., Westwood, MA) equipped with a 5-W argon laser. Entry into cell cycle was assessed by measuring the increase in RNA content (red fluorescence) indicative of

entry into G_1 (26). Hydroxyurea (2 mM) was routinely included in the cultures to prevent cell division. For detection and delineation of doublets and higher aggregates of stained cells, the integrated red (RNA) fluorescence intensity was measured as a function of the pulse width of the green (DNA) signal as described by Sharpless et al. (27). The cell concentration in stained samples was adjusted to $3–5 \times 10^5$ cells/ml. A flow rate of 100 cells/s was generally maintained.

Determination of Relative Membrane Potential. Cytofluorimetric determination of the relative membrane potential of individual B lymphocytes was performed using the procedure of Shapiro et al. (28) with some modifications. Briefly, cells were stimulated in media containing 10% FCS. After stimulation for the duration indicated, the cells ($1–3 \times 10^5$) were centrifuged and resuspended in 1.0 ml of medium without FCS but containing the stimulating antigens or mitogens. 10 μl of 5.0 μM 3,3′-dipentyloxacarbocyanine iodide ($DiOC_5[3]$) (a gift from Dr. Howard Shapiro, Sidney Farber Cancer Institute, Boston, MA) in dimethylsulfoxide was added to each sample and allowed to equilibrate for 10 min at room temperature. After incubation, the cells were analyzed by flow cytofluorimetry using 488 nm illumination at 250 mW power. Integrated fluorescence (520–550 nm) was used as a measure of relative membrane potential. Depolarized cells contain less cell-associated dye than control cells and thus exhibit decreased fluorescence intensity. Forward, narrow-angle light scatter was used as a second parameter to facilitate gating and thereby elimination of dead cells from the analysis (29). In experiments where membrane potential changes were examined <15 min after addition of the stimulating agent, cells were preequilibrated by exposure to dye for 15 min before stimulation. Cells preequilibrated with dye in this way release dye upon depolarization, which results in decreased individual fluorescence intensity relative to control cells (30). For experiments designed to assess the effect of 50 mM K^+ on B cell membrane potential, 20 μl of a 2,500 mM KCl aqueous solution was added per milliliter of media. Results obtained in these experiments were equivalent to those in which media tonicity was corrected after addition of K^+ (data not shown).

Results

Anti-Fab Induces Changes in B Lymphocyte Membrane Potential. It has been demonstrated using microelectrodes (31) and membrane-potential–sensitive dyes (19) that mitogen activation of T and B lymphocytes is accompanied by depolarization of the plasma membrane. It has been suggested (18) that a causal relationship may exist between plasma membrane depolarization and entry into cell cycle. In view of the potential importance of membrane depolarization in activation, we investigated the effect of anti-Fab on B cell membrane potential. We used a method described by Shapiro et al. (28) that allows measurement of relative membrane potential by flow cytometry using membrane-potential–sensitive, charged dyes (30, 32). In particular, we used the dye $DiOC_5[3]$, a lipophilic, cationic dye that partitions between cells and their suspending medium in a membrane-potential–dependent fashion. More dye is taken up by cells upon hyperpolarization, when the interior becomes more electronegative with respect to the exterior. Conversely, less dye is taken up by cells that have undergone depolarization.

As a control in these studies, we incubated murine B lymphocytes in medium supplemented with 50 mM K^+ for 10 min, followed by staining and cytofluorimetric analysis. This concentration of external K^+ has been shown to cause depolarization of murine spleen cell membranes (33). As shown in Fig. 1, the K^+-treated B cells exhibit significantly less fluorescence intensity relative to the control B cells in normal media. Comparison of the histograms for control and K^+-treated B cells reveals that the entire curve is shifted to the left, which suggests that virtually all of the cells have decreased plasma membrane potential as a result of this treatment.

We then tested the effect of anti-Fab on the relative membrane potential of B cells

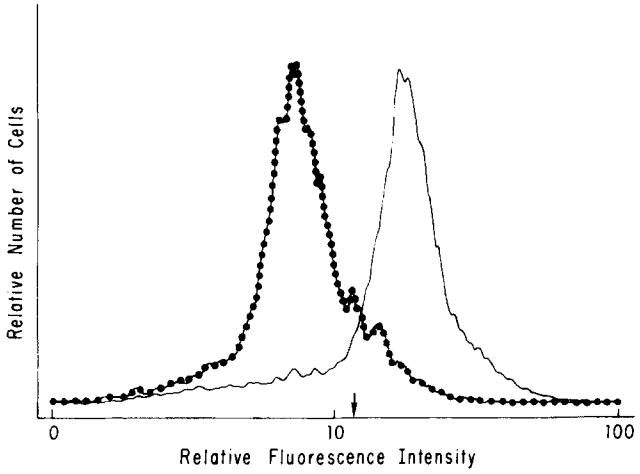

FIG. 1. K^+-induced depolarization of murine B lymphocytes. Murine B cells (2×10^5) were cultured for 2 h in 1 ml of media supplemented (●) or not supplemented (——) with 50 mM K^+. After incubation, the cells were stained with the membrane-potential-sensitive dye $DiOC_6[3]$ and analyzed cytofluorimetrically using the parameters forward, narrow angle light scatter to facilitate exclusion of dead cells from the analysis and the log of integrated green fluorescence for relative membrane potential. For each analysis, 10,000 cells were analyzed.

by incubating the cells with 25 μg/ml of anti-Fab and conducting cyanine dye analysis 2 h later. As shown in Fig. 2*a*, anti-Fab induced a decrease in the B cell plasma membrane potential within 2 h of exposure to ligand that was comparable to the decrease induced by potassium. However, in contrast to the histogram for the K^+-depolarized B cells, which exhibited a uniform shift, the histogram for the anti-Fab-depolarized cells was bimodal. The population with higher fluorescent intensity exhibits staining equivalent to unstimulated control cells, which suggests that this subpopulation of cells was not depolarized by the anti-Fab reagent. By integrating the histograms above and below the inflection point between cells with high fluorescence intensity and those with low fluorescence intensity (depolarized), the proportion of cells depolarized within a given population can be determined. Such an analysis of cell populations shown in Fig. 2*a* revealed that 80% of the population was depolarized in response to anti-Fab antibody exposure.

To determine whether the observed anti-Fab-induced B cell depolarization was the result of some attribute of $F(ab')_2$ fragments of rabbit Ig unrelated to their antigen specificity, we assessed the effect of $F(ab')_2$ fragments of normal rabbit Ig on membrane potential. As can be seen in Fig. 2*b*, when used at doses equivalent to those at which anti-Fab causes B cell depolarization, $F(ab')_2$ fragments of normal rabbit Ig had no detectable effect on B cell membrane potential.

To determine whether divalent antibody interaction with any membrane protein moiety is sufficient to cause membrane depolarization, we incubated $B6D2F_1$ B cells with 25 μg/ml of hybridoma anti-Qa-2 or alloantibody against $H\text{-}2D^d$. The monoclonal anti-Qa-2 reagent binds ~70% of B cells from $B6D2F_1$ mice (J. Monroe, unpublished observations). As can be seen in Fig. 2*b*, anti-Qa-2 had no effect on membrane potential, which suggests that anti-Fab-induced depolarization is due to specific interaction with membrane Ig. Similarly, when used at the optimal staining concentration, alloantisera against $H\text{-}2D^d$ also had no detectable effect on membrane

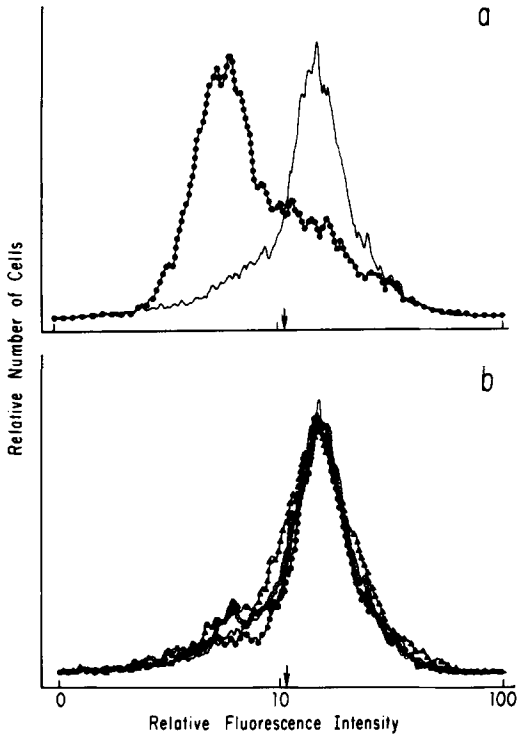

FIG. 2. Effect of anti-Ig on relative B lymphocyte membrane potential. (*a*) B6D2F$_1$ B cells (2×10^5/ml) were cultured for 2 h in media with (●) or without (——) 25 μg/ml of F(ab')$_2$ fragments of rabbit anti-mouse Fab. After incubation, the cells were suspended in 1 ml of media without FCS but containing anti-Ig and stained for membrane potential as described in the text. Stained cells were analyzed cytofluorimetrically using the parameters forward light scatter to facilitate exclusion of dead cells from the analysis and the log of integrated fluorescence for relative membrane potential. (*b*) B6D2F$_1$ B cells were treated as above except with no stimulus (——), 25 μg/ml anti-Qa-2 (○), 25 μg/ml of F(ab')$_2$ fragments of normal rabbit Ig (●), or an alloantisera anti-H-D^d (▲). In both panels the arrow designates the inflection point between depolarized (left) and nondepolarized (right) cells. In all cases, 10,000 cells were analyzed.

potential (Fig. 2*b*). Finally, in results not shown, F(ab')$_2$ fragments of rabbit anti-H-2K^k had no effect on membrane potential.

Dose-Response Relationship for Anti-Fab-induced Membrane of Depolarization Differs from the Dose-Response Observed for Thymidine Uptake. We next determined the dose-response relationship for anti-Fab-induced membrane depolarization. In Fig. 3 are the results of this experiment. Fig. 3*a* depicts frequency histograms generated after membrane potential analysis of B cells stimulated with various doses of anti-Fab. The results demonstrate that doses of anti-Fab ≥1 μg/ml are effective in inducing maximal frequencies of depolarized cells. At 0.1 μg/ml a significant but lower frequency of cells exhibits decreased membrane potential. Finally, no detectable change in membrane potential is observed at a dose of 0.01 μg/ml. It is noteworthy that anti-Fab-induced B cell depolarization appears to be an all-or-none event. The membrane potential of the cells depolarized by 0.1 μg/ml of anti-Fab is indistinguishable from that induced by higher doses. Furthermore, at doses below 0.1 μg/ml there is a sudden shift in absolute membrane potential towards resting potential levels rather than a

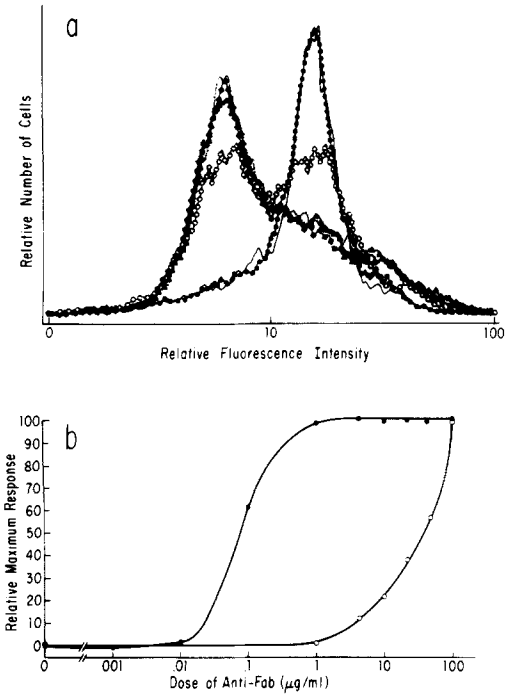

FIG. 3. Dose-response relationships for anti-Fab-induced membrane depolarization and [^{3}H]thymidine incorporation. (*a*) B6D2F$_1$ B cells (2×10^5/ml) were cultured in media for 2 h with 0 μg/ml (——), 0.01 μg/ml (●), 0.1 μg/ml (○), 1 μg/ml (▲), 10 μg/ml (–··–), and 50 μg/ml (◆) of anti-Fab. After incubation, the cells were stained with the membrane-potential-sensitive dye DiOC$_5$[3] and analyzed cytofluorimetrically using the parameters forward, narrow angle light scatter to facilitate exclusion of dead cells from the analysis and the log of integrated fluorescence for relative membrane potential. For each analysis, 10,000 cells were analyzed. (*b*) Relative membrane potentials were determined as described for *a*. Frequency histograms were generated and the frequency of cells depolarized was determined by superimposition of the histograms generated by stimulated and unstimulated cultures, followed by integration of the portion of the curve to the left (low fluorescence) of the inflection point. Backgrounds from unstimulated companion cultures were subtracted. Values are reported relative to the frequency depolarized by the maximum dose of anti-Fab, 100 μg/ml. For each point, 10,000 cells were analyzed. For relative [^{3}H]thymidine uptake, 2×10^5 B cells per culture were stimulated with varying doses of anti-Fab. After incubation, the incorporated radioactivity was determined as described in Materials and Methods. Backgrounds of unstimulated companion cultures were subtracted. The values reported here are relative to the maximum response to anti-Fab observed in this experiment, that elicited by 100 μg anti-Fab/ml. Each point is the mean of triplicate cultures.

gradual shift as the dose of anti-Fab is decreased. This result is observed even when membrane potential is assessed at doses intermediate between 0.01 and 0.1 μg/ml (data not shown).

In Fig. 3*b* is depicted a quantitative assessment of the relative frequencies of cells depolarized by various doses of anti-Fab. As discussed for Fig. 3*a*, 0.1 μg/ml of anti-Fab is sufficient to stimulate a suboptimal but nonetheless significant (61% maximal) proportion of the cells to exhibit decreased plasma membrane potential. Doses of anti-Fab ≥1 μg/ml stimulate maximal numbers of cells to become depolarized. These results indicate that levels of anti-Fab that are theoretically sufficient to saturate the B cell surface Ig (i.e., 1–5 μg/ml) are able to induce a maximum response as determined by membrane depolarization.

These results appear inconsistent with those of others (15) that demonstrate that doses of anti-Ig sufficient to saturate receptor Ig do not stimulate significant levels of B cell activation as assessed by thymidine uptake. To determine whether the inconsistency between these two observations was related to a difference in the ability of our anti-Ig preparation to act as a B cell stimulant, we determined the relative response of B cells to various doses of anti-Fab as measured by [^{3}H]thymidine incorporation. The results are presented in Fig. 3*b*. As can be seen, we too observed suboptimal stimulation as measured by thymidine incorporation at the lower doses of anti-Fab (1–5 μg/ml). Although maximal levels of depolarization are observed in this dose range, both in terms of frequency and magnitude (Fig. 3*a*), only very low levels of thymidine incorporation are stimulated (12% maximal at 5 μg/ml). These results, therefore, indicate that our anti-Ig reagent is similar to others (15) in terms of its dose dependence for B cell stimulation as measured by thymidine incorporation and further suggests that signaling requirements for depolarization and thymidine incorporation may differ.

Kinetics of Anti-Ig-induced Membrane Depolarization. To determine how rapidly B cells are depolarized in response to anti-Fab, we exposed B cells for varied lengths of time to 25 μg/ml of anti-Fab before analysis of relative membrane potential. The results (Fig. 4) indicate that by the earliest time of analysis (5 min), a significant frequency of cells was depolarized. The frequency of cells depolarized begins to plateau by 60 min after anti-Fab exposure. Consistent with observations in studies using mitogens (19), cells begin to repolarize within 6 h after stimulation.

Anti-Fab-mediated Membrane Depolarization Is Dependent upon Receptor Cross-linking. To determine whether receptor cross-linking by anti-Fab is required to facilitate membrane depolarization, we performed the following set of experiments. B cells were incubated with 0.1 μg/ml divalent anti-Fab in the form of the F(ab′)$_2$ fragments or monovalent Fab fragments of the same anti-Fab preparation, followed by staining

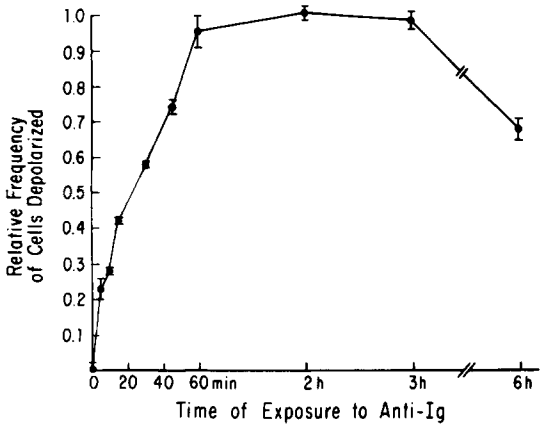

FIG. 4. Time course of anti-Ig induced membrane depolarization. B6D2F$_1$ B cells were cultured for various times with 25 μg/ml of anti-Fab before staining and cytofluorimetric determination of relative membrane potential. Cells to be stimulated for <15 min were preequilibrated with the dye before stimulation. Results are expressed as the mean of three independent experiments ± SEM relative to the frequency of cells depolarized after 2 h stimulation. Backgrounds of unstimulated companion cultures were subtracted. The frequency of cells depolarized was determined by integration of the fluorescence histograms as described in Fig. 3*b*. In all cases, 10,000 cells were analyzed.

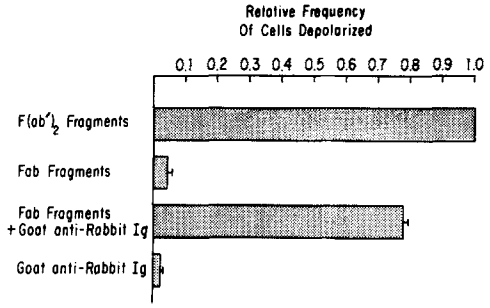

FIG. 5. Receptor cross-linking requirements for anti-Ig-induced membrane depolarization. B6D2F$_1$ B lymphocytes (2×10^6/ml) were cultured for 2 h in medium containing the indicated stimuli before staining and cytofluorimetric determination of relative membrane potential. F(ab')$_2$ and Fab fragments of the same preparation of rabbit anti-mouse F(ab')$_2$ were used at 0.1 μg/ml. Goat anti-rabbit Ig antiserum was used at a 1:20 dilution. Results are presented as the frequency of cells depolarized relative to that induced in control cultures by F(ab')$_2$ fragments of anti-Fab. Backgrounds of unstimulated companion cultures were subtracted. Proportion of cells depolarized was determined as described for Fig. 3*b*. Each data point represents the mean of three separate experiments ± SEM. Fab fragments and goat anti-rabbit Ig were not significantly different from background ($P > 0.1$). In all cases, 10,000 cells were analyzed.

and cytofluorimetric determination of relative membrane potential. As can be seen in Fig. 5, divalent molecules stimulated a significantly ($P < 0.001$) higher frequency of cells to undergo depolarization than did the monovalent antibody. The frequency of cells depolarized after exposure to Fab fragments was not significantly greater than background ($P > 0.1$). However, a significant ($P < 0.001$) proportion of cells underwent depolarization when the Fab-stimulated B cells were washed and then exposed to goat anti-rabbit Ig. The observed depolarization was not the result of B cell interaction with the secondary antibody alone, as the goat anti-rabbit Ig reagent had no significant ($P > 0.1$) influence on the observed membrane potential. Taken together, these results indicate that receptor cross-linking is required for anti-Fab-mediated plasma membrane depolarization.

Membrane Depolarization Precedes G_0–G_1 Transition of B Lymphocytes. Although these studies with anti-Fab and others with mitogens (19, 20) demonstrate membrane depolarization by cells in the responding populations, it is unclear whether this depolarization has any relevance to B cell proliferation and differentiation. To address this point, we sought to determine whether B cells, stimulated to enter cell cycle, first underwent membrane depolarization. We compared the frequency of B cells from two strains of mice that were depolarized by anti-Fab or LPS-DxSO$_4$ with the frequency that was stimulated to enter cell cycle under the same conditions. Entry into cycle was determined by AO cell cycle analysis as described previously (34). Cells were incubated in the presence of 2 mM hydroxyurea to prevent cell division and thus allow a more accurate determination of the proportion of cells entering cycle. An increase in cellular RNA content relative to cultured, unstimulated control cells was considered indicative of entry into cycle (26). The relative frequency of cells depolarized was determined as described for Fig. 2*a*. The results of three separate experiments are presented in Fig. 6. B cells from BALB.k mice are low responders to LPS-DxSO$_4$ relative to B6D2F$_1$ mice (J. Monroe and J. Cambier, unpublished observations). This difference is reflected by the frequency of cells stimulated to enter cycle (30 and 50%, respectively), which is paralleled by the frequency depolarized by these agents (36

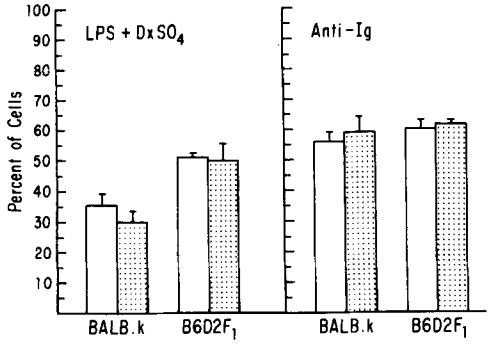

FIG. 6. Correlation between the frequency of cells depolarized by anti-Ig or mitogens and the frequency that undergoes G_0–G_1 transition. B cells from two strains of mice, BALB.k and B6D2F$_1$, were cultured in media containing LPS-DxSO$_4$ (50 and 20 μg/ml, respectively) or anti-Ig (25 μg/ml). Depolarization (light bars) was determined 2 h after initiation of cultures by cytofluorimetric analysis of DiOC$_5$[3]-stained cells. The proportions of cells depolarized were determined as described for Fig. 3*b*. Backgrounds from unstimulated companion cultures were subtracted. Frequencies of cells stimulated to undergo G_0–G_1 transition were determined 24 h after initiation of culture by cytofluorimetric analysis of AO-stained cells. The frequency of cells with increased RNA content indicative of entry into G_1 was determined by integration of red fluorescence (RNA) histograms using the Cytofluorograf distribution analyzer. Backgrounds of unstimulated companion cultures were subtracted. Values represent the means of three separate experiments ± SEM. In all cases, the frequency of cells depolarized is not significantly ($P < 0.01$) different from the frequency that undergoes G_0–G_1 transition. In all cases, 10,000 cells were analyzed per experiment.

and 51%, respectively). However, both strains respond equally well to anti-Fab, a point reflected by the frequency of cells from both strains stimulated to enter cycle (60 and 63%, respectively), which is again paralleled by the frequency depolarized (59 and 61%, respectively). In all cases, the frequency of cells depolarized by a given stimulus was not significantly different from the frequency that subsequently underwent G_0–G_1 transition ($P < 0.01$). These results suggest a correlation between plasma membrane depolarization and B cell activation as determined by G_0–G_1 transition. Further, these results suggest that transition from G_0 to G_1 does not occur unless the cell is first depolarized. However, consistent with the data presented in Fig. 3*b*, depolarization of the B cell plasma membrane alone is insufficient to stimulate G_0–G_1 transition, as 50 mM K^+, in the absence of additional stimuli, is unable to mediate this event (data not shown).

Discussion

In this report we describe studies which indicate that cross-linking of mIg by anti-Fab results in a decrease in B cell plasma membrane potential (depolarization). Furthermore, our results suggest that this depolarization event correlates with subsequent entry into cell cycle (G_0–G_1 transition) by anti-Fab- and mitogen-stimulated B cells. We conclude that cross-linking of surface Ig transmits a very early signal for B cell activation in the form of or resulting in membrane depolarization. Although the physiologic relevance of membrane depolarization is unknown, it has been suggested (35) that in pituitary cells, membrane depolarization facilitates the opening of Ca^{+2} channels, allowing Ca^{+2} influx. This hypothesis suggests a possible role for membrane depolarization in lymphocyte activation in view of the fact that Ca^{+2} is a likely candidate for the second messenger in lymphocyte activation (for review, see reference

36). Such a second messenger role for Ca^{+2} is suggested by studies indicating inhibition of phytohemagglutinin (PHA)-stimulated mitogenesis in Ca^{+2}-free media (36, 37). Ca^{+2}, via calmodulin, is believed to regulate pyruvate oxidation (38), protein kinase activity (39), and adenylate cyclase activity (38, 40), all of which are of critical importance in lymphocyte activation (36).

This report is not the first to link membrane depolarization with cell activation. Meissner and Schmelz (17) have demonstrated that pancreatic cells stimulated by glucose exhibit a slow, then rapid, depolarization to a plateau level before insulin release. This depolarization is followed by a repolarization and, finally, a slight hyperpolarization. Our results (Fig. 5) using anti-Ig-stimulated B lymphocytes are similar to those of Meissner and Schmelz (17) in that the observed depolarization requires 1 h to reach maximum levels. After 1 h, it has reached a plateau level that is maintained until 3 h post-stimulation, at which time a slow repolarization phase begins.

A relationship between membrane potential and mitotic activity has been suggested by Cone (18, 41, 42). His work demonstrates that cultured cells maintained at high membrane potential have depressed mitotic activity. In contrast, active proliferation occurs when there is a pronounced decrease, below a threshold level, in membrane potential (depolarization). Using concanavalin A (Con A)- and LPS-stimulated murine splenocytes, Kiefer et al. (19) demonstrated that responding cells undergo an early depolarization followed by repolarization and, finally, hyperpolarization. Again, we observe a similar repolarization beginning at 3 h after anti-Ig stimulation (Fig. 4).

An important role for membrane depolarization is also suggested by studies that demonstrate inhibition of PHA-stimulated blastogenesis of treated human peripheral blood lymphocytes by the K^+ ionophore valinomycin. The effect of valinomycin is believed to be inhibition of mitogen-induced changes in membrane potential (20). In data not shown, we have demonstrated that valinomycin inhibits anti-Fab-induced membrane depolarization (J. Monroe and J. Cambier, manuscript submitted for publication). These results, taken together, support our contention that an intimate relationship exists between depolarization and activation of anti-Fab- and mitogen-stimulated B cells, and, more specifically, that early depolarization of the plasma membrane is a prerequisite for activation or entry into cell cycle of stimulated cells. The significance of this initial depolarization event is unknown, although, as discussed above, it has been suggested that it mediates Ca^{+2} influx.

Some studies of changes in membrane potential after mitogen stimulation of T lymphocytes argue against depolarization being an event in lymphocyte activation. Tsien et al. (43), using an anionic oxonal dye, have demonstrated hyperpolarization but no detectable depolarization by mouse thymocytes in response to Con A and PHA. This result is in direct conflict with the results of Shapiro et al. (28), using cyanine dyes, Kiefer et al. (19), using a lipophyllic cation dye, and Taki (31), using microelectrodes; all demonstrated Con A- and/or PHA-induced depolarization of human and murine T lymphocytes. Tsien et al. (43) and Rink et al. (44) rationalize their results in the case of the cyanine dyes by claiming that the cationic dyes themselves depolarize lymphocytes. However, this depolarization is thought to occur only at dye concentrations >5,000 nM (30). Rink et al. (44) only demonstrate significant depolarization of mouse splenocytes at a final dye concentration >500 nM. In all of our experiments, a final dye concentration of 50 nM is used. Although the

conflict between the results of Tsien et al. (43) and others (19, 28, 31) has yet to be resolved, their results at present stand alone in light of the results from other laboratories using a variety of assay systems, all demonstrating an initial depolarization event during mitogen-induced lymphocyte activation.

It is not clear why all of the cells in populations stimulated with anti-Ig do not express decreased membrane potential (see Fig. 2*a*). Although the majority do, we generally observe that 5–20% of the population do not. It may be that this population is composed of non-Ig-bearing cells that would be unable to interact with the anti-Ig. Alternatively, these cells may be depolarized, but because they are slightly larger than most cells and therefore take up more stain, they fail to be scored as depolarized by our method. We believe that both of these alternatives contribute to the observed responses of these populations of B cells to anti-Ig.

In contrast to thymidine uptake, depolarization induced by 1 μg anti-Ig per ml is maximal both in the frequency of responding cells and the magnitude (degree of membrane depolarization). These results suggest that, unlike anti-Ig-induced thymidine uptake (11, 15), levels of anti-Fab which do not saturate surface receptors (i.e., <5 μg/ml) stimulate maximal responses as assessed by plasma membrane depolarization. These findings indicate that receptor Ig binding and the resultant membrane depolarization alone are insufficient to initiate thymidine uptake. It should also be noted that the low doses (0.1–1.0 μg/ml) of antibody which induce membrane depolarization do not induce capping, suggesting that mIg capping is unnecessary for generation of this signal.

Although the term depolarization has been used throughout this report in reference to an induced decrease in membrane potential, we do not wish to imply that the cells are completely depolarized (i.e., have a membrane potential of 0). This is surely not the case, as the anti-Ig-stimulated B cells, upon staining, are always significantly more fluorescent than dead cells, which suggests their absolute membrane potential is >0. However, the degree of depolarization is equivalent to that obtained by supplementation with 50 mM K^+. Our results are only interpreted to indicate that a change in membrane potential occurs upon anti-Ig cross-linking, which is in the direction of decreased membrane potential. The results of Cone et al. (18, 41) demonstrate that while cells entering cycle are depolarized relative to resting cells, they still maintain a low membrane potential difference with the cell interior being electronegative with respect to the exterior.

Summary

We report analyses of the effect of anti-Fab antibodies on plasma membrane potential of mouse B lymphocytes. Results indicate that divalent fragments of anti-Fab antibodies mitogenic for B cells stimulate membrane depolarization detectable by cytofluorometric analysis of 3,3′-dipentyloxacarbocyanine iodide-stained cells. Depolarization is detectable within 5 min of exposure to ligand and maximal within 1 h of exposure when ≥80% of splenic B cells exhibit decreased membrane potential. The ineffectiveness of monovalent Fab antibody fragments in inducing this event suggests that receptor immunoglobulin cross-linking is essential. Frequencies of cells induced to enter cell cycle, as assessed by acridine orange cell cycle analysis, are equal to those induced to depolarize by lipopolysaccharide plus dextran sulfate or anti-Fab, which suggests a relationship between these events. However, membrane depolariza-

tion is itself an insufficient signal to promote subsequent thymidine uptake, as evidenced by the fact that doses of anti-Fab that are suboptimal for thymidine uptake induce maximal depolarization. These results suggest that cross-linking of surface immunoglobulin on B cells may provide an initial signal for activation but is itself insufficient to drive B cell proliferation.

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