Spatial Relationships of Microtubule-organizing Centers and the Contact Area of Cytotoxic T Lymphocytes and Target Cells

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ABSTRACT Specific binding (conjugation) of cytotoxic T lymphocytes (CTL) to target cells (TC) is the first step in a multistage process ultimately resulting in dissolution of the TC and recycling of the CTL. We examined the position of the microtubule organizing center (MTOC) of immune CTL bound to specific TC. Immunofluorescence labeling of freshly prepared CTL-TC conjugates with tubulin antibodies indicated that the MTOC in essentially all conjugated CTL but not in the conjugated TC were oriented towards the intercellular contact site. This finding was corroborated by electron microscopy examination of CTL-TC conjugates fixed either immediately after conjugation or during the lytic process. Antibody-induced caps of membrane antigens of CTL such as H-2 and Thy 1, did not show a similar relationship to the MTOC. Incubation of CTL-TC conjugates, 10-15 min at room temperature, resulted in an apparent deterioration of the microtubular system of conjugated CTL. It is proposed that the CTL plasma membrane proximal to the MTOC is particularly active in forming stable intercellular contacts, resulting in CTL-TC conjugation, and that subsequent modulation of the microtubular system of the CTL may be related to the cytolytic response and to detachment of the effector cell.

A prominent manifestation of cell-mediated immunity is the lytic interaction of cytotoxic T lymphocytes (CTL) with appropriate target cells (TC). This process is believed to be relevant to virus, tumor, and transplantation immunity (4, 13, 42). The first step in CTL-mediated lysis is the binding of CTL and TC (conjugation) mediated through specific CTL cell surface receptor(s) and TC major histocompatibility complex determinants. Binding is followed by a lethal hit step delivered by the CTL, ultimately leading to lysis. Following lysis of the TC, effector CTL detach and can recycle to start a new lytic interaction (see references 4, 7, 8, 18, 19, 23, 28 for reviews).

Several observations suggest that the CTL are polar from at least a functional point of view. It has been shown that the lethal hit is strictly unidirectional, i.e., it affects only CTL-conjugated TC without causing damage to the effector CTL (40). This “immunity” of the CTL cannot be attributed to an inherent resistance towards the cytolytic process, since CTL of a given type can be readily killed by other specifically immunized CTL (14, 17). Moreover, it has been observed that although an individual CTL can bind a number of TC simultaneously, lysis of individual TC occurs sequentially (40, 41). These and other (17) results suggest that the lethal hit, whatever its nature, is expressed in a polar, unidirectional fashion.

In this study we examine the possibility that the unidirectional killing activity of CTL is related, at least in part, to a specific polar arrangement of the cytoskeletal system of either the CTL or the TC. We present evidence suggesting polarity by showing that CTL bind predominantly through a cell surface region proximal to the microtubule organizing center (MTOC). Moreover, we demonstrate that following CTL-TC binding, the CTL microtubular system becomes partially deteriorated. The significance of these findings to the mechanism of cytotoxic interactions is discussed.

MATERIALS AND METHODS

Animals and Tumors

Highly inbred, C57BL/6 (major histocompatibility H-2d) and BALB/c (H-2b) mice were provided by the Weizmann Institute animal-breeding center. Leukemia EL4 and mastocytoma P815 cells were maintained in ascitic form by weekly transfer of about 25 X 10⁶ cells into syngeneic recipients (C57BL/6 and DBA/2 respectively).

Generation of CTL

A system to generate and study alloimmune peritoneal exudate CTL has been described before (5). Briefly, 10-12 d after intraperitoneal injection of 25 X 10⁶ EL4 leukemia cells, BALB/c anti-EL4 mice were killed by CO₂ narcosis and...
their peritoneal cavities rinsed with PBS supplemented with 10% fetal calf serum (PBS-FCS). The peritoneal cells were centrifuged 10 min at 250 g at 4°C, resuspended in 15 ml PBS-FCS per four to seven mice, and incubated on nylon wool columns at 37°C for 45 min (5) to remove adherent cells (macrophages and others). Nonadherent cells containing 30-40% (9) CTL were eluted by raising the columns with 20 ml cold PBS-FCS.

**CTL-TC Binding (Conjugation)**

The method involved centrifugation of mixtures containing CTL and TC, leading to formation of CTL-TC clusters, referred to as conjugates, which can be examined and studied individually under the microscope (6). 1 ml suspensions containing 10⁶ CTL and 10⁶ TC in PBS-FCS, in 12 × 75 mm tubes were centrifuged at 170 g for 10 min at room temperature. The pellets were resuspended vigorously with a Pasteur pipette and the number of CTL-TC conjugates was counted in a hemocytometer.

**CTL-mediated Lysis of TC**

The chromium-release technique (12, 13) was used to measure the cytolytic capacity of CTL. EL4 or P815 TC were removed from the peritoneal cavity of syngeneic hosts and 3 × 10⁵ cells in 1 ml PBS-FCS were incubated with 200 μCi Na₂⁵¹CrO₄ (Amersham CJS 1P, Amersham Corp., Arlington Heights, IL) for 45 min at 37°C with occasional shaking, and washed twice with PBS-FCS. Labeled TC (1 × 10⁶ cells) were mixed with CTL (3 × 10⁵ cells) in 1 ml aliquots in 75 × 12 mm test tubes, centrifuged at 170 g for 10 min to promote CTL-TC contact, and incubated at 37°C in a humidified atmosphere. At the end of the incubation period the radioactivity of the supernatant was assayed in a well-type gamma scintillation counter. Released radioactivity is expressed as the percent of total releasable radioactivity, determined by repeated freezing and thawing of labeled target cells. Results are corrected for spontaneous release (10-15% of total radioactivity).

**Electron Microscopy**

BALB/c anti-EL4 CTL (1 × 10⁶) were mixed with EL4 TC (3-10⁶) in 1 ml PBS-FCS in Falcon (Oxnard, CA) 75 × 12 mm plastic tubes at room temperature. Cells were centrifuged at 170 g for 10 min at room temperature to promote CTL-TC conjugate formation. Medium was removed and carefully replaced by prewarmed (37°C) fixative (2.5% glutaraldehyde in 0.09 M cacodylate buffer, pH 7.2, containing 2.5 mM CaCl₂). Prewarming of the fixative was important to prevent retraction of cellular projections due to temperature change. Cells were centrifuged at 170 g for 10 min at room temperature, as described (6). Microscopic examination of the cells with differential interference contrast or phase-contrast optics revealed a large number of CTL-TC conjugates comprised of one CTL bound to one TC (Fig. 2 C). EL4 and P815 TC, as monitored by the release of chromium, increased with time resulting in a progressive decrease in the amount of conjugates. After 60 min of incubation only 14.5% conjugated TC could be detected.

**The Spatial Relationships of MTOC and CTL-TC Contact Areas**

**Immunofluorescence Microscopy:** Specific conjugates of BALB/c anti-EL4 CTL and EL4 cells (TC) were prepared by centrifuging mixtures containing CTL and TC at room temperature, as described (6). Microscopic examination of the cells with differential interference contrast or phase-contrast optics revealed a large number of CTL-TC conjugates comprised of one CTL bound to one TC (Fig. 2 C). EL4 cells (average diameter of 15-20 µm) could easily be distinguished from the much smaller CTL (7-10 µm) (5, 6). The microtubular system of nonconjugated (free) CTL consisted of about 10-30 visible, fluorescently-labeled microtubules or microtubule bundles. Careful examination of the labeled cells revealed a single microtubule organizing center (MTOC) in essentially every cell (Fig. 2 E). EL4 cells exhibited a dense microtubular network (Fig. 2 F) originating in a single MTOC. In CTL-TC conjugates we found that essentially all effector CTL were bound to EL4 through a membrane region proximal to the MTOC. This polar orientation was observed in over 95% of the cells fixed immediately after onset of conjugation by centrifugation (out of over 500 conjugated cells analyzed). Conjugated TC on the other hand, exhibited a random orientation of their contact zone with respect to the MTOC. A single CTL-TC conjugate is

| Incubation Time (min) | 0 | 5 | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 45 | 50 | 55 | 60 |
|----------------------|---|---|---|----|----|----|----|----|----|----|----|----|----|
| % SlCr released      | 0 | 5 | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 45 | 50 | 55 | 60 |

**FIGURE 1** Conjugation and cytolyis of EL4 TC by BALB/c Anti-EL4 CTL. Cells (3:1 TC:CTL) were mixed, centrifuged 170 g for 10 min at room temperature, and incubated at 37°C. At various times thereafter percent lysis was monitored by the ⁵¹Cr-release technique and percent conjugation was determined microscopically. Data based on five independent experiments. Solid line, % ⁵¹Cr released. Broken line, % conjugation.
shown in Fig. 2A and 2B. The cells were photographed at two focal planes showing the MTOC of the effector CTL (Fig. 2A) and the TC (Fig. 2B).

At effector cell excess we frequently observed complex conjugates consisting of a number of effector cells bound to one target (see also reference 6). In Fig. 2D, three types of conjugates (CTL 1-TC 1; CTL 2-TC 1; CTL 3-TC 1) are shown. In these, the MTOC of all bound CTL were oriented towards the target, while the MTOC of the latter was randomly distributed.

**ELECTRON MICROSCOPY:** The results outlined above were corroborated by transmission electron microscopy. Examination of a large number of CTL-TC conjugates, sectioned across intercellular contact sites, indicated that the centrioles as well as the pericentriolar array of microtubules of the CTL were located proximal to the contact area. This was found in conjugates at early stages of the cytotoxic interaction (Fig. 3A, B, and F) or in relatively late stages where the damage to the TC was clearly apparent (Fig. 3C and D). Due to the extensive membrane interdigitation at the contact area and the random plane of sectioning (with respect to the contact plane) we could not determine whether the axes of the centrioles themselves were oriented toward the cell membrane. In high magnifications (Fig. 3B, D, and F) we could detect a large number of microtubules emerging from the centriole complex. In some
CTL we detected elements of the Golgi complex in the vicinity of the contact area in the CTL (for example the cell shown at the top of Fig. 3 E and in Fig. 3 F). Nevertheless, this was not nearly as prominent as the presence of the MTOC in that region.

The centrioles of the EL4 TC were apparently randomly distributed with respect to the intercellular contact area as demonstrated in Fig. 3 E. It should be pointed out that the number of MTOC detected in CTL in ultrathin sections selected for the CTL-TC contact area was considerably higher (more than twofold) than that detected in unselected sections or unconjugated cells.

**Ligand-induced Surface Caps are not Spatially Related to the MTOC**

Incubation of BALB/c anti-EL4 CTL carrying both the H-2a and Thy-1 antigens with H-2d and Thy-1 antibodies followed by rhodamine-labeled goat anti-mouse IgG resulted in redistribution of the respective molecules into patches and subsequently into polar caps (Fig. 4 A and D). Usually 20-30 min of incubation at 37°C were required for maximal cap formation.

Capped, fixed cells were permeabilized with Triton X-100 and reacted with a rabbit antitubulin antibody followed by fluorescein-labeled goat anti-rabbit IgG. The results (Fig. 4) indicate that the polar caps formed in the two systems (A and D) were not spatially related to the MTOC (B and E). Moreover, in most cases (ca. 60-75%) the MTOC were localized opposite to the cap. Comparison of the microtubular system of cells before and after capping suggested that some deterioration of the microtubule network occurred during capping (compare for example Fig. 4 B and E with Fig. 2 E).

**Modulation of the Microtubular System in TC-bound CTL**

CTL-mediated lysis is highly temperature-dependent; incubation of CTL-TC conjugates at room temperature resulted in virtually no killing (6). Nevertheless, we noticed that after 5–10 min incubation at room temperature (or at 37°C) the microtubule system of TC-bound CTL underwent substantial deterioration. This was usually manifested by a decrease in number or complete disappearance of defined microtubules (Fig. 5). Documentation of this effect required examination of the cells...
such changes observed in noneonjugated CTL. At several focal planes. NO changes were noted in the organi-

gates, fixed after 15 rain at room temperature. The conjugate in A and B was photographed at two focal planes to demonstrate that whereas the MTOC can still be detected near the contact area, the microtubular system of the CTL appears deteriorated.

**FIGURE 5** Indirect immunofluorescence labeling for tubulin of conjugates, fixed after 15 min at room temperature. The conjugate in A and B was photographed at two focal planes to demonstrate that whereas the MTOC can still be detected near the contact area, the microtubular system of the CTL appears deteriorated. (C) Unconjugated CTL which was exposed to the same incubation. Bar, 10 μm. X 950.

at several focal planes. No changes were noted in the organization of the microtubular systems in the target cells nor were such changes observed in nonconjugated CTL.

**DISCUSSION**

Extensive efforts were invested in recent years to characterize the mechanisms involved in the interactions of cytotoxic T lymphocytes with specific targets (for reviews see 4, 13, 18, 19, 23, 28). Many studies focused on the early steps of the process and the nature of the CTL receptor for TC recognition, whereas others explored the lytic process itself. Although the cytotoxic mechanism of CTL has not yet been defined at the molecular level, several models have been considered to account for the lethal hit, ranging from secretion of toxic substances, through mechanical deformation, to induction of local instabilities in the TC membrane. Whichever working hypothesis better presents the cytotoxic effect, it must be compatible with a fundamental feature of this process, that is, the unidirectionality of the killing. It has been established that after the target cell has been damaged, the CTL can recycle to start a new lytic interaction (40). The killer cell, however, is not inherently immune to killing, as it has been shown that CTL can be killed when exposed to appropriately immunized effector cells (17).

A finding related to unidirectional killing by CTL is that effector CTL bound simultaneously to more than one TC kill the TC sequentially (41). This suggested that the delivery of the lethal hit not only requires direct intercellular contact but preferentially occurs at defined regions along the CTL plasma membrane. Electron microscopy examination of CTL-TC conjugates revealed extensive interdigitation of the plasma membranes at the contact region (26, 29), suggesting that membrane-folding forces are generated at CTL-TC contact zones. In view of the involvement of cytoskeletal elements in the mechanical responses of cells (20, 25, 35), we explored the possibility that specific organization of such intracellular networks might be related to the unidirectionality of the cytotoxic process. It has been recently shown by immunofluorescence microscopy that the CTL contact area is enriched with actin (27), in analogy to the increase in actin labeling under lectin- or antibody-induced surface caps (9, 15, 30, 33). Actin enrichment at CTL-TC contact regions is compatible with the extensive membrane interdigitations observed at this site (26, 29).

The observations reported here were related to the microtubule system and its organizing center. They may be summarized as follows: (a) immune CTL interact with and bind to TC predominantly through a membrane region proximal to the MTOC and the centrioles; (b) unlike CTL, CTL-bound TC do not display their MTOC proximal to the contact region; (c) after incubation at room temperature (or 37°C), the micro-
tubules of TC-bound CTL undergo progressive deterioration; (d) antibody-induced caps of H-2 and Thy-1 antigens do not localize proximal to the MTOC; often they are found in the opposite pole of the cell.

The two phenomena, namely polar orientation of the MTOC and deterioration of microtubules seem to be related to distinct, sequential events and will be considered separately. The results indicate that the plasma membrane proximal to the MTOC is engaged in binding to the target, at least in conjugates composed of one CTL bound to one TC. This spatial relationship was found in essentially all CTL-TC conjugates fixed immediately after onset of conjugation. It is still unclear whether it is related to an uneven distribution of relevant membrane components or to adhesive properties of the cell membrane adjacent to the MTOC which favor the formation of stable intercellular contacts. It may be related to the more general role of the centriole in cellular dynamics (for discussion see references 1, 2). It has been shown that in mobile cells the centrioles are localized in front of the nucleus, towards the leading edge of the cell membrane (3, 21). The membrane in this area exhibits an increased protrusive and deformational potential which may render it more compatible for the formation of stable intercellular contacts.

Another organelle found localized in the vicinity of CTL contact regions is the Golgi complex. This is in line both with reports by Zagury et al. (40) and by Bykovskaya et al. (10, 11) and with the notion that the Golgi apparatus is usually localized in the vicinity of the centrioles (32, 34). However, analysis of large numbers of electron micrographs of CTL-TC conjugates suggests that the contact area is primarily related to the centrioles and that the localization of the Golgi system is only secondarily related to the contact site.

Patching or capping of surface components on the CTL plasma membrane which might occur during CTL-TC inter-

action could not in itself account for the orientation of the MTOC inasmuch as surface caps induced by H-2 or Thy 1, antibodies, and rhodamine-labeled goat anti-mouse IgG were
not proximal to the MTOC and were often localized at the opposite pole. It was shown (31) that capping of these antigens occurred predominantly in the area opposite the Golgi region (in contrast to anti-IgG or Con A induced caps [24, 31, 36]).

The second phenomenon reported above was the deterioration of the microtubule network of conjugated effector cells. This process, though difficult to evaluate quantitatively, was quite rapid and occurred progressively after incubation of cell conjugates for 5–15 rain at room temperature or at 37°C. Similar modulation of microtubule organization was often found in cells that undergo patch or cap formation (Fig. 4).

These observations are in line with the report of Yahara and Kakimoto-Sameshima (39) on the modulation of microtubule organization by capping of surface Ig in mouse spleen lymphocytes. Unlike CTL-TC conjugation which requires Mg ++, the lytic process is strictly Ca ++ dependent (23). However, it is still unclear whether this Ca ++ dependence is related to well-known effects of Ca ++ on microtubule disassembly.

The significance of modulation of microtubule organization in CTL and its relevance to the cytocytic response are at present not clear. One may consider, however, the distinct and often conflicting actions of microtubules and actin-containing microfilaments in living cells. It has been shown that paralysis of the lateral mobility of surface components induced by high concentrations of Con A could be abolished by the microtubule-disrupting drug colchicine (37, 38). It may be proposed that the intact network of microtubules restrains dynamic, actin-dependent processes of the membrane such as those that might be involved in cytocytic interaction. 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