THE SPECIFIC INTERACTION OF HELPER T CELLS AND ANTIGEN-PRESENTING B CELLS

IV. Membrane and Cytoskeletal Reorganizations in the Bound T Cell as a Function of Antigen Dose

By ABRAHAM KUPFER and S. J. SINGER

From the Department of Biology, University of California at San Diego, La Jolla, California 92093

Over the last several years, we have been investigating the nature of specific cellular interactions in immunology by studying the properties of individual interacting cell couples, formed in vitro, either between NK cells or CTL and their targets, or between Th cells and APC of the B cell type (B-APC)[1] (1). In this paper, we deal with an important extension of our earlier experiments with Th/B-APC cell couples. In the earlier experiments (2–4), cloned specific Th cells were used along with APC that were B cell lymphomas or hybridomas. These B-APC were non-antigen (Ag) specific; that is, while they expressed the appropriate class II MHC molecules on their surfaces, they lacked the surface Ig (sIg) receptor for the particular Ag to which the Th corresponded. To form specific couples with a given Th cell, the non-Ag-specific B-APC were dosed with very large concentrations of the appropriate Ag. Sufficient, but unknown, amounts of the Ag were then processed by the B-APC and expressed on the cell surface. In conjunction with the appropriate class II MHC, the Ag/class II MHC complex ligand on the B-APC was then able to elicit specific responses when the Th cell was bound to the B-APC in cell couples.

The specific responses that were monitored and detected by immunofluorescence observations of these individual cell couples included: (*a*) the rapid reorientation of the microtubule organizing center (MTOC) and the Golgi apparatus (GA) inside the Th cell to face the cell contact region with the B-APC (2, 3); (*b*) the accumulation of the cytoskeletal protein talin under the membrane of the Th cell where it was bound to the B-APC (3, 4); and (*c*) the coclustering of the TCR and CD4 (4), and in preliminary experiments, LFA-1 (Kupfer, A., S. L. Swain, and S. J. Singer, unpublished observations), on the surface of the Th cell where it contacted the B-APC. These were unidirectional responses, occurring only in the bound Th cell and not in the B-APC, and were strictly specific, not occurring if the appropriate Ag was not processed or if the class II MHC on the B-APC was not the specific one for the Th under study.

This work was supported by U.S. Public Health Service grants AI-23764 to A. Kupfer and AI-06659 to S. J. Singer, who is an American Cancer Society Research Professor. A. Kupfer's present address is the National Jewish Center for Immunology and Respiratory Medicine, 1400 Jackson Street, Denver, CO 80206.

[1] *Abbreviations used in this paper:* Ag, antigen; B-APC, APC of the B cell type; MTOC, microtubule organizing center; GA, Golgi apparatus; sIg, surface Ig.

To explore further the molecular bases for these specific cellular responses, we next wished to use as a B cell-APC one that was specific for the Ag, in place of the non-Ag-specific B lymphoma and hybridoma cells previously used. This would allow a careful study to be made of the effects of Ag dose on the cell/cell interaction. For this purpose, we chose the cell line A20-HL (5). A20-HL is the A20 B hybridoma (IA^d) cell line that was stably transfected with the rearranged genomic DNA for the H and L chains of a surface IgM with binding specificity for the 2,4,6-TNP hapten. A20-HL cells therefore recognize and process any proteins suitably modified with TNP, or its crossreactive hapten, 2,4-DNP. In the experiments reported in this paper, DNP-modified OVA (DNP-OVA) was added at varying concentrations to A20-HL cells, and these B-APC were then used to form cell couples with cloned Th cells specific for OVA peptides in the context of IA^d. In these Th/B-APC couples, significant separations occurred of the several specific responses mentioned above, depending on the Ag dose. These and related results have led us to propose that at least two distinct signals are transmitted into the Th cell by binding to the specific B-APC, the first occurring at low Ag doses, and the second requiring higher Ag doses. The first signal stimulates a specific LFA-1-mediated intercellular adhesion, but is insufficient to induce Th cell proliferation, an indicator of T cell activation, for which the second signal is necessary.

Materials and Methods

Cells. Cloned Th cell lines were derived from the draining lymph nodes of OVA-immunized B10.D2 mice. The preparation and properties of the clone D20.36 (D36), specific for the peptide 323–339 of OVA and for IA^d, which was generously provided to us by Dr. Dale Wegmann (Lilly Research Laboratories, La Jolla, CA), will be described in detail elsewhere (Wegmann, D., manuscript in preparation). The cells were stimulated with Ag and PMA-induced EL4-supernants on a biweekly schedule. The transfected B lymphoma line A20-HL was a generous gift of Dr. N. Hozumi and was maintained as described (5).

Antibodies. The affinity-purified rabbit antibodies specific for chicken gizzard talin and chick tubulin, and the rat mAb anti-CD4, GK1.5, were described before (2–4). The rat mAb I21 (6), specific for LFA-1 on murine T cells, was a generous gift of Dr. Ian Trowbridge from the Salk Institute (La Jolla, CA). The secondary affinity-purified Abs, rhodamine-labeled $F(ab')_2$ fragments of goat anti-rabbit IgG and biotinylated $F(ab')_2$ fragments of goat anti-rat IgG (both from Jackson Immuno Research, Avondale, PA) were further purified as described (7). Fluorescein-labeled streptavidin was obtained from Amersham Corp., Arlington Heights, IL.

Cell Couples and Procedures for Immunofluorescence Microscopy. OVA or DNP-OVA in DME was added to A20-HL cells for 18–20 h, and unbound Ag was then washed away. The formation of 1:1 cell couples with the Th cells, and the processing of these cell mixtures for immunofluorescence microscopic observations, were carried out as described (2–4). At least 100 cell couples were scored on each coverslip. In our attempts to quantify these microscopic observations, we noticed that the efficiency of cell coupling could vary when experiments were repeated on different dates. However, independent of the total number of cell couples, the fraction of cell couples displaying any of the properties that are reported here was found to be very reproducible.

Ag and Proliferation Assays. OVA and Conalbumin were obtained from Sigma Chemical Co., St. Louis, MO. DNP-OVA was prepared as described (8), and had, on the average, 5.5 DNP groups per OVA molecule. The proliferation of the D36 cells was assayed by coculturing for 3 d irradiated (25,000 rad) A20-HL cells (5×10^4 cells/well) with the D36 cells (2×10^4 cells/well) in the presence of the indicated concentrations of DNP-OVA. In some experiments the A20-HL cells were pulsed overnight with DNP-OVA, and after washing away the unbound Ag, the A20-HL cells were irradiated and cocultured with D36 cells in the ab-

sence of freshly added DNP-OVA. [^{3}H]Thymidine (1 μCi/well; New England Nuclear, Boston, MA) was present during the last 6 h of the coculture. The cells were harvested and the incorporated [^{3}H]thymidine was determined by liquid scintillation spectrometry.

Results

The Coclustering of LFA-1 and Talin at the Surfaces of Th Cells in D36/A20-HL (Th/B-APC) Couples. A20-HL cells were pulsed with concentrations of DNP-OVA ranging from 0 to 1,000 ng/ml and mixed at a 1:1 ratio with D36 cells. The fixed cell mixtures were then double-immunofluorescently labeled using the primary reagents anti-LFA-1 mAb I21 and polyclonal rabbit anti-talin antibodies. We have previously shown (3, 4) that the accumulation of talin under the Th cell membrane where it is in direct contact with the B-APC serves as a criterion for the formation of a specific Th/B-APC couple. At concentrations of 25 ng/ml DNP-OVA or greater, both talin and LFA-1 were found to be maximally (>95% of the couples) colocalized on the D36 cell surface where it was in contact with the APC (as in Fig. 1, *A–C*). This represents a ~2,000-fold lower concentration for the effective processing and expression of the specific (DNP-OVA) vs. the nonspecific (OVA) Ag by the same B-APC (results not shown). When concentrations of DNP-OVA <25 ng/ml were used, there was a

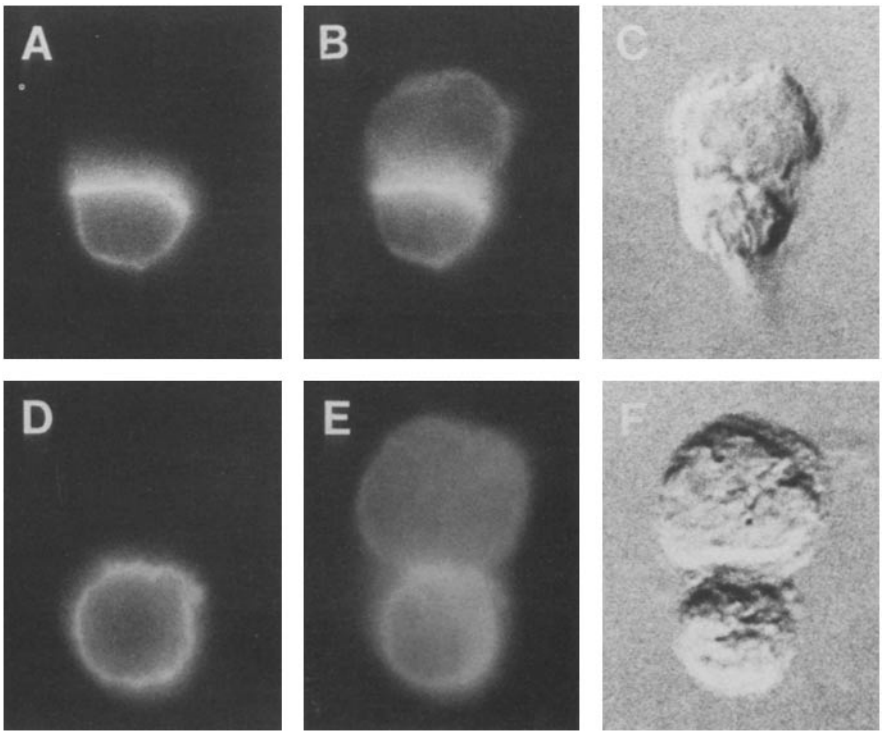

FIGURE 1. Double-immunofluorescence labeling of LFA-1 and talin in D36/A20-HL couples pulsed with 5 ng/ml DNP-OVA. (*A* and *D*) LFA-1 labeling; (*B* and *E*) talin labeling; and (*C* and *F*) the corresponding Nomarski pictures. The couple in *A–C*, with both proteins concentrated at the cell contact region, represents ~70% of the couples in the cell mixture, and that in *D–F*, with neither protein redistributed, represents the remaining 30%. Same magnification as in Fig. 6.

progressive decrease in the numbers of cell couples formed, and of those, an increasing fraction showed no talin or LFA-1 redistribution (as in Fig. 1, *D–F*). These dose-response results are summarized in Fig. 2. It is significant that in those couples that displayed a clustering of talin in the cell contact region, LFA-1 was always coclustered with the talin (Fig. 2), and furthermore, the labeling intensity of these clusters was about the same at both low and high doses of DNP-OVA. In other words, the talin/LFA-1 redistributions were always coordinate, and in that fraction of the couples where they occurred, showed the same extent of redistribution independent of the Ag dose. It was also observed that at the light microscopic level of resolution, the morphological appearances of the specific cell couples, and in particular their cell/cell contact regions as viewed with Nomarski optics, were indistinguishable whether the Ag expressed was specific or nonspecific for the B-APC (not shown). The effective cell couples often appeared, however, to show larger areas of cell/cell contact than the ineffective ones (compare Fig. 1, *C* with *F*).

The Effect of Ag Dose on the Reorientation of the Th MTOC in Th/B-APC Couples. In our previous experiments, we showed that in addition to the talin redistribution discussed above, a second criterion for the formation of a specific Th/APC couple was the reorientation of the MTOC (coordinately with the GA) inside the Th cell to face towards the region of cell contact (3). On the other hand, the coupling of these two events was not stringent. In particular, the MTOC reorientation was shown to be generally dependent on extracellular Ca^{+2}, while the talin redistribution was not (3). We therefore examined by double-immunofluorescence labeling the effect of DNP-OVA concentration on both the LFA-1 distribution and the MTOC orientation in individual D36/A20-HL couples. The results are shown in Figs. 3 and 4. At sufficiently large concentrations of DNP-OVA (>100 ng/ml), in essentially all (>95%) of the couples, the LFA-1 was concentrated at the cell contact area (as in Fig. 3 *A*), and simultaneously, the MTOC in the Th cell was oriented to face the cell contact (as in Fig. 3 *B*, *arrow*). With decreasing concentrations of DNP-OVA, in the range of 100 to 10 ng/ml, the LFA-1 was still concentrated at the cell contact area in essentially all of the couples, but the extent of the MTOC reorientation progressively decreased (Fig. 4). An example of a cell couple in which the LFA-1 redistribution occurred (Fig. 3 *D*) while the MTOC orientation in the Th cell remained random (Fig. 3 *E*, *arrow*) is shown. Finally, in the absence of any DNP-OVA, generally neither the LFA-1 redistribution nor the MTOC reorientation was observed in any of the couples (Fig. 3, *G–I*). These results confirm that the LFA-1 redistribution

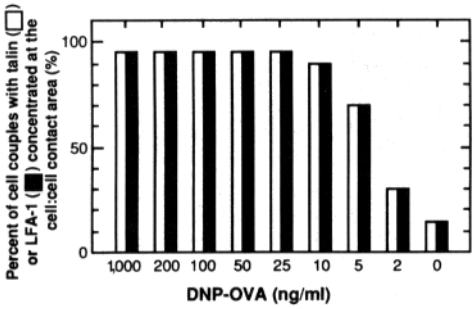

FIGURE 2. The effects of the concentration of the DNP-OVA on the distribution of LFA-1 (*open bars*) and talin (*shaded bars*) in D36/A20-HL cell couples. A20-HL were pulsed overnight with the indicated concentrations of DNP-OVA and mixed with equal numbers of D36 cells. The cell mixtures were double-immunofluorescently labeled for talin and LFA-1, as in Fig. 1. The percentage of cell couples that displayed a redistribution of LFA-1 or talin into the contact area, as shown in Fig. 1, *A–C*, was scored for each cell mixture.

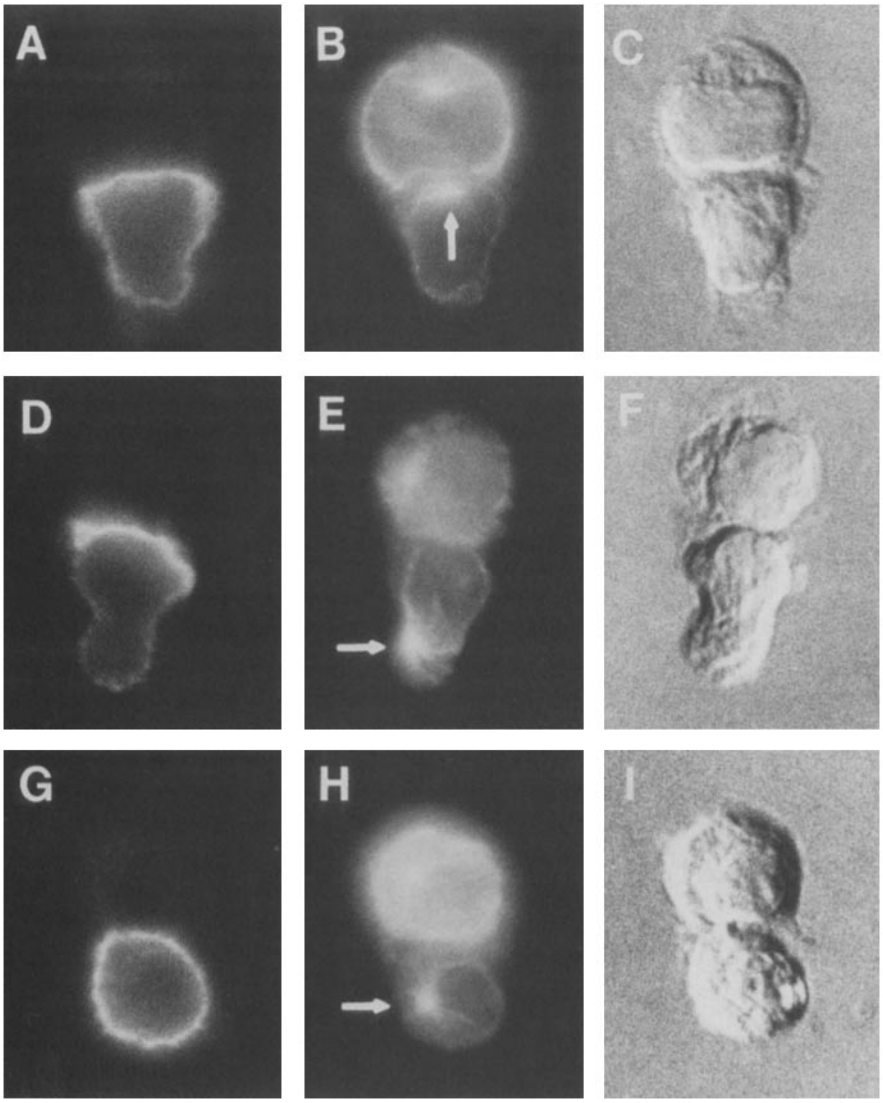

FIGURE 3. Double-immunofluorescence labeling of the MTOC (*B*, *E*, and *H*) and LFA-1 (*A*, *D*, and *G*) in D36/A20-HL conjugates that were pulsed with different concentrations of DNP-OVA. The Normarski pictures of the corresponding cells are shown in *C*, *F*, and *I*. The cell couple in *A–C* was formed with A20-HL pulsed with 100 ng/ml DNP-OVA. This cell couple represents the majority (90%) of the cell couples in this cell mixture. Note that LFA-1 shows a concentration at the contact area and the D36-MTOC (*arrow*) is facing the A20-HL cell. The cell couple in *D–F* was formed with A20-HL pulsed with 25 ng/ml DNP-OVA. This cell couple represents ~35% of the cell couples in this cell mixture, with the other 65% appearing as in *A–C*. Note that in *D–F*, LFA-1 is clustered to the contact area but the D36-MTOC (*arrow*) is facing away from the A20-HL cell. The cell couple in *G–I* was formed in the absence of any DNP-OVA, and it represents about half (45%) of the population of cell couples in this cell mixture. Note that LFA-1 is uniformly distributed on the D36 cell and that the D36-MTOC (*arrow*) is not facing toward the A20-HL. An entirely random orientation of the MTOC corresponds to 50% by our scoring method. The magnification is the same as in Fig. 6.

and the MTOC reorientation in the Th cell are both dependent on the presentation of the specific Ag by the B-APC, but demonstrate, however, that the two phenomena are not tightly coupled to one another, the LFA-1 redistribution persisting at low Ag concentrations where the MTOC reorientation no longer occurs.

The Effects of Ag Dose on the Proliferation of the Th Cells. The finding of Ag-specific Th/B-APC cell couples in which LFA-1 and talin were redistributed but the Th-MTOC did not reorient raised the question of what are the states of activation of the Th cell induced by such interactions. One of the major functional consequences of the interactions of Th cells with B-APC is the induction of Th proliferation. The proliferation of the D36 cells, as determined by [^{3}H]thymidine incorporation following two different experimental protocols, was assayed as a measure of T cell activation. In one set of experiments a conventional proliferation assay was performed, whereby irradiated A20-HL cells were mixed with D36 cells in the continuous presence of the indicated concentrations of DNP-OVA. In another set of experiments, corresponding to the above described microscopic studies, the A20-HL cells were pulsed overnight with DNP-OVA, and after washing away the unbound Ag, the A20-HL cells were irradiated as above. These A20-HL cells were then mixed with D36 cells without adding fresh DNP-OVA to the cell mixtures. The results of such prolifer-

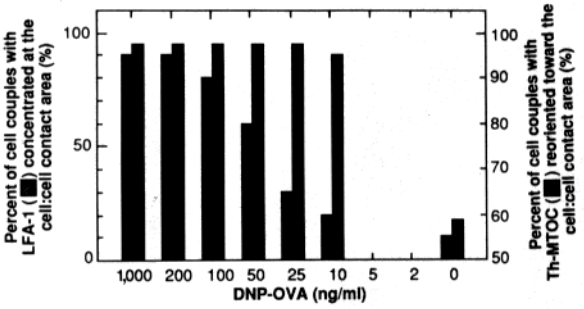

FIGURE 4. The effects of the concentration of DNP-OVA on the orientation of the Th-MTOC (*filled bars*) and the distribution of LFA-1 (*shaded bars*) in D36/A20-HL cell couples. Cell couples were formed with A20-HL pulsed with the indicated concentration of DNP-OVA. The cells were doubly labeled as described in the legend to Fig. 3. Each cell couple was scored for the surface distribution of LFA-1 and for the position of the D36-MTOC. Note that at concentrations of Ag <100 ng/ml, there is a gradual increase in the proportion of cell couples with the labeling patterns shown in Fig. 3, *D–F*. An entirely random orientation of the MTOC corresponds to 50% by our scoring method.

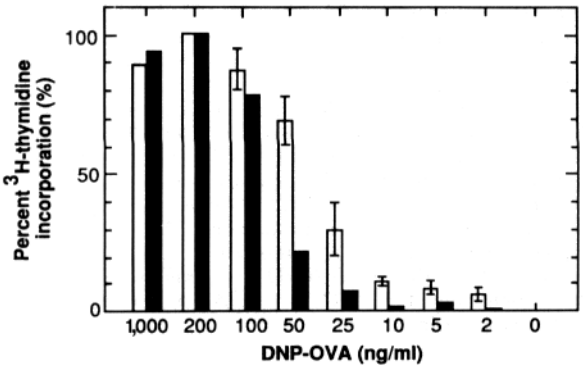

FIGURE 5. The effects of the concentrations for DNP-OVA on the proliferation of the D36 cells. The details of these assays are described in Materials and Methods. The open columns show the results of conventional proliferation assays done in continuous presence of DNP-OVA; the shaded columns show the results of assays done with DNP-OVA-prepulsed A20-HL cells.

ation assays are shown in Fig. 5. The dose-response curves as measured by the two methods are not entirely coincident, but this does not obscure the main conclusions to be derived from the results. It is clear that as the concentration of DNP-OVA was descreased between 100 and 10 ng/ml, there was a progressive decrease in the proliferation of the population of D36 cells by the B-APC. Furthermore, if we compare the proliferation results in Fig. 5 with the phenomenological data in Fig. 4, it is evident that the Th cell proliferation is better correlated with the extent of the MTOC reorientation than with the LFA-1 redistribution on the Th cells.

The Effects of Ag Dose on the Distribution of CD4 on the Th Cells in Th/B-APC Couples. Another observation made in our previous experiments using non-Ag-specific APC and large doses of Ag was that both the TCR and CD4 became clustered on the surface of the Th cell where it was bound to the specific APC (4). In the absence of the Ag, the cell couples that formed showed neither the TCR nor CD4 to be clustered at the cell contact region. It was therefore of interest to determine to what extent these coclustering effects depended on Ag dose, and whether they were more highly correlated with the LFA-1/talin clustering on the same Th cells or with the MTOC reorientation inside them. We did not have available a suitable Ab reagent specific for the TCR on D36 cells. Accordingly, only CD4 clustering was examined. A20-HL cells were pulsed with different concentrations of DNP-OVA, and these B-APC were used as above to form 1:1 complexes wih the D36 Th cells. The cell mixtures were then double-immunofluorescently labeled using the primary antibodies GK1.5, a rat mAb-specific for CD4, together with either rabbit anti-talin or rabbit anti-tubulin antibodies. With A20-HL pulsed with DNP-OVA between 50 and 10 ng/ml, most of the cell couples showed the expected talin clustering (Fig. 6, *B* and *E*), but in an increasing fraction of the couples, the CD4 distribution remained essentially uniform (compare Fig. 6, *D* with *A*). No strong correlation between talin redistribution and CD4 clustering was observed. On the other hand, a stricter correlation was found between the MTOC reorientation and CD4 clustering. Over the entire range of concentrations of DNP-OVA, in every cell couple that displayed CD4 clustering to the cell contact, the D36-MTOC faced the bound A20-HL (Fig. 7, *A–C*) and, conversely, whenever the D36-MTOC was not reoriented toward the A20-HL cell, CD4 was not clustered to the same cell contact area (Fig. 7, *D–F*).

Discussion

In our previous studies of individual cell couples formed between cloned Th cells and B-APC that were not Ag specific (reviewed in reference 1), several different phenomena were shown to accompany and characterize specific Th/B-APC interactions at high Ag doses: (*a*) the reorientation of the MTOC inside the Th cell (but not the MTOC inside the B-APC) to face the region of cell/cell contact; (*b*) the accumulation of the cytoskeletal protein talin under the Th cell membrane where it was in contact with the B-APC; and (*c*) the clustering of the independent membrane proteins TCR, CD4, and LFA-1 within the Th cell membrane to become concentrated where the Th was in contact with the B-APC. Our objectives in undertaking the present study were to understand the possible connections between these phenomena, what mechanisms induce them to occur, and what functions they may serve in the Th/B-APC interaction.

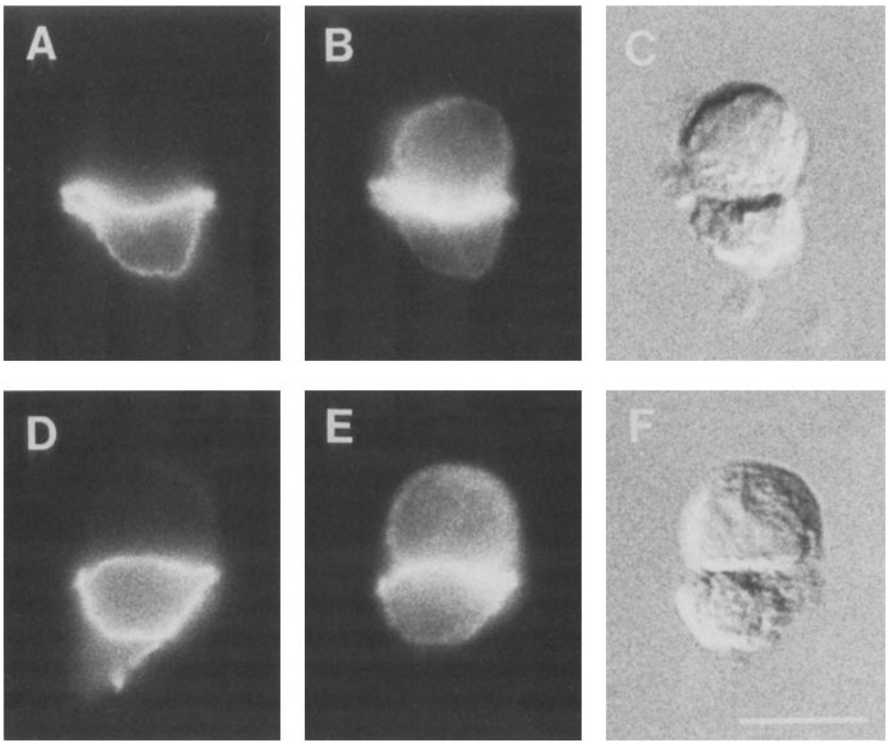

FIGURE 6. Double-immunofluorescence labeling of CD4 (*A* and *D*) and talin (*B* and *E*) in D36/A20-HL cell couples, which were pulsed with 50 ng/ml DNP-OVA. *C* and *F* show the Nomarski pictures of the corresponding cells. Note that in both cell couples talin is concentrated at the contact area but CD4 is concentrated to the same contact areas only in the *A–C* couple. The bar in *F* represents 10 μm.

In these studies we have used a B-APC that is Ag specific, namely A20-HL, a B lymphoma cell line produced by Hozumi and his colleagues (5), expressing anti-TNP sIg on its surface. These investigators showed that A20-HL cells can process and present a TNP-modified protein Ag added at very low concentrations. By studying Th cell proliferation in whole mixed cell populations of Th and Ag-dosed A20-HL, they found that a TNP-modified protein was presented ~2,000 times more effectively than the unmodified protein. In the present paper we found at the level of individual Th/B-APC couples, applying the cell biological criteria that we have previously developed to demonstrate a specific Th/B-APC interaction, that effective D36/A20-HL interactions occurred at concentrations of DNP-OVA ~2,000 times smaller than that required for OVA itself (not shown). The quantitative agreement between the two quite different types of experiments, on the one hand with entire cell populations, and on the other with individual cell couples, is striking. This correspondence, therefore, justified the use of the D36/A20-HL system to make a systematic study of the effect of Ag dose on the Th/B-APC interaction with individual cell couples. In addition, because our previous findings (2–4) were obtained with Th cells that secrete IL-4 and are classified as Th2 cells (9), the fact that the Th

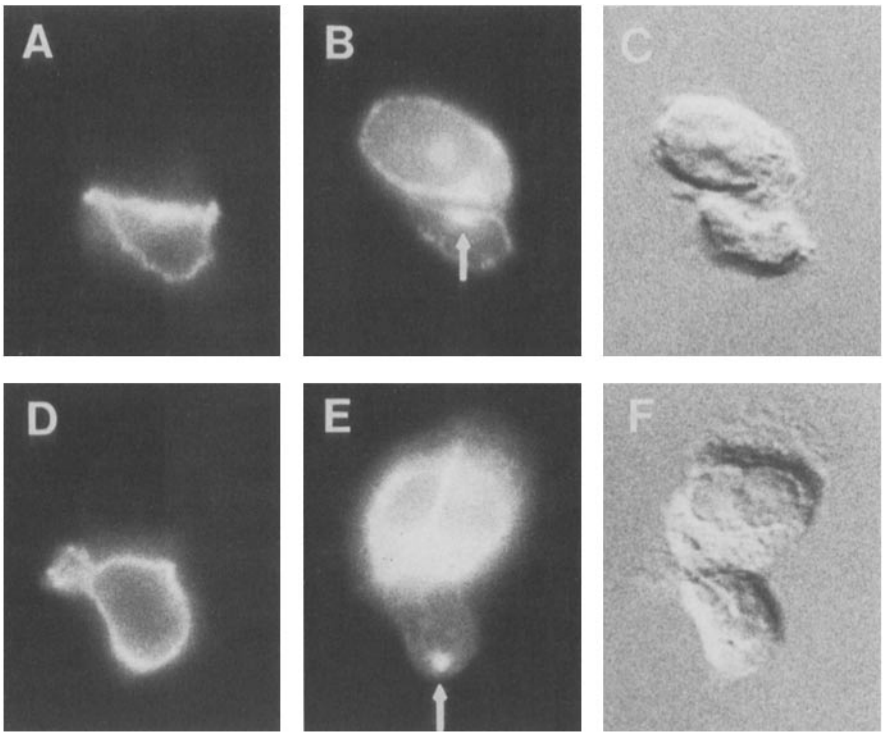

FIGURE 7. Double-immunofluorescence labeling of CD4 (*A* and *D*) and the D36-MTOC (*arrows* in *B* and *E*) in D36/A20-HL couples. The Nomarski pictures of the corresponding cells are shown in *C* and *F*. (*A–C*) A cell couple formed with A20-HL, which was pulsed with 50 ng/ml DNP-OVA, shows both a CD4 concentration and MTOC reorientation in the Th cell. (*D–F*) A cell couple formed with A20-HL pulsed with 20 ng/ml DNP-OVA; neither CD4 concentration nor MTOC reorientation is observed. The magnification is the same as in Fig. 6.

cells used in the present study secrete IL-2, but not IL-4, and are therefore Th1 cells (9), allows us to generalize our previous results to cover both Th classes (see also reference 10).

The Th-MTOC Reorientation. A remarkable finding in all of our previous studies, not only of Th/B-APC cell couples, but also of couples formed by NK cells and CTL with their target cells, was the very strong correlation between a specific, effective cell interaction and the reorientation of the MTOC inside the effector T cell to face the bound target (1). It was proposed that the principal function of the MTOC reorientation was to cause the simultaneous reorientation of the Golgi complex, because the MTOC and GA appear to be physically linked to one another (11). In view of the fact that vesicles that contain secretory components in their lumen and plasma membrane proteins in their membranes are derived from the GA, and go on to fuse with the plasma membrane to release their secretory contents at the cell exterior, a coordinate reorientation of the MTOC and GA would serve to direct secretion from the effector T cell exclusively to its bound target (12). In the case of Th/B-APC interactions, the directed and exclusive secretion of T cell-derived growth and differentiation factors, such as IL-4 and IL-5, to the specifically bound

B-APC could be an important contributor to specific B cell proliferation and differentiation (1).

The results obtained for the Th-MTOC reorientation in the present study are in agreement with our earlier findings. Within experimental uncertainties, there was a good correlation between the Th-MTOC reorientation in specific D36/A20-HL couples (Fig. 4) and the extent of Th cell proliferation in mixed populations of D36 and A20-HL cells (Fig. 5), as a function of DNP-OVA concentration. This is consistent with the conclusion that the MTOC (and GA) reorientation inside the bound Th is closely coupled to an effective Th/B-APC interaction.

LFA-1 and Talin. LFA-1 is a monomorphic integral protein molecule on the surface of all leukocytes and is a member of the family of related membrane proteins called integrins (13, 14). Integrin molecules consist of two transmembrane polypeptide chains, α and β. On the cytoplasmic face of the plasma membrane, integrin molecules are linked in some as yet unknown manner to actin microfilaments of the cytoskeleton (15). With lymphocytes, there is strong evidence that LFA-1 molecules play an important role in intercellular adhesion and interactions (16). Thus, mAbs to LFA-1 markedly inhibit different T cell effector functions. Furthermore, in humans with genetically deficient LFA-1, many immunological functions dependent on cell/cell interactions are impaired (16). There is evidence that LFA-1 on a T cell may bind transcellularly to a particular ligand on a target cell, called I-CAM-1 (16), thereby participating in intercellular adhesion. In addition, two unusual rat mAbs specific for the α chain of LFA-1 have been described (17, 18); one of which (17) induced proliferation and lymphokine secretion of cloned T cells, and the other mAb (18) mimicked the physiological responses of IL-4 (see also reference 19). While the information available clearly implicates LFA-1 as an important contributor in T cell functions, the precise mechanisms for its involvement are not understood.

Talin is a 215-kD cytoplasmic protein that was originally isolated from smooth muscle (20), but is widely distributed among different cell types. Talin is often found inside cells at specialized regions where actin filaments are known to be attached to the cell membrane; such regions generally function in cell/cell or cell/substratum adhesions. The induced accumulation of talin under T cell membranes, where they come in contact with specific target cells (as in Fig. 1 *B*), is a phenomenon specific to talin; several other actin-associated cytoskeletal proteins do not show such clustering (1).

Because the TCR, CD4, and LFA-1 transmembrane integral proteins and the cytoplasmic protein talin were all found to be coclustered into the specific cell contact sites in our earlier studies of Th/B-APC interactions (1), we considered the possibility that one or more of these transmembrane molecules might be linked to talin inside the Th cell. There was a report that a member of the chicken integrin family and talin interacted weakly with one another in vitro (21). If such an interaction existed in vivo, we reasoned that the capping of integrin molecules on living cells with a specific anti-integrin antibody should collect talin with the integrin caps. Following up on our earlier capping experiments with integrin and talin on chicken lymphocytes (22), we found that upon capping LFA-1 on the D36 Th cells, talin codistributed with the LFA-1 caps, only, however, if the cells were first treated with the phorbolester PMA (Kupfer, A., P. Burn, and S. J. Singer, manuscript in preparation). This PMA-induced cocapping of talin with LFA-1 was specific to talin, not occurring with either vinculin or α-actinin, and was specific to LFA-1, not occurring

with CD4. Since PMA is an activator of protein kinase C (23), the conclusions to be drawn from these experiments are that in mouse T cells, LFA-1 and talin are normally not associated with one another, but upon appropriate stimulation of protein kinase C, or similar activity, the two proteins become directly or indirectly linked to one another inside the cell.

The results of the present study are entirely consistent with these conclusions. LFA-1 and talin were always found in the same distribution in a D36 cell that was coupled to a B-APC; either both proteins were clustered together (as in Fig. 1, *A–C*; see also Fig. 2), or both were dispersed (as in Fig. 1, *D–F*). Furthermore, their coclustering occurred only upon specific Th/B-APC interactions, and did not occur if the specific Ag was not presented by the B-APC (Fig. 1, *D–F*; Fig. 2). Thus, a specific signal into the Th cell from the bound B-APC is apparently required for the linkage and coclustering of talin and LFA-1.

CD4 in Th/B-APC Couples. CD4 is an important accessory molecule in most Th/B-APC interactions, as indicated for example by the fact that mAb directed to CD4 often inhibits Th cell activation (for a recent discussion, see reference 24). CD4 is an integral protein of the cell membrane (25). Because it is closely associated with class II MHC restriction, it has been suggested (26) that CD4 molecules on the Th cell form transcellular bonds to an invariant site on class II MHC molecules on the surface of the B-APC. A weak interaction between CD4 and class II MHC molecules has indeed been reported (27). On the other hand, other evidence has suggested that CD4 interacts with the TCR (or TCR/T3) within the Th cell membrane (compare references 24, 28–33). It is therefore still uncertain exactly how CD4 exerts its influence on Th/B-APC interactions.

Our earlier finding (4) with Th/B-APC couples, that CD4 is coclustered with the TCR in the Th cell membrane where it is in contact with the specific B-APC, strongly supports the idea that CD4 serves a crucial role in Th/B-APC interactions. In the present study, this idea is further strengthened by the strong correlation between CD4 clustering at Th/B-APC contact sites and the MTOC reorientation inside the bound Th cell (Fig. 7), and hence, with Th cell activation (Figs. 4 and 5). We could not directly examine the TCR distribution in these experiments, but because the TCR and CD4 were always specifically coclustered in our earlier experiments (4), we infer that this was also true in the present study.

In recent investigations, we (7), and others (33a), have provided some additional information about CD4 clustering, and in particular, CD4-TCR coclustering in Th cell membranes. Using a clonotypic mAb against the TCR of D10 Th cells, both studies showed that this mAb (called 3D3) could itself induce the TCR on isolated D10 cells to become clustered (whereas other nonclonotypic anti-TCR mAbs could not), and furthermore, that CD4 was coclustered with these 3D3-induced TCR clusters. 3D3 mAb had previously been found to be capable of activating the D10 cells itself, without requiring a secondary antibody (34, 35). The interpretation given to these results was that the 3D3 mAb, recognizing only the clonotypic TCR on D10 Th cells, probably bound to a determinant on or close to the unique ligand-binding site on the TCR. Such 3D3 binding, it was suggested, caused a conformational change in the TCR that promoted both the clustering of the TCR by 3D3 mAb crosslinking, and the association of CD4 with the TCR clusters, within the D10 cell membrane.

The dual findings just discussed, that (*a*) CD4 clustering into specific Th/B-APC contact sites is correlated with Th cell activation; and (*b*) under specific circumstances, CD4 coclusters with TCR within the Th cell membrane, although both molecules are normally independent of one another, are utilized in the mechanistic proposals presented next.

More than One Signal Is Received by the Th Cell Bound to its Specific B-APC. The results of this study clearly show that at least two signals are transmitted into the Th cell when it is bound to its specific B-APC. Both require Ag presentation by the B-APC. The first signal, but not the second, is stimulated at low Ag doses. It results in the formation of a direct or indirect linkage between talin and LFA-1 molecules, and their joint massive accumulation at the Th cell membrane where it is in contact with the B-APC. The first signal is sufficient to produce a strong specific cell/cell adhesion (see below), but is not sufficient to activate the Th cell to proliferate. The latter requires a second signal that is transmitted only at higher Ag doses. The second signal is associated with several events: (*a*) the clustering of the TCR, and coclustering of CD4 with the TCR, into the Th membrane region that is in contact with the B-APC, and where LFA-1 is already clustered; (*b*) the reorientation of the Th-MTOC to face the cell contact; and (*c*) the activation of the Th cell to proliferate.

What are the molecular mechanisms involved in this complex succession of events? At this stage, there is still much that is unknown, but the following plausible scenario of membrane events is at least consistent with all of our observations.

The First Signal. At low doses, only a small number of specific Ag/class II MHC ligands are expressed on the surface of the B-APC, and these bind to an equivalently small fraction of the total number of TCR on the Th cell. This small number of TCR/Ag/class II MHC bonds is sufficient to transmit the first signal into the Th cell. This signal may be mediated by a conformational change that is induced in the TCR molecule upon binding to its specific ligand (34, 35), and, based on our studies of the PMA-mediated linkage of talin and LFA-1 described above, results in stimulating a protein kinase C-like activity inside the Th cell. In turn, this enzyme stimulation, presumably via appropriate protein phosphorylations, induces the linkage of talin to LFA-1 at the cytoplasmic face of the Th cell membrane. Even at low Ag doses the extent of this linkage is already maximal (Fig. 2). To account for the clustering of LFA-1 into the specific Th/B-APC contact site, we propose that another consequence of the first signal is to so alter the LFA-1 molecule that it acquires a significant increase in affinity for its ligand I-CAM-1 (16) on the surface of the B-APC. The LFA-1 molecules on the Th cell, and the I-CAM-1 molecules on the B-APC that is in transient contact with the Th cell, then bind to one another transcellularly and undergo "mutual capping" into the area of cell contact. Mutual capping (36, 37) is a process whereby receptor molecules on one cell membrane, and their specific ligand molecules on a second cell membrane, upon binding to one another, are mutually collected by diffusion into a forming cell/cell contact, eventually producing a stable intercellular adhesion over an extended area of cell contact (as in Fig. 1, *A–C*).

A possibility other than mutual capping to explain the concentration of LFA-1 at the Th surface, where it is in contact with the APC, is that it arises by the directed insertion of new membrane mass containing LFA-1, as a result of GA-derived vesicles

from the cell interior fusing with the plasma membrane. This possibility is ruled out, however, by two observations. One is that LFA-1 accumulation at the cell/cell contact site occurs at low Ag doses even when there is no MTOC reorientation (Figs. 4 and 5), which is required to direct membrane mass insertion to the contact site (12). The second observation is that if these immunofluorescence experiments with cell couples were carried out in the presence of monensin, an ionophoric drug that blocks the insertion of new membrane mass into the plasma membrane (38), LFA-1/talin clustering was unaffected (unpublished experiments).

This part of the scenario therefore provides an explanation for the fact that LFA-1 is not clustered into contact sites formed in nonspecific couples (i.e., in the absence of Ag); namely, a signal mediated by specific TCR/Ag/class II MHC binding is required to enhance LFA-1 binding to I-CAM-1 so as to produce their mutual capping into the contact site. Furthermore, the first signal–induced mutual capping of LFA-1 and I-CAM-1 is proposed to be the primary source of the specific Th/B-APC adhesion, because other potential transcellular binding contributions to cell/cell adhesion (e.g., TCR/Ag/class II MHC binding) are less significant at low Ag doses. This scheme therefore explains why LFA-1 is so critically important to Th/B-APC interactions; without intact LFA-1 function, specific intercellular adhesion may not occur.

This suggested mechanism for LFA-1 involvement in specific Th/B-APC adhesion is related to the "adhesion-strengthening" mechanism that was proposed for the interactions of CTLs with their target cells (16, 39), in which transcellular interactions involving the CTL TCR would contribute little to the overall strength of the adhesion but would activate the adhesion mediated by other molecules such as LFA-1. On the other hand, we do not find evidence for, and our suggested mechanism does not attribute, a similar role for LFA-1 in nonspecific adhesions, as proposed by others (40, 41).

The Second Signal. The proposed first signal–induced mutual capping of LFA-1 and I-CAM-1, however, is not sufficient to activate the Th cell to proliferate (Figs. 4 and 5). This requires higher Ag doses. At such doses, an increased number of Ag/class II MHC ligands on the B-APC engage a correspondingly larger number of TCR molecules on the Th cell. We propose that this allows a "mutual cocapping" of the TCR with its Ag/class II MHC ligand into the intercellular contact sites already formed by the prior mutual capping of LFA-1 with I-CAM-1. In mutual cocapping (37), conditions are not adequate for a particular receptor-ligand pair (here the TCR/Ag/class II MHC) to undergo its own independent mutual capping. For example, the bond between the receptor and ligand may be too weak, or their concentrations in the membrane too low. However, if a stable intercellular contact already exists, then the mutual capping of that receptor-ligand pair into the existing contact site is favored. As a consequence of such mutual cocapping, TCR molecules are clustered in the Th cell membrane into the region of cell contact. As discussed in the previous section, the specific ligand-induced clustering of the TCR produces a coclustering of CD4 with the TCR clusters in the Th cell membrane. Because of these events, CD4 molecules are therefore collected into the cell contact region on the Th cell, and Ag/class II MHC ligands are collected (by mutual cocapping with the TCR) into the cell contact region on the B-APC. The important consequence of these processes is that the local concentrations of CD4 and class II MHC

molecules in the cell contact site might thereby become sufficiently large to result in the formation of a significant number of transcellular CD4/class II MHC bonds that would otherwise be too weak (27) to form.

It is attractive to suggest that such transcellular CD4/class II MHC bonds, formed only because of this complex series of events in specific Th/B-APC interactions, are required for a critical second signal to be transmitted into the Th cell bound to the B-APC. Such a CD4-mediated signal would explain how and why CD4 is important in class II MHC-mediated cell activation processes (26). It is also possible that still other signals are transmitted into Th cells as a consequence only of the clustering of the TCR itself, within the Th cell membrane, not necessarily involving CD4. The TCR clustering, as suggested above, would result from its mutual cocapping with Ag/class II MHC ligands at high Ag doses. That TCR clustering alone can be important in Th cell activation is demonstrated by the fact that the capping of TCR on isolated Th cells by a combination of primary and secondary antibodies results in Th cell activation (34, 35). In such general antibody-induced TCR clustering and activation, in contrast to the case referred to above involving the 3D3 mAb, CD4 is not coclustered with the TCR (4, 7).

Such second and possibly additional signals beyond the first signal must somehow be responsible for the Th-MTOC reorientation and Th cell proliferation, but the molecular mechanisms involved in producing these effects are still obscure. There is evidence (42, 43) that CD4 is complexed to a particular protein tyrosine kinase, $p56^{lck}$, in Th cll membranes, and it is possible that a CD4-mediated signal involves the activation state of this kinase. Another factor to consider is Ca^{+2}. It is known that the first signal, which induces the formation of talin/LFA-1 linkages and their collection into the Th/B-APC contact site, is independent of external Ca^{+2}, but the signal(s) for MTOC reorientation and cell activation generally requires external Ca^{+2} (3).

Finally, all of the events and mechanisms that we have proposed for the involvement of CD4 and class II MHC molecules in Th/B-ABC interactions may be paralleled by those for CD8 in conjunction with class I MHC molecules in the interactions of CTL and their target cells.

Summary

We have used double-immunofluorescence labeling to determine the surface distributions of LFA-1 and CD4, and the intracellular distributions of the cytoskeletal protein talin and of the microtubule organizing center (MTOC) of cloned Th cells in 1:1 cell couples with antigen (Ag)-specific APC of the B cell type (B-APC). The Th cell was directed to a peptide fragment of the Ag OVA in the context of IA^d. The B-APC was the transfected A20 B hybridoma cell A20-HL, bearing on its surface a surface Ig specific for the hapten TNP, and pulsed with different concentrations of DNP-OVA. At sufficiently high doses of DNP-OVA (>100 ng/ml), in essentially all couples, LFA-1, CD4, and talin were each concentrated at the Th cell membrane where it was in contact with the B-APC, and the MTOC inside the Th cell was reoriented to face the contact region. At lower doses of DNP-OVA (between 50 and 10 ng/ml), in all couples, LFA-1 and talin were concentrated at the Th/B-APC contact region, but the extent of CD4 clustering, MTOC reorientation, and

Th cell proliferation all decreased with decreasing Ag dose. With no Ag, none of these effects was observed. These and other data indicate that two distinct signals are received by the Th cell that is specifically bound to its B-APC. The first signal, at low Ag doses, stimulates a linkage of LFA-1 and talin in the Th cell, and a specific LFA-1-mediated intercellular adhesion; the second signal, at higher Ag doses, is required to induce Th cell proliferation, with which the Th-MTOC reorientation and CD4 clustering are correlated.

We thank Mrs. Hannah Kupfer and Mrs. Margie Adams for excellent technical assistance; Dr. Ian Trowbridge for the I21 mAb; and Dr. N. Hozumi for the gift of the A20-HL cell line. Special thanks are due to Dr. Dale Wegmann for providing us with Th clones and for performing the proliferation assays.

Received for publication 23 May 1989 and in revised form 18 July 1989.

References

1. Kupfer, A., and S. J. Singer. 1988. Cell biology of cytotoxic and helper T cell functions. Immunofluorescence microscopic studies of single cells and cell couples. *Annu. Rev. Immunol.* 7:309.
2. Kupfer, A., S. L. Swain, C. A. Janeway, Jr., and S. J. Singer. 1986. The specific direct interaction of T helper cells and antigen-presenting B cells. *Proc. Natl. Acad. Sci. USA.* 83:6080.
3. Kupfer, A., S. L. Swain, and S. J. Singer. 1987. The specific interaction of helper T cells and antigen-presenting B cells. II. Reorientation of the microtubule organizing center and reorganization of the membrane-associated cytoskeleton inside the bound helper T cells. *J. Exp. Med.* 165:1565.
4. Kupfer, A., S. J. Singer, C. A. Janeway, Jr., and S. L. Swain. 1987. Coclustering of CD4 (L3T4) molecule with the T-cell receptor is induced by specific direct interaction of helper T cells and antigen-presenting cells. *Proc. Natl. Acad. Sci. USA.* 84:5888.
5. Watanabe, M., D. R. Wegmann, A. Ochi, and N. Hozumi. 1986. Antigen presentation by a B-cell line transfected with cloned immunoglobulin heavy- and light-chain genes specific for a defined hapten. *Proc. Natl. Acad. Sci. USA.* 83:5247.
6. Trowbridge, I. S., and M. B. Omary. 1981. Molecular complexity of leukocyte surface glycoproteins related to the macrophage differentiation antigen Mac-1. *J. Exp. Med.* 154:1517.
7. Kupfer, A., and S. J. Singer. 1988. Molecular dynamics in the membranes of helper T cells. *Proc. Natl. Acad. Sci. USA.* 85:8216.
8. Ovary, Z., and B. Benacerraf. 1963. Immunological specificity of the secondary response with dinitrophenylated proteins. *Proc. Soc. Exp. Biol. Med.* 114:72.
9. Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clone. 1. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* 136:2348.
10. Sanders, V. M., R. Fernandez-Botran, R. L. Coffman, T. R. Mosmann, and E. S. Vitetta. 1988. A single antigen-specific B cell can conjugate to either a type 1 or a type 2 helper T cell. *Proc. Natl. Acad. Sci. USA.* 85:7724.
11. Rogalski, A., and S. J. Singer. 1984. Associations of elements of the Golgi apparatus with microtubules. *J. Cell Biol.* 99:1092.
12. Singer, S. J., and A. Kupfer. 1986. The directed migration of eukaryotic cells. *Annu. Rev. Cell Biol.* 2:337.

13. Hynes, R. O. 1987. Integrins: a family of cell surface receptors. *Cell.* 48:549.
14. Buck, C. A., and A. F. Horwitz. 1987. Cell surface receptors for extracellular matrix molecules. *Annu. Rev. Cell Biol.* 3:179.
15. Rogalski, A. A., and S. J. Singer. 1985. An integral glycoprotein associated with membrane attachment sites of actin microfilaments. *J. Cell Biol.* 101:785.
16. Springer, T. A., M. L. Dustin, T. K. Kishimoto, and S. D. Marlin. 1987. The lymphocyte function-associated LFA-1, CD2, and LFA-3 molecules: cell adhesion receptors of the immune system. *Annu. Rev. Immunol.* 5:223.
17. Pircher, H., P. Groscurth, S. Baumhutter, M. Aguet, R. M. Zinkernagel, and H. Hengartner. 1986. A monoclonal antibody against altered LFA-1 induces proliferation and lymphokine release of cloned T cells. *Eur. J. Immunol.* 16:172.
18. Mishra, G. C., M. T. Berton, K. G. Oliver, P. H. Krammer, J. W. Uhr, and E. S. Vitetta. 1986. A monoclonal anti-mouse LFA-1α antibody mimics the biological effects of B cell stimulatory factor-1 (BSF-1). *J. Immunol.* 137:1590.
19. Noesel, van C., F. Miedema, M. Brouwer, M. A. de Rie, L. A. Aarden, and R. A. W. van Lier. 1988. Regulatory properties of LFA-1 α and β chains in human T-lymphocyte activation. *Nature (Lond.).* 333:850.
20. Burridge, K., and L. J. Connell. 1983. A new protein of adhesion plaques and ruffling membranes. *J. Cell Biol.* 97:359.
21. Horwitz, A., K. Duggan, C. Buck, M. C. Beckerle, and K. Burridge. 1986. Interaction of plasma membrane fibronectin receptor with talin: a transmembrane linkage. *Nature (Lond.).* 320:531.
22. Burn, P., A. Kupfer, and S. J. Singer. 1988. Dynamic membrane-cytoskeletal interactions: Specific association of integrin and talin arises *in vivo* after phorbol ester treatment of peripheral blood lymphocytes. *Proc. Natl. Acad. Sci. USA.* 85:497.
23. Nishizuka, Y. 1986. Studies and perspectives of protein kinase C. *Science (Wash. DC).* 233:305.
24. Ledbetter, J. A., C. A. June, P. S. Rabinovitch, A. Grossmann, T. T. Tsu, and J. B. Imboden. 1988. Signal transduction-through CD4 receptors: stimulatory versus inhibitory activity is regulated by CD4 proximity to the CD3/T cell receptor. *Eur. J. Immunol.* 18:525.
25. Littman, D. R. 1987. The structure of the CD4 and CD8 genes. *Annu. Rev. Immunol.* 5:561.
26. Swain, S. L. 1983. T cell subsets and the recognition of MHC class. *Immunol. Rev.* 74:129.
27. Doyle, C., and J. L. Strominger. 1987. Interaction between CD4 and class II MHC molecules mediates cell adhesion. *Nature (Lond.).* 330:256.
28. Anderson, P., M.-L. Blue, and S. F. Schlossman. 1988. Evidence for a specific association between CD4 and approximately 5% of the CD3:T cell receptor complexes on helper T lymphocytes. *J. Immunol.* 140:1732.
29. Weyand, C. M., M. J. Goronzy, and C. G. Fathman. 1987. Modulation of CD4 by antigenic activation. *J. Immunol.* 138:1351.
30. O'Neil, H. C., M. S. McGrath, J. P. Allison, and I. L. Weissman. 1987. A subset of T cell receptors associated with L3T4 molecules mediate C6VL leukemia cell binding of its cognate retrovirus. *Cell.* 49:143.
31. Saizawa, K., J. Rojo, and C. A. Janeway, Jr. 1987. Evidence for a physical association of CD4 and the CD3:α:β T-cell receptor. *Nature (Lond.).* 328:260.
32. Rivas, A., S. Takada, J. Koide, G. S. McDevitt, and E. G. Engleman. 1988. CD4 molecules are associated with the antigen receptor complex on activated but not resting T cells. *J. Immunol.* 140:2912.
33. Owens, T., B. Fazekas de St. Groth, and J. F. A. P. Miller. 1987. Coaggregation of the T-cell receptor with CD4 and other T-cell surface molecules enhances T-cell activation. *Proc. Natl. Acad. Sci. USA.* 84:9209.

33a. Rojo, J. M., K. Saizawa, and C. A. Janeway, Jr. 1989. Physical association of CD4 and the T-cell receptor can be induced by anti-T-cell receptor antibodies. *Proc. Natl. Acad. Sci. USA.* 86:3311.

34. Tite, J. P., J. Kaye, K. M. Saizawa, J. King, M. E. Katz, L. A. Smith, and C. A. Janeway, Jr. 1986. Direct interactions between B and T lymphocytes bearing complementary receptors. *J. Exp. Med.* 163:189.

35. Rojo, J. M., and C. A. Janeway, Jr. 1988. The biologic activity of anti-T cell receptor V region monoclonal antibodies is determined by the epitope recognized. *J. Immunol.* 140:1081.

36. Singer, S. J. 1976. The fluid mosaic model of membrane structure: some applications to ligand-receptor and cell-cell interactions. *In* Surface Membrane Receptors, Interface between Cells and their Environment. R. A. Bradshaw, W. A. Frazier, R. C. Merell, D. I. Gottlieb, and R. A. Hogue-Angeletti, editors. Plenum Publishing Corp., New York. 1–24.

37. Singer, S. J., and A. Kupfer. 1988. On membrane dynamics in cell-cell interactions, with application to immunology. *In* The T-Cell Receptor. M. Davis and J. Kappler, editors. Alan R. Liss, Inc., New York. 361–376.

38. Tartakoff, A. M., and P. Vassalli. 1977. Plasma cell immunoglobulin secretion. Arrest is accompanied by alterations of the Golgi complex. *J. Exp. Med.* 146:1332.

39. Martz, E. 1987. LFA-1 and other accessory molecules functioning in adhesions of T and B lymphocytes. *Hum. Immunol.* 18:3.

40. Spits, H., W. van Schooten, H. Keizer, G. van Seventer, M. van Rijn, C. Terhorst, and J. E. de Vries. 1986. Alloantigen recognition is preceeded by nonspecific adhesion of cytotoxic cells and target cells. *Science (Wash. DC).* 232:403.

41. Shaw, S., G. E. G. Luce, R. Quinones, R. E. Gress, T. A. Springer, and M. E. Sanders. 1986. Two antigen-independent adhesion pathways used by human cytotoxic T-cell clones. *Nature (Lond.).* 323:262.

42. Rudd, C. E., J. M. Trevillyan, J. D. Dasgupta, L. L. Wong, and S. F. Schlossman. 1988. The CD4 receptor is complexed in detergent lysates to a protein-tyrosine kinase (pp58) from human T lymphocytes. *Proc. Natl. Acad. Sci. USA.* 85:5190.

43. Veillette, A., M. A. Bookman, E. M. Horak, and J. B. Bolen. 1988. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase $p56^{lck}$. *Cell.* 55:301.