Calcium Response of Helper T Lymphocytes to Antigen-Presenting Cells in a Single-Cell Assay

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ABSTRACT We developed a dynamic, single-cell assay involving alternating differential interference contrast and fluorescence microscopy, together with digital imaging, for both viewing the physical interaction of live helper T lymphocytes (Th cells) with antigen-presenting cells (APCs) and monitoring the increases in the intracellular free calcium concentration of the Th cell, an early event in Th cell activation. We obtained Th-APC conjugates by allowing the Th cells to migrate toward and interact with APCs that either settled nearby or had been micromanipulated in close proximity to the Th cells. Th cell motility played an important role in initiating Th-APC contacts but not in determining the Th cell calcium response. We found that the intracellular calcium responses of individual Th cells are heterogeneous and an all-or-none phenomenon, independent of antigen concentration. However, the fraction of Th-APC conjugates involving responding Th cells is an increasing function of the antigen concentration. Finally, we measured some characteristics of the developing Th-APC contact area. We used all of these data together with previously developed mathematical models to estimate that only 1 to 20 major histocompatibility class II-antigen complexes are required in the initial Th-APC contact area to elicit a Th cell calcium response.

INTRODUCTION

Helper T lymphocytes (Th cells) within the immune system play a crucial role in defending the body against invasion by antigen. Native antigen cannot be recognized by a Th cell; the protein antigen must be processed and presented in a recognizable form by an antigen-presenting cell (APC), typically a macrophage, B lymphocyte, or dendritic cell, which internalizes, degrades, and complexes a peptide fragment of the antigen with a class II major histocompatibility complex (MHC II) molecule (Brodsky and Guagliardi, 1991). MHC II-antigen (MHC-Ag) complexes are presented on the surface of the APC where they can be recognized by T cell receptors (TCRs) on the Th cell surface. In addition to TCR/MHC-Ag binding, the binding of other complementary Th-APC adhesion molecules is involved in the Th-APC interaction. For example, LFA-1 and CD2 on the Th cell bind to ICAM-1/ICAM-2/ICAM-3 and LFA-3, respectively, on the APC (Springer, 1990; Shimizu and Shaw, 1990; Janeway and Golstein, 1993). Upon TCR/MHC-Ag binding, a cascade of signaling events begins within the Th cell, including the activation of the enzyme phospholipase C, which hydrolyzes phosphatidylinositol-4,5-bisphosphate into the second messengers inositol-1,4,5-triphosphate and diacylglycerol within ~30 s of TCR triggering (Klausner and Samelson, 1991). Inositol-1,4,5-triphosphate then releases calcium from intracellular stores, causing an increase in the intracellular free calcium concentration, $[Ca^{+2}]_i$ (Segal, 1991). This series of signaling events continues, transferring signals from the T cell surface to the nucleus, where genes necessary for Th cell activation are transcribed (Altman et al., 1990). Upon activation, the Th cell enhances the overall immune response by both direct and indirect methods; lymphokines such as interleukin-2 (IL-2) secreted by the Th cell indirectly induce cytotoxic T cells and Th cells to proliferate, and direct Th cell contact and secreted lymphokines both induce B lymphocytes to proliferate and differentiate. A precise understanding of the events in Th cell activation is important if a greater understanding of the immune system is to be obtained, for example, for purposes of manipulation of the immune response at the level of antigen presentation.

Th cell activation assays are typically performed on populations of Th cells. These population studies often involve Th cells activated nonphysiologically, using antibodies instead of APCs for example. In addition, they often involve monitoring late events in the Th cell activation signaling cascade, such as IL-2 secretion. Although these types of population studies provide valuable information on Th cell activation, they cannot provide dynamic information on early Th cell activation events occurring within individual Th-APC interactions; rather, single-cell Th-APC studies, although more tedious than population studies, are necessary in order to provide this type of insight into Th cell activation.

During the past few years, several single-cell Th-APC studies have been performed. Some of these studies have dealt with the cytoskeletal reorganizations, clustering of receptors, and polarized synthesis of cytokines that occur within the Th cell as a result of specific Th-APC interaction (Kupfer et al., 1991; Kupfer and Singer, 1989a,b). Others have used electron microscopy to investigate details of the physical Th-APC interaction (Concha et al., 1988, 1993; Sanders et al., 1986). However, all of these studies involved fixed cells, so dynamic interactions were not followed within the same Th-APC conjugates. On the other hand,

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recent studies by Donnadieu et al. (1992) and Røtnes and Bogen (1994) dynamically followed the changes in $[Ca^{+2}]_i$ of live Th cells involved in Th-APC interactions. Both of these studies have provided information on early Th cell activation events occurring within two different Th-APC systems. Nevertheless, data on the interaction of live Th cells with their physiological ligand, live APCs bearing processed antigen, remain scarce.

Our overall objective was to quantify the responses of individual Th cells to their physiological stimuli. To do this, we developed a dynamic, single-cell assay involving alternating differential interference contrast and fluorescence microscopy, together with digital imaging, for both viewing the Th-APC interaction and monitoring the increases in $[Ca^{+2}]_i$ of the Th cell. We used a well characterized Th-APC-Ag system consisting of murine 3A9 hybridoma T cells, murine TA3 hybridoma B cells as APCs, and the whole antigen hen egg-white lysozyme, which requires processing. Because the stimulus that the Th cell actually "sees" (namely the MHC-Ag complexes presented by the APC and bound by TCRs in the Th-APC contact area) is a quantity that is technically difficult, if not impossible, to measure experimentally, we used the antigen concentration with which the APCs have been incubated, which affects the number of MHC-Ag complexes presented, as the experimental variable. We used the intracellular calcium response of the Th cell as the experimental measure of Th cell response because it is an early event in the activation of the Th cell. We hypothesize that a threshold number of MHC-Ag complexes may be required to be present within the initial Th-APC contact area in order to elicit a Th cell calcium response. Previously developed mathematical models (Linderman et al., 1994; Singer and Linderman, 1990, 1991), in combination with our experimental data, enabled us to estimate this threshold number of MHC-Ag complexes. Finally, because of the scarcity of single-cell Th-APC data, we extracted as much information as possible from our single-cell Th-APC experiments. Therefore, in addition to our intracellular calcium studies, we also analyzed the direction of motion of Th cells in establishing Th-APC contact and measured and analyzed some characteristics of the developing Th-APC contact area.

MATERIALS AND METHODS

Cell culture

Murine 3A9 hybridoma T lymphocytes and murine TA3 hybridoma B lymphocytes, which express I-A^k and I-A^d molecules, were provided by Dr. Roderick Nairn (University of Michigan, Department of Microbiology and Immunology). 3A9 T cells, which we refer to throughout this paper as simply "Th cells," are known to be specific for a hen egg-white lysozyme (HEL) peptide (residues 46–61) bound to the I-A^k molecule (Allen et al., 1987; Harding and Unanue, 1989; Lambert and Unanue, 1989). HEL, 3× crystallized, was purchased from Sigma Chemical Company (St. Louis, MO). This particular Th-APC cell system has previously been employed in many population studies that have contributed to the understanding of some of the events involved in antigen processing and presentation (Allen et al., 1984, 1987; Allen and Unanue, 1984; Esch, 1989; Glimcher et al., 1983; Harding et al., 1989; Harding and Unanue, 1989, 1990; Nelson et al., 1994); hence we selected this same cell system to further investigate the Th-APC interaction at the single-cell level.

Cells were maintained separately in RPMI 1640 (Sigma) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (Hyclone Laboratories, Logan, UT), 1.0% (vol/vol) antibiotic antimycotic solution (penicillin, streptomycin, amphotericin B) (Sigma), and 0.5% (vol/vol) 0.1 M β-mercaptoethanol (Sigma) in a 5% CO_2 environment at 37°C. Because the antigen processing time for B cells has been shown experimentally (Lakey et al., 1988) and predicted theoretically (Singer and Linderman, 1990) to range from 6 to 8 h for native antigen, TA3 cells were incubated with varying concentrations of native HEL in Hanks' balanced salt solution (Sigma) for at least 8 h before the experiments. Immediately before each experiment, TA3 cells were rinsed by centrifugation and resuspension in experimental media to wash away unbound antigen. The experimental media consisted of RPMI 1640 containing no phenol red (Sigma) supplemented with 1.0% (vol/vol) antibiotic antimycotic solution, 20 mM HEPES (Sigma), and 0.6 mM $CaCl_2$ (Sigma).

Method of Th-APC conjugate formation

Fluorescently labeled Th cells (loading procedure described below) and unlabeled APCs were introduced into a home-built acrylic flow chamber that was placed on the stage of the microscope. A schematic diagram of the flow chamber is shown in Fig. 1. A hole in the center of the flow chamber provided the viewing area for the cells. To prevent leakage, a seal was maintained at the bottom of the flow chamber with a rubber gasket, a treated glass coverslip, and a metal gasket, sandwiched together with layers of vaseline and secured with four nuts and screws. The glass coverslips were either treated with 0.10 mg/ml poly-D-lysine (Sigma) followed by rinsing to facilitate cell attachment or cleaned thoroughly by washing with a chromic-sulfuric acid solution. A major feature of the flow chamber was a stainless steel coil that ran along the inside edge of the open viewing area of the chamber. The coil was attached to a circulating water bath that allowed the cells to be maintained at a physiological temperature. Although temperature gradients existed in the vertical direction within the flow chamber, the temperature at the top of the coverslip, where the cells later settled, was able to be maintained at 37°C, as determined by using a thermocouple.

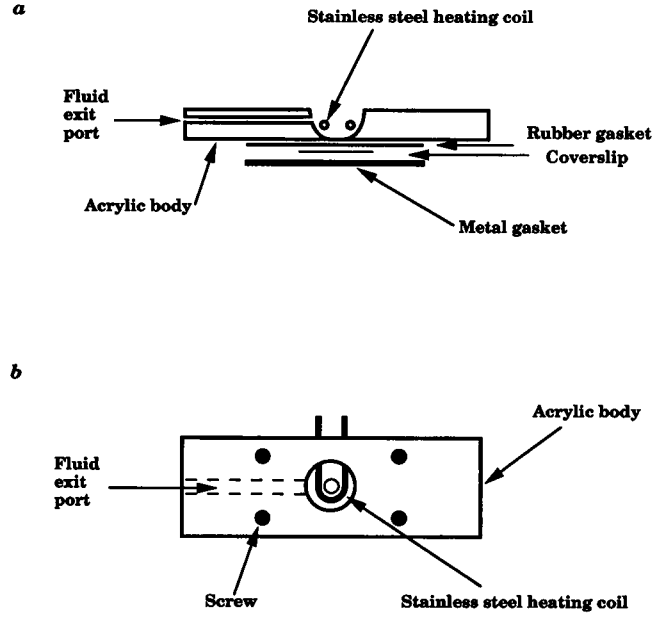

FIGURE 1 Schematic diagram of flow chamber. (*a*) Cut-away side view; (*b*) top view

To initiate Th-APC conjugate formation, fluorescently labeled Th cells were added dropwise to the flow chamber, which already contained experimental media at 37°C. After allowing sufficient time (~5 min) for most Th cells to settle onto the coverslip, APCs were added dropwise to the flow chamber. These APCs had been incubated earlier with varying concentrations of antigen, as previously described. As the cells settled, Th-APC conjugates were sometimes formed. In a few experiments, however, the order of cell addition was reversed; Th cells were added to the flow chamber, which already contained settled APCs. In both cases, because the Th cells were fluorescently labeled and the APCs were not, the two cell types were distinguishable and monitoring the formation of potential Th-APC conjugates was possible.

To enhance the probability of obtaining Th-APC conjugates, micromanipulation was sometimes used to pick up individual APCs and move them in close proximity to (but not touching) a Th cell. This was accomplished by using a microinjector system (Tritech Research, Los Angeles, CA) to suction part of an APC into a pipette that had been made by pulling and then fire-polishing a 1.0-mm OD thin-wall glass pipette (World Precision Instruments, Sarasota, FL) using a Sachs-Flaming type patch pipette/micropipette puller (PC-84; Sutter Instrument Co., Novato, CA). Before use, pipette tips were treated with a 0.25% (wt/wt) solution of Aquasil siliconizing fluid (Pierce, Rockford, IL) in order to prevent cells from adhering to the glass pipettes. Manipulation was performed by using a Nikon/Narishige 3D manual coarse manipulator (MN-188; Nikon Inc., Garden City, NY) and a Nikon/Narishige 3D hydraulic micromanipulator (MO-188; Nikon Inc.).

Imaging system

An imaging system was used to collect two different types of images; fluorescence images were collected to monitor the changes in $[Ca^{+2}]_i$ within the Th cells, and Nomarski images were collected to view the physical interactions between the APCs and the Th cells. Fluorescence images were visualized with a Nikon Diaphot-TMD inverted microscope (Nikon Inc.) equipped with a 100-W mercury arc lamp (Oriel Corp., Stratford, CT), a computer-controlled dual filter wheel assembly (Ludl Electronic Products, Hawthorne, NY) with 365-nm and 334-nm band pass filters (Oriel), a 10% transmission neutral density filter (Newport, Irvine, CA), Nikon TMD-EF epifluorescence attachments, a 400-nm dichroic mirror, a 510-nm long-pass barrier filter, and a Nikon 40× NA1.3 oil objective. Additional attachments required for visualizing Nomarski images included a 50-W halogen lamp, a Uniblitz VS25 series shutter (Vincent Associates, Rochester, NY), and Nikon Nomarski DIC TMD-NT2 attachments (Mager Scientific). All images were collected with a slow-scan, cooled, charge-coupled device camera (Photometrics Ltd., Tucson, AZ) equipped with a 384 × 576 pixel Thomson CSF TH7882 CDA chip, which was programmed to perform a 2×2 bin on each of the images. Both data acquisition and subsequent image analysis were accomplished using ISee imaging software (Inovision Corp., Durham, NC) on a Sun SPARCstation 4/330 computer and a Sun SPARCstation 2 computer (Sun Microsystems, Mountain View, CA).

Intracellular calcium measurements

Th cells were incubated with the calcium-sensitive fluorescent dye Fura-2/AM (Molecular Probes, Eugene, OR) in dimethyl sulfoxide at a concentration of 5 μM Fura-2/AM at 37°C. After ~30 min, excess dye was rinsed from the Th cells, and they were resuspended in experimental media. The fluorescent dye served a dual role; it provided a means not only for quantifying changes in $[Ca^{+2}]_i$ within the Th cell but also for distinguishing Th cells from APCs.

Fluorescence images were collected for two excitation wavelengths, 334 nm and 365 nm. The 334-nm and 365-nm images were collected back-to-back so that each pair of images approximated one time point (1.5 s/image pair, with exposure times of 0.1 s and 0.3 s for the 365-nm and 334-nm excitation wavelengths, respectively). Pairs of fluorescence images were collected every 10 to 15 s, and Nomarski images required for monitoring the conjugate formation were taken approximately every minute.

Calibration measurements for the fluorescence images were obtained by imaging three additional solutions within the flow chamber. A minimum calcium image was obtained from a solution of 2 mM EDTA in phosphate-buffered saline with 10 μM Fura-2 free acid (Molecular Probes). A maximum calcium image was obtained from a saturated solution of 1.8 mM $CaCl_2$ in HEPES (Sigma) with 10 μM Fura-2 free acid. A background image was obtained from experimental media (no Fura-2 free acid).

To calculate $[Ca^{+2}]_i$, images were first corrected for background fluorescence. Then, a ratio image R was generated for each time point, where $R = F_{334}/F_{365}$ = background-subtracted fluorescence emission for 334/background-subtracted fluorescence emission for 365. Calibration images were used to generate $R_{min} = F_{334}/F_{365}$ (EDTA solution), $R_{max} = F_{334}/F_{365}$ (saturated Ca^{+2} solution), and $\beta = F_{365}$ (EDTA solution)/F_{365}(saturated Ca^{+2} solution). The average value of the fluorescence in each of these ratio images was determined for individual cells; these average values were then used to calculate $[Ca^{+2}]_i$ in the cells, according to Grynkiewicz et al. (1985):

$$[Ca^{+2}]_i = K_d \beta \frac{(R - R_{min})}{(R_{max} - R)}$$

where K_d, the equilibrium dissociation constant for Fura-2 and Ca^{+2} at 37°C, is 224 nM.

Directional analysis of Th cell motility

The direction of motion was analyzed for all Th cells by using a modification of a method used by Zigmond (1977). As illustrated in Fig. 2, the direction of motion of a Th cell was determined from the direction of pseudopod extension relative to the APC. In some cases a Th cell extended pseudopods in directions of several different APCs during an experiment; each instance of pseudopod extension was analyzed independently for each Th cell. A Th cell was classified as having migrated in a directed manner if it migrated only toward the quadrant containing the APC for each pseudopod extension. A Th cell was classified as having migrated in a seemingly random manner if it migrated toward a quadrant not containing the APC. A limitation of this method was that sometimes, depending on the number of cells present within the field of view, the selection of the APC in the analysis was ambiguous.

Contact area measurements

Initial and maximal Th-APC contact areas were estimated and expressed both in μm^2 and as percentages of the APC surface area. The Th-APC

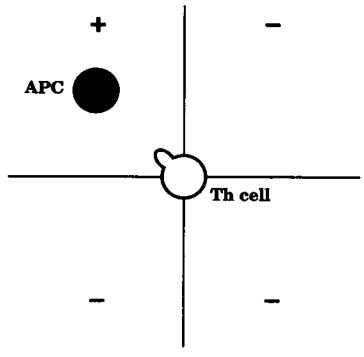

FIGURE 2 Directional analysis of Th cell motility. The Th cell was placed at the origin of the coordinate system, and the APC was placed in the center of one of the quadrants. The motion of the Th cell was classified as "directed" if pseudopod extensions occurred only toward the quadrant containing the APC.

contact area was assumed to form a circle with a diameter equal to the straight-line contact length between the Th cell and the APC. These straight-line contact lengths were measured in pixels using the ISee imaging software at the observed initial Th-APC contact and at the observed maximal Th-APC contact. The pixel values were then converted to actual length measurements in microns. To estimate the APC surface areas, cells were assumed to be spheres with diameters equal to the average diameter of the cell image.

Definition of conjugate and responder

A conjugate was defined as a Th-APC interaction involving a Th cell that actively migrated toward and contacted an APC. This definition was imposed in order to eliminate Th cells and APCs in which the APCs merely settled on top of the Th cells. A Th cell involved in a conjugate was considered a responder if it exhibited a sharp increase in $[Ca^{+2}]_i$ of at least 50% over baseline levels, typically within ~1 min of conjugate formation, whereas a Th cell involved in a conjugate was considered a nonresponder if it did not exhibit such an increase in $[Ca^{+2}]_i$.

Statistical analysis

It was assumed that there were no differences between experiments at the same antigen concentration; in other words, stage of cell cycle, age of cells, and length of antigen incubation >8 h were assumed to have negligible effects compared to antigen concentration in day-to-day experiments. Therefore, experimental results for each antigen concentration were pooled. Analysis of variance (ANOVA) was then used on responding Th cells to determine whether various characteristics of the calcium responses, such as the percentage increase in $[Ca^{+2}]_i$ over basal levels, response duration, and number and period of oscillations, were statistically different between antigen concentrations. ANOVA was also used on all conjugates to determine whether initial and maximal contact areas were statistically different between responding and nonresponding conjugates. In addition, ANOVA was used on all conjugates to determine whether initial and maximal contact areas were statistically different between antigen concentrations.

RESULTS

By using the combination of differential interference contrast and fluorescence microscopy together with digital imaging, we dynamically monitored at the single-cell level both the physical interaction of live Th cells with live APCs and an early event in the subsequent activation of the Th cells, an increase in $[Ca^{+2}]_i$ in the Th cells. We investigated the specificity, the heterogeneity, and the antigen dose-dependence of the Th cell intracellular calcium response. In addition, we studied the role of Th cell motility in the initiation of the Th-APC contact as well as some characteristics of the developing contact area between the Th cell and the APC. Finally, we used our previously developed mathematical models, along with our experimental data, to estimate the threshold number of MHC-Ag complexes required in the initial Th-APC contact area to elicit a Th cell calcium response.

Few Th cells respond without specific APC interaction

To determine whether observed intracellular calcium responses are attributable solely to specific Th-APC interaction, two types of control experiments were performed. First, the effect of different coverslip treatments on the Th intracellular calcium responses was determined. To do this, Fura-loaded Th cells were added to the flow chamber and allowed to settle onto coverslips that were either poly-D-lysine-coated or acid-washed. Neither APCs nor antigen were introduced in these experiments. A sharp increase in $[Ca^{+2}]_i$ was found in 17% (15/87) of Th cells added to poly-D-lysine-coated coverslips and 3% (2/59) of Th cells added to acid-washed coverslips. Second, the effect of nonspecific APC contact on the Th intracellular calcium responses was determined. To achieve this, conjugate-forming experiments were performed with APCs that were not exposed to antigen. These experiments were performed on both poly-D-lysine-coated and acid-washed coverslips. A sharp increase in $[Ca^{+2}]_i$ was found in 28% (9/32) of Th cells involved in Th-APC conjugates on poly-D-lysine-coated coverslips and 9% (4/44) of Th cells involved in Th-APC conjugates on acid-washed coverslips.

On the basis of these two types of control experiments, we conclude that basal fractions of Th cells respond with increases in $[Ca^{+2}]_i$ even without specific APC contact, possibly because of the coverslip treatments or nonspecific APC contact or both. Therefore, in conjugate-forming experiments involving APCs that have been incubated with antigen, only fractions of Th cells responding above these basal levels may be attributed to specific APC contact. Because the basal fractions of spontaneously responding Th cells were lower in control experiments on acid-washed coverslips, we focused primarily on conjugate-forming experiments on these coverslips.

Intracellular calcium responses of individual Th cells are heterogeneous

The intracellular calcium responses of individual Th cells involved in Th-APC conjugates were observed to be heterogeneous; the calcium responses consisted of either a single peak, oscillations, or miscellaneous behavior. An example of each of these calcium responses is shown in Fig. 3. As can be seen, calcium responses that were classified as miscellaneous are those that clearly cannot be considered either single-peaked or oscillatory. Because the Th cells that displayed an increase in $[Ca^{+2}]_i$ all exhibited a sharp increase in $[Ca^{+2}]_i$ of at least 50% over basal levels, typically within ~1 min of conjugate formation, we defined a responding Th cell as a Th cell that was involved in a conjugate and had these characteristics. From the pooled data at all antigen concentrations, consisting of 86 responding Th cells, 37% of all calcium responses consisted of a single peak, 50% consisted of oscillations, and 13% consisted of miscellaneous responses. In addition, the type of calcium response obtained did not have any clear dependence on the coverslip treatment.

The calcium responses were analyzed, both pooled and individually, to determine the effect of antigen concentra-

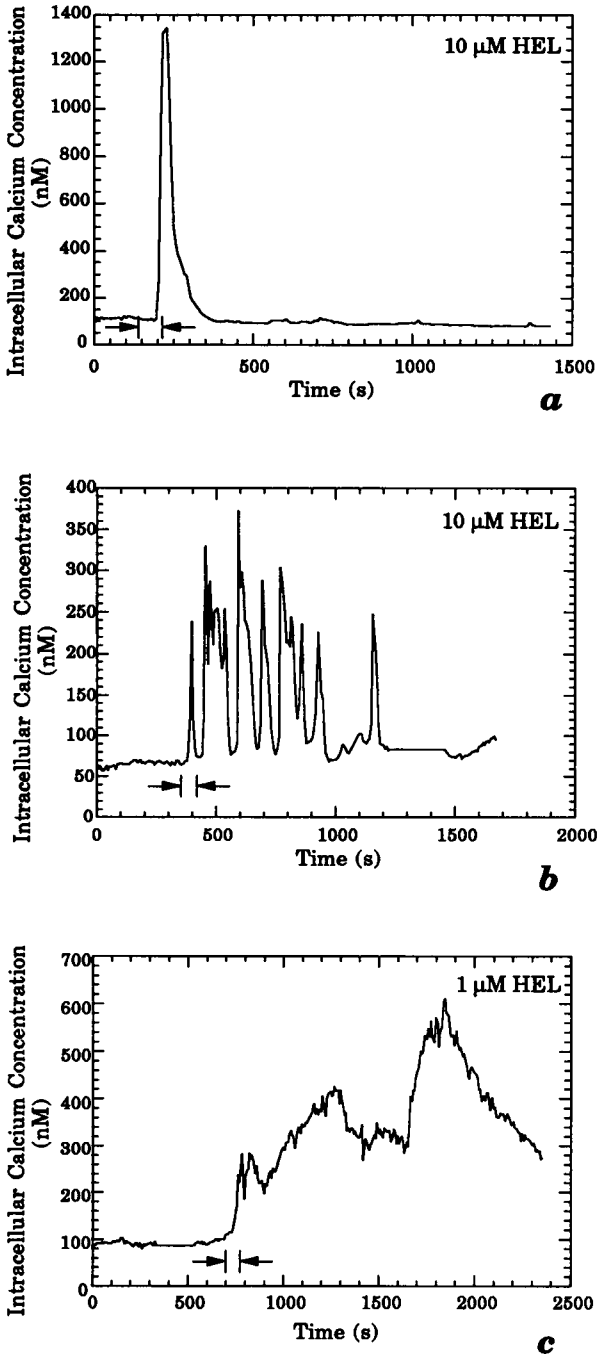

FIGURE 3 Heterogeneity of intracellular calcium responses of individual Th cells in Th-APC conjugate-forming experiments. The calcium responses of the Th cells consisted of either (*a*) a single peak, (*b*) oscillations, or (*c*) miscellaneous behavior. All of the responses involved a sharp increase in $[Ca^{+2}]_i$ of at least 50% over basal levels, typically within ~1 min of conjugate formation. Conjugate formation occurred within the windows shown by arrows.

tion on particular characteristics of the calcium responses. First, the data were pooled, without regard to the type of calcium response, for the maximum percentage increase in $[Ca^{+2}]_i$ over basal levels for each antigen concentration. To determine whether the average percentage increase was statistically different between antigen concentrations, ANOVA was then performed. No statistical difference between antigen concentrations was seen at a 10% level of significance. Similarly, data were pooled for calcium response durations for each antigen concentration, where the response duration was defined as the time lapse between the initial sharp increase in $[Ca^{+2}]_i$ and the return to a steady $[Ca^{+2}]_i$, usually basal level. In this case, however, only the single-peaked and oscillatory responses were pooled because in most miscellaneous responses the ending of the calcium response was not clearly discernible. Again, no statistical difference between antigen concentrations was seen at a 10% level of significance. Thus, no correlations were observed between antigen concentration and these particular characteristics of the calcium responses when the data were pooled without regard to the type of response.

Next, the three types of calcium responses were analyzed individually to determine the effect of antigen concentration on particular characteristics of the individual calcium responses. For single-peaked responses, the percentage increase in $[Ca^{+2}]_i$ over basal levels ranged from 100% to 1400% and response durations ranged from 50 s to 1200 s. However, no statistical difference between antigen concentrations was seen at a 10% level of significance in either the percentage increase in $[Ca^{+2}]_i$ or the response duration. For oscillatory responses, the percentage increase in $[Ca^{+2}]_i$ ranged from 50% to 1500%, the response duration ranged from 150 s to 1050 s, the number of oscillations ranged from 2 to 14, and the oscillation period ranged from 40 s to 250 s. Again, no statistical difference between antigen concentrations was seen at a 10% level of significance in any of these characteristics of the oscillatory response. For miscellaneous responses, the percentage increase in $[Ca^{+2}]_i$ ranged from 50% to 1100%. Again, no statistical difference between antigen concentrations was seen at a 10% level of significance in the percentage increase in $[Ca^{+2}]_i$. Thus, no correlations were observed between antigen concentration and any particular characteristics of the individual calcium responses.

Fraction of Th-APC conjugates involving responding Th cells is an increasing function of antigen concentration

Fig. 4 shows a series of Nomarski images taken at different time points during one particular conjugate-forming experiment. The Th cell of interest is labeled "T," and surrounding APCs are labeled "A." At 737 s, the Th cell is not in contact with any other cell, but at 811 s, the Th cell extends a pseudopod and contacts an APC; however, this Th-APC contact is no longer present at 885 s. By 1332 s, the Th cell has migrated (images not shown) toward an APC that has since settled onto the coverslip; at 1407 s, a Th-APC conjugate is clearly visible. Fig. 5 shows the intracellular calcium response of the Th cell as a function of time. No significant change in $[Ca^{+2}]_i$ results from the first brief

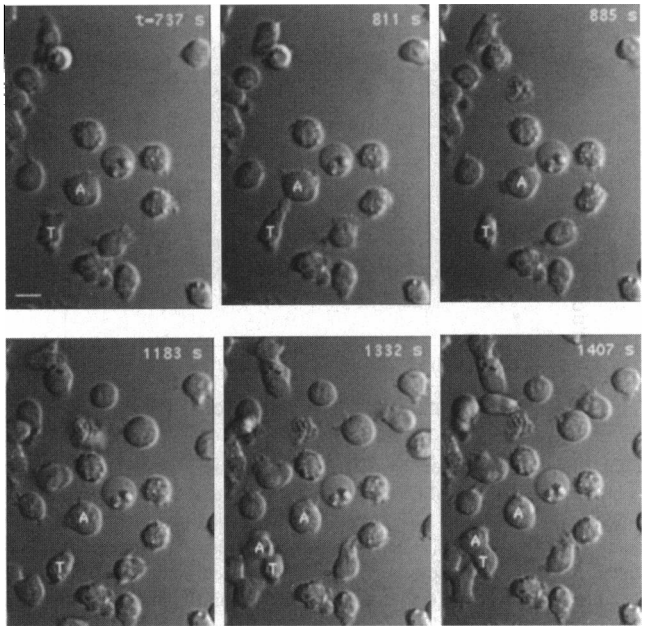

FIGURE 4 Time series of Nomarski images taken from one particular Th-APC conjugate-forming experiment. The Th cell of interest is labeled "T," and surrounding APCs are labeled "A." In this experiment, 10 μM HEL and poly-D-lysine-coated coverslips were used. The bar shown in the first image is 10 μm in length.

contact with an APC at 811 s. However, the formation of the second conjugate is accompanied by a sharp increase in $[Ca^{+2}]_i$, seen after 1300 s. In addition, the conjugate remains intact despite three attempts at rinsing the coverslip within the flow chamber. Thus, the Th cell of interest failed to exhibit an increase in $[Ca^{+2}]_i$ upon one Th-APC contact but did exhibit an increase in $[Ca^{+2}]_i$ upon another Th-APC contact.

Fig. 6 shows another time series of Nomarski images during a different conjugate-forming experiment in which two conjugates involving different Th cells were formed. The Th cells of interest are labeled "T1" and "T2," and the corresponding APC is labeled "A." At 103 s, T1 is beginning to extend a pseudopod toward A, and by 162 s, T1 has made contact with A; however, this Th-APC contact is no longer present by 220 s. T2, which settled onto the coverslip by 162 s, migrates toward the same APC some time after 743 s; by 858 s, a Th-APC conjugate is clearly visible. Fig. 7 shows the intracellular calcium response of both Th cells as a function of time. No increase in $[Ca^{+2}]_i$ results from the T1-A conjugate. However, the T2-A conjugate formation is accompanied by a sharp increase in $[Ca^{+2}]_i$, seen after 800 s. Because both Th cells were migrating during the experiment but only one Th cell responded, it seems that cell motility alone does not cause calcium responses. Hence, the increase in $[Ca^{+2}]_i$ seen in T2 is most likely caused by the specific APC contact rather than the motility used to establish the contact.

From these two experiments, it is clear that not all Th-APC contacts result in an increase in $[Ca^{+2}]_i$ within the Th cells; therefore, we tallied the number of responding and nonresponding Th cells at each antigen concentration, where a responder was defined as a Th cell involved in a conjugate that exhibited a sharp increase in $[Ca^{+2}]_i$ of at least 50% over basal levels, typically within ~1 min of

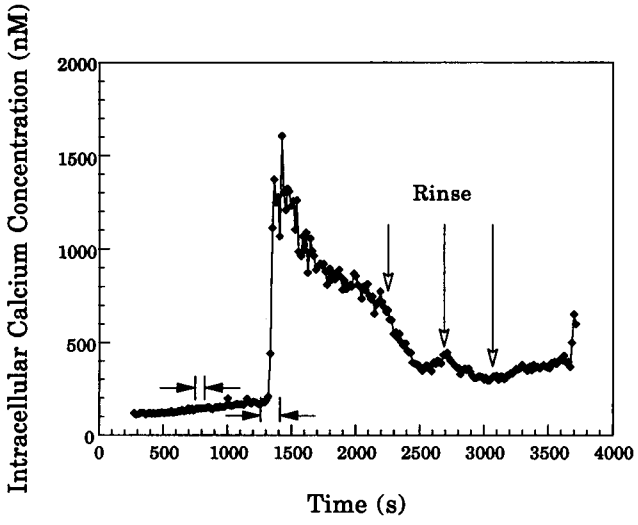

FIGURE 5 Intracellular calcium response of "T" from the Th-APC conjugate-forming experiment shown in Fig. 4. Conjugate formation occurred within the windows shown by arrows. Experimental medium was added to the flow chamber to rinse the coverslip at the times indicated by the open arrows.

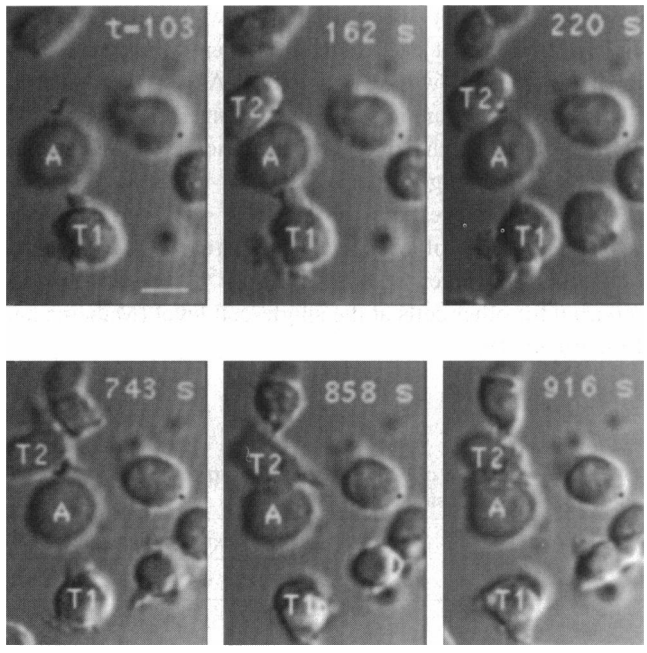

FIGURE 6 Time series of Nomarski images taken from one particular Th-APC conjugate-forming experiment. The Th cells of interest are labeled "T1" and "T2," and the corresponding APC is labeled "A." In this experiment, 10 μM HEL and poly-D-lysine-coated coverslips were used. The bar shown in the first image is 10 μm in length.

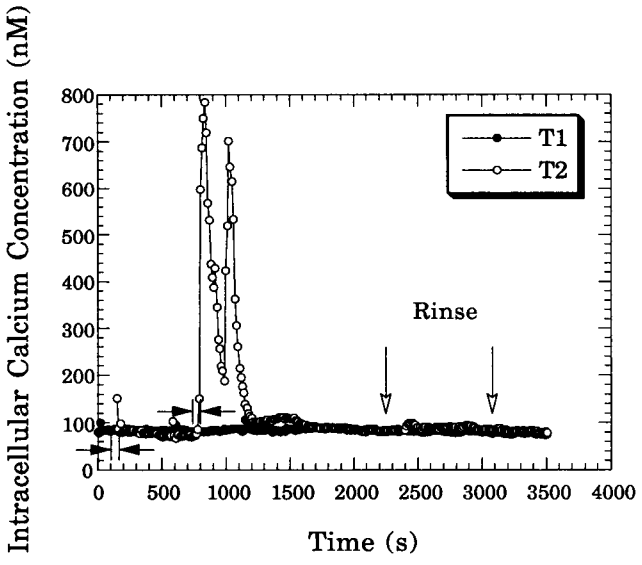

FIGURE 7 Intracellular calcium response of "T1" and "T2" from the Th-APC conjugate-forming experiment shown in Fig. 6. Conjugate formation occurred within the windows shown by arrows. Experimental medium was added to the flow chamber to rinse the coverslip at the times indicated by the open arrows.

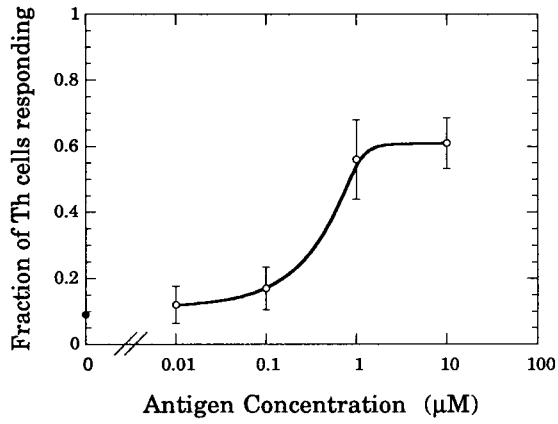

FIGURE 8 Fraction of Th cells responding as a function of antigen concentration in Th-APC conjugate-forming experiments. Open data points (○) represent the fraction of Th cells responding for experiments on acid-washed coverslips, where error bars represent standard deviations calculated from the binomial distribution for each antigen concentration. The closed data point (●) represents the fraction of Th cells responding during control Th-APC conjugate-forming experiments on acid-washed coverslips involving no antigen. The curve shown is the result of a curve fit to the data.

conjugate formation, and a nonresponder was defined as a Th cell involved in a conjugate without an accompanying increase in $[Ca^{+2}]_i$. Table 1 summarizes the data for the fraction of Th cells responding at each antigen concentration for experiments on acid-washed coverslips. Similar results were found for experiments on poly-D-lysine-coated coverslips (data not shown). Fig. 8 shows the fraction of Th cells responding at each antigen concentration for experiments on the acid-washed coverslips. As can be seen from Fig. 8, the fraction of cells responding is ~10% at low antigen doses. As the antigen dose is increased, the fraction of cells responding increases sharply, possibly in a step-function manner, reaching a plateau at ~60%. It is unclear why the fraction of cells responding reaches a plateau at only 60%; however, similar response behavior has been reported for other cells at the single-cell level (Mahama and Linderman, 1994).

TABLE 1 Fraction of Th cells responding on acid-washed coverslips

	Antigen concentration (μM)			
	0.01	0.10	1.00	10.00
Conjugates	34	35	18	41
Responders	4	6	10	25
Apparent spontaneous responders*	5	8	8	9
Total Th cells observed	146	147	110	120
Fraction of cells responding	0.12	0.17	0.56	0.61

* Spontaneous responders are defined as Th cells that exhibit a sharp increase in $[Ca^{+2}]_i$ without specific APC contact.

Th cell motility plays an important role in Th-APC conjugate formation

As was observed in the Nomarski images in Figs. 4 and 6, Th cell motility seems to play a role in Th-APC conjugate formation. This Th cell motility involves, for the most part, the extension of pseudopods rather than a net movement of the Th cell from one point to another. Because of the apparent importance of Th cell motility, a conjugate was defined throughout all experiments as a Th-APC interaction involving a Th cell that actively migrated toward and contacted an APC. This method eliminated Th cells and APCs in which the APCs merely settled next to the Th cells.

In the experiments shown in Figs. 4 and 6, all of the Th cells of interest seem to migrate in the "right" direction, the direction of a nearby APC, whereas in other experiments (not shown) the Th cells seem to extend pseudopods in random directions, regardless of APC position; therefore, the direction of motion was examined for all Th cells, both conjugated and unconjugated. Of all Th cells not involved in conjugates, 17% (102/616) migrated in a directed manner toward APCs, 6% (38/616) migrated in a seemingly random manner, and the remainder generally migrated minimally or not at all. Of all Th cells later involved in Th-APC conjugates, 67% (105/157) migrated in a directed manner toward APCs, 11% (18/157) migrated in a seemingly random manner, and the remainder generally migrated very minimally, meaning that the APCs settled so closely to the Th cells that the Th cells did not migrate far in establishing contact. Upon closer analysis of the Th cells involved in conjugates, we observed that the distribution of the direction of motion was similar for both responders and nonresponders. From these data, we conclude that directed Th cell motility is crucial in establishing Th-APC contact, although it has no apparent

role in determining whether or not the Th cell responds to the resulting Th-APC contact.

Size of Th-APC contact area increases with time

We observed that the size of the Th-APC contact area increases with time up to a maximum area for both responding and nonresponding conjugates. The duration of the maximum Th-APC contact ranged from 4 to 5 min, with the division between the Th cell and APC often appearing indistinguishable in Nomarski images (see Fig. 4, 1407 s, and T2-A in Fig. 6, 916 s.). In addition, for responding conjugates, the maximum Th-APC contact occurred on average ~1–2 min after the initial calcium response of the Th cell. After the maximum Th-APC contact, the Th-APC contact area decreased to varying extents; 78% of the conjugates remained intact for the duration of the experiments, up to 55 min, whereas 22% of the conjugates became visibly detached, on average ~4 min and 13 min after the initial Th-APC contact for nonresponding and responding Th cells, respectively.

The initial and maximal Th-APC contact areas were estimated for all conjugates, as described in the Materials and Methods section. Estimated initial contact areas ranged from 4 μm^2 to 112 μm^2, with an average of 32 μm^2, and estimated maximal contact areas ranged from 11 μm^2 to 180 μm^2, with an average of 80 μm^2. Expressed as a percentage of the APC surface area, estimated initial contact areas ranged from 0.6% to 22%, with an average of 5.3%, and estimated maximal contact areas ranged from 2% to 29%, with an average of 14%.

ANOVA was used to determine whether the initial and maximal contact areas were statistically different between responding and nonresponding conjugates and between antigen concentrations. No statistical difference was seen in initial contact areas between responding and nonresponding conjugates at a 10% level of significance; however, the maximal contact area was found to be larger for responding conjugates (at a 10% significance level). The contact area data for the responding and nonresponding conjugates are summarized in Table 2. For both the responding and nonresponding conjugates, no difference was seen in either initial or maximal contact areas between antigen concentrations at a 10% level of significance. Furthermore, for the responding conjugates, no correlation was found between the size of the contact areas and the type of calcium response (single-peaked, oscillatory, or miscellaneous).

TABLE 2 Summary of Th-APC contact areas

	Combined	Nonresponding conjugates	Responding conjugates
Initial contact area (μm^2)	31.5 ± 19.0*	30.4 ± 18.6	33.2 ± 19.6
Initial contact area (% of APC surface area)	5.3 ± 3.4	5.3 ± 3.8	5.3 ± 2.9
Maximal contact area (μm^2)	80.1 ± 32.2	71.2 ± 32.6	92.2 ± 27.7
Maximal contact area (% of APC surface area)	13.5 ± 5.8	12.3 ± 5.9	15.1 ± 5.3

* Values are expressed as mean ± SD.

Few MHC-Ag complexes are calculated to be required in the initial Th-APC contact area to elicit a Th cell calcium response

We hypothesize that a Th cell calcium response will occur only if a threshold number of MHC-Ag complexes (or more) is present within the initial Th-APC contact area. We estimated this threshold number of complexes by using the combination of 1) the assumption that MHC-Ag complexes are randomly (not uniformly) distributed on the APC surface, 2) a simple probabilistic calculation, and 3) an estimate of the expected number of MHC-Ag complexes on the APC as a function of antigen concentration.

We used a simple model that allows calculation of the probability of a Th cell response as a function of antigen concentration. The probability of Th cell response, p, is

$$p = \sum_{c=c_{\text{thresh}}}^{Q} p_c \tag{1}$$

where c_{thresh} is the number of MHC-Ag complexes that must be "seen" by a Th cell (i.e., be present in the contact area) in order to induce a calcium response, and p_c is the probability that c complexes are present in the initial Th-APC contact area. p_c is given by the binomial distribution

$$p_c = \frac{Q!}{(Q-c)!\,c!} A^c (1-A)^{Q-c} \tag{2}$$

Q is the total number of MHC-Ag complexes present on the APC surface, and A is the fractional APC surface area involved in the contact area. Equations 1 and 2 were used to generate a plot of the probability of Th cell response, p, as a function of the average number of MHC-Ag complexes in the contact area, QA, for different values of c_{thresh}. This theoretical plot is shown in Fig. 9, where the curves represent the different assumed values of c_{thresh}.

We used our experimental data to estimate the value of c_{thresh} for our experimental system. To do this, we assume that our cells are identical and that they all respond identically under the same conditions. The value of A was obtained from our experimental Th-APC contact area measurements (Table 2). The values of p were obtained by using our experimental fraction of Th cell responding data (Fig. 8), subtracting background levels of Th cell response, and scaling the probability of Th cell response to fall between 0 and 1. Q was estimated as a function of antigen concentration by using the antigen processing model developed by Singer and Linderman (1990, 1991). In particular, we used the synthesis/cycle model with 110 μM competing antigen (representing the 10% fetal calf serum in our culture media). Parameter values are the same as those used by Singer and Linderman with the exception of the following values obtained from literature on TA3 cells (in the notation of Singer

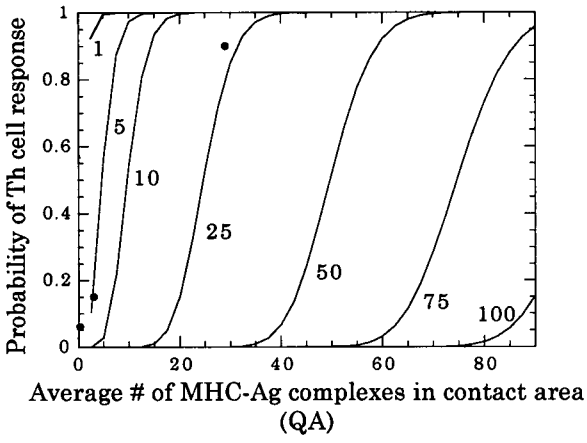

FIGURE 9 Calculated probability of a Th cell calcium response as a function of the average number of MHC-Ag complexes in the contact area, *QA*, for different values of the threshold number of complexes, c_{thresh}. The closed data points (●) represent experimental probabilities of Th cell calcium response as a function of the predicted number of MHC-Ag complexes in the contact area.

and Linderman): $A_0 = 2 \times 10^5$ (Harding and Unanue, 1990), $k_v = 1\ \mu m^3$/cell/min (Selby et al., 1995), $k_{-v} = 0.10$ min^{-1} (Selby et al., 1995), $K_d = 1\ \mu M$ (Rothbard and Gefter, 1991), $k_r = 5.22 \times 10^{-5}$ min^{-1} (Rothbard and Gefter, 1991), $k_f = 52.2\ M^{-1}\ min^{-1}$ (Rothbard and Gefter, 1991), $k_{shed} = 1 \times 10^{-3}$ min^{-1} (Harding and Unanue, 1990), and $SA_c = 630\ \mu m^2$ (our data).

To estimate our value of c_{thresh}, we plotted our experimental values of p and QA over our theoretical curves in Fig. 9. Because our experimental values, indicated by the closed data points, seem to lie along a curve somewhere between the theoretical curves of $c_{thresh} = 1$ and $c_{thresh} = 20$, we estimated the value of c_{thresh} for our experimental system to be on the order of 1–20 MHC-Ag complexes.

DISCUSSION

We used live APCs, previously incubated with whole protein antigen, to physiologically stimulate live Th cells and dynamically monitored both the physical Th-APC interaction and an early event in the subsequent activation of the Th cells, the increase in $[Ca^{+2}]_i$ in those cells. Our method involves using alternating differential interference contrast and fluorescence microscopy together with digital imaging. Hence we are able to observe the timing of the intracellular calcium response of the Th cell in relation to the time of Th-APC conjugate formation. However, because we collected Nomarski images only every minute, we could monitor the formation of Th-APC conjugates only within that accuracy. Figs. 3, 5, and 7 show examples of the timing of the increase in $[Ca^{+2}]_i$ in relation to the time of conjugate formation. Typically, the increase in $[Ca^{+2}]_i$ of the Th cells, which consisted of a sharp increase of at least 50% over basal levels, occurred within ~1 min (one Nomarski image) of Th-APC conjugate formation.

In addition to monitoring the timing of the Th intracellular calcium response, we both measured and statistically analyzed the intracellular calcium responses of individual Th cells involved in Th-APC conjugates at varying antigen doses. We found that not all Th cells involved in Th-APC conjugates respond with an increase in $[Ca^{+2}]_i$. However, for the Th cells involved in conjugates that do respond, we found that the Th cell calcium responses are heterogeneous and that no correlations exist between antigen concentration and any particular characteristics of the individual calcium responses, suggesting that the intracellular calcium response of individual Th cells is an all-or-none phenomenon. In other words, Th cells involved in conjugates either respond or do not respond, and if they do respond, they respond in a heterogeneous manner, independent of antigen concentration. The response heterogeneity that we observed could be attributable to several factors: the efficiency of APC processing and presentation or Th cell response may vary depending on the stage of cell cycle, the age of the cells, or the length of the antigen incubation period >8 h. Nevertheless, the calcium response heterogeneity that we observed in our single-cell experiments is not unique. Similar cell-to-cell variability has been reported, for example, in porcine arterial smooth muscle cells (Linderman et al., 1990), BC_3H1 smooth muscle-like cells (Mahama and Linderman, 1994), and human peripheral T cells (Dolmetsch and Lewis, 1994; Wacholtz and Lipsky, 1993). Similarly, all-or-none type calcium response phenomena have been reported for other cell systems studied at the single-cell level (Mahama and Linderman, 1994; Mahoney et al., 1992). We note, however, that these examples of calcium response heterogeneity and all-or-none calcium responses are for systems in which soluble ligands, not cells, are used to stimulate single cells.

We found that the fraction of Th-APC conjugates involving responding Th cells is an increasing function of the antigen concentration. This type of concentration-dependent cellular response has been reported in soluble ligand/cell systems studied at the single-cell level (Mahama and Linderman, 1994; Mahoney et al., 1992). Taken together, our results suggest that the overall enhancement of the Th cell response that is typically observed at the population level when the antigen concentration is increased (Vidard et al., 1992; Ozaki et al., 1988) is caused not by an increase in the magnitude of individual Th cell responses, which in our system is not dependent on antigen concentration, but rather by the increased fraction of Th cells responding, which in our system is dependent on antigen concentration.

Our observation that only a fraction of Th cells involved in Th-APC conjugates responds with an increase in $[Ca^{+2}]_i$ leads us to question why some Th cells respond to APC stimulation and others do not. One obvious possibility is that either the Th cell or the APC is defective in nonproductive conjugates. Although it is unusual for us to observe the situation of multiple APCs interacting with a single Th cell or multiple Th cells interacting with a single APC, we do observe an example of each of these types of multiple

interactions in Figs. 4 and 6. In both cases, one of the Th-APC interactions results in a responding conjugate, whereas the other interaction, which involves one of the cells from the responding conjugate, does not. Hence our data do not support the hypothesis that defective cells are the sole cause of nonproductive conjugates.

Instead, we speculate that the Th cell must recognize a threshold number of MHC-Ag complexes in order to initiate a calcium response. Experimental data suggest that APC expression of as few as 100–300 MHC-Ag complexes is sufficient to activate Th cells (Harding and Unanue, 1990; Demotz et al., 1990). However, even fewer are likely to be bound by TCRs, inasmuch as we find that only ~5% of the APC surface area is involved in the initial Th-APC contact. Because of the low number of MHC-Ag complexes involved, probabilistic effects may be important. We obtained an estimate of this threshold number of MHC-Ag complexes that must be seen by a Th cell by using our experimental data in conjunction with our previously developed mathematical models and literature data on antigen processing parameters. Because the Th cell calcium response is an early event in the Th-APC interaction, we considered only the initial Th-APC contact area and neglected diffusion of complexes. We also neglected removal of complexes as well as binding kinetics. For reasonable parameter values, we estimate that 1–20 MHC-Ag complexes are needed within the initial Th-APC contact area in order to produce a Th cell calcium response. We note that this threshold number of complexes may vary for different Th-APC-Ag systems. For example, the threshold number of complexes required in related cytotoxic T cell systems has been reported to range from fewer than 10 to several thousand complexes per target cell (Kageyama et al., 1995). It would not be surprising if different APCs/target cells have a different threshold number of complexes required to elicit a T cell response inasmuch as the affinities of TCRs are known to vary for different MHC-Ag complexes (Kageyama et al., 1995; Sykulev et al., 1994). It would be of interest, then, to determine whether the number of bound TCRs required to elicit the T cell response is the same for APCs/target cells that express different threshold numbers of complexes.

We analyzed the role of Th cell motility in the initiation of Th-APC contacts. We found that Th cell motility seems to play an important role in establishing Th-APC contact but not in determining the Th cell calcium response. The high percentage of Th cells later involved in Th-APC conjugates that migrated in a directed fashion toward APCs in our experiments suggests a possible role of chemotaxis in establishing the Th-APC contact; it is possible that the Th cells can sense and travel up a chemical gradient produced by substances released or secreted by the APCs. Th cells are known to be attracted to some of the same chemoattractants that attract neutrophils and monocytes. These chemoattractants are believed to aid in lymphocyte recirculation and trafficking within the body (Springer, 1994). However, our experiments were not designed to measure chemotaxis because we did not specifically place APCs so as to create a chemotactic gradient and because we periodically added media to the flow chamber, potentially disturbing any chemotactic gradient that might have been present.

Our experimental Th-APC contact area observations are consistent with the idea of a two-stage immune surveillance mechanism in which antigen-independent Th-APC adhesion precedes specific TCR/MHC-Ag recognition (Davis and Chien, 1993). It is likely that Th-APC adhesion mediated through complementary receptors located on both cells precedes TCR/MHC-Ag binding (Springer, 1990) because the affinity of the TCR for the MHC-Ag complex is fairly low, with the K_d ranging from 10^{-4} to 10^{-5} M (Davis and Chien, 1993; Premack and Gardner, 1992; Williams and Beyers, 1992), and the affinities of other T cell-APC adhesion molecules, such as CD2/LFA-3 and CD28/B7, are considerably higher (Davis and Chien, 1993). Our experimental observation that the initial Th-APC contact areas are the same for both responding and nonresponding conjugates is consistent with this idea. In addition, experimental data suggest that diffusion of adhesion receptors into the contact area between the cells is essential in stabilizing the intercellular adhesion (Singer, 1992). In particular, the TCR and CD4 have been shown to co-cluster, along with LFA-1, on the Th cell surface into the Th-APC contact area (Kupfer and Singer, 1989). This diffusion of adhesion molecules into the Th-APC contact area is consistent with our observation that the Th-APC contact area increases with time for both the responding and nonresponding conjugates. Upon specific triggering of the TCR, cell-cell adhesion is known to become greatly enhanced. The reason for this enhanced adhesion is a transient TCR-induced increase in the affinity of LFA-1 for its complementary receptors ICAM-1 and ICAM-2 (Dustin and Springer, 1989a, b). The high affinity state of LFA-1 peaks after 5–10 min, and the normal low affinity state returns within 30 min to 2 h. The return to the low affinity state could provide a means for the eventual de-adhesion of the Th-APC conjugate (Dustin, 1990). Again, our observation that the maximal Th-APC contact area is greater for responding conjugates than for nonresponding conjugates is consistent with this TCR-induced enhanced adhesion.

Why do we find that most of our Th-APC conjugates remain intact for >55 min when the initial increase in $[Ca^{+2}]_i$ of the Th cell begins typically within 1 min of Th-APC contact and ends within 20 min? We speculate that although the Th cell may require a threshold number of MHC-Ag complexes to be bound in order to initiate a cellular response (e.g, the calcium response), the Th cell may require the binding of more MHC-Ag complexes in order to continue a cellular response (e.g., the intracellular signaling events leading to the secretion of IL-2). During the prolonged Th-APC contact, there is likely to be sufficient time for the diffusion of most MHC-Ag complexes into the contact area, followed by trapping of these complexes through binding to TCR (Linderman et al., 1994). Other adhesion and signaling molecules on both cells may also diffuse into the contact area. Thus the prolonged contact

may allow the Th cell to "count" the total number of MHC-Ag complexes bound at steady state and respond accordingly. In other words, although we find the intracellular calcium response of individual Th cells involved in Th-APC conjugates to be an all-or-none phenomenon, later individual cell responses, such as cell proliferation and IL-2 secretion, may in fact depend on antigen concentration. A parallel may be drawn to the epidermal growth factor receptor system, in which initial receptor/ligand binding is sufficient to generate some signals (e.g., calcium increases and ion fluxes) but not final commitment, and in which the cell proliferation rate has been found to be linearly related to the number of occupied receptors at steady state (Knauer et al., 1984).

Two other single-cell Th-APC calcium studies have been reported, and we would like to compare and contrast some of our experimental results with those from these studies. We will first briefly describe some of the differences in cell systems between our work and that of these two other groups, Donnadieu et al. (1992) and Røtnes and Bogen (1994). We used a homolog murine hybridoma Th-APC cell system, and our antigen is whole and requires processing by APCs before Th-APC coupling experiments. However, Donnadieu et al. used a heterolog cell system consisting of a human Th cell clone and a transfected murine fibroblast, although they too used whole antigen that required APC processing. Røtnes and Bogen used a homolog murine cell system involving a Th1 clone. Like Donnadieu et al., their APC was a transfected murine fibroblast. Unlike both Donnadieu et al. and us, Røtnes and Bogen used synthetic peptides as antigen, which required little or no APC processing. We will discuss the effects that these different cell systems might have on the experimental results.

We observed that the increase in $[Ca^{+2}]_i$ of the Th cells occurred within ~1 min of Th-APC conjugate formation, and Røtnes and Bogen (1994) observed the increase to occur on the order of only a few seconds to a few minutes after conjugate formation, which is in agreement with our findings. However, Donnadieu et al. (1992) observed the increase in $[Ca^{+2}]_i$ to occur 6 min after Th-APC contact. We believe that the discrepancy in the timing of the Th cell response could be attributable to the heterolog (human-mouse) Th-APC system used by Donnadieu et al. This system is thought to involve no complementary adhesion molecules other than the TCRs and CD4 molecules on the Th cells and MHC-Ag complexes on the APCs. In Th-APC cell systems that do possess complementary adhesion molecules, it is known that the affinity of the TCR for the MHC-Ag complex is fairly low, whereas the affinities of other T cell-APC adhesion molecules are considerably higher (Davis and Chien, 1993). Presumably then, binding of the higher affinity Th-APC complementary adhesion molecules would facilitate the binding of the weaker affinity TCR/MHC-Ag (Singer, 1992), and the absence of such higher affinity complementary adhesion molecules could increase the time required for a threshold number of MHC-Ag complexes to be bound by TCRs.

Although our Th cell calcium responses consisted of three types of behavior, the responses observed by Donnadieu et al. (1992) and primarily reported by Røtnes and Bogen (1994) consisted of oscillations. When oscillations were observed, the average oscillation period was similar in all three studies (~100 s.). However, some other characteristics of the oscillatory responses were slightly different. The oscillations observed by Donnadieu et al. and Røtnes and Bogen lasted for 30–40 min and ~25 min, respectively; afterward, oscillations ceased but the $[Ca^{+2}]_i$ typically remained elevated above basal levels. The oscillations that we observed lasted for a shorter period of time, 2–20 min, after which the oscillations ceased and the $[Ca^{+2}]_i$ typically returned to basal levels, unlike in the previous two Th-APC studies.

Finally, we found that the intracellular calcium responses of individual Th cells are heterogeneous and that the intracellular calcium response is an all-or-none phenomenon, independent of antigen concentration; however, using their Th-APC system, Røtnes and Bogen (1994) reported that the individual Th intracellular calcium response is dependent on antigen concentration. Although they observed considerable diversity between different Th cells stimulated at the same peptide concentration, the maximum and mean increase in $[Ca^{+2}]_i$ and the frequency and period of oscillations correlated with the amount of peptide used for pulsing their fibroblast APCs. In addition, they observed that the calcium response latency and increase rate also correlated with the peptide concentration.

We suspect that these dramatically different results could be attributable to different experimental conditions or more likely to the different Th-APC cell systems that were used; however, it is not clear exactly what aspects of the different cell systems may be responsible for the different Th cell responses. Ideally, cellular studies should be performed with physiologically relevant cells, such as cells taken directly from the human body or some other organism. However, single-cell Th-APC interactions cannot be easily studied using freshly isolated cells because of the Th-APC-Ag specificity required. Alternative cell systems such as clones and hybridomas are often used for studies of antigen presentation. Hybridomas are more convenient to use because they are more easily maintained than are clones, which require periodic restimulation. However, some caution must be taken in that hybridomas and clones may respond differently (Ozaki et al., 1988), and few studies have compared freshly isolated cells with clones or hybridomas. We chose to use the 3A9-TA3 hybridoma cell system in our studies because the system has been well characterized, and thus our data can be interpreted in the context of other information on antigen processing and presentation already available with this cell system. Additional studies are needed to provide more confidence in the physiological relevance of data collected using various types of cell systems. In addition, more studies are needed to elucidate the roles that adhesion molecules and antigen (e.g., whole versus peptide)

play in determining the characteristics of the Th cell response in these various cell systems.

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