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A SCANNING ELECTRON MICROSCOPY ANALYSIS OF HUMAN CYTOTOXIC CELL SUBSETS AND OF THEIR MODE OF CONJUGATION WITH TUMOR CELL TARGETS

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Abstract

In this study we describe the surface features of non-MHC (Major Histocompatibility Complex)-restricted cytotoxic cells isolated from human peripheral blood. Purified populations of $CD3^-$, natural killer (NK) cells were allowed to interact with NK-sensitive (K562) and NK-resistant (THP-1-0) tumor cell targets. The type of effector to target cell binding was investigated by scanning electron microscope (SEM) analysis. A different interaction with the effectors is described for NK-resistant targets in comparison with NK-susceptible tumor cells. SEM was also used to investigate the relationship between interleukin 2 (IL2)-activated cytotoxic cells (lymphokine-activated killer, LAK, cells) and the tumor targets. We also describe the unique growth features of certain clones of cytotoxic T cells expressing γδ antigen receptors which support the contention that these cells may have a special ability of homing into tissues. We conclude that non-specific cytotoxic cells constitute a diverse population of effectors which differ not only for the expression of surface antigens, but also for their ability to interact with tumor cell targets and to home into the peripheral tissues where they may exert their lytic functions.

Key Words: Non-specific cytotoxic effectors; Tumor target cells; Interleukin-2; γδ T cell receptors; Effector/target conjugates.

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Introduction

The elimination of cells infected by intracellular pathogens or undergoing neoplastic transformation is accomplished by the immune system through a variety of defense mechanisms which include soluble factors (i.e., complement components) and cell-mediated lysis (Trinchieri and Perussia, 1984; Podack, 1985). Cell-mediated cytotoxicity occurs via two distinct effector mechanisms: i.e., antigen-specific, MHC-restricted target cell lysis, a function of T cells which recognize antigens in the context of MHC class I molecules, and non-specific, non-MHC-restricted cytotoxicity. The latter cytolytic function is exerted by cells which belong to several lymphocyte subsets. However, the bulk of non specific cytolytic effectors is represented by non-T ($CD3^-$) cells which can kill targets both at the resting state (NK cells) and, more efficiently, following stimulation with Interleukin 2 (IL2) (Henney et al., 1981; Ferrarini and Grossi, 1984; Trinchieri et al., 1984). The latter cells are commonly referred to as lymphokine-activated killer (LAK) cells (Grimm et al., 1982). Certain T cell subsets may also lyse tumor targets efficiently, provided that they receive the appropriate activating signals. Among T cells, the cytotoxic cell precursors are predominantly lymphocytes with granular morphology (large granular lymphocytes, LGL) and they are comprised in the subset of cytotoxic cells which express the surface antigens CD3, CD8, CD11b and CD57 (Clement et al., 1984; Lanier and Loken, 1984). More recently, a novel subset of T cells has been described which rearrange gene sets different from those encoding for the classic αβ T cell receptor (TcR). These cells express a surface heterodimer consisting of γδ chains (Brenner et al., 1986; 1987; Borst et al., 1987; Moingeon et al., 1987; Lanier et al., 1987). γδ TcR^+ cells, displaying granular lymphocyte morphology, do not exert NK function but may respond to a variety of stimuli (including IL2) by acquiring non-specific cytotoxic activity (Ferrini et al., 1989).

Phenotypic and functional analyses of a

Table 1. SUSCEPTIBILITY OF THE LEUKEMIA-DERIVED CELL TARGETS, K562 AND THP-1-0, TO THE LYTIC EFFECT OF NK CELLS AND LAK CELLS[1]

Target cells	K562		THP-1-0	
Effector cells	NK	LAK	NK	LAK
E:T = 40:1	30.5 ± 6.2[2]	83.9 ± 8.1	1.9 ± 0.6	22.7 ± 12.4
E:T = 20:1	20.9 ± 4.4	73.5 ± 8.0	2.0 ± 1.3	14.8 ± 9.3
E:T = 10:1	14.6 ± 3.1	71.5 ± 5.2	1.8 ± 1.1	11.9 ± 7.1

1. The effector cells were freshly isolated PBL (NK assay) or PBL cultured for 3 days with human recombinant IL2 (LAK assay).
2. % specific ^{51}Cr release at the different Effector (E) to Target (T) cell ratios. Means ± SD from 4 to 12 distinct experiments.

number of tumor cell targets have shown that the resistance of some tumor cell lines to the lytic effect of non-specific cytotoxic cells is either due to ineffective binding (i.e., lack of conjugate formation between effector and target cells) or to defective activation of the effector cells bound to the target (Zarcone et al., 1987 a,b). These data suggest that, after interacting with each other, the target cells play an active role in delivering signals to the effector cells. Furthermore, morphologic observations of the effector-target cell interaction also suggest that the target cell plays an active role for its own killing (Arancia et al., 1989; Pitsillides et al., 1988).

Previous scanning electron microscope (SEM) studies have described ultrastructural changes during T lymphocyte-mediated cytolysis of tumor targets; a close relationship has been established between release of ^{51}Cr and target cell blebbing (Liepins et al., 1977; 1978). In this study, conjugates formed between NK cells and LAK cells with two different tumor targets, the erythroleukemic cell line K562 (NK-sensitive) and the THP-1-0 monocytic cell line (NK-resistant), were analyzed by SEM. Differences in the mode of interaction are described for the two target cells. In addition, we report certain unique growth features of clonal microcultures of γδ TcR^+ ytotoxic cells as detected by SEM analysis. Our observations indicate that a high degree of morphologic heterogeneity exists among non-specific cytotoxic cells.

Materials and Methods

Cytotoxic cell preparations

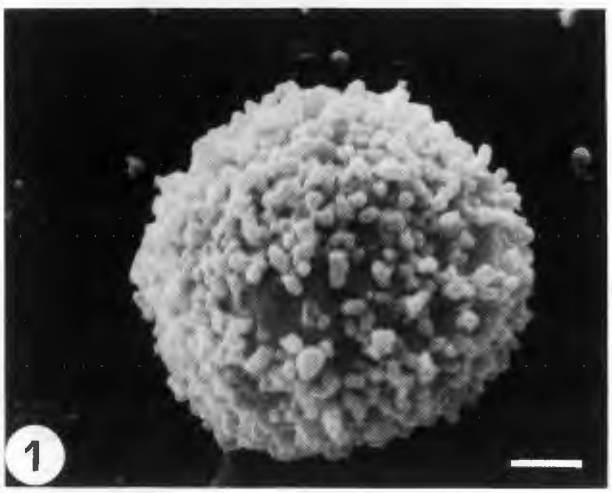
Human peripheral blood lymphocytes (PBL) were isolated from normal adult donors by Ficoll-Hypaque density gradient centrifugation, were depleted of monocytes as described (Zarcone et al., 1987 b), and further processed for cytotoxic cell enrichment. NK cell-enriched fractions were obtained by methods previously described, including E-rosetting, antibody plus complement depletion, and Percoll discontinuous gradients (Timonen et al., 1981; Zarcone et al., 1987 b). The purity of the cell populations was evaluated by phenotypic analyses of surface antigen expression (CD3, CD16, CD56), using the monoclonal antibodies Leu 4, Leu 11b and Leu 19 (Becton Dickinson, Mountain View, CA) followed by affinity-purified goat anti-mouse Ig antibodies labeled with FITC (Fluorescein isothiocyanate)(Southern Biotechnology Associates, Birmingham, AL).

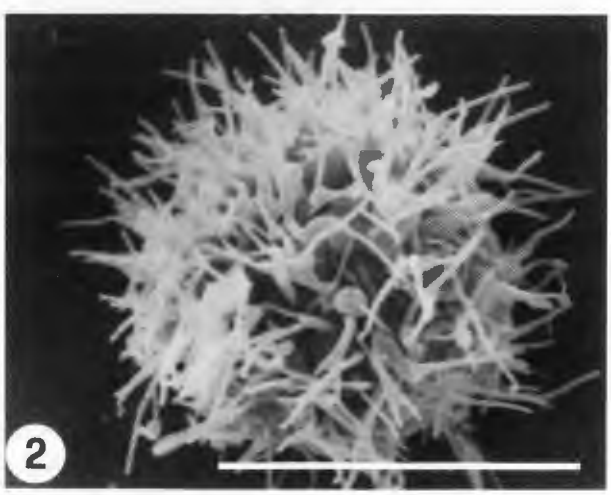
For the preparation of LAK cells, PBL fractions were cultured for 3 days at 10^5 cells/ml in RPMI 1640 supplemented with 2 mM glutamine and 10% heat-inactivated fetal calf serum (FCS). Parallel cultures in the same media were supplemented with 100 IU/ml human recombinant IL2 (Cetus Corporation, Emeryville, CA). At the end of the culture period, cell preparations were enriched for $CD3^-$ lymphocytes by incubation with anti-CD3 antibody and rabbit complement (Cedarlane, Rockville, MD).

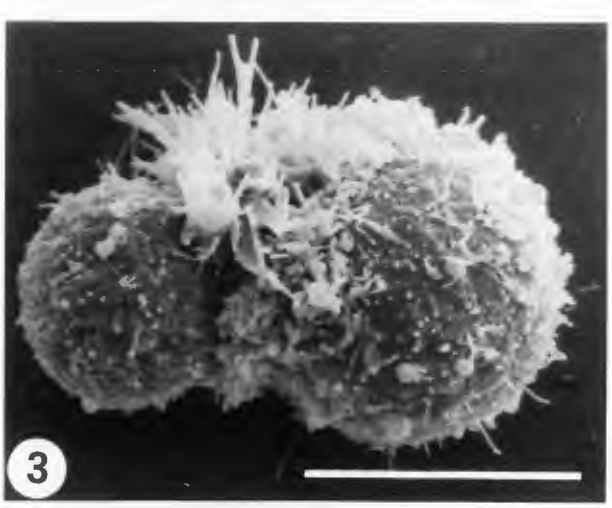
For the isolation of γδ TcR^+ cells we used a method described elsewhere (Ferrini et al., 1989). Briefly, PBL were allowed to form rosettes with sheep erythrocytes and E-rosetting cells were isolated and incubated with a mixture of anti-CD4 and anti-CD8 monoclonal antibodies, followed by treatment with rabbit complement. These cell preparations were subsequently cloned using the limiting dilution method (from 10 to 0.5 cells/well). Clones were expanded in the presence of IL2 and their phenotype was assessed using two monoclonal antibodies, BB3 and A13, which recognize two distinct, non-overlapping, subsets of γδ TcR^+ cells (Ciccone et al., 1988; Bottino et al., 1988).

Target cell lines

Two human tumor cell lines were used as targets for cytotoxic cells. The K562 erythroleukemic cell line is a standard target highly susceptible to NK cells and LAK cells. The other target was the monocytic cell line THP-1-0 which has been described previously (Zarcone et al., 1987 a). THP-1-0 cells bind to NK cells without subsequent target cell lysis and display low susceptibility to the







lytic effect of LAK cells. This resistance of THP-1-0 cells to non-specific cytotoxic effectors is due to a lack of effector cell activation by the target (Zarcone et al., 1987 a). Both cell lines were maintained in standard culture conditions (RPMI-FCS) and were used when in active growth phase.

Conjugate formation and SEM analysis

The ability of cytotoxic cells to interact with the targets was tested using a previously described binding assay (Velardi et al., 1985). Effector (E) and target (T) cells were admixed at 5:1 = E:T ratios, centrifuged at 200 g for 7 min and incubated for 10 min at 37° C as pellets. For SEM analyses, cells were resuspended and seeded on polylysine-coated coverslips. Following adhesion to glass surface, cells were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 20 min, postfixed 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 (15 kV).

Cytotoxic assays

The cytotoxic activity of freshly isolated PBL and of PBL cultured with IL2 was evaluated against K562 and THP-1-0 cells using a 4 h ^{51}Cr-release assay at E:T ratios ranging from 5:1 to 40:1. ^{51}Cr-labelled target cells (5×10^5) were added to various numbers of effector cells in a final culture volume of 200 µl/well using U-bottom 96 well microtiter plates. After 4 h culture, 100 µl of supernatant were removed from each well and counted in a gamma counter. The percent specific ^{51}Cr release was determined as previously described (Zarcone et al., 1987 a).

Results

Susceptibility of K562 and THP-1-0 cells to NK cell- and LAK cell-mediated lysis

Data in Table 1 show that consistent differences in the susceptibility to the lytic effect of NK cells and LAK cells exist between

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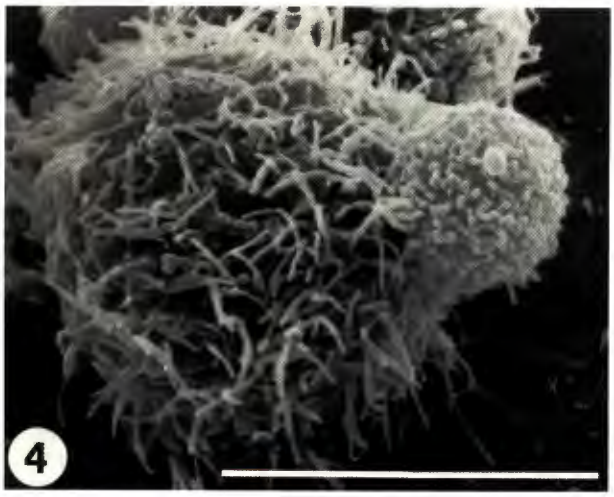
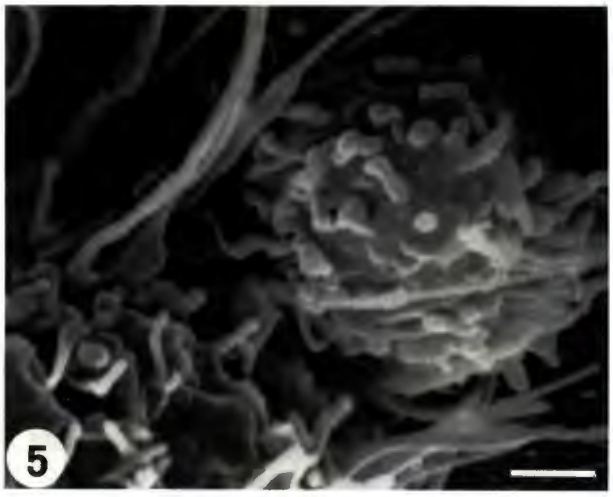
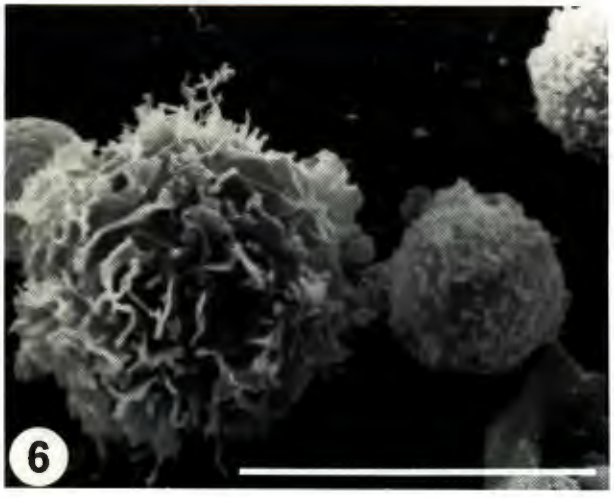
Fig. 1. Surface morphology of an NK cell from human peripheral blood. Numerous short microvilli are seen over the entire cell surface. Bar=1µm.

Fig. 2. K562 target cell with long protrusions randomly distributed over the cell surface. Bar=10µm.

Fig. 3. A conjugate formed between an NK cell and a K562 target cell. Surface structures are rearranged on both partners. The effector cell (to the left) shows a smoother surface as compared to the unconjugated cell shown in Fig. 1. The target cell (to the right) exhibits long microvilli, small blebs and ruffled membranes oriented towards the effector cell. Bar=10µm.







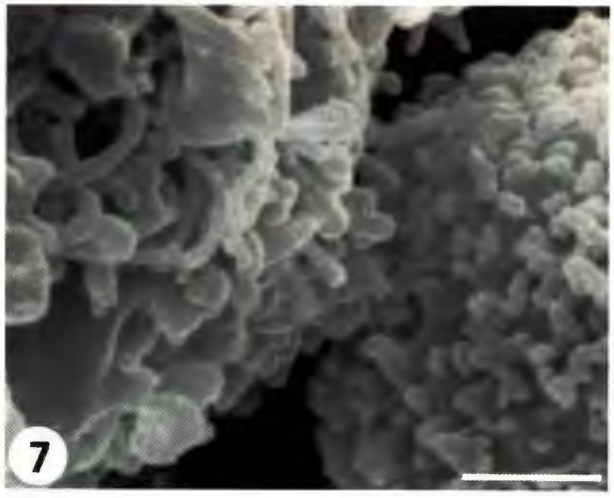


Fig. 4. Lymphokine activated killer (LAK) cell forming a conjugate with a K562 target cell. In the early phase of conjugation both the target (to the left) and the effector (to the right) do not show significant morphologic changes. However, in the contact region, the two cells show a very close interaction. Bar=10μm.

Fig. 5. Higher magnification of the contact region in a LAK-K562 conjugate. The target cell (to the left) wraps the effector cell with well developed cellular protrusions. Bar=10μm.

Fig. 6. LAK cell conjugated with a THP-1-0 target cell. The effector cell (to the right) binds to the target (to the left); the interaction occurs to a very limited extent. Bar=10μm.

Fig. 7. A higher magnification of the contact region in an LAK-THP-1-0 conjugate. The tightly intertwined binding observed in both NK-K562 (Fig. 3) and LAK-K562 (Fig. 5) conjugates is not detectable in conjugates formed between activated effectors and relatively resistant targets. Bar=1μm.

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the two cell targets used in this study. K562 cells are lysed efficiently by NK cells and more so by LAK cells. In contrast, THP-1-0 cells are resistant to NK-mediated lysis and are killed to some extent only by LAK cells.

Phenotypic analysis of cell preparations enriched for cytotoxic effectors

Freshly isolated PBL were enriched for NK cells by fractionation on discontinuous Percoll gradients or by antibody plus complement depletion of non-relevant cell subsets (Timonen et al., 1981; Ferrini et al., 1989). The degree of purity so obtained was determined by immunofluorescence staining with monoclonal antibodies anti-CD3 and anti-CD16 or anti-CD56. Preparations obtained by fractionation on discontinuous Percoll gradients contained 65% to 85% $CD3^-$-$CD16^+$-$CD56^+$ cells, whereas the range of positivity for the

above phenotype was detected in 55% to 78% of the cells in antibody plus complement depleted preparations.

PBL cultured with IL2 for the generation of LAK cells were similarly treated with anti-CD3 antibody plus complement, to enrich for $CD3^-$-derived LAK cells. Less than 10% of residual $CD3^+$ cells were detected following treatment of the LAK cell preparations with CD3 plus complement. The purity of $\gamma\delta$ TcR^+ cells was determined, prior to cell cloning, by phenotypic analyses using mixtures of A13 and BB3 monoclonal antibodies. Cell preparations with <65% $\gamma\delta$ TcR^+ cells were not considered for cloning experiments.

SEM analysis of the interaction between non-specific cytotoxic effectors and tumor cell targets

The surface morphology of the effector cells (NK and LAK cells) and of the target cells is shown in Figs. 1-7. Freshly isolated NK cells are round in shape and display short microvilli evenly distributed on their surface (Fig. 1). LAK cells show similar surface features (Figs. 4-7). The surface morphology of tumor cell targets is shown in Figs. 2 and 4 for K562 cells, and in Fig. 6 for THP-1-0 cells. K562 cells exhibit numerous surface projections consisting of long and thin microvilli (Fig. 2), whereas ruffled membranes are predominant on the surface of THP-1-0 cells, an expected morphologic feature for cells of monocytic lineage (Fig. 6).

SEM analysis of conjugates formed between cytotoxic effector cells and tumor cell targets

The aim of these studies was to investigate changes in the surface morphology which occur in the early phase of conjugate formation. Thus, morphologic changes were analyzed within the first hour of target to effector cell interaction. As depicted in Fig. 3, a redistribution of surface cell protrusions occurs in both the target and the effector cell, shortly after conjugate formation. A definite polarization of microvilli takes place in the area where a large cell to cell contact is established (Fig. 3). These morphologic changes determine a broader contact which forms a "close chamber" between the target and the effector cell. This type of interaction has been observed for both NK and LAK effectors when they encounter the susceptible tumor target, K562. When conjugate formation between NK cells and LAK cells with THP-1-0 cells was analyzed by SEM, the morphologic features of the interaction were completely different; namely, no redistribution of surface microvilli occurred in both cells. In addition, the area of contact was always quite narrow. These target-related differences are shown in Figs. 6 and 7 (LAK cells forming conjugates with THP-1-0 cells) as compared to Figs. 4 and 5 (conjugates between LAK cells and K562 targets).

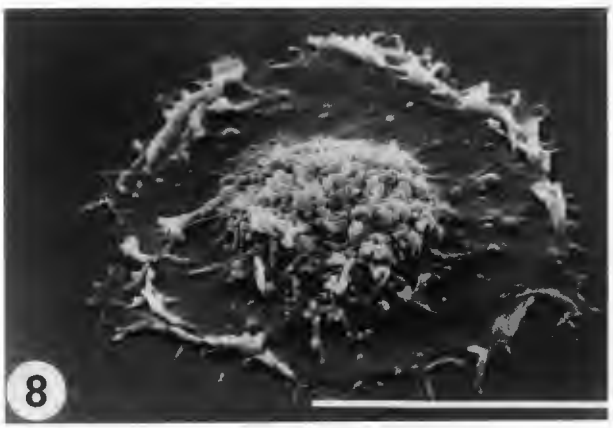
SEM analysis of cytotoxic cell clones expressing the $\gamma\delta$ TcR

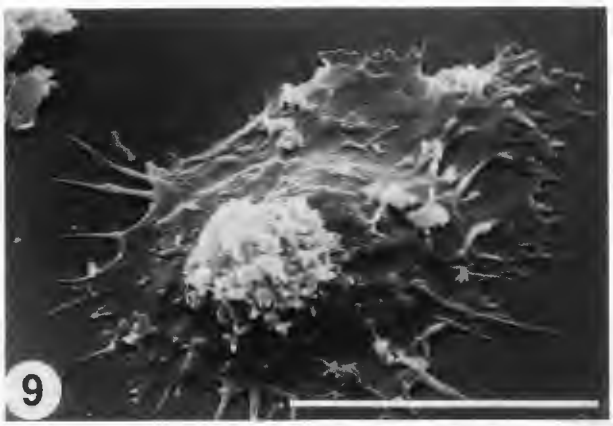
Cloning of purified $\gamma\delta$ TcR^+ cells from human peripheral blood showed that certain $\gamma\delta$ TcR^+ clones expressing the C γ 2-encoded $\gamma\delta$ TcR (i.e., A13/ δ $TCS1^+$ cells) had unique growth features. Cells adhered promptly to the substrate and displayed the sequence of morphologic changes shown in Figs. 8-13. These changes can be summarized as follows: a) cells adhere to surface and display peripheral ruffles similar to those seen in all types of adherent cells (Fig. 8); b) next, cells form a distinct uropod and emit numerous microspikes (Figs. 9, 10); c) in the final phase of adherence, cells display two polar filopodia ending with adhesion plaques, as shown in Figs. 11-13. These in vitro growth features have been so far observed only for a minority of $\gamma\delta$ TcR^+ clones, and are distinctly different from those of the majority of T cell clones which grow as non-adherent cell clusters (Grossi et al., 1983).

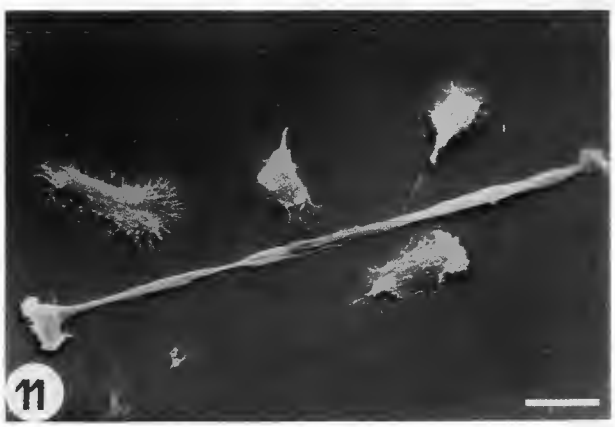
Discussion

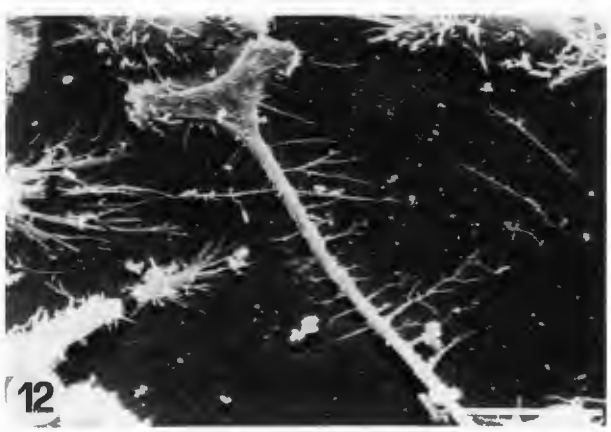
This study describes the surface features detected by SEM analysis of a variety of non-specific cytotoxic effector cells such as freshly isolated NK cells, IL2-cultured LAK cells (largely derived from NK cells), and $\gamma\delta$ TcR^+ T cells which may function as non-specific cytotoxic effectors and respond to IL2 stimulation by increasing their cytotoxic potential (Ferrini et al., 1989).

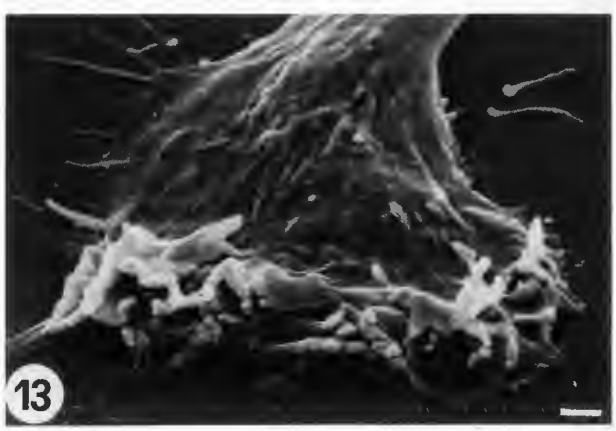
A sequence of surface morphologic changes occurs following interaction between the effector and the target cell, and these changes are related to the susceptibility or resistance of the target to the lytic function of the effector. When the interaction between a resting or an activated effector cell with a highly susceptible target is analyzed, a broad cell to cell contact is established, and a redistribution of surface structures in both components of the cell pair is observed. These changes suggest that a reciprocal signalling occurs. It is conceivable that the susceptibility of the cell target is not related simply to its ability to deliver activation signals. It appears, rather, that the target may play an active role in the enhancement of the lytic effect by establishing the appropriate microenvironment where cytotoxic factors are released by the effector. This contention is further supported by the observation that tumor cell targets which are resistant to NK cells and poorly susceptible to LAK cells do not undergo redistribution of their surface structures and, albeit being able to bind cytotoxic cells, they form cell to cell contacts of very limited extension. In view of the necessity of establishing complementary receptor to ligand interactions between molecules expressed on the surface of the effector and of the target cell, an extensive cell to cell contact should allow the











Figs. 8-13. A sequence of morphologic changes detected by SEM analysis of growing clonal microcultures of γδ TcR^+ cells.
Fig. 8. Round cell with a central nucleus and several membrane ruffles. Bar=10μm.
Fig. 9. Adherent cell with polar nucleus. Numerous microvilli are found in the nuclear region. Bar=10μm.

Figs. 10-11. Uropodia and filopodia are often observed. These structures do not bear microvilli on their surface. Bars=10μm.
Figs. 12-13. At higher magnification, filopodia and lamellipodia show lateral branches (Fig. 12) and ruffling membranes (Fig. 13). Bar=10μm in Fig. 12; Bar 1μm in Fig. 13.

delivery of a more efficient activation signal.

Previous studies have shown that a subset of T cells express a surface γδ heterodimer which functions as the receptor for antigen (see Refs. in the Introduction). Molecular genetic and phenotypic analyses indicate that several forms of γδ TcR's exist which differ for the expression of C γ 1 or C γ 2 gene sets. Monoclonal antibodies are available which identify a subset of cells expressing a disulphide-linked form of the receptor (encoded by C γ 1 genes and recognized by BB3 monoclonal antibodies) and another subset of γδ TcR^+ cells which express a non-disulphide linked γδ TcR (encoded by C γ 2 genes and recognized by A13/ δ TCS1 monoclonal antibodies).

In this study we describe the growth features of certain γδ TcR^+ clones which exert non-specific cytotoxic functions. These clones are distinctly different from the majority of the T cell clones growing as microcultures in that the cells adhere promptly to the substratum, form uropodia and assume a neuron-like shape, with the emission of polar filopodia ending with adhesion plaques. In view of the cytotoxic activity of this cell subset, it is tempting to speculate that lymphocytes with these properties may have special homing abilities, namely that of seeding into the epithelia where they may exert immune surveillance functions against infected or transformed epithelial cells (Grossi et al., 1989).

Altogether, morphological analyses suggest that the polymorphism shown here for a variety of cytotoxic cells investigated both at the resting and at the activated state may subserve different functions which can be related to the properties of the cell target encountered by the different effectors, and also to the tissue environment where the cytolytic functions are exerted.

Acknowledgements

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Discussion with Reviewers

E. De Harven: If this reviewer understands the way "conjugates" were prepared, E and T cells interacted within a cell pellet for 10 minutes? You should explain a) why you decided not to allow the cells to interact in a mixed cell suspension, and b) why you didn't perform a time-sequence study at 10, 20, 30, 60 min, etc?

Authors: The conjugate formation procedure used in this study is fairly standardized and derives from previous experience on single cell cytotoxicity assays in agar. More specifically, E/T interaction in cell suspension does not allow optimal conjugate formation due to conjugate splitting during the effector cell activation phase that follows conjugate formation. This explains the need to have the cells immobilized such as it occurs in agar or in a cell pellet.

A time sequence study was not performed because our aim was that of analyzing the mode of conjugate formation between effector cells and a variety of tumor targets. Conjugate formation takes place in seconds following E/T interaction and an increase of the incubation period leads to damage of the target cells due to triggering of the cytolytic machinery which occurs in minutes.

E. De Harven: You seeded the cells, after they had a chance to interact for 10 minutes in a cell pellet, on poly-L-lysine coated glass coverslips. Did you also fix them in suspension, i.e., prior to attachment to the coverslip, in order to evaluate how much surface morphology has been influenced by attachment to a positively charged surface?

Authors: In the present experiments we have used the technique described by M.A.Hayat ("Substrates for particulate specimens". In: Introduction to biological scanning electron microscopy. pp. 213-218; University Park Press, London, 1978). Conjugates were also fixed prior to their seeding onto coverslips and the morphological changes occurring at the effector/target interface were identical to those observed in cell pairs fixed after attachment to glass surface. Similar results were also obtained when collagen-coated coverslips were used.

V.P.Collins: In brief, what are the transmission electron microscopic findings at the cytotoxic cell-tumor target cell interface?

Authors: TEM studies have demonstrated that several changes occur in the effector cell following its binding to the tumor cell target. Uropod formation and redistribution of the Golgi apparatus and of the cytoskeletal structures are the major events. At the interface between the target and the effector cell, long microvilli are seen which appear to increase the cell to cell surface contact. Apparently, some sort of close chamber is formed where the granule content of the effector cell appears to be released.

V.P.Collins: In what way do you think these findings help us understand cytotoxicity at the molecular level?

Authors: Our bias is that analysis of cell to cell contact in this experimental model is relevant because the physical interaction is preliminary to the interaction between several complementary molecules expressed on the surface of the effectors and of the targets (e.g. CD2/LFA3, LFA1/ICAM1). Some of these molecules (e.g. CD2), upon interaction with their ligand may trigger pathways of effector cell acti-

vation.

Reviewer III: The results of this study rely heavily on subjective interpretation of surface morphology. How could you be affirmative as to the SEM type of cells described as NK or LAK cells if there were only 50-60% of such cells in a preparation?
Authors: Percentages of $CD3^-$-$CD16^+$-$CD56^+$ cells in each NK cell preparation are now reported in detail, as well as percentages of $CD3^-$ cells in LAK cell preparations treated with CD3 antibody and complement. In most cases, the cell purity largely exceeded 50-60%, being even over 90%. Changes of surface morphology described here are representative of observations of greater than 80% of the conjugates analyzed by SEM. We believe that this indicates that we have indeed described phenomena mediated by $CD3^-$ effector cells.

Reviewer III: The pictures show one effector cell conjugated to a target cell. What was the actual number of effector cells seen conjugated to each target? How could you distinguish under the SEM between cells forming specific conjugates with the targets and cells which are non-specifically adhering to these cells?
Authors: The average conjugate consisted of one effector cell bound to one target. Occasionally, we observed two-three effectors bound to one target in our experimental conditions. Since we did not label the effector cells, our criteria for cell identification were solely based on the purity of the cell preparations.

P.M. Grimley: In the Results section you state that cell preparations with greater than 50% of cells bearing specified phenotypic markers were considered "appropriate" for conjugation experiments with tumor cell targets. In the case of quantitative assays for target cell lysis, this may be statistically justified; but how well does this apply to cytologic analyses where the sampling is more limited? Obviously, the specific fields illustrated can only be representative, so correct identification might be no more than 50% based upon binomial theory. Can you provide any quantitative data to support the cytologic impressions: for example, number of cells examined and proportions showing specific surface features?
Authors: As stated above, percentages of $CD3^-$ cells are now indicated for all of the preparations used for conjugation experiments. More than 200 conjugates were analyzed by SEM for each experiment and over 80% of the cell to cell contacts exhibited the features shown in the illustrations, namely membrane ruffling at the interface. Occasional contacts of limited extension, without ruffling of the target and of the effector cell membranes, were also observed. These may correspond to conjugates formed between the target and lymphocytes of lineage other than NK.

P.M. Grimley: Several previous studies by Kang and colleagues (Anat. Rec. 217:274-289 and 290-304,1987) as well as a paper in Scanning Microscopy (2:1567-1586, 1988) demonstrated the feasibility of labelling cytotoxic cells with immunogold or carboxylate bead techniques. Was this approach considered for ascertaining cell identities in present studies?
Authors: We considered the approach suggested by the Reviewer. However, we decided not to pursue it because a preliminary labelling of the effector cells could either decrease their binding ability (e.g. anti-CD56) or activate them (e.g. anti-CD2 or anti-CD16).