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Immunology

Molecular dynamics in the membranes of helper T cells

(T-cell receptor/CD4/mutual capping/immunofluorescence microscopy)

ABRAHAM KUPFER* AND S. J. SINGER

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

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ABSTRACT **We provide evidence that redistributions and interactions of integral proteins in the fluid membranes of helper T (Th) cells may play important roles in Th-cell activation. A particular monoclonal antibody, 3D3, directed to a clonotypic determinant on the T-cell receptor (TCR) of the cloned Th-cell line D10, had previously been shown to be distinctively capable of directly activating D10 cells at low concentrations. We demonstrate here by immunofluorescence experiments that it is also distinctively able itself to produce a clustering (capping) of the TCRs on the D10 cell surface. Simultaneously, by means of double-immunofluorescence experiments, we find that the 3D3-induced clustering of the TCRs distinctively produces a co-clustering of the accessory molecule CD4 with the TCR clusters, although the CD4 and TCR molecules are normally independent of one another in the D10 cell membrane. These results, and related ones previously obtained from studies of the interactions of D10 Th cells with antigen-presenting cells, are analyzed to suggest that the membrane clustering of TCRs and the induced TCR–CD4 interactions are critical to the signaling events in Th-cell activation.**

The direct interaction of helper T (Th) cells with specific antigen-presenting B cells (APCs) has been implicated in the proliferation and ultimate differentiation of APCs into antibody-secreting plasmacytes as well as in the proliferation of, and induction of lymphokine secretion by, Th cells. That such a direct cell–cell interaction occurs was previously inferred from the fact that a Th cell expresses on its surface a clonotypic T-cell receptor (TCR) with specific affinity for a unique ligand on the surface of an APC, a ligand consisting of a fragment of an antigen molecule attached to a molecule of the polymorphic class II major histocompatibility complex (MHC), and was supported by studies of specific binding of Th hybridoma cells to monolayers of APCs (1). That specific binary Th–APC interactions indeed occur has been demonstrated by immunofluorescence microscopy experiments with individual cell–cell couples formed between cloned Th cells and APCs (2–4), and also by specific cell–cell binding observations (5). Two phenomena were shown to identify a specific Th–APC couple and distinguish it from a nonspecific cell couple: (*i*) massive reorganizations of cytoplasmic organelles and of cytoskeletal proteins occur inside the Th cell (2, 3), and (*ii*) the TCRs become concentrated into the region of the Th cell surface that is in contact with the APC (4).

In addition to the TCR, the accessory molecules CD4 and lymphocyte function-associated antigen 1 (LFA-1) on the Th cell are thought to play important roles in the Th–APC interaction (see *Discussion*), but the precise nature of those roles is not yet known. Of considerable interest, therefore, are the observations that both CD4 (4) and LFA-1 molecules (A.K., S. L. Swain, and S.J.S., unpublished experiments) are also clustered with the TCR into the region of the Th-cell membrane that is in contact with the specific APC, but not in regions of nonspecific couples.

Among the questions raised by these observations are the following. (*i*) Is this clustering of the TCRs important in generating the signals that are transmitted into the Th cell? (*ii*) What factors are responsible for CD4 and LFA-1 molecules co-clustering with TCRs into the contact site? To investigate these questions, we have now examined the effects of several monoclonal anti-TCR antibodies on the surface properties of the Th cells. It is known that many of the physiological consequences on the Th cell of its interaction with specific APCs can be simulated by the appropriate direct interaction of the Th cells with anti-TCR antibodies. Janeway and his colleagues (6, 7) have produced and characterized a number of monoclonal antibodies (mAbs) directed to the TCR of the cloned Th-cell line D10. Among these mAbs is 3D3, a mAb specific for a clonotypic determinant of the D10 TCR, that is unusual in that it activated D10 cells directly at very low concentrations. Other anti-TCR mAbs generally required cross-linking by a secondary antibody to induce activation. By immunofluorescence microscopy, we now show that the direct addition of mAb 3D3, but not of other anti-TCR mAbs, to D10 cells causes a rapid and massive clustering of TCRs on the cell surface and that CD4 molecules are co-clustered with TCRs under these circumstances, although CD4 is normally independent of the TCR. These clustering effects of the activating 3D3 mAb on the Th cell, therefore, mimic the effects observed upon the specific interaction of an APC on the Th cell. The implications of these and related findings for the molecular events resulting from Th–APC interactions are discussed.

MATERIALS AND METHODS

Cells and Antibodies. The cloned Th-cell line D10.G4.1 (D10) (8) was kindly provided to us by Jonathan Kaye (University of California at San Diego). D10 cells were stimulated with antigen and maintained as described (8). The following mAbs were utilized: 3D3 (8), a mouse mAb specific for a clonotypic determinant of the D10 TCR, and its Fab fragment (both the gifts of Charles Janeway, Jr.; Yale University); F23.1 (9) and KJ16 (10), a mouse mAb and a rat mAb, respectively, specific for $V_\beta 8$ determinants of the TCR; GK1.5 (11), a rat mAb specific for the mouse CD4 glycoprotein; and I21 (12), a rat mAb specific for mouse LFA-1 on T cells. Affinity-purified rabbit anti-2,4-dinitrophenyl (anti-DNP) antibodies were as prepared (13). The rhodamine-conjugated and the biotinylated $F(ab')_2$ fragments of goat anti-rat, -mouse, and -rabbit IgG were obtained from Jackson ImmunoResearch (Avondale, PA), and fluorescein-conjugated streptavidin was from Amersham. Each of these

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Abbreviations: Th cell, helper T cell; TCR, T-cell receptor; APC, antigen-presenting B cell; MHC, major histocompatibility complex; LFA-1, lymphocyte function-associated antigen 1; mAb, monoclonal antibody; DNP, dinitrophenyl.

*Present address: National Jewish Center for Immunology and Respiratory Medicine, 1400 Jackson Street, Denver, CO 80206.

secondary antibodies was passed through the appropriate columns containing rat, mouse, or rabbit IgG to eliminate any cross-species reactivities.

Coupling of DNP-Propionimidate to mAb. In several double-immunofluorescence experiments, to avoid artifactual labeling, one of the two primary mAbs was coupled with the hapten DNP for use in a modified hapten sandwich technique. Purified IgG fractions of the mAbs GK1.5, KJ16, and I21 were prepared from the ascites fluids by differential ammonium sulfate precipitations and chromatography on DEAE-cellulose. DNP-propionimidate was conjugated to GK1.5, KJ16, and I21 as described (14), resulting in the coupling of 5.9, 5.5, or 7.7 mol of DNP per mol of IgG for GK1.5, KJ16, or I21, respectively.

Surface Labeling, Capping, and Immunofluorescence Microscopy. For immunofluorescence single-label experiments (Fig. 1), 1–2 × 10^6 D10 cells were incubated for 1 hr at 4°C with one of the following mAbs: 3D3 (at a 1:400 dilution of the ascites fluid), F23.1 (at a 1:400 dilution of ascites fluid), KJ16 (culture supernatant), and GK1.5 (a 1:1500 dilution of ascites fluid). The unbound mAbs were washed away, and the cells were transferred to an incubator at 37°C. At the indicated times, cells were plated on poly(D-lysine)-treated coverslips and fixed with 3% (wt/vol) paraformaldehyde. For immunofluorescence detection the cells were labeled with the appropriate rhodamine-labeled secondary antibodies.

In some experiments (Fig. 2) where the distribution of a second surface determinant was to be studied by double immunofluorescence, 3D3-treated cells that had been fixed were labeled with a biotinylated $F(ab')_2$ fragment of goat anti-mouse antibody. The cells were fixed again with 3% (wt/vol) paraformaldehyde. To block nonspecific labeling, the cells were then treated with normal rat IgG (80 μg/ml). All subsequent labelings were done in the presence of normal mouse or rat IgG. The cells were labeled with either DNP-modified GK1.5 (15 μg/ml) or DNP-modified I21 (15 μg/ml), followed by rabbit anti-DNP (10 μg/ml). The cells were finally double-labeled with rhodamine-conjugated $F(ab')_2$ fragments of goat anti-rabbit IgG and fluorescein-modified streptavidin.

In another series of experiments (Fig. 3), TCRs were induced to undergo capping by 3D3 only after the addition of a secondary antibody reagent. Control experiments of a similar type were carried out with mAb 11.4.1 (15), a mAb

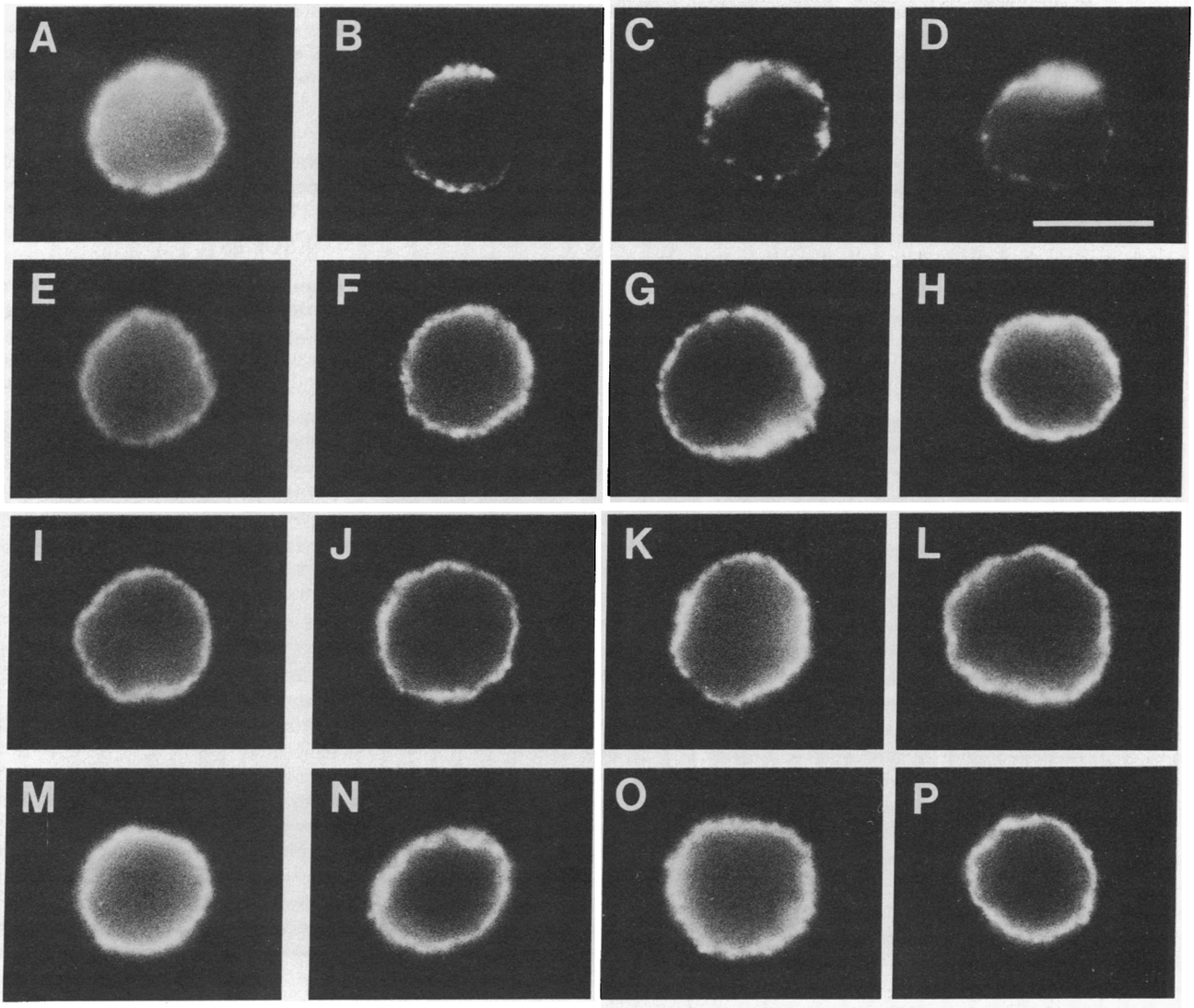

FIG. 1. Immunofluorescence observations of the distribution of the TCRs (*A–L*) and CD4 molecules (*M–P*) on the surfaces of D10 cells labeled with the mAb 3D3 (*A–D*), F23.1 (*E–H*), KJ16 (*I–L*), and GK1.5 (*M–P*). The D10 cells were labeled at 4°C with one of these mAbs, and after washing, were transferred to 37°C and processed. Four micrographs are shown for each mAb. (*A*, *E*, *I*, and *M*) Cells fixed just before the temperature was shifted to 37°C. All other cells were fixed 30 min after the temperature was shifted to 37°C. Note the extensive clustering of the TCRs in the 3D3-treated cells (*B–D*). (Bar in *D* = 10 μm.)

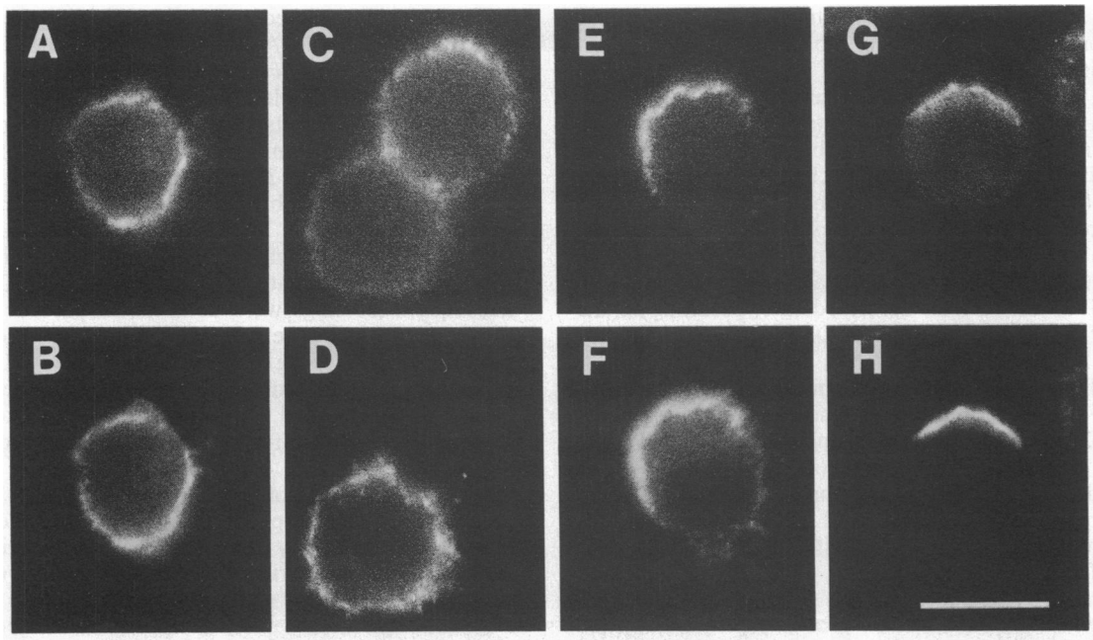

FIG. 2. Double-immunofluorescence observations of the codistribution of CD4 molecules (*B*, *D*, *F*, and *H*) with 3D3-induced clusters of TCRs (*A*, *C*, *E*, and *G*, respectively). D10 cells were treated with the mAb 3D3 as in Fig. 1, and samples were removed and double-labeled with DNP-GK1.5 mAbs 15 min (*A–D*) and 30 min (*E–H*) after the cells were transferred to 37°C. The cell in *C* and *D* at the upper right exhibits TCR clustering in *C*, but in *D* exhibits no labeling for CD4. (Bar = 10 μm.)

specific for H-2K^k, instead of 3D3. After the excess unbound 3D3, or 11.4.1, was washed away, the living cells were treated with biotinylated F(ab')$_2$ fragments of goat anti-mouse IgG for 30 min at 4°C, and the unbound secondary antibody was washed away. The cells were then transferred to a 37°C incubator for 30 min to induce capping and then plated and fixed as before. The cells were then labeled with either DNP-GK1.5 or DNP-KJ16, followed by rabbit anti-DNP and rhodamine-conjugated F(ab')$_2$ fragments of goat antirabbit IgG and fluorescein-streptavidin as above.

All fluorescence observations were made with a Zeiss Photoscope III epifluorescence instrument.

RESULTS

The Binding of the Anti-TCR mAb 3D3 Is Distinctively Capable of Directly Triggering the Clustering and Capping of TCRs. At 4°C each of the four mAbs, 3D3, D23.1, KJ16, or GK1.5 initially displayed a similar uniform surface labeling of D10 cells (Fig. 1 *A*, *E*, *I*, and *M*), but, after allowing the cells

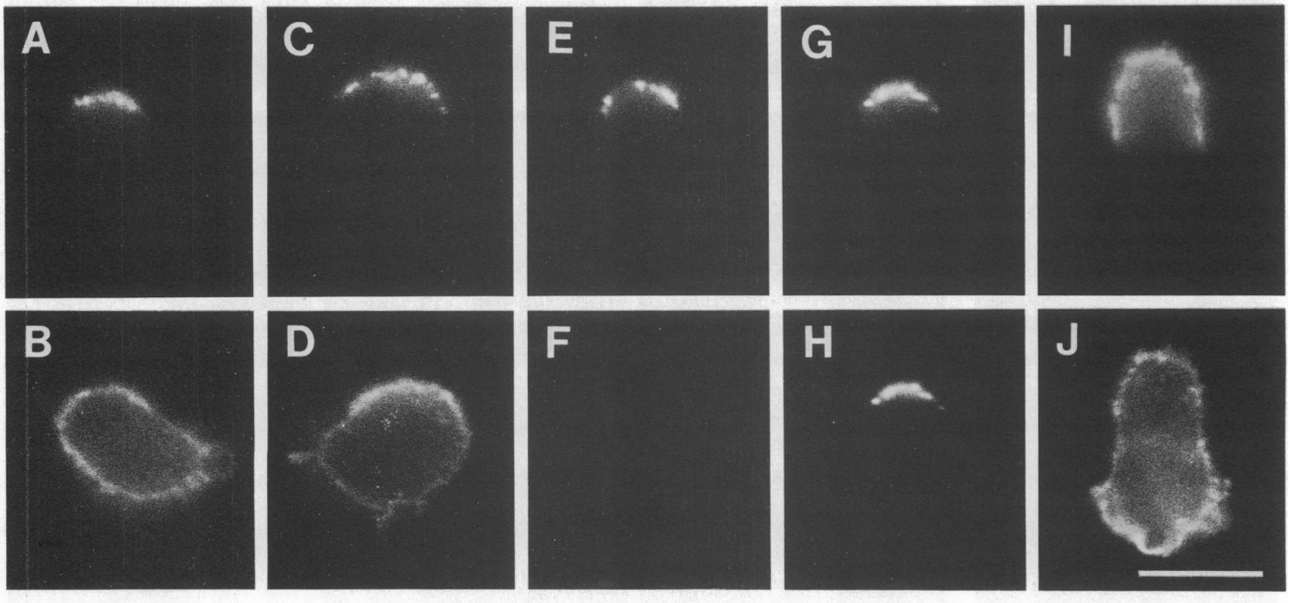

FIG. 3. Double-immunofluorescence experiments, in which D10 cells were first treated at 4°C with either 3D3 for the TCR (*A*, *C*, *E*, and *G*) or 11.4.1 for H-2K^k (*I*). The TCR and the H-2K^k molecules were subsequently clustered only after addition of the secondary goat anti-mouse IgG antibodies. The same cells were double-labeled with either DNP-GK1.5 for CD4 (*B*, *D*, *F*, and *J*) or DNP-KJ16 for the $V_\beta 8$ determinant on the TCR (*H*). The TCR cap in *A* shows no corresponding clustering of CD4 molecules in *B*, in contrast to Fig. 2. In some cases (*C* and *D*), however, an apparent redistribution of CD4 molecules was observed (*D*) but was not precisely codistributed with the TCR cap (*C*). (*E* and *F*) Another example of a cell that showed TCR capping (*E*) but no CD4 labeling (*F*) is shown. The capping of H-2K^k (*I*) caused no change in the distribution of CD4 molecules (*J*). (Bar = 10 μm.)

to warm to 37°C, a major difference emerged. After 30 min at 37°C, >50% of the cells treated with mAb 3D3 had their TCRs clustered into large patches or caps (Figs. 1 *B*, *C*, and *D* and 2 *E* and *G*). No such clustering of the TCRs was observed with either of the two other anti-TCR mAbs or with CD4 molecules that reacted with anti-CD4 antibodies (Fig. 1 *F–P*). When the Fab fragment of mAb 3D3 was used in place of intact 3D3, no clustering of TCRs was observed (data not shown).

CD4 Molecules Are Co-clustered with 3D3-Induced Clusters of TCRs. D10 cells, incubated with mAb 3D3 in the cold, were then transferred, after washing, to 37°C. After 15 or 30 min, the surface distribution of TCR and CD4 molecules was examined by double-immunofluorescence microscopy. Within 15 min, when 3D3 showed early signs of clustering, the labeling with the anti-CD4 appeared to co-localize with that of 3D3 (Fig. 2 *A* and *B*). Interestingly, a small fraction (<5%) of the cloned D10 cells did not appear to have any detectable CD4 on their cell surfaces, even though they expressed normal levels of the TCR. In these cells binding of the 3D3 mAbs also resulted in the clustering of TCRs (Fig. 2 *C* and *D*). By 30 min in essentially all the cells where the TCRs had become clustered, CD4 molecules were found to be coclustered with the TCRs (Fig. 2 *E–H*).

In similar experiments, in which 3D3-induced clusters of TCRs were examined for possible co-clustering of LFA-1 molecules with DNP-I21 mAb, LFA-1 molecules were generally found not to be clustered with the TCRs on the Th cell (data not shown), in contrast to the results with CD4 molecules.

CD4 Molecules Do Not Usually Co-cluster with the TCRs. We have shown (3) that when the TCRs on the D10 cells were clustered with mAb F23.1, directed against a $V_\beta 8$ region of the TCR, followed by secondary goat anti-mouse IgG antibodies, CD4 was generally found not to co-cap with the TCRs (3). Because of the differences with the results obtained in this report using 3D3 alone to cluster the TCRs, we studied the distribution of CD4 molecules on D10 cells whose TCRs were collected only by the combined action of 3D3 mAb and goat anti-mouse IgG. Under these conditions, in most cells CD4 molecules were not co-capped with the TCRs (Fig. 3 *A* and *B*). In some cells (Fig. 3 *C* and *D*), it appeared as if CD4 molecules were partially co-capped with the TCR caps, but close examination of the pictures revealed that the distribution of CD4 labeling was not exactly superimposed on that of the TCR labeling. The surface distribution of CD4 was affected almost to the same extent when the mAbs 3D3, F23.1 (4), or 11.4.1 (an anti-H-$2K^k$ mAb) (Fig. 3 *I* and *J*) were collected by secondary goat anti-mouse IgG antibodies. Two additional control results are shown. With the small fraction of the D10 cells that were CD4-negative (Figs. 3 *E* and *F* and Fig. 2 *C* and *D*), it was demonstrated that the observed co-capping of CD4 and TCR molecules induced by 3D3 was not an artifact due to cross reactions of antibody reagents. In a second control experiment, we addressed the possibility that a co-capping of CD4 and the TCR molecules might not have been detected because of inaccessibility of the TCR caps to the CD4 antibodies. 3D3-capped cells, similar to those in Fig. 3 *A–F*, were labeled with another anti-TCR antibody, KJ16. As expected, the labeling for KJ16 matched exactly that obtained with 3D3 (Fig. 3 *G* and *H*), indicating that even a second mAb directed to a second antigenic determinant on the TCR had ready access to the capped molecules.

DISCUSSION

The Role of TCR Clustering. Th cells can be induced to proliferate and to secrete lymphokines by two quite different stimuli: when they interact with specific APCs and when they are appropriately treated with anti-TCR antibodies. It should, therefore, be possible to compare the molecular events at the Th cell surface resulting from these two stimuli, to assess which events are critical to cell activation generally. When soluble anti-TCR mAbs are used, they generally have to be cross-linked and clustered by secondary anti-mAb reagents to produce these responses. In our studies of individual Th–APC couples, we observed that the TCRs were clustered into the cell–cell contact region (4) only for specific couples. These two sets of results suggest that the clustering of the TCRs in the Th membrane, although achieved by different mechanisms in the two types of stimulation, might be a requirement for a signal to be transmitted into the Th cell to induce its activation. 3D3 is an anti-TCR mAb that stimulates D10 Th cells alone, without requiring a secondary antibody. If TCR clustering was critical to activation, then 3D3 alone should, uniquely among anti-TCR mAbs tested, induce TCR clustering. This we demonstrate to be the case (Fig. 1). The Fab monovalent fragment of 3D3 neither activates D10 cells (6) nor induces TCR clustering detectable by immunofluorescence observations.

Why should 3D3 mAb binding induce extensive TCR clustering? 3D3 is a clonotypic mAb; that is, it appears to bind only to the TCR of D10 cells, against which it was raised (8). This suggests that 3D3 is directed against a determinant that is part of the active site of the D10 TCR and, upon binding, might induce a conformational change in the TCR, as has been proposed (7). This conformational change might in turn directly or indirectly promote an aggregation of the TCRs (or the TCR–T3 complexes) in the Th membrane, which aggregation is then driven by cross-linking of the TCRs by 3D3 mAbs. The 3D3-induced clustering of the TCRs does not depend upon an interaction with CD4 (Fig. 2 *C* and *D*).

We conclude that there is a critical requirement for a clustering of the TCRs in signal transmission into the Th cell. Such a requirement has also been invoked in the activation of antigen-specific B-cell differentiation by antigen-bridged Th cells (16) and in the induced exocytosis of secretory components by cytotoxic T cells (17).

The Involvement of CD4. The productive interactions of Th cells and APCs involve, in addition to the clonotypic TCR, a number of monomorphic Th-cell membrane proteins, such as CD4 and LFA-1. That these so-called accessory proteins play important roles has been deduced from the capacity of mAbs directed against them to inhibit Th-cell activation by APCs. These roles, however, are not clear. In particular, it has often been suggested (see ref. 18) that CD4, because of its association with class II MHC-restricted T cells, forms an intercellular bond to monomorphic determinants on the class II MHC molecules of APCs, whereas CD8, which is confined to class I MHC-restricted T cells, forms an intercellular bond to class I MHC molecules of the target cell. Evidence for an intercellular binding of CD4 to the class II MHC has been obtained (19). On the other hand, there are several lines of evidence suggesting that CD4 may interact with the TCR or the TCR–T3 complex within the Th-cell membrane (20–26).

In our studies of Th–APC couples (4), we found that CD4 becomes co-clustered with the TCRs in the contact region between the two cells, in spite of the fact that CD4 and TCR molecules are normally independent of one another in the Th membrane. In the present study, we found that the 3D3-induced clustering of TCRs on D10 cells (Fig. 1) induced a co-clustering of CD4 with the TCRs in the absence of an APC (Fig. 2). Similar results have been obtained by J. M. Rojo, K. Saizawa & C. A. Janeway, Jr. (personal communication). The suggestion is that CD4 becomes bound to TCRs, or TCR–T3 complexes, only upon the conformational change, and perhaps subsequent clustering, of the TCRs that is induced by 3D3. That this co-clustering is not a simple matter, however, is indicated by the additional finding that if TCR

clustering is induced, not by 3D3 alone, but by 3D3 in combination with a secondary antibody, CD4 molecules are now not significantly co-distributed with the TCRs (Fig. 3). A possible explanation of this difference is kinetic; the TCRs may be too rapidly aggregated into large clusters in the presence of 3D3 and a secondary antibody to be able to bind CD4.

The nature of LFA-1 involvement in Th–APC interactions will not be discussed here, but LFA-1 clearly has different properties from CD4 since LFA-1 molecules are not co-clustered with 3D3-induced TCR clusters.

On the Mechanisms of Th–APC Interactions. In our studies of Th–APC couples (4), we observed a clustering of the TCRs into the region of specific cell–cell contact. This was attributed to a "mutual capping" (4, 27) of the TCRs with the antigen–class II MHC ligands on the APC. This phenomenon can in principle be simple: if the concentrations of the two freely diffusible receptor and ligand molecules in their respective membranes are sufficiently large, and the rate of dissociation of their intermolecular bond is slow enough, a clustering of the receptor–ligand pairs (mutual capping) into the cell–cell contact region is to be expected without the mediation of other factors. However, the present results suggest that additional factors may be involved. The initial binding of the antigen–class II MHC ligand on the APC to the active sites of its specific TCR on the Th cell might induce a conformational change in the TCR molecule, as has been suggested to occur upon binding the 3D3 mAb to the same, or nearby, domain of the TCR molecule (7). This conformational change might directly or indirectly promote a clustering of the TCR molecules within the Th-cell membrane that would greatly enhance the mutual capping of the TCRs with the antigen–class II MHC ligands into the region of Th–APC contact. The resultant clustering of the TCRs in the Th membrane is regarded to be essential for an activating signal to be transmitted into the APC-bound Th cell as discussed above. In addition, the mutual capping of TCR–ligand pairs would contribute to a stable cell–cell adhesion.

At the same time, the co-clustering of CD4 molecules with the TCRs within the Th membrane would lead to CD4 molecules also being concentrated into the specific Th–APC contact region. This could explain the experimental observation of CD4 accumulation in the contact region of specific cell couples (4). CD4 apparently possesses an affinity, but only a very weak one, for a monomorphic determinant on class II MHC molecules (19). Such weak CD4–class II MHC bonding might not of itself be capable of producing a mutual capping of these two molecules into the cell–cell contact region if both CD4 and class II MHC existed as freely diffusible monomeric molecules in their respective membranes. [This would explain why CD4 molecules are not accumulated into the contact region of nonspecific couples (4)]. But if CD4 molecules were concentrated with the ligand-bound TCRs into the contact region of specific Th–APC couples and if antigen-linked class II MHC molecules on the APC surface were simultaneously concentrated in the contact region by their binding and mutual capping with the TCRs, the formation of a significant number of intercellular CD4–class II MHC bonds might then, and only then, occur. Such stable CD4–class II MHC bonds not only would increase the cell–cell adhesion but they might also be required for an additional signal to be passed between the Th cell and its bound APC (the first signal having been transmitted by the clustering of the TCRs in the Th-cell membrane). Such an additional CD4-mediated signal would be consistent with the findings and conclusions of Ledbetter *et al.* (25) and Gay *et al.* (28). These conjectures provide a plausible mechanism to explain the direct class II MHC-restricted involvement of CD4 in Th–APC interactions. Furthermore, anti-CD4 antibodies that block the Th–APC response (29) might work by interfering with the essential step of co-clustering of the CD4 molecules with the TCRs.

Entirely parallel considerations apply concerning the roles of CD8 and class I MHC molecules in cytotoxic T-cell–target-cell interactions, as have here been discussed for CD4 and class II MHC molecules in Th–APC interactions (cf. refs. 30 and 31).

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