Distinct Patterns of Transmembrane Calcium Flux and Intracellular Calcium Mobilization after Differentiation Antigen Cluster 2 (E Rosette Receptor) or 3 (T3) Stimulation of Human Lymphocytes

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Abstract

We evaluated CD2 (E rosette) and CD3 (T3)-triggered activation of resting lymphocytes by measuring the intracellular free calcium concentration ($[Ca^{2+}]i$) of individual cells. The $[Ca^{2+}]i$ of indo-1-loaded cells was measured by flow cytometry and responses were correlated with cell surface phenotype. Stimulation with anti-CD3 antibody caused an increase in $[Ca^{2+}]i$ in >90% of $CD3^+$ cells within 1 min, and furthermore, the response was restricted to cells bearing the CD3 marker. In contrast, stimulation of cells with anti-CD2 antibodies produced a biphasic response pattern with an early component in $CD3^-$ cells and a late component in $CD3^+$ cells. Thus, the CD2 response does not require cell surface expression of CD3. In addition, stimulation of a single CD2 epitope was sufficient for activation of $CD3^-$ cells, whereas stimulation of two CD2 epitopes was required for activation of $CD3^+$ cells. Both the CD2 and CD3 responses were diminished in magnitude and duration by EGTA. However, ~50% of T cells still had a brief response in the presence of EGTA, indicating that the increased $[Ca^{2+}]i$ results in part from intracellular calcium mobilization, and furthermore demonstrates that extracellular calcium is required for a full and sustained response. Our results support the concept that CD2 represents the trigger for a distinct pathway of activation both for T cells that express the CD3 molecular complex and for large granular lymphocytes that do not.

Introduction

Current models of T cell activation propose a requirement for at least two signals, one for expression of interleukin 2 (IL-2)[1] receptors and another for IL-2 synthesis and secretion (1–4). Primary activation signals may be initiated through ligand binding to either of two different cell surface molecules, the CD3 (T3) complex or the CD2 (E rosette) receptor (1, 4–9). The CD3 complex is closely associated with the T cell receptor (TcR) and is thus found only on T cells (10). The CD2 molecule is found both on T cells and on larger granular lymphocytes (LGL) (11). Thus, CD3-induced stimulation has been proposed as an antigen-specific mechanism of T cell activation, while CD2-initiated activation might be independent of specific antigen (7, 8).

An increase in intracellular free calcium concentration ($[Ca^{2+}]i$) is thought to represent one mode of signal transmission from the cell surface to the cytosol (12). In T lymphocytes, binding of monoclonal antibody to the TcR or CD3 complex was shown to increase $[Ca^{2+}]i$ (13–15). More recently, binding of antibody to certain epitopes on the CD2 molecule has likewise been shown to increase $[Ca^{2+}]i$ in T cell clones (16). The increase in $[Ca^{2+}]i$ that occurs after antibody binding to the CD3 complex occurs within seconds, and is among the earliest biochemical responses detected. Increased $[Ca^{2+}]i$ can occur through at least two mechanisms. With CD3-triggered activation, there is evidence for a calcium flux across the plasma membrane (17, 18), and Borst et al. have suggested that the CD3 complex functions as a calcium channel (19). There is also evidence indicating mobilization of an intracellular calcium pool after cell surface binding of anti-CD3 antibody (20).

To date, there is no published evidence that unequivocally demonstrates that CD3 and CD2 represent trigger points for distinct pathways of T cell activation. For example, $CD3^+$ natural killer clones can be stimulated to proliferate by CD2 antibodies, whereas $CD3^-$ clones cannot (21). Both CD2- and CD3-driven proliferation are IL-2–dependent (6, 7). However, previous studies have shown that the proliferation induced by anti-CD2 antibodies is prevented if CD3 molecules are removed from the cell surface by antigenic modulation (7). In addition, others have found that CD3-induced changes in $[Ca^{2+}]i$ depend on membrane potential (22). In preliminary studies, we found that the increased $[Ca^{2+}]i$ and proliferation induced by anti-CD2 antibodies were both prevented by antigenic modulation of CD3, and furthermore, that both CD2- and CD3-induced changes in $[Ca^{2+}]i$ depend on membrane potential (unpublished data). CD3-induced proliferation requires the presence of accessory cells, whereas CD2-induced proliferation can occur in the absence of accessory cells, depending on the CD2 epitopes triggered (7, 9). Thus, other than possible differences in the requirements for accessory cells, the available evidence can be most easily interpreted to indicate that a single pathway is triggered by two different signals.

The purpose of this study was to evaluate CD2- and CD3-triggered activation of resting lymphocytes by measuring the $[Ca^{2+}]i$ of individual cells. This has been made possible with the development of indo-1 (23), a new fluorescent calcium indicator

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1. *Abbreviations used in this paper:* $[Ca^{2+}]i$, intracellular free calcium concentration; CD, clusters of differentiation antigens; IL-2, interleukin 2; LGL, large granular lymphocyte; NWT, nylon wool-purified T cell; PBL, peripheral blood lymphocyte; PE, R-phycoerythrin; TcR, T cell receptor.

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that is well adapted for flow cytometry. We provide direct evidence that CD2-triggered activation is indeed distinct and separable from CD3-triggered activation. In addition, LGL respond to CD2 but not CD3 stimulation, and moreover, after stimulation by CD2, the kinetics and epitope specificity of $CD3^-$ cells are distinct from those of $CD3^+$ cells.

Methods

Cells and reagents. Human peripheral blood lymphocytes (PBL) were isolated from heparinized blood by Ficoll-Hypaque density gradient centrifugation. T cells were enriched by filtration over nylon wool columns (24). Cells were suspended in RPMI-1640 containing 25 mM Hepes, pH 7.4, (Gibco, Grand Island, NY) and 2% heat-inactivated human AB serum (Pel-Freez, Brown Deer, WI). The total calcium concentration was 0.5 mM in this medium. The ionized calcium concentration was measured with an ion-selective electrode (model 1AC1, Radiometer, Copenhagen) and found to be 0.44 mM. In calcium depletion experiments, the usual extracellular to intracellular calcium concentration gradient was abolished by bringing the medium to 5 mM EGTA. This concentration of EGTA was found to decrease the ionized calcium concentration of the medium in the absence of cells to ~15 nM using quin2 (Calbiochem-Behring Corp., San Diego, CA) exactly as described (25).

Monoclonal antibodies. Cell surface antigens defined by the antibodies used in this study are described according to the Cluster Designation (CD) nomenclature adopted by the First and Second International Workshops on Leukocyte Differentiation Antigens (26). The following azide-free antibodies were used for experiments to study the effect on the $[Ca^{2+}]i$ on cells (Table I). Anti-CD3 [T, gp19-29] antibody G19-4 (Balb/c, IgG1) (27) was purified by DEAE chromatography and was used at 3 μg/ml, the minimum dose required to elicit a maximal calcium flux in normal T cells as was previously determined with the quin2 assay (unpublished data). For anti-CD2 stimulation, antibodies 9.6 and 9-1 were used alone or added simultaneously in combination. This combination of antibodies causes vigorous proliferation of T cells (unpublished data and reference 28). Anti-CD2 [T, gp50] antibody 9.6 (Balb/c, IgG2a) (29) was purified by protein A sepharose and used at a concentration of 10 μg/ml. Anti-CD2 antibody 9-1 ascites (Balb/c, IgG3) (28) was used at a final dilution of 1:2,000. This corresponds to a concentration of ~3 μg/ml as determined by protein electrophoresis of the ascites. Anti-CD7 [T, gp41] antibody G3-7 (Balb/c IgG1) (27) was used as a binding, nonactivating control either as dilutions of ascites or after purification by DEAE chromatography.

Direct immunofluorescence studies. The following azide-free antibodies were purified and conjugated with R-phycoerythrin (PE) as previously described (30): anti-CD2 PE-9.6 (29), anti-CD5 [T, gp67] PE-10.2 (31), anti-CD16 [Fc gamma receptor, p50-70] PE-FC-2 (27), anti-CD4 [T, gp55] PE-G19-2 (27), anti-CD8 [T, gp32] PE-G10-1 (27), and anti-CD20 [B, p35] PE-2H7 (32). Direct immunofluorescence analysis was done by incubating saturating amounts of PE antibody with indo-1-loaded PBL or nylon wool-purified T cells (NWT) at 22°C for 20 min. The cells were washed once and immediately analyzed. This procedure was used because it had no measurable effect on the basal $[Ca^{2+}]i$. In addition, excellent discrimination between PE-positive and negative cells was achieved with the mean fluorescence of positive cells at least 13-fold brighter than negative cells.

Table I. Monoclonal Antibodies Used in This Study

Antibody used	Cluster designation	Cell distribution	Similar antibodies
9.6	CD2	Pan T, some LGL (E rosette receptor)	$T11_1$, Leu 5
9-1	CD2	Same, activation epitope	$T11_3$, D66
G19-4	CD3	Mature T cells	T3, Leu 4
10.2	CD5	Mature T cells	T101, UCHT2
G3-7	CD7	Mature T cells	3A1
FC-2	CD16	LGL, neutrophils	Leu 11
2H7	CD20	Pan B	B1

Indo-1 assay. The synthesis and spectral properties of indo-1 were described by Grynkiewicz et al. (23). Our procedure for the indo-1 assay will be described in detail elsewhere (32a). Briefly, cells were loaded with the acetoxymethyl ester of indo-1 (Molecular Probes, Junction City, OR) using an initial concentration of 8 μM. This resulted in an intracellular indo-1 concentration of ~50 μM. Viability of indo-1-loaded cells exceeded 98% as determined by propidium iodide exclusion. After the loading procedure, the cells were washed, placed in fresh medium at 2.5×10^6/ml, and stored in the dark at room temperature until analysis. In some experiments, cells were stained with PE antibodies immediately before analysis. For each assay, indo-1-loaded cells were diluted to 1×10^6/ml with medium and equilibrated at 37°C. The cells were analyzed by flow cytometry at 400 cells/s, although this could vary by as much as 100 cells/s during the course of a 20-min experiment.

Flow cytometry. For three-color flow cytometry, an Ortho Cytofluorograph 50HH cell sorter was used with a 2150 computer (Ortho Diagnostic Systems Inc., Westwood, MA). Ultraviolet excitation was from an argon ion laser (Spectra Physics, Mountain View, CA) using 80 mW at 351–364 nm. Blue (480–520 nm, Corion Optics, Holliston, MA) and violet (383–407 nm, Omega Optical Co., Brattleboro, MN) band pass filters were used to collect indo-1 fluorescence emission after separation with a 430-nm dichroic mirror (Ortho Diagnostic Systems). 35 μs later, cells passed through the beam of a krypton ion laser (Spectra Physics, 531 nm excitation, 180 mW output) and the red (PE) fluorescence was monitored at 564–588 nm. Forward, narrow angle light scatter was used to gate on lymphocytes. Red (PE) fluorescence was displayed as a log-amplified signal while violet and blue (indo-1) fluorescence and forward scatter were recorded as a linear signal. The indo-1 ratio of violet to blue fluorescence is directly related to the $[Ca^{2+}]i$ and was digitally calculated in real time for each individual cell using a linear scale. The ratio was multiplied by a factor of 50 in order scale the data to the display. At the beginning of each experiment, the blue and violet photomultiplier settings were routinely adjusted so that the basal ratio was 0.4 (displayed as 20). The ratio subsequently did not vary by >4% during the course of a 4-h experiment.

The $[Ca^{2+}]i$ can be calculated from the indo-1 violet/blue ratio using the formula derived by Grynkiewicz et al. (23): $[Ca^{2+}]i = K_d \cdot (R - R_{min})/(R_{max} - R) \cdot (f2)/(b2)$, where $[Ca^{2+}]i$ = intracellular ionized calcium concentration (nanomolars); K_d = 250 nM for the intracellular dye; R = indo-1 violet/blue ratio; R_{min} = indo-1 violet/blue ratio of calcium-free dye; R_{max} = indo-1 violet/blue ratio of calcium-saturated dye; $f2$ = blue fluorescence intensity of calcium-free dye; and $b2$ = blue fluorescence intensity of calcium-saturated dye. We found the basal $[Ca^{2+}]i$ of T cells to be 131±8 nM (mean±SD) using this technique (32a), which compares favorably with the value obtained by the quin2 technique (25). For the purposes of this study, results are presented as the primary indo-1 blue/violet ratio rather than the derived calcium concentration.

Data were collected on the 2150 "time mode," in which the y-axis of a histogram represents the indo-1 violet/blue fluorescence ratio, the x-axis represents units of time, and the z-axis represents cell number (Fig. 1 *A*). For isometric displays, small variations in the cell analysis rate were arithmetically normalized and the data smoothed before display. In experiments in which cells were also stained with PE-antibodies, the indo-1 violet/blue ratio was displayed for cells falling within specified regions of red (PE) fluorescence vs. time. The histograms were analyzed by programs that calculated the mean indo-1 violet/blue fluorescence ratio vs. time. In addition, the percent responding cells vs. time was analyzed by programs that first determined the value of the indo-1 ratio, which was 2 SD above the mean ratio of control cells, and then plotted the percent of cells above this threshold value vs. time. There are 100 data points on the x (time) axis on plots of either mean indo-1 ratio or percent responding cells.

Results

The $[Ca^{2+}]i$ of unstimulated T cells measured by the indo-1 ratio had a normal distribution, and there was no detectable change during the first 18 minutes after the addition of an anti-CD7 pan T cell control antibody, whether tested in the form of purified antibody or as dilutions of ascites (Fig. 1 *B*). Thus, in contrast to the spectrofluorimetric assays, which commonly employ quin2, there was no detectable interference from the fluorescent pigments in ascites.

The time course for the development of changes in $[Ca^{2+}]i$ after CD3 or CD2 stimulation is shown in Fig. 1, *C* and *D*. A rapid onset response occurred within 1 min after addition of anti-CD3 antibody, and this reaction was completed within 10 min (Fig. 1 *C*). In contrast, a biphasic response was observed after addition of CD2 antibodies that required ~15 min to complete. The first phase of the response was evident within 3 min, and the second phase occurred after six min (Fig. 1 *D*). The first response involved fewer cells and was lower in magnitude than the second response. The time to 50% maximal response after CD3 or CD2 stimulation in 12 independent experiments was 0.9±0.2 min (mean±SD) and 6.5±2.0 min, respectively. This kinetic difference was present in the cells from each donor tested. When cells were stimulated with suboptimal doses of CD3 antibody, the response was always more rapid than the CD2 response (data not shown). In addition, the CD3 response was never biphasic.

The cell surface phenotype of the responding cells was determined by three-color flow cytometry with the use of PE antibodies and a second laser. We found that all PBL responding to the CD3 stimulus were $CD5^+$ (Fig. 2, *A* and *B*). The CD5 antigen was chosen as a nonactivating marker for $CD3^+$ T cells because there is virtually 100% coordinate expression of the two antigens on normal T cells (27). The change in indo-1 ratio of the CD5-positive cells accounted for the entire response of the combined population of $CD5^+$ and $CD5^-$ cells (Fig. 2 *C*). When the response of individual cell populations was analyzed, >90% of the $CD5^+$ cells had an increase in $[Ca^{2+}]i$, while there was no detectable response in the $CD5^-$ cells (Fig. 2 *D*). Thus, only $CD3^+$ cells respond to CD3 stimulation, and furthermore, there appear to be few, if any, $CD3^+$ cells that do not respond to CD3 stimulation.

It was possible that the biphasic CD2 response consisted of two populations of cells that could be distinguished by the kinetics and magnitude of the response. Alternatively, it was possible that a single population of cells responded in a biphasic pattern. Consistent with this interpretation is the fact that only a single wave of depletion of cells from the baseline indo-1 ratio was evident (Fig. 1 *D*). When CD2 antibodies were added to PE–CD5-labeled cells, a response occurred in both $CD5^+$ and $CD5^-$ populations (Fig. 3, *A* and *B*). The increase in mean $[Ca^{2+}]i$ was slight in $CD5^-$ cells (Fig. 3 *C*). However, at the single cell level, ~40% of the $CD5^-$ cells had responded (Fig. 3 *D*). The response in $CD5^-$ cells occurred within 2 min after the addition of CD2 antibodies and reached a plateau by 4 min. In contrast, the response of $CD5^+$ was later in onset, higher in magnitude, and required 15 min to reach a plateau (Fig. 3, *A, C,* and *D*). Approximately 90% of $CD5^+$ cells responded to CD2 stimulation. Thus, the CD2 response of PBL is biphasic, with the early and late components entirely accounted for by temporally distinct

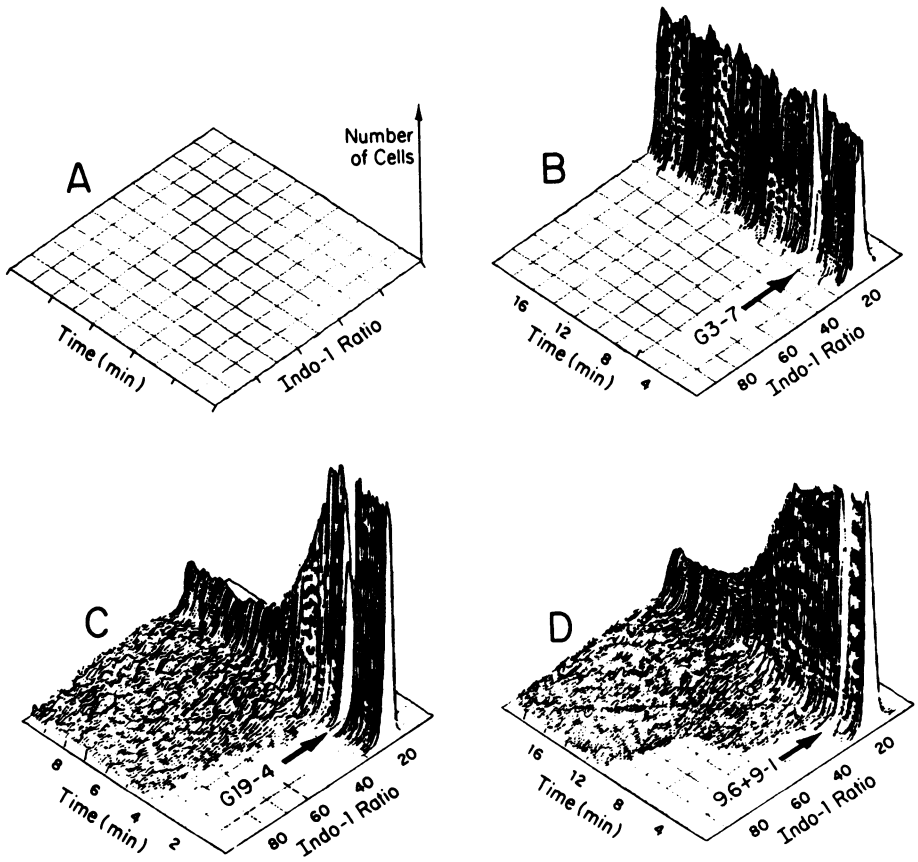

Figure 1. Time course for the development of increased $[Ca^{2+}]i$ in indo-1–loaded PBL. (*A*) Data are displayed as time vs. indo-1 ratio of violet/blue emission (proportional to $[Ca^{2+}]i$) vs. number of cells. (*B*) Histogram of $[Ca^{2+}]i$ in resting PBL. At time = 4 min, control CD7 antibody G3-7 was added (1:100 dilution of ascites). (*C*) CD3 antibody G19-4 and (*D*) CD2 antibodies 9.6 plus 9-1 were added at time = 2 min at the concentration indicated in Methods.

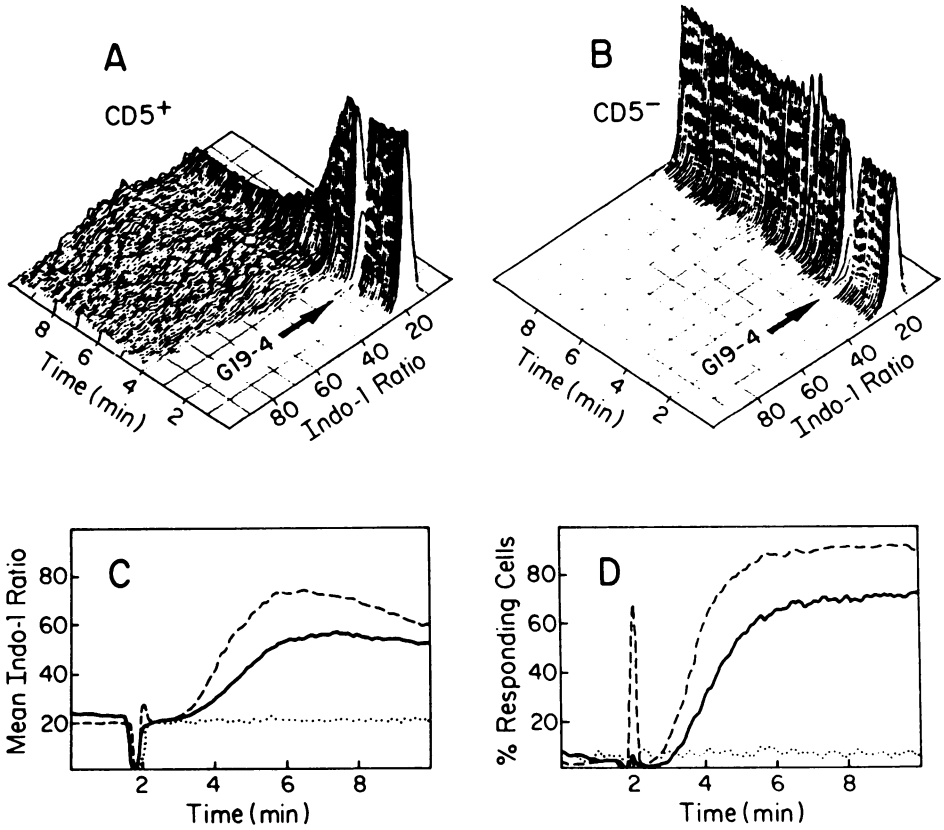

Figure 2. Isometric plot of the effect of CD3 stimulation on $[Ca^{2+}]i$ in (*A*) $CD5^+$ or (*B*) $CD5^-$ lymphocytes. CD5 is coordinately expressed with CD3. Indo-1–loaded PBL were first stained with CD5 PE-10.2 antibody and then stimulated with CD3 antibody G19-4 as in Fig. 1. Cells were analyzed for the violet/blue indo-1 emission ratio and the red (PE) positive and negative cells displayed in panels *A* and *B*, respectively. (*C*) Time course for the development of an increased mean indo-1 fluorescence ratio and (*D*) percent responding cells above a threshold ratio of 25 are displayed (——, total cell population analyzed; – – –, $CD5^+$ cells; and · · · · ·, $CD5^-$ cells). CD5 antibody PE-10.2 had no effect on the basal $[Ca^{2+}]i$. See the Methods section for calculation of percent responding cells. The phenotype of the PBL used for these experiments was 78% $CD2^+$, 65% $CD3^+$, 66% $CD5^+$, 6% $CD16^+$, 15% monocytes, and 9% B cells. Transient changes in the indo-1 ratio that occur immediately after addition of the stimulating antibody are artifacts caused by reagent addition.

responses in $CD5^-$ and $CD5^+$ cells, respectively. The early response in $CD5^-$ cells had a lower plateau, while the response of $CD5^+$ cells had a higher magnitude and accounted for most of the change in mean indo-1 ratio of the total population.

In order to exclude the possibility that the response of $CD5^+$ cells was delayed by the PE-CD5 conjugate, we analyzed the CD2 response with the use of PE–FC-2–labeled cells. The FC-2 antibody is similar to Leu-11 and recognizes CD16, the Fc

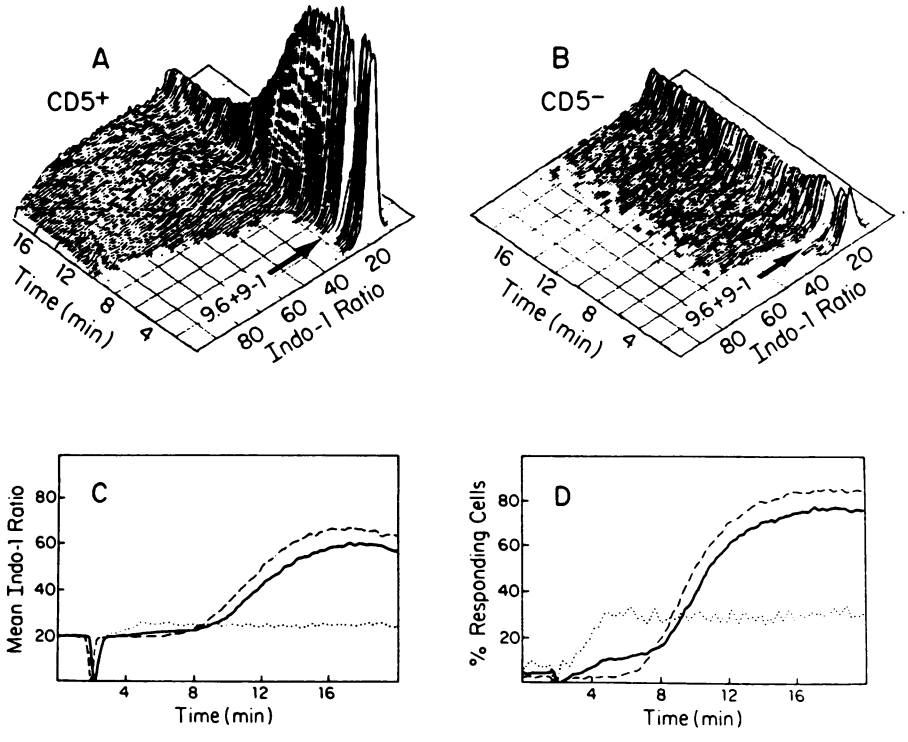

Figure 3. Isometric plots of the effect of CD2 stimulation on $[Ca^{2+}]i$ on $CD5^+$ or $CD5^-$ lymphocytes in *A* and *B*, respectively. Indo-1–loaded PBL were stained with CD5 antibody PE-10.2 as in Fig. 2 and stimulated with CD2 antibodies 9-1 and 9.6 as described in Methods. (*C*) Time course for the development of an increased mean indo-1 fluorescence ratio and (*D*) percent responding cells above a threshold ratio of 26 are displayed (——, total cell population analyzed; – – –, $CD5^+$ cells; · · · · ·, $CD5^-$ cells). See Fig. 2 legend for phenotype of the cells.

gamma receptor expressed on granulocytes and LGL. CD16 is not co-expressed with CD3 or CD5, and thus represents a reciprocal and nonoverlapping marker (27, 33). When PE–CD16-labeled cells were stimulated with CD2 antibodies, there was an early onset, low-magnitude response in $CD16^+$ cells and a later onset, high-magnitude response in $CD16^-$ cells (Fig. 4, *A* and *B*). The increase in mean $[Ca^{2+}]i$ was slight in $CD16^+$ cells (Fig. 4 *C*). However, at the single cell level, ~40% of the $CD16^+$ cells had responded (Fig. 4 *D*). These results are consistent with the previous experiments using CD5-labeled cells. However, for the early responding cells, positive selection by CD16 appeared to be a more specific marker than negative selection for CD5, because ~60–70% of $CD16^+$ responded (Fig. 4 *D*) as compared with 30–40% of $CD5^-$ cells. Thus, we conclude that only $CD5^+$ cells respond to CD3 stimulation, whereas two populations consisting of $CD5^-/CD16^+$ and $CD5^+/CD16^-$ cells respond to CD2 stimulation.

Previous studies have shown that combinations of CD2 antibodies recognizing two different epitopes are able to induce T cell proliferation (7–9) or cause an increase in $[Ca^{2+}]i$ in T cell clones (17). When using indo-1–loaded NWT cells, we confirmed that both antibodies 9.6 and 9-1 were required in order to activate $CD16^-$ cells, and that neither antibody used alone was sufficient to alter the $[Ca^{2+}]i$ of $CD16^-$ cells (Fig. 5 *B*). Unexpectedly, it was found that antibody 9-1 alone was sufficient to activate 50–60% of $CD16^+$ cells (Fig. 5 *A*). Anti-CD16 antibody was not required for this effect, because in separate experiments using CD5-labeled cells, a similar proportion of $CD5^-$ cells responded to antibody 9-1 alone (data not shown).

To distinguish between extracellular transport and intracellular mobilization as mechanisms for the increase in $[Ca^{2+}]i$, we investigated whether extracellular calcium was required for the CD3 and CD2 responses. The increase in indo-1 ratio after CD2 or CD3 activation was almost totally ablated by EGTA (Figs. 6 and 7, *A* and *B*). However, ~65% of the EGTA-treated NWT did have a slight, transient CD3 response (Fig. 6 *D*), although the mean indo-1 response was nearly ablated (Fig. 6 *C*). Similarly, ~35% of NWT transiently responded to CD2 in the presence of EGTA (Fig. 7 *D*), although the mean response was hardly detectable (Fig. 7 *C*). Thus, the CD2 and CD3 responses were made transient; the number of responding cells decreased; and the magnitude of the response diminished by EGTA, indicating that the response depends in part on extracellular calcium. However, extending the EGTA pretreatment time from 5 to 30 min did not further ablate the CD3 or CD2 responses, and in addition, increasing the concentration of EGTA to 10 mM also failed to ablate the responses, which indicated that the response, in part, results from intracellular calcium mobilization. When the EGTA-resistant CD3 response was analyzed by subsets, ~50% of $CD4^+$ and 25% of $CD8^+$ cells showed an increase in $[Ca^{2+}]i$ (data not shown).

Discussion

Our study supports the hypothesis that the CD2 and CD3 complexes activate separate pathways for cell activation. First, $CD3^-$ cells could respond to CD2 stimulation. Second, the CD3 and CD2 responses had different kinetics: the CD3 response occurred within 1 min, whereas the CD2 response of $CD3^+$ cells showed a 6-min lag phase. Interestingly, the CD2 response of $CD3^-/CD16^+$ cells did not show a lag phase. Finally, $CD3^-/CD16^+$ cells could be activated by stimulation of the 9-1 CD2 epitope alone, whereas activation of $CD3^+$ cells required stimulation of both the 9-1 and 9.6 CD2 epitopes.

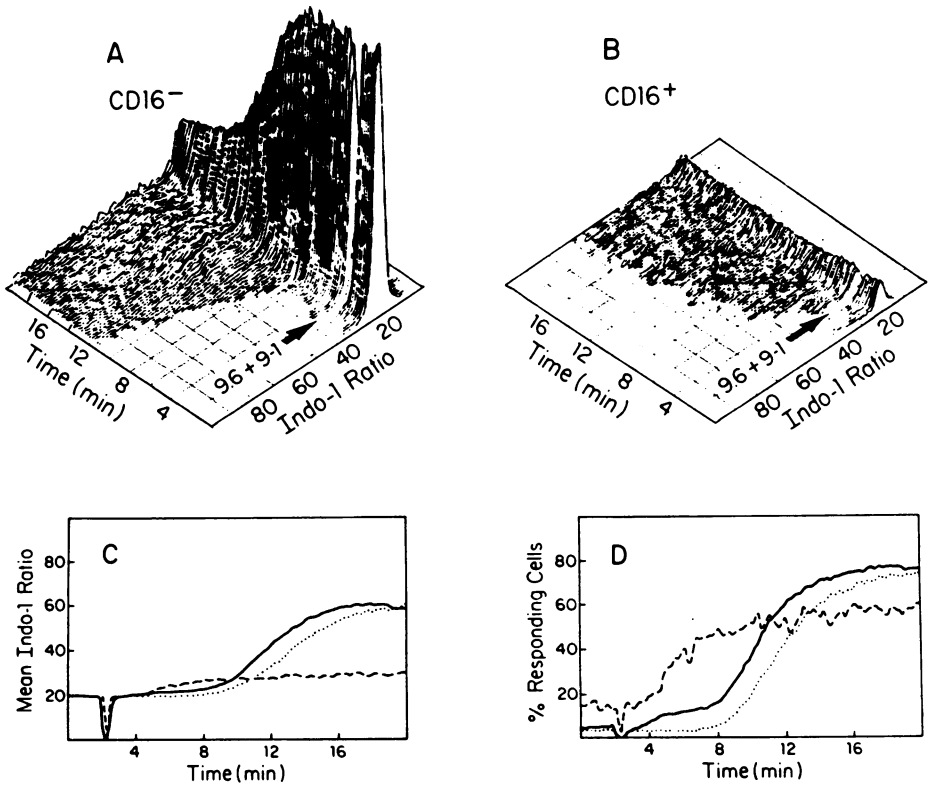

Figure 4. Isometric plots of the effect of CD2 stimulation on $[Ca^{2+}]i$ of $CD16^-$ or $CD16^+$ lymphocytes. Indo-1–loaded NWT were stained with CD16 antibody PE–FC-2, which indentifies a population of LGL that do not express the CD3 antigen, and stimulated with CD2 antibodies as described in Methods. The red (PE) negative and positive cells are displayed in *A* and *B*, respectively. (*C*) Time course for the development of increased mean indo-1 ratio and (*D*) percent responding cells above a threshold ratio of 26 are displayed (——, total cell population analyzed; – – –, $CD16^+$ cells; · · · · ·, $CD16^-$ cells). Antibody FC-2 had no effect on the basal $[Ca^{2+}]i$. The phenotype of the cells used for this experiment was 99% $CD2^+$, 95% $CD3^+$, and 2% $CD16^+$.

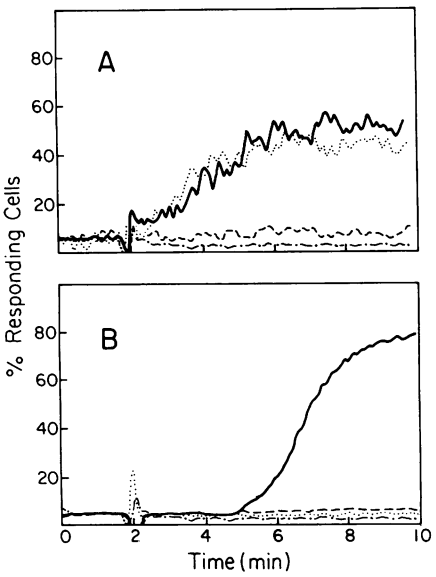

Figure 5. Differential requirement for anti-CD2 activation of $CD16^+$ or $CD16^-$ lymphocytes. Indo-1-loaded NWT cells were stained with CD16 PE-FC-2 as in Fig. 4 and the percent responding $CD16^+$ or $CD16^-$ cells displayed in *A* and *B*, respectively, after stimulation with (- - -) control antibody G3-7; (· · · · ·) antibody 9-1; (— • —) antibody 9.6; and (——) 9.6 + 9-1.

It is important to note that we used resting PBL or NWT for our study. Most previous studies of CD3- or CD2-induced changes in $[Ca^{2+}]i$ have employed either T cell clones or leukemic cell lines, cells that are already in a nonresting state. Several investigators have shown that exposure to CD3 antibody causes a rapid increase in $[Ca^{2+}]i$ in T cell clones or lines (13, 14, 16, 20, 22). Weiss et al. (17) have demonstrated that CD2 antibodies increase $[Ca^{2+}]i$ in $CD3^+$ T cell clones. The antibodies used in that study bind to the CD2-$T11_2$ and CD2-$T11_3$ epitopes, whereas the antibodies we used bind to the CD2-9.6/$T11_1$ and CD2-9-1 epitopes (28). It remains to be determined whether stimulation of the CD2-$T11_3$ epitope is also sufficient to activate $CD3^-$ cells. In the only previous study that examined responses in resting PBL, O'Flynn et al. (15) found that only E rosette-positive cells responded to CD3 stimulation.

The biphasic response of CD2-stimulated cells was unexpected because our previous experiments using the quin2 assay had shown only a uniphasic response (unpublished data). This apparent discrepancy in results most likely reflects the inability of the quin2 system to detect changes in minor populations. Further experiments are needed to determine the cause for the delayed CD2 response of $CD3^+/CD5^+$ cells. The delayed response was evident at all concentrations of CD2 antibodies tested (data not shown). In addition, the lag phase was still present when the CD2 antibodies were added sequentially rather than simultaneously, suggesting that the delay does not simply reflect differences in antibody avidity or time-dependent interactions between one of the antibodies and the CD2 molecule (data not shown). The two-epitope requirement may indicate that two separate pathways must be initiated, which together result in a transmembrane calcium flux, perhaps through the CD3 molecule. It is also possible that the delayed response of $CD3^+$ cells depends on an interaction with the $CD3^-/CD16^+$ population of early responding cells.

Our results are consistent with the notion that all $CD3^+$ cells respond to CD3 stimulation and that nearly all $CD2^+$ cells respond to CD2 stimulation. In separate studies, the $[Ca^{2+}]i$ increased in >97% of $CD4^+$ cells after CD3 or CD2 stimulation

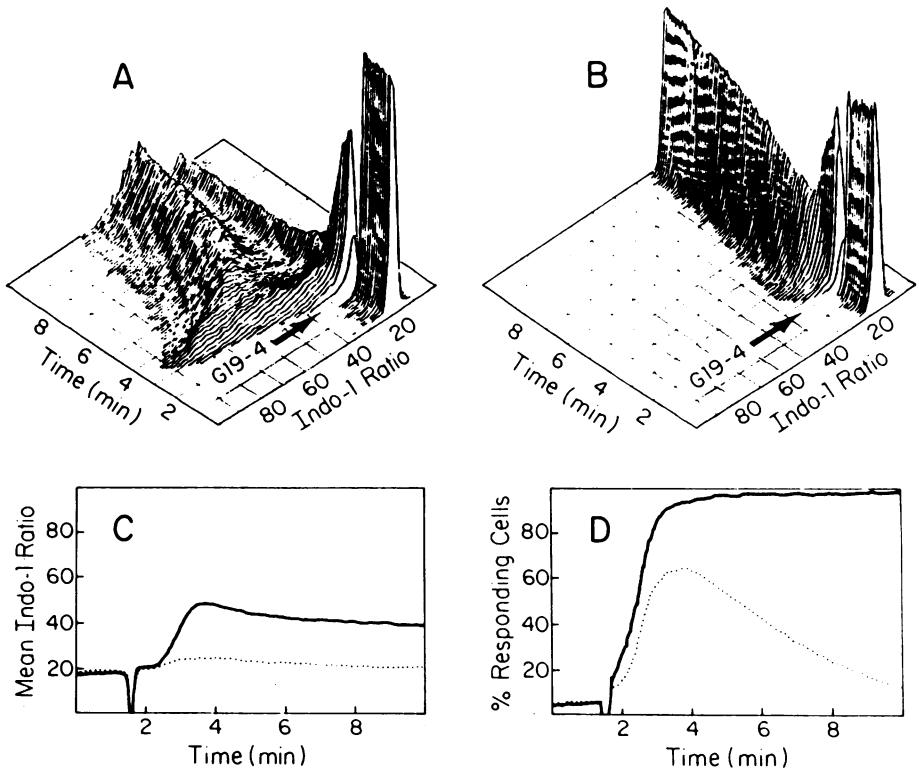

Figure 6. Effect of extracellular calcium depletion on the CD3 response. Indo-1-loaded NWT were stimulated with CD3 antibody G19-4 as in Fig. 1 in the presence or absence of 5 mM EGTA (*B* and *A*, respectively). Mean indo-1 fluorescence ratio is displayed in *C*, and percent responding cells in *D* (——, no EGTA; - - -, 5 mM EGTA). The threshold ratios for responding cells in *A* and *B* were 22 and 19, respectively. The concentration of EGTA used in the calcium depletion experiments did not affect antibody binding to cells (data not shown).

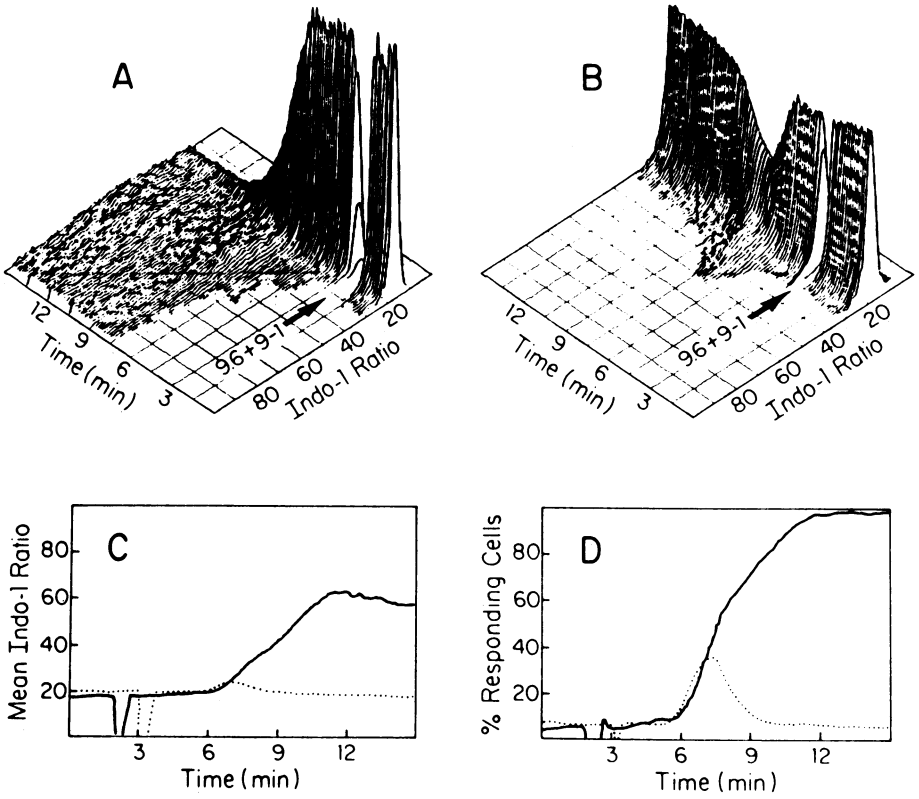

Figure 7. Effect of extracellular calcium depletion on the CD2 response. Indo-1–loaded NWT were stimulated with CD2 antibodies 9.6 and 9-1 as in Fig. 1 in the presence or absence of 5 mM EGTA (*B* and *A*, respectively). Mean indo-1 fluorescence ratio is displayed in *C*, and percent responding cells in *D* (——, no EGTA; - - -, 5 mM EGTA). The threshold ratios for responding cells in *A* and *B* were 21 and 23, respectively.

(data not shown). Among $CD8^+$ cells, the relative number of cells responding to anti-CD2 antibody (72%) was slightly greater than the number responding to anti-CD3 antibody (66%). A much greater disparity was found in the proportions of $CD16^+$ cells responding after stimulation with CD2 and CD3 (57% and <3%, respectively). Approximately 70–90% of $CD16^+$ cells co-express CD2 (27, 33) but few, if any, of these cells co-express CD3.

The changes in $[Ca^{2+}]i$ could have resulted from either extracellular transport or intracellular mobilization. The latter has been shown to result from hydrolysis of phosphatidyl inositol in the plasma membrane, which releases inositol triphosphate, a polar compound proposed to mediate release of intracellular calcium stores (34). Imboden and Stobo (20) have shown in the Jurkat T cell line that CD3 stimulation results in the production of inositol triphosphate. The CD2 pathway has not yet been studied in this manner, but our results predict that in comparison with CD3 stimulation, small amounts of inositol triphosphate will be produced with a delayed kinetic pattern after CD2 stimulation. However, quantitatively, most of the calcium mobilization after CD2 or CD3 stimulation of resting T cells appears to be due to extracellular transport.

Adaptation of indo-1 to flow cytometry permits examination of responses in single cells. The excellent red (PE) discrimination allowed a quantitative and kinetic analysis of changes in $[Ca^{2+}]i$ in small subsets of cells. For example, we were able to detect a CD2 response in $CD16^+$ cells which comprised only 2–8% of the total population. Our results also help to interpret some apparently contradictory studies of the effect of EGTA on the CD3 response (18, 20, 22). With the quin2 assay, Oettgen et al. (22) found that the CD3 response of the HPB-ALL cell line was ablated by EGTA (22), while Imboden and Stobo found that the CD3 response of the Jurkat cell line was only slightly diminished by EGTA (20). When measured as a change in mean fluorescence, we found that the response of resting NWT cells was nearly ablated by EGTA, although a diminished and transient response was clearly evident when measured in single cells. Thus, the CD3 response of the HPB-ALL cell line appears to be similar to that of resting T cells, whereas the response of the Jurkat cell line may be less dependent upon extracellular calcium. The explanation for the transient nature of the CD2 and CD3 responses in the presence of EGTA requires further examination. Under physiologic conditions, the responses may have components of both intracellular mobilization and transmembrane flux. The response may be initiated by the former and maintained by the latter, thereby accounting for the transient nature of the response in the presence of EGTA. Alternatively, if in fact the CD3 complex can act as a calcium channel, then it is possible that the direction of calcium transport might be reversed under conditions of extracellular calcium depletion, thereby blunting the response.

The association of CD3 with the T cell receptor makes it likely that CD3 triggering initiates an antigen-specific pathway of T cell activation. Meuer et al. (7) have proposed that CD2 triggering might initiate an antigen-independent pathway (8). Our results are consistent with that hypothesis, and further suggest that the CD2 pathway provides the potential for separate or conjunct antigen nonspecific activation of LGL or T cells via the CD2-9-1 and/or CD2-9.6 epitope(s). Such activation might regulate growth or effector functions. For example, T cell proliferation may either be stimulated or inhibited, depending on the CD2 epitopes triggered (7–9, 35). In addition, CD2 antibodies have been shown to inhibit a variety of functions, including natural killing (36), a function mediated by LGL, and antigen-specific cytotoxicity (37), a function mediated by T cells. More recently, CD2 antibodies were shown to stimulate "promis-

cuous" killing by cytolytic T cell clones and natural killer cell clones (38). Thus, the CD2 molecule, through regulation of $[Ca^{2+}]i$, might be involved with both proliferative and effector functions of T cells and LGL. Taken together, our findings support the concept that CD2 stimulation indeed initiates an alternative pathway of activation involving both T cells expressing the CD3/TcR molecular complex and LGL cells that do not.

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