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ROLE OF ADHESION MOLECULES IN THE MECHANISM OF NON-MHC (MAJOR HISTOCOMPATIBILITY COMPLEX) RESTRICTED CELL-MEDIATED CYTOTOXICITY

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Abstract

Adhesion molecules involved in the interaction between immune system effector cells and tumor targets are surface molecules which contribute to the formation of cell-to-cell contacts and belong to the integrin family. In this paper, the role played by the adhesion molecules in the process of cell-mediated cytotoxicity is reviewed. Furthermore, the contact area between effector and target cells has been analyzed by scanning electron microscopy. This region, termed "closed chamber", seems to contribute to killing efficiency by creating an intimate contact region in which cytotoxic factors can easily induce lethal hit in target cell. Thus, the extension of the closed chamber seems to be positively related to effector cell killing potential as well as to target cell sensitivity and, in this context, the adhesion molecules prove to play a pivotal role. In fact, a receptor-ligand interaction occurs between CD11a/CD18 (LFA-1) and CD2 molecules, expressed on the effector cells, and the respective counterparts on target cells, i.e., ICAM-1, ICAM-2, or LFA-3. Treatment with antibodies against such molecules strongly modifies closed chamber formation without inhibiting cell-to-cell binding. Nevertheless, in these conditions, the killing ability of different effector cells toward tumor targets appears to be strongly impaired. Hence, the adhesion molecules seem to be strongly involved in the formation of the closed chamber as well as in the activation of effector cell killing machinery.

Key Words: Natural killer cells, lymphokine activated killer cells, cytotoxicity, adhesion molecules.

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Introduction

Cell-mediated cytotoxicity is a process mediated by cells of T lineage or NK lineage which ensures defence against viral infection or neoplastic transformation. Cells infected by virus or expressing tumor-specific antigens can be recognized and eliminated by cytotoxic T cells (CTL) which specifically recognize antigens in the context of class I major histocompatibility complex (MHC) molecules. NK cells may kill virus-infected or transformed cells by mechanisms which are, so far, not defined, since these cells do not express "classic" antigen receptors, such as surface Ig or T cell receptor (TcR), and exert their cytolytic function in a MHC-unrestricted fashion (Savary and Lotzova, 1986; Sprent and Webb, 1987; Young and Cohn, 1987).

As a general rule, cell-mediated cytotoxicity is a multistep process in which recognition, binding and killing of the target cells (TC) occur in sequence. MHC-unrestricted cytotoxic cells belong predominantly to the NK lineage which exert their lytic activity as resting, freshly isolated cells (Stutman, 1981; Trinchieri and Perussia, 1984; Bonavida and Right, 1986; Herberman *et al.*, 1986; Kang *et al.*, 1987; Young, 1989). The cytotoxic potential of NK cells is enhanced by Interleukin 2 (IL-2) stimulation which leads to the generation of the so-called lymphokine activated killer (LAK) cells (Rosemberg, 1986; Dianzani *et al.*, 1989; Ferrini *et al.*, 1989). In addition, NK cytotoxicity can also be modulated or enhanced by various agents (Robertson and Ritz, 1992).

Morphologically, most cells exhibiting NK activity as well as the LAK cell precursors of T lineage are characterized by the presence of cytoplasmic azurophilic granules and are termed large granular lymphocytes (LGL) (Grossi *et al.*, 1982; Liepins *et al.*, 1977; Grossi and Ferrarini, 1982). In humans, cells exhibiting NK function express multiple surface phenotypes. Most LGL (about 90%) are represented by cells of $CD3^-$ $CD16^+$ $CD56^+$ phenotype, however, $CD16^-$ (approximately 5%) or $CD56^-$ NK cells can also be detected (Timonen *et al.*, 1979; Trinchieri and Perussia, 1984; Zarcone *et al.*, 1987a).

Both CTL and NK/LAK cells kill their targets by

a multistage mechanism that is initiated by the binding of the effector cell to the target cell (conjugate formation). Binding is accompanied by a rearrangement of cell surface microvillar structures (Liepins *et al.*, 1977; Carpen, 1987; Arancia *et al.*, 1991a) and cytoskeletal structures (polarization toward the target) followed by the activation of the effector cell and the release of molecules (e.g., perforins) in the intercellular space capable of inducing the target cell lysis (Plaut *et al.*, 1973; Strom *et al.*, 1973; Rayser *et al.*, 1982). In this space, the cell adhesion molecules (CAMs) such as LFA-1 (CD11a/CD18) and CD2, could easily interact with corresponding molecules in the target cell [e.g., ICAM-1 (CD54) and LFA-3 (CD58)] (Martz, 1987; Patarroyo *et al.*, 1990; Zarcone *et al.*, 1992a). The LFA-1 protein is a member of the integrin family that binds to the ICAM-1 and ICAM-2 molecules, members of the immunoglobulin super-family, expressed in a wide variety of cells from different tissues (Selvaraj *et al.*, 1987; Boyd *et al.*, 1988). Binding of LFA-1 to ICAM, in addition to the CD2-LFA-3 interaction, induces antigen-independent adhesion between effector and target cell (Springer *et al.*, 1987; Shaw and Shimizu, 1988; Springer, 1990).

It has been suggested that the contact region between conjugated cells, with the formation of a so-called "closed chamber", might have an important role in the activity of effector cells by modulating their efficiency (Sung *et al.*, 1986, 1988; Malorni *et al.*, 1989; Arancia *et al.*, 1989, 1991a). During T cell-target cell interaction, the LFA-1 molecules co-migrate to the site of cell-cell contact with the TcR molecules, but a direct binding of LFA-1 and the TcR complex has not been demonstrated (Shaw and Luce, 1987; Takai *et al.*, 1987). As mentioned above, the CD2 glycoprotein on the surface of thymocytes and T lymphocytes binds to the lymphocyte function antigen (LFA-3) on target cells and antigen-presenting cells. It has been suggested that the physical association of CD2 and TcR complex can likely be required for T cell triggering (Selvaraj *et al.*, 1987; Takai *et al.*, 1987). Furthermore, the intimate contact resulting from such an interaction system seems to be involved in the mechanism of cytotoxicity by improving the "efficiency" of cytotoxic factors produced by immune effectors and released in the contact region (Podak, 1985; Clark, 1988; Stanley and Luzio, 1988; Arancia *et al.*, 1990).

In order to evaluate the role of the closed chamber features in the killing efficiency, a study has been performed on cell surface and cytoskeleton of conjugates formed between different effector cells and various tumor targets, by means of fluorescence microscopy and scanning electron microscopy. In addition, a quantitative morphometric analysis has been carried out on the structural characteristics of such a contact region. The effect of antibodies against CAMs has also been evaluated in order to establish their involvement in the closed chamber formation and related killing activity. Results obtained clearly provide a general indicative method for the evaluation of the cell-to-cell interaction in cell mediated immunity and seem to be suggestive for a key role of adhesion molecules, not only in the binding process, but also in the activation mechanisms.

Materials and Methods

Cytotoxic effector cells

Several effector cell preparations were used in this study. Peripheral blood lymphocytes (PBL) were isolated from heparinized blood samples of normal adult donors by Ficoll-Hypaque density gradient centrifugation and depleted of monocytes by Percoll gradient centrifugation. Cytochemical staining for acid esterase activity showed that monocyte contamination of PBL preparation was always less than 3%. PBL were used as effector cells for the determination of NK cell-mediated cytotoxicity. For the generation of LAK cells, PBL were cultured for 3 days in RPMI 1640 (with 10% fetal calf serum and 50 μg/ml gentamycin) containing 100 IU/ml Interleukin 2 (IL-2) (Biogen, Lausanne, Switzerland).

For the scanning electron microscopy (SEM) analysis of conjugates formed between effector cells and tumor cell targets (see below) we used purified populations of NK cells and of LAK cells. To this end, freshly isolated or IL-2-cultured PBL were allowed to form high affinity E rosettes, at 29°C, with untreated sheep erythrocytes. Non-rosetting cells were recovered by Ficoll-Hypaque density gradient centrifugation and incubated with a mixture of anti-CD3 and anti-CD2 mouse monoclonal antibodies. Cells were subsequently admixed with immunobeads coated with goat anti-mouse immunoglobulin (Dynabeads, Oxoid, Unipath, Milano) at a bead to cell ratio of 40 to 1. Cells which did not adhere to the beads were recovered and the percentage of NK cells was evaluated by flow cytometry (FACScan, Becton Dickinson, Mountain View, CA), following staining with anti-CD16 or CD56 antibodies and affinity purified, FITC-labelled goat anti-mouse immunoglobulin antiserum (Southern Biotechnology Associates, Birmingham, AL). Purified populations of NK and LAK cells always contained > 80% $CD16^+$ cells.

Tumor target cells

The NK-sensitive erythroleukemic cell line K562 (Landay *et al.*, 1987) was used in all of the experiments. To determine LAK activity, we used a variant of the promyelocytic cell line HL60, HL60R, which does not bind NK cells and consequently is NK-resistant (Zarcone *et al.*, 1987b). This cell line was also used as a control for the effector to target cell binding experiments.

Antibodies

NK cells, defined as $CD16^+$ cells, and their IL-2-activated progeny were enumerated by FACS analysis using FITC-labelled Leu 11b antibodies (Becton Dickinson). The same antibody was employed to determine the binding of $CD16^+$ cells to tumor targets. Other antibodies used in these studies were directed against CD2 (Leu 5b), CD3 (Leu 4) and CD56 (Leu 19) (all from Becton Dickinson). The anti-LFA1 (CD11a/CD18) antibody was selected among several anti-LFA-1

monoclonal reagents produced in our laboratory for its ability to determine maximal inhibition of NK cell-mediated cytotoxicity.

Cytotoxicity assays

NK activity was measured as the ability of fresh PBL to lyse K562 cells. LAK activity was determined using IL-2-cultured PBL against K562 cells or the NK-resistant cell line HL60R. NK and LAK cell-mediated cytotoxicity was determined in five distinct experiments. The lytic function (cytotoxicity) of 30 $CD3^-$ clones was tested against K562 cells. Target cells were labelled with ^{51}Cr by incubation with 200 μCi of $Na^{51}CrO_4$ (Amersham International, Buckinghamshire) for 1 hour at 37°C, as described (Zarcone *et al.*, 1989). Effector cells were admixed with target cells at 40:1 ratio for NK and LAK cells and at 20:1 ratio for $CD3^-$ cell clones. Cell mixtures were incubated for 4 hours at 37°C in round-bottomed 96 well microtiter plates and centrifuged at 200 rpm for 5 minutes at the end of the incubation period. After centrifugation, 100 μl of supernatant were harvested from each well and counted in a gamma counter.

Spontaneous release, maximum release and % specific ^{51}Cr release were determined as previously described (Tilden *et al.*, 1991).

The effect of antibodies to CD16, CD56, CD2, CD11a and CD2+CD11a on the cytotoxicity mediated by NK cells, LAK cells and $CD3^-$ cell clones was determined through the decrease of specific ^{51}Cr release and expressed as % inhibition. Target cells were added to the effector cells at the ratios indicated above and the 4 hour cytotoxicity assay was performed in the presence of the antibodies.

Flow cytometric determination of NK/LAK cell binding to tumor target cells

Percentages of $CD16^+$ cells in freshly isolated or IL-2-cultured PBL were determined by flow cytometric analyses after staining with FITC-labelled monoclonal antibody (mAb) of IgM isotype (Leu 11b). Cells were subsequently allowed to form conjugates with tumor cells for an immunofluorescence binding assay. Briefly, PBL were admixed with tumor cells (K562 or HL60R) at 5:1 ratio and cell pellets obtained by centrifugation at 200 rpm were incubated for 10 minutes at 37°C. Pellets were gently resuspended and analyzed by FACS. Dot plot analyses showed two distinct cell populations consisting of unbound lymphocytes and of target cells (with or without bound lymphocytes), respectively. Gates were set on the unbound lymphocyte populations and percentages of $CD16^+$ cells were determined. The extent of effector to target cell binding was indicated by decrease in the percentage of $CD16^+$ cells among unbound lymphocytes. Thus, percentages of conjugate-forming $CD16^+$ cells could be calculated. As a control for the assay, we used the HL60R cell line, previously shown to be unable of binding NK cells (Zarcone *et al.*, 1987b).

The effect of anti-adhesion molecule antibodies was tested using the same assay. Effector cells were separately incubated with anti-CD2, anti-CD11a/CD18 or anti-CD56 (10 mg protein/10^6 cells), or with mixtures of these mAb, for 30 minutes on ice. Target cells were subsequently added and the binding assay was performed for 5 minutes at room temperature. Negative controls were performed by using anti-Leu 19 (CD56) and anti-Leu 11 (CD16), IgG 1 isotype (about 0.1% inhibition).

Scanning electron microscopy (SEM) analysis

For SEM analysis, conjugates between effector and target cells were seeded on polylysine-coated coverslips. After a few minutes, to allow adhesion to the glass surface, cells were fixed with 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) at room temperature for 20 minutes, post-fixed with 1% OsO_4 in the same buffer, dehydrated through a graded ethanol series, critical-point dried with CO_2 and gold-coated by sputtering. Samples were examined with a Philips 515 scanning electron microscope.

Fluorescence microscopy analysis

Samples were applied to polylysine-coated glass slides and fixed in 3.5% formaldehyde for 20 minutes at room temperature. After washing in PBS buffer, the cells were permeated with 0.5% Triton X-100 (Sigma T-6878) in the same buffer for 5 minutes at room temperature. For actin detection, cells were stained with fluorescein-phalloidin (NBD-phallacidin, Molecular Probe) (final dilution 1:100 in PBS) at 37°C for 30 minutes. For tubulin labelling, incubation with a mixture of monoclonal anti-alpha and anti-beta tubulin antibodies (Amersham) (final dilution 1:500 in PBS) at 37°C for 30 minutes were performed. Washed cells were then incubated with a sheep anti-mouse IgG fluorescein-linked whole antibody (Amersham, final dilution 1:20 in PBS). After washing coverslips were mounted with glycerol-PBS (2:1) and analyzed with a Nikon Optiphot fluorescence microscope.

Quantitative evaluation of the conjugates contact area

In order to evaluate the extent of the contact area between effector cells (NK, LAK) and different target cells (K562, HL60R) conjugates were examined by SEM. About 50 micrographs at the same magnification (6500x) were taken for each sample. Measurements were performed either on control conjugates or on cell pairs after exposure to antibodies to CD2 and CD11a/CD18. Before recording every picture, the specimen was tilted to put the axis of the cell pair perpendicular to the detector in order to avoid the overlapping of the two cell types. The length of the contact region was determined by an interactive image analyzer (IBAS II, Kontron), measuring the distance between the limit points of the adhesion area between effector and target cells. The contact area was calculated by roughly assuming it circular in shape. The mean values and the relative standard deviations were then calculated for each sample. The significance of the difference between the mean values recorded in control and MoAbs-treated conjugates was evaluated by Student's T test.

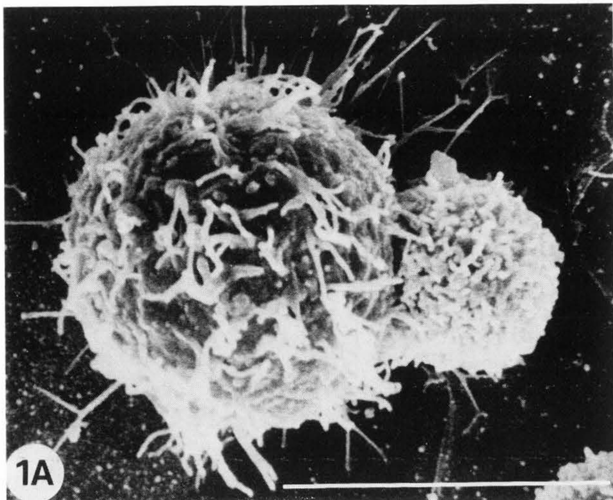
Results and Discussion

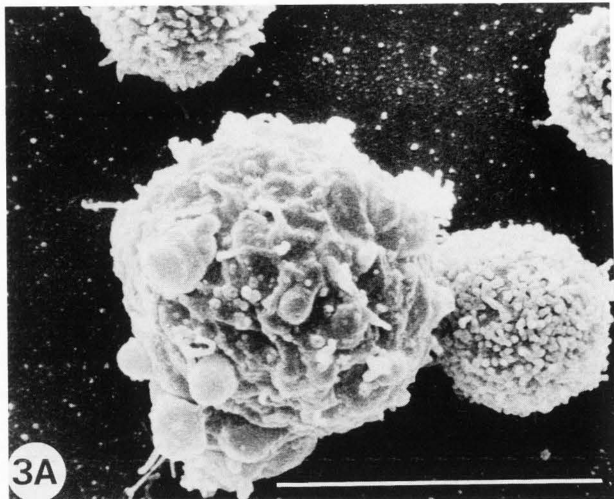
Previous studies (Arancia *et al.*, 1989, 1991a) showed that cytotoxic lymphocytes can exert a powerful lytic activity when a wide and intimate contact area is established with the target cell. Such a close interaction leads to the formation of the so-called "closed chamber" between effector and target cell, in which the various lytic factors find ideal conditions for enhancing their lethal potential (Zychlinsky *et al.*, 1988; Arancia *et al.*, 1991a). This was also supported by morphological studies performed by comparing the interaction of effector cells with target cells displaying different NK susceptibility (Arancia *et al.*, 1989, 1991a). In fact, in conjugates formed with sensitive target cells, the cell pair contact regions appeared to be large and characterized by the presence of numerous intertwined protrusions; conversely, when resistant target cells were employed, the contact area appeared to be narrow and no significant involvement of the cell surface structures could be detected (Carpen 1987; Kang *et al* 1987; Arancia *et al* 1991a). Furthermore, if NK-resistant target cells, forming a weak contact with NK cells, were conjugated with LAK cells, an increased interaction surface was revealed.

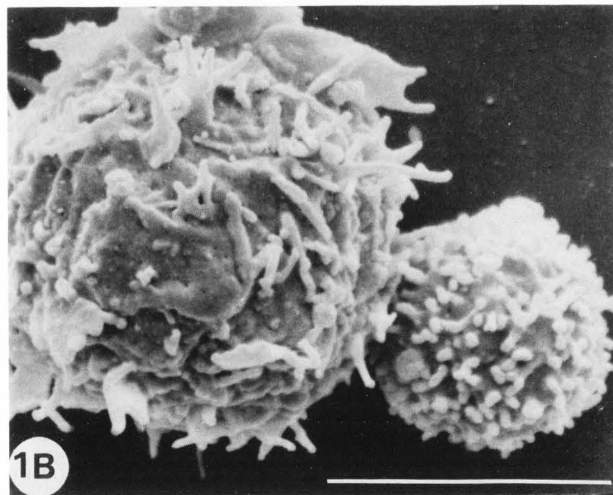
We report here data obtained using two different tumor targets: the NK-sensitive K562 cell line and the NK-resistant, LAK-sensitive, HL60R cell line. The contact area between effector and target cells has been studied by SEM and a quantitative evaluation of the extension of the contact region has been carried out. Such an analysis revealed that a close interaction occurred between effector and target cells in both experimental conjugates examined: NK/K562 and LAK/HL60R (Figs. 1a and 1b, respectively). Furthermore, the quantitative evaluation showed that no significant difference occurred between the two different cell pairs. In fact, the average length of the closed chamber in NK/K562 conjugates was about 4.01 μm with respect to 3.53 μm measured in LAK/HL60R pairs (Fig. 2). The cell contact area was calculated by assuming it to be circular and smooth. However, it is known that the cell surface of both effector and target cells is characterized by numerous intertwined microvilli and invaginations in the contact region. Thus, it is not possible to measure the real extent of the adhered cell membranes.

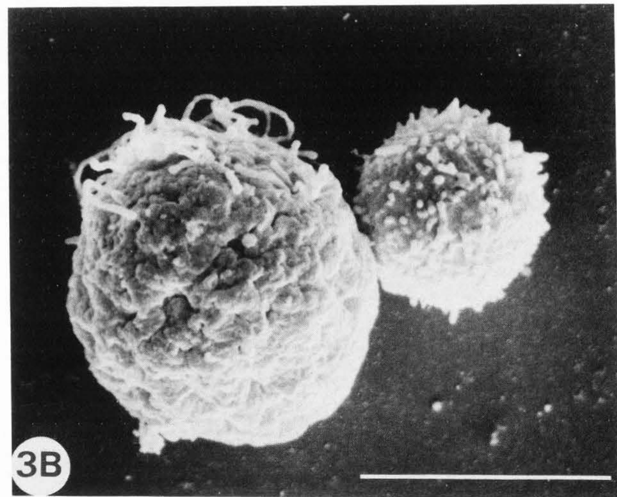
Several studies have been carried out to investigate the involvement of the cytoskeletal components in the binding and killing processes (Plaut *et al.*, 1973; Sirianni *et al.*, 1988; Malorni *et al.*, 1989; Arancia *et al.*, 1991a). In fact, the configuration of the intercellular space between effector and target cell also depends on the cytoskeletal arrangement of both cell types. In particular, the binding of the NK or LAK cells to the target cells depends on microfilaments, whereas target cell lysis is known to require microtubule assembly and rearrangement in both effector and target cells (Kupffer *et al.*, 1983; Kupffer and Dennert, 1984; Sirianni *et al.*, 1988; Arancia *et al.*, 1991a). In this context, the role of the target cell in the interaction with the effector cell has also to be evaluated. An "induced suicide" model has been suggested by Ucker (1987) who hypothesized a suicide program that can be activated in the target cell by several different stimuli. Other authors also focused on the possibility of the target cell playing a direct role in its own death (Russel, 1983; Goldstein, 1987; Pitsillides *et al.*, 1988; Chayen *et al.*, 1990). Thus, different susceptibilities found in the tumor target cells could also depend on a different suicide program and different degree of "permissiveness" in allowing their death (Suthanthiran *et al.*, 1984; Kramer and Simon, 1987). Moreover, these data indicated that the target cell contributes actively in determining the formation of an extended and intimate contact with the killer cell (Kang *et al.* 1987; Arancia *et al.*, 1991a). The changes occurring in cytoskeletal elements of target cell seem to support the challenging opinion of an induced suicide mechanism. In fact, the lytic process seems to occur differently in the conjugated target cells studied by an ultrastructural SEM analysis. A cell surface phenomenon, known as blebbing, is easily appreciated in K562 cells, highly sensitive to NK activity (Fig. 3a). By contrast, the NK-resistant HL60R cells, even though recognized and bound by effector cells, undergo surface smoothing and appear to be simply adjacent to the killer cells (Fig. 3b). Blebbing process, widely recognized as a peculiar marker of injured or dying cells, was usually associated with ion deregulation and free radical formation (Allen, 1987; Bellomo *et al.*, 1990; Malorni *et al.*, 1991a). The finding that OH scavengers inhibit NK cell activity, suggested that OH, possibly generated via the lipooxygenase pathway of arachidonic acid metabolism, can contribute directly to target cell lysis or to being generated in the inter-plasma membrane space between target cells and effector cells (Suthanthiran *et al.*, 1984). These data can also be related to recent results which involve target cell triggering by exogenous mechanisms (Di Virgilio *et al.*, 1990). However, dying target cells showing different sensitivity to immune effectors exhibit different morphological features which could probably be further important markers of cell behaviour in terms of susceptibility (Liepins *et al.*, 1977; Arancia *et al.*, 1989). This could also depend on the availability of different targets to be "turned on" in their "suicide program"-cascade.

To evaluate the role played by the adhesion molecules in the formation of the closed chamber and to verify if the cell-to-cell interaction could be modified by altering CAM function, the NK and LAK cells were incubated with anti-CD2 or anti-LFA-1 monoclonal antibodies (alone or in association) before conjugation with the different target cells. In fact, some studies hypothesized that antibodies to CAMs expressed on the target cells or on the effector cells inhibit, to a variable extent, the target cell lysis induced by both CTL and NK/LAK cells (Robertson *et al.*, 1990; Springer, 1990; Van Seventer *et al.*, 1990). The general hypothesis on the mechanism of such inhibition was that it depends primarily on a reduced binding, i.e., pair number, or to an altered conjugate formation, i.e., an impairment of inter-









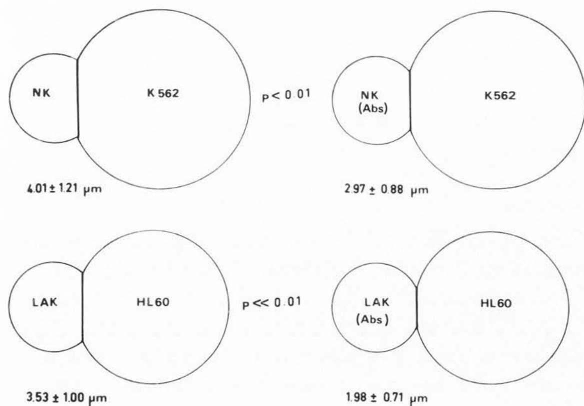


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Figure 1. NK/K562 (**a**) and LAK/HL60R (**b**) conjugates observed by SEM. In both cases, a wide and intimate contact region is established between effector (to the right) and target cells (to the left). Bars = 10 μm (Fig. 1a) and 5μm (Fig. 1b).

Figure 2. Scheme showing the average length of the contact region in the various cell pairs examined. In both cell conjugates (NK/K562 and LAK/HL60R) the extent of the cell-to-cell contact appears to be significantly reduced after treating the effector cells with anti-CD2 and anti-CD11a/CD18 monoclonal antibodies.

Figure 3. Morphological aspects of the target cell death after binding with NK cell. K562 cells, highly sensitive to NK activity, undergo lysis by showing an evident surface blebbing phenomenon (**a**). In NK-resistant HL60R cells the surface blebbing was not observed and the dying cells showed a surface smoothing with a complete loss of microvilli (**b**). Bars = 10 μm (Fig. 3a) and 5μm (Fig. 3b).

cellular contact. In our experience, when LAK cells were incubated with anti-CAM antibodies before conjugation with the target cells (HL60R), most cell pairs showed a modification of the closed chamber features resulting in a remarkable reduction and narrowing of contact surface which became sometimes "punctiform" (Fig. 4). In particular, a reduction of effector cell cytotoxicity, but not of binding, was detected after exposure to anti-CAMs either if administered solely or in combination. However, when treatment was performed with both antibodies the effector cell function appeared even more impaired (Table 1). The same effect was obtained in NK-K562 conjugates, albeit to a lesser extent.

The quantitative morphometric evaluation performed on SEM images showed statistically significant differences between control and antibody treated samples (Fig. 2). In particular, in NK-K562 conjugates the average cell contact area appears to be reduced of about one half after preincubation of effector cells with anti-CD2 and anti-LFA-1 antibodies. In LAK-HL60R conjugates this effect was even more evident, the cell contact area resulting to be about one third in antibody-treated samples when compared to control ones (Fig. 5).

Results obtained seem to suggest that both CD2 and LFA-1 play an important role in the mechanism of interaction and that the combined action of these two surface molecules contributes to the formation of a wide "closed chamber" between effector and target cell, probably improving killing ability. In fact, the pretreatment of both resting and IL-2-activated NK cells with antibodies to LFA-1 and CD2, alone or in combination, appeared to yield the highest level of inhibition. However, there are large differences in the level of inhibition which appear to be related to the degree of target cell sensitivity to NK/LAK cells. In conclusion, the killing process, rather than binding, seems to be a CAM-dependent phenomenon.

Treatment of NK and LAK cells with antibodies to CD2 and LFA-1 produces modifications only in the microtubule network when compared to control effector cell/target cell pairs. In fact, actin microfilaments appear to be marginalized in both control (Fig. 6a) and MoAbs exposed effector cells (Fig. 6b). In contrast, microtubular apparatus appears to be polarized toward target cell in control conjugates only (Fig. 6c), whereas it appears to be diffuse in pairs from MoAbs exposed effectors (Fig. 6d). These data are in accord with the functional results above mentioned and strongly suggest a role of adhesion molecules not merely in the binding process but in the killing too (Bierer *et al.*, 1989; Figdor *et al.*, 1990; Makgoba *et al.*, 1988, 1989; Springer, 1990; Van Seventer *et al.*, 1990). The changes in the cytoskeletal arrangement observed in the effector cell treated with MoAbs, after binding with the target cell, seem in fact to contribute to both the important aspects of the cytotoxic mechanism: the formation of a close cell-to-cell contact between effector and target cell but, most of all, the delivery of the lytic substances against the target cell.

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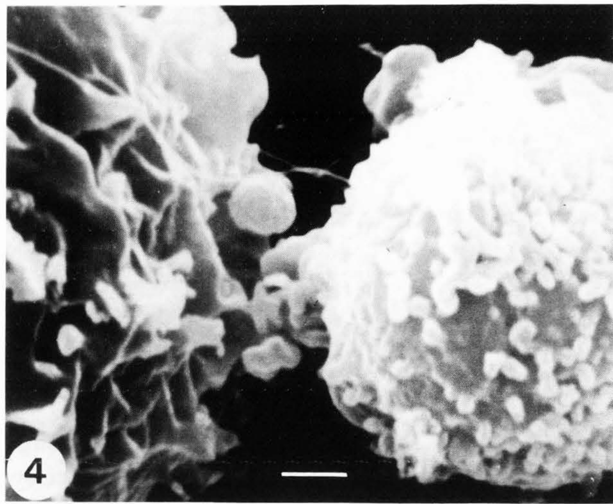

CLOSED CHAMBER SURFACE (μm^2)

12.6

6.9

NK-K562

NK(Abs)-K562

9.8

3.1

LAK-HL60

LAK(Abs)-HL60

5

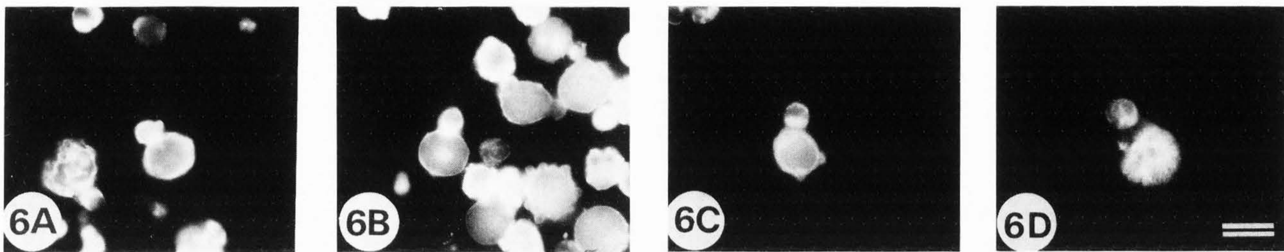

Figure 4. The contact area in a LAK/HL60R cell pair after treatment of the effector cells with anti-CAM antibodies before conjugation. In this case the contact surface appears to be remarkably reduced when compared to that of control conjugates (see Fig. 1b). Bar = 1μm.

Figure 5. Scale-drawing of the closed chamber surface in both control (to the left) and antibodies-treated (to the right) conjugates. In LAK/HL60R cell pairs, after pre-incubation of effector cells with anti-CD2 and anti-LFA-1 antibodies, the average cell contact surface resulted to be about one third (3.1 μm^2) when compared to control conjugates (9.8 μm^2).

Figure 6. Effects of the antibody treatments on the actin (**a** and **b**) and tubulin (**c** and **d**) distribution in effector cell/ target cell pairs as revealed by fluorescence microscopy. Actin microfilaments appear to be marginalized in both control (a) and MoAbs-treated effector cells (b). In contrast, the microtubular apparatus appears to be polarized toward target cell in control conjugates (c) and to be diffused in pairs from MoAbs-treated effector cells (d). Bar = 10 μm.

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Table 1: Effect of anti adhesion molecule antibodies on binding and killing of tumor target cells.

	% Inhibition of binding	% Inhibition of killing
NK-K562		
CD2	10	14
LFA-1	9	52
CD2+LFA-1	17	79
LAK-HL60R		
CD2	ND	27
LFA-1	ND	44
CD2+LFA-1	0	92

Nd = not determined.

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Discussion with Reviewers

E. de Harven: Was the effect of antibodies demonstrated to be dose-dependent, and what irrelevant antibodies were used as controls in these experiments?

Y.H. Kang: The authors do not mention how much of each antibody was used to inhibit the cytotoxicity of NK cells and LAK cells. The authors also did not address whether the inhibition by the antibodies is dose-related.

Authors: Antibody concentrations were those used in immunofluorescence assays for staining cells, i.e., 10 mg protein/10^6 cells. More importantly, this concentration represents the plateau dose for efficient inhibition of cytotoxicity. Negative controls were performed by using anti-Leu 19 (CD56) and anti-Leu 11 (CD16), IgG1 isotype (about 0.1% inhibition).

E. de Harven: You appropriately described cell-mediated cytotoxicity as a multistep process. Surprisingly, the time factor is barely mentioned in your experiments. Are the "conjugates" observed by SEM 2, 10, 30 or 120 minutes after the effector cells (NK or LAK) have had an opportunity to make contact with the target cells? Admittedly, it is difficult to precisely synchronize the encounter between effector and target cells. However, if all the SEM observations are made at one arbitrarily chosen time after conjugation, please explain the rationale for such a static approach to the study of a dynamic cell-cell interaction process.

Authors: The binding phase occurs in seconds to minutes, the killing phase in minutes to hours. We think

that a time course study would be interesting but not useful considering the ability of the effectors to "recycle" themselves continuously to kill their targets. In our opinion, such a study would refer to the conjugation process *per se*. Different incubation times (30 minutes, 1, 2 and 4 hours) were in fact used. Unfortunately, conjugate formation, once it is occurring, proceeds continuously.

L.H. Graf: Under the experimental conditions employed, CD2 and LFA-1 of the effector cells do not seem to contribute in a major way to the formation of the observed conjugates. In the case of the temperature- and Mg^{2+}-dependent formation of conjugates between ICAM-1 and LFA-1 (Mac-1), the incubation conditions (room temperature, unspecified Mg^{2+} concentration) might be a factor. Information about the percents of $CD16^{+}$ cells involved in the conjugates in the absence of antibodies (Abs) would be useful, as the possibility that the experimental conditions are not permitting a physiologically critical component of effector to target recognition to occur, hence allowing only "backup" adhesion mechanisms to be studied, must be considered.

E. de Harven: Cells were incubated with anti-adhesion molecule antibodies for one hour at room temperature. How much of these Abs/CAM complex is already internalized at that time? And, if most of these complexes are already internalized, how could the CAM molecules still be determinant facts in the subsequent binding assays you performed?

Authors: In the conjugate formation assay and in order to determine the effect of antibodies, we proceeded as follows: Effector cells were incubated with antibodies to CD2 and LFA-1 for 30 minutes on ice and subsequently mixed with target cells. The target-effector mixture was pelleted and incubated at room temperature for 5 minutes. The mixture was resuspended and conjugates were counted. In these conditions, the percentage of conjugate-forming cells was unchanged as compared to controls. Our observations according to the literature data indicate that 50-70% of CD16-positive cells are capable of forming a conjugate with K562 cells under the experimental conditions describe above (see also Zarcone *et al.*, 1992). We also agree that several other ligand-receptor pairs may influence the binding of effectors to target cells as is also suggested by our recent studies (Zarcone *et al.*, 1992).

Y.H. Kang: Which target or tumor cells express CD54?

Authors: We refer you to Table 1 in Zarcone *et al.* (1992) for the expression of adhesion molecules on tumor cell targets.

Y.H. Kang: Do some tumor tissues also express integrins? If so, what cells?

Authors: For this important question, please refer to papers by R.O. Hynes (Hynes, 1987, 1992).

Y.H. Kang: How were NK cells purified? Which NK-specific antibodies were used?

Authors: NK cells were purified by negative selection using B and T cell depletion with anti-CD3 and CD19 antibodies following treatment with complement or magnetic beads. Monocytes were previously eliminated by Percoll gradients.

Y.H. Kang: What is the definition of "closed chamber"? Is this term created by the authors based on their own ultrastructural observations? Ultrastructure of the NK-K562 conjugates does not really reveal that the contacts between NK cells and K562 target cells form a chamber-like structure.

Authors: The term "closed chamber" was used in order to define the contact region between effector cells and target cells. In this region, a wide intertwining of microvillous structures leads to the formation of a sort of chamber in which perforins as well as other "cytotoxic" factors can easily reach the target cell with minimal dispersion in the environment. We think that SEM analysis can provide useful information about this region. Kang *et al.* (1987) have used transmission electron microscopy (TEM) to analyze effector-target cell conjugates. Under these circumstances only a study of serial sections may define the existence, or not, of a closed chamber. Otherwise, the effector/target cell contacts are selected at random.

Y.H. Kang: The authors assume that the contacts between effector and target are circular. How did the authors derive such an assumption?

Authors: We think that two spherical cells could only interact by forming a round-shaped contact area, as revealed by SEM or light microscopy (including confocal microscopy). Only serendipity could provide such an image in a singe section in a TEM (see Liepins *et al.*, 1977, or Carpen, 1987).

Y.H. Kang: The authors intend to correlate their observations on the alterations in the organization of actin filaments and microtubules in both effector cells and target cells with the lytic process. The reorganization of actin filaments is known to be regulated by surface integrins. The authors do not mention the effect of the anti-CAM antibodies (anti-LFA-1) on the reorganization of actin filaments. In this respect, does anti-ICAM-1 antibody affect the reorganization of the cytoskeleton and result in impairment of "close chamber" formation and killing? ICAM-1 is a specific ligand for LFA-1 receptor.

Authors: Papers by Kupffer and colleagues, e.g., Kupffer *et al.* (1989), and by our group (Arancia *et al.*, 1985, 1989, 1991a, 1991b; Malorni *et al.*, 1987, 1989, 1991b; Sirianni *et al.*, 1988; Zarcone *et al.*, 1992a, 1992b) have tried to approach this question of "dynamic changes" occurring in two cells capable of binding themselves and interacting so closely.