ON THE MECHANISM OF UNIDIRECTIONAL KILLING IN MIXTURES OF TWO CYTOTOXIC T LYMPHOCYTES

Unidirectional Polarization of Cytoplasmic Organelles and the Membrane-associated Cytoskeleton in the Effector Cell

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When NK cells or CTL are mixed with their specific target cells (TC),1 the TC are lysed in a process that requires the binding of the effector to the target cell (for review, see 1). The effector cell is not damaged in this process, since a single effector cell is able to successively lyse several TC (2). Such unidirectional killing could conceivably be an intrinsic property of CTL or NK cells that is triggered whenever such an effector cell is appropriately bound to a susceptible target. That this simple mechanism is not the case is demonstrated with mixtures of two CTL, such as a anti-b and b anti-c (3); only the b anti-c is killed. These and related results (1) suggest that a directionality of lysis exists which is determined by the engagement of the specific receptors on the surface of the effector cell by the antigen molecules on the surface of the TC. The question then arises, by what mechanisms does this unidirectional engagement result in unidirectional lysis?

There is now increasing evidence (4-11) that the mechanism by which NK cells and CTL lyse TC involves the secretion of one or more cytotoxic components from the effector cell to the TC bound to it. We have previously shown (7-9) that, in cell conjugates formed between NK or CTL and their TC, there occurs inside the effector cell, but not inside the TC, a rapid and coordinate reorientation of the perinuclear Golgi apparatus (GA) and the microtubule organizing center (MTOC) to face in the direction of the bound TC. The purpose served by such a reorientation, we suggested, was to direct secretion derived from the GA inside the effector cell to the plasma membrane at the site of TC binding. One could ask whether this organellar reorientation is simply a property of NK and CTL effector cells that is exhibited whenever a TC is bound to them, or whether it is a consequence of the unidirectional engagement of their specific receptors for antigen. To answer this question, we have examined by immunofluorescence microscopy the orientation of the MTOC in cell conju-

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1 Abbreviations used in this paper: GA, Golgi apparatus; MTOC, microtubule organizing center; TC, target cell.
gates formed between two CTL of the type a anti-b with b anti-c. We report here that in such conjugates, the MTOC inside the a anti-b cell was oriented to face the site of cell contact with b anti-c, but the MTOC inside the b anti-c cell was randomly oriented. In other words, the unidirectional recognition between the two CTL was followed rapidly by the unidirectional polarization of the MTOC (and presumably the GA) inside the effector cell of the pair. These results therefore suggest that one aspect of the mechanisms responsible for unidirectional killing is the unidirectional polarization of the GA and MTOC inside the effector cell, with the consequent unidirectional secretion of cytotoxic components from the effector cell towards the bound TC.

One might expect, however, that even if secretion is unidirectional, any cytolytic components would be released into the narrow extracellular space between the surfaces of the effector cell and the TC in the regions where the two cells are in contact. If these components damage the TC by, for example, inserting permeable channels into the cell membrane (5, 6), why doesn’t this happen to the proximal membrane of the effector cells as well? This is particularly relevant where both the effector and the target are killer cells and therefore must have closely similar membrane properties. It seemed possible that, following the unidirectional engagement of the receptor molecules on the effector CTL with the antigen molecules on the target CTL, the membranes of the two cells, originally closely similar, become grossly differentiated in the regions of contact of the two cells, thereby rendering the membrane of the effector CTL less susceptible to lysis than the adjoining membrane of the target CTL. It is well known that in many instances where cell surface receptors become bound by their specific ligands, elements of the cytoskeleton become associated with the affected regions of the cell membrane (as for example in capping or phagocytosis, see 12) and modulate the properties of those membrane regions. It has previously been reported for cell conjugates formed by CTL (13) and NK cells (14) with their TC that actin appeared to be concentrated under the contacting membrane of the effector cell. In both of these studies, however, the target was not a T lymphocyte, and the cytoskeletal properties of the TC could well have been intrinsically different from those of a CTL or NK cell. In this study, both the effector and the target cells were CTL. We have examined their conjugates for the intracellular distributions of several cytoskeletal proteins that have been shown in a variety of cell types to be associated with sites of interaction of actin microfilaments with the cell membrane. These cytoplasmic proteins included α-actinin (15, 16), vinculin (17, 18), talin (19), the 200 kD protein (20), and fimbrin (21). We have found that the protein talin was concentrated at the membrane of the effector CTL where it was in contact with the target, but seemed to remain uniformly distributed in the target CTL. In contrast, the other four cytoskeletal proteins remained essentially uniformly distributed in both cells of such conjugates. In addition, a similar concentration of talin occurred inside NK cells bound to their TC. These results suggest that the contacting membranes of the effector cells become specifically and grossly differentiated upon unidirectional recognition and cell-cell binding.
Materials and Methods

Cells. CTL cell lines were established from mixed lymphocyte cultures and were grown in the presence of supernatant from Con A-stimulated splenocytes and irradiated stimulator splenocytes (6). H-2^d CTL lines specific for H-2^d (d anti-b) or for H-2^k (d anti-k) were derived from BALB/c anti-C57BL/6 or BALB/c anti-CBA cultures, respectively. H-2^d CTL lines specific for H-2^b (b anti-d) or H-2^k (b anti-k) were derived from C57BL/6 anti-BALB/c or anti-C3H cultures, respectively. The cytolytic activities of the CTL lines were checked at regular intervals, using as targets the BALB/c myeloma cell S194 (H-2^d), the C3H myeloma cell C1.18.4 (H-2^a), and the C57BL/6 thymic lymphoma cell EL4 (H-2^b). All target cells were grown in DMEM supplemented with 10% horse serum. NK clone B6.1B10 was derived from C57BL/6 splenocytes as described previously (22).

Antibodies. The mouse mAb specific for the H-2D^d antigen, 34.5.8, has been described (5). The rat mAb against Thy-1, T24/31.7, and the affinity-purified rabbit antibodies against chick brain tubulin have been used in previous studies (7-9). Affinity-purified rabbit antibodies directed to chicken α-actinin were obtained as described (23), and affinity-purified rabbit antibodies specific to fimbrin were prepared by similar procedures. Talin was purified from chicken gizzard smooth muscle by a modification of a published method (19); the mixture of proteins that was enriched in talin, instead of being passed over a phosphocellulose column, was subjected to preparative SDS gel electrophoresis on a 6% polyacrylamide slab gel. After light staining with Coomassie blue to define the location of the protein bands, the part of the gel containing the protein with a molecular mass of 215 kD was excised. The protein extracted from this excised gel was used as an immunogen in rabbits, and was also used to prepare a talin-bound affinity column. The affinity-purified rabbit antitalin antibodies were passed through two additional affinity columns, one containing bound chicken gizzard myosin (200 kD) and the other chicken gizzard filamin (240 kD), to remove any possible contaminating antibodies to these proteins. The resulting purified antibodies were monospecific for talin, as judged by immunoblotting of gel overlays of whole chicken gizzard extracts, and by immunofluorescence microscopic labeling of chick embryo fibroblasts and normal rat kidney cells (19). The affinity-purified rabbit antibodies to vinculin (17) and to the 200 kD protein (20) have been described.

Immunofluorescent Labeling and Detection. 35 min after mixing the cells, the CTL were immunolabeled on their surfaces with the 34.5.8 mouse mAb. In the case of the NK-S194 cell mixtures, NK labeling was carried out with a rat anti-Thy-1 mAb. After 10 min at 37°C, the cells were fixed with 3% formaldehyde, then permeabilized by brief treatment with Triton X-100 and immunolabeled for one of the cytoskeletal proteins. The cells were further doubly labeled with a rhodamine conjugate of goat antibodies to rabbit IgG, and a fluorescein conjugate of an F(ab')2 fragment of goat antibodies to mouse or rat IgG. Immunofluorescence microscopy was performed as described previously (7).

Effector-target Conjugation and Cytotoxicity Assays. CTL-CTL or NK-TC pairs were formed by mixing an equal number of effector and target cells, centrifuging the cells, and plating the cell mixtures on poly-L-lysine-coated coverslips, as described previously (7). Cytotoxicity was measured by a 51Cr-release assay (22).

Results

MTOC Orientation in Cell Couples of Two CTL. Four allogeneic CTL lines were used in these studies. In direct cytotoxicity assays with mixtures of the d anti-b CTL and b anti-k CTL, as expected, the latter were lysed, while the former were not. Similarly, in mixtures of the b anti-d CTL and the d anti-k CTL, only the latter cells were lysed (Table I). Viable cell couples made by mixing equal numbers of the d anti-b CTL with b anti-k CTL were fixed and then double immunofluorescently labeled with mouse mAb specific for H-2D^d, to distinguish the effector cell of the couple, and with rabbit antibodies to tubulin to detect the MTOC inside the two cells. In nearly all (96%) such couples, the MTOC in the
**Table I**

Lysis of Normal and T Killer Targets by T Killer Cell Lines

| Effector cells (H-2 specificity) | Target cells (H-2) | Percent cytotoxicity at E/T ratios of: |
|----------------------------------|--------------------|----------------------------------------|
|                                 |                    | 100:1  | 30:1  | 10:1  |
| C57BL/6 Anti-BALB/c (H-2^b anti-H-2^d) | S194 (H-2^d) | 93     | 80    | 66    |
| BALB/c Anti-CBA (H-2^d anti-H-2^d)  | H-2^d             | 83     | 71    | 65    |
| C1.18.4 (H-2^d)                     |                    | 70     | 43    | 21    |
| C57BL/6 Anti-BALB/c (H-2^d)         | <1                | <1     | <1    |
| BALB/c Anti-C57BL/6 (H-2^d anti-H-2^d) | EL4 (H-2^d) | 73     | 45    | 31    |
| C57BL/6 Anti-C3H (H-2^d)            |                    | 70     | 43    | 31    |
| C57BL/6 Anti-C3H (H-2^d anti-H-2^d) | C1.18.4 (H-2^d) | 28     | 21    | 13    |
| BALB/c Anti-C57BL/6 (H-2^d)         | <1                | <1     | <1    |

**Figure 1.** Effector CTL–target CTL couples doubly labeled with antitubulin antibodies (A, D) and anti-H-2^D^d antibodies (B, E), 35 min after cell mixing. The Nomarski pictures of the same cells are shown in C and F. A–C, equal numbers of cells from two CTL cell lines, H-2^d^ anti-H-2^b^ and H-2^b^ anti-H-2^d^, were mixed together. The CTL labeled with the anti-H-2^D^d (B) antibodies is the effector cell in this cell couple. Note that the MTOC (small arrow) in the effector CTL is facing the contact area, and the MTOC in the target CTL (large arrow) is facing away. D–F, equal numbers of cells from two CTL cell lines, H-2^d^ anti-H-2^b^ and H-2^b^ anti-H-2^d^, were mixed together. In this case, the H-2^D^d-positive cell (E) is the target cell. Note that again the MTOC in the effector CTL (small arrow) but not in the target CTL (large arrow) is facing toward the contact area. K stands for the effector and T for the target cell. The bar in C represents 10 μm.

The effector CTL of the pair was seen to face the contact area with the target CTL (Fig. 1, A–C). In the same cell couples, the MTOC in the target CTL of the pair remained essentially randomly oriented, with 58% of the cells exhibiting an MTOC oriented towards the effector CTL (Fig. 1, A–C). A random distribution
FIGURE 2. Light microscopic observations in cell couples between H-2\textsuperscript{a} anti-H-2\textsuperscript{b} CTL and H-2\textsuperscript{b} anti-H-2\textsuperscript{a} CTL, 35 min after cell mixing. The Nomarski pictures of the same cells are shown in C and F. The cells were doubly immunofluorescently labeled with either antibodies specific for talin (A) or a-actinin (D), and anti-H-2D\textsuperscript{d} antibodies (B, E). The H-2D\textsuperscript{d}-labeled cells (marked K in B and E) are the effector cells in these cell couples. Note that a-actinin (D) is uniformly distributed in the two cells, but while talin (A) is uniformly distributed in the target CTL, it is concentrated to the contact area in the effector CTL. The bar in C represents 10 \mu m.

would have been scored as 50%. A similar set of experiments was carried out with couples formed between b anti-d CTL and d anti-k CTL. In this combination, the H-2D\textsuperscript{d}-positive cells were the targets and the H-2D\textsuperscript{d}-negative cells the effectors. Again, in essentially all such couples, the MTOC in the effector CTL faced the contact area with the target CTL, whereas the MTOC in the target CTL remained randomly oriented (Fig. 1 D–F).

Cytoskeletal Protein Distributions in Cell Couples of Two CTL. Couples formed between d anti-b CTL and b anti-k CTL were surface labeled with mouse mAb specific for H-2D\textsuperscript{d} to mark the effector cell, and then were labeled intracellularly with rabbit antibodies for one of the cytoskeletal proteins: talin, a-actinin, vinculin, the 200 kD protein, or fimbrin. In such conjugates, the talin was regularly found to be highly concentrated inside the effector CTL at the site of binding to the target, whereas the talin inside the target CTL appeared in most cases to be uniformly distributed (Fig. 2A). On the other hand, a-actinin (Fig. 2D), as well as vinculin, the 200 kD protein, and fimbrin (not shown), remained essentially uniformly distributed inside both cells of the conjugate. A similar concentration of talin within effector CTL at the cell contacts was also observed with couples formed between b anti-d CTL and d anti-k CTL (not shown).

Cytoskeletal Protein Distributions in NK-TC Couples. Because of the apparent similarities between CTL and NK cell killing (5, 6), the distributions of cytoskeletal proteins were also examined in NK-TC couples. In this case, however, targets
were used that were not NK cells, since NK cells cannot be induced to lyse one another. With couples formed between the cloned NK line B6.1B10 and S194 targets, in which the NK cell was recognized by surface labeling with anti-Thy-1 antibody, intracellular immunofluorescent labeling for one of the five cytoskeletal proteins was simultaneously performed. Talin (Fig. 3D) was found to be concentrated inside the NK cell at the area of contact with the TC, but was uniformly distributed inside the TC. In contrast, α-actinin labeling (Fig. 3A) was essentially uniform inside both the NK and bound TC, as was the labeling for vinculin, the 200 kD protein, and fimbrin (not shown). The specific polarization of talin inside the NK cell at the site of target binding therefore closely resembles the results obtained in the effector cell of couples made with two CTL.

Discussion

In our previous studies (7–9) of the intracellular polarization of organelles in cytotoxic effector cells, we have shown that the productive binding of a susceptible target to the cytotoxic cell resulted in a rapid and coordinate reorientation of the GA and MTOC inside the effector cell to face the area of contact with the bound target. Such rapid organelle reorientation was strictly correlated with subsequent lysis of the TC, which led to the proposal (9) that the reorientation is a prerequisite to lysis. We presumed that the function served by this GA/MTOC reorientation is to direct the secretion of cytotoxic component(s)
inside the effector cell to the area of contact with the target (4–11). It is possible that the signal that leads to the reorientation also stimulates the synthesis and/or processing of such cytotoxic component(s) inside the effector cell. We predicted, therefore, that in the case where both the effector and target cells are CTL, such as of the types a anti-b plus b anti-c, in which it is known that lysis is unidirectional (3), a reorientation of the GA and MTOC would occur inside the a anti-b effector CTL to face the bound b anti-c target CTL, but not inside the target. This prediction has been confirmed herein, by observations of the MTOC with two independent sets of CTL-CTL couples (Fig. 1). Although only the MTOC was immunolabeled in these experiments and not the GA (because simultaneous immunolabeling for a cell surface marker was carried out to identify the effector CTL of a couple) ample evidence has been obtained in previous studies (7, 24, 25) that the GA is always coordinately localized and reoriented with the MTOC in interphase cells. Our results strongly suggest, therefore, that the GA/MTOC reorientation that occurs unidirectionally (only in the effector CTL of the pair) is the consequence of the unidirectional engagement of the specific T cell receptors on the effector CTL by the H-2 antigen on the target CTL.

Such unidirectional polarization of the GA and MTOC inside effector CTL bound to target CTL does not provide a complete explanation, however, for the fact that lysis is unidirectional. If the proposal is, for example, that cytolytic components are secreted by the effector CTL into the confined intercellular space where the effector CTL is bound to the target CTL (5, 6), why is it that only the target CTL membrane is susceptible to the effects of such cytolytic component(s)? We therefore examined the possibility that the two CTL membranes in contact become differentially restructured so as to make either the target CTL membrane more susceptible, or the effector CTL membrane more resistant, to the cytolytic components. The localization of several cytoskeletal proteins known to be associated with microfilament-membrane attachments in a variety of cells was therefore determined inside cell couples made of two CTL. We found that the protein talin was strongly concentrated at the membrane of the effector CTL, but was not concentrated at the membrane of the target CTL, where the two membranes were in contact (Fig. 2 A–C). This effect is specific, because four other cytoskeletal proteins that we examined showed no comparable redistribution inside either the effector or the bound target CTL (Fig. 2 D–F). The selective redistribution of talin inside a cytotoxic effector cell, to become largely associated with the membrane region in contact with a susceptible target, appears to occur in NK cells (Fig. 3) as well as CTL.

Talin is a 215 kD cytoplasmic protein that was originally extracted from chicken gizzard smooth muscle (19), and shown by immunofluorescence microscopy to be localized to the focal contacts formed between cultured fibroblasts and their substrata. The focal contacts are sites where, inside the cell, actin microfilaments appear to terminate at the cell membrane (26), and where the other cytoskeletal proteins that we examined in this study; vinculin (17), the 200 kD protein (20), α-actinin (16), and fimbrin (21) are also localized. This colocalization of all five proteins at the focal contacts might suggest that they are all actin microfilament–associated proteins, perhaps involved in the attachment of
the microfilaments to the membrane. It is therefore of interest that among the
five proteins, only talin is concentrated inside the cytotoxic cell at the membrane
in contact with the target. This concentration of talin is probably accompanied
by a unidirectional redistribution of actin in the effector CTL, but we did not
examine actin distributions in the present study. Previous studies have shown
that actin is concentrated inside CTL (13) and NK cells (14) at the sites of contact
with TC, but as these targets were not themselves cytotoxic cells, it is not clear
whether such actin redistributions were truly unidirectional; i.e., characteristic
only of effector cells and not their targets. However, for this discussion, we
assume that the actin redistributions are indeed unidirectional. A concentration
of both talin and actin at the membrane of the effector cell, but not of the target
cell, would reflect a rather gross structural differentiation of the two contacting
membranes. While the relatively unaltered contacting membrane of the target
cell remained susceptible, these cytoskeletal changes might render the contacting
membrane of the effector cell resistant to the effects of any cytolytic components
secreted by the effector cell. One possibility among many is that the enhanced
local cytoskeletal interactions with the contacting membrane of the effector cell
might markedly decrease the local fluidity of that membrane and prevent the
intercalation of ion-permeable tubular complexes (5, 6) into the bilayer. Apart
from these speculations, however, the important point is that we have shown
that correlated with the unidirectional killing that occurs in mixtures of two
CTL, there is a unidirectional polarization of the GA and MTOC inside the
effector CTL, accompanied by a unidirectional gross cytoskeletal reorganization
of the contacting membrane of the effector CTL.

Summary

In mixtures of two CTL of the type a anti-b and b anti-c, only the latter is
lysed; i.e., killing is unidirectional. Here, we show that two profound types of
changes occur in the effector CTL but not in the target CTL upon formation of
couples between them. One is that the microtubule organizing center (and
presumably the Golgi apparatus that is invariably colocalized with it) is reoriented
inside the effector CTL to face the bound target CTL. This unidirectional
reorientation, it is proposed, serves to direct putative cytotoxic secretory com-
ponents derived from the Golgi apparatus of the effector cell to the site of cell-
cell binding. The second unidirectional change is in the membrane-associated
cytoskeleton of the effector CTL in the area of target cell binding. The cyto-
skeletal protein talin, but not any of four other such proteins assayed, is highly
concentrated at the contacting membrane of the effector CTL, while it is
uniformly distributed over the entire membrane of the bound target CTL. This
localized, massive cytoskeletal reorganization may reflect a mechanism to protect
the membrane of the effector CTL from the effects of putative cytotoxic
components secreted by the effector cell into the intercellular space between it
and the target cell.

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