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Scanning electron microscopic study of natural killer cell-mediated cytotoxicity

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Summary. The interaction between human natural killer (NK) cells and NK-susceptible target cells, as well as the mechanism involved in target cell lysis, were studied with scanning electron microscopy (SEM). Low density human peripheral blood lymphocytes, highly enriched with large granular lymphocytes (LGL), were used as effector cells, and K562-cells were used as NK-susceptible target cells. The surface features of LGL/NK cells were examined under SEM. In the area of interaction, NK/target-cell conjugates showed microvilli and/or filipodia, and extensive areas of intercellular contact. In addition, the effector cells in some NK/target-cell conjugates were polarized toward the target cell. Changes in target cell surface features included loss of microvilli, large surface blebs and the appearance of small pore-like lesions on the cell membrane. Our findings show that target cell lysis occurred by apoptosis and plasma membrane lesions analogous to those seen during complement-mediated cytotoxicity.

Key words: NK-cell-mediated cytolysis, Cell conjugate, Surface feature, Scanning electron microscopy

Introduction

Recent studies have suggested the existence of three types of human cytotoxic lymphocytes: 1) antigen-specific, major histocompatibility complex (MHC)-restricted cytotoxic T-lymphocytes (CTL), 2) MHC-unrestricted CTL, and 3) natural killer (NK) cells (Lanier et al., 1986; Lanier and Phillips, 1988). Human NK-cells were initially characterized by their ability to lyse a large variety of target cells without previous sensitization or MHC restriction (Trinchieri and Perussia, 1984). Human NK-cells are currently considered a subpopulation of peripheral blood leukocytes characterized morphologically as large granular lymphocytes (LGL). Phenotypically, these cells express the leukocytic differentiation antigen CD16 in the absence of a rearrangement of T-receptor genes, and do not express the CD3/Ti surface complex (Perussia and Trinchieri, 1988).

The mechanism of lymphocyte-mediated cytotoxicity has been investigated by many workers in recent years (Henkart, 1985; Podack, 1986; Young and Cohn, 1986; Brunet et al., 1987; Berke, 1988; Henkart and Yue, 1988; Meuer and Dienes, 1989). A number of authors have attempted to analyze the ultrastructural characteristics of cytotoxic lymphocyte-mediated cell lysis (Liepins et al., 1977; Sanderson and Glauert, 1977, 1979; Roder et al., 1978; Matter, 1979; Sanderson, 1981; Russell et al., 1982; Foa et al., 1988), and have shown lysis of the target cell to take place through two different pathogenetic mechanisms; necrosis and apoptosis (Wyllie et al., 1980), each of which is correlated with different pathogenetic processes (Searle et al., 1982; Stacey et al., 1985; Bishop et al., 1987). However, little is known of the morphological, immunological and molecular phenomena (Heberman and Callewaert, 1985) related to target cell lysis by NK-cells. This morphological SEM analysis of the interaction between isolated human NK-cells and susceptible K562 target cells was designed to establish not only the morphological mechanisms of cell lysis but also to determine the possible analogies between this process and lysis by CTL on the one hand, and complement-mediated lysis on the other (Dourmashkin et al., 1980; Russell et al., 1980; Dennert and Podack, 1983; Podack, 1986; Engelhard et al., 1988; Podack et al., 1988).

Materials and methods

Isolation of effector cells

Human peripheral blood mononuclear cells (PBMC) were isolated from whole blood obtained by venipuncture

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from healthy donors according to Boyum (1986). The blood was diluted to 1:2 (v/v) in Hank's balanced salt solution (HBSS, Gibco Laboratories, UK) and centrifuged on gradients of Ficoll-Paque (d: 1077 g/ml) (Pharmacia Fine Chemicals, Sweden). The PBMC were resuspended in HBSS at a concentration of 3.5 x 10^6 cells/ml, and adherent cells were depleted by incubating the suspension in tissue culture flasks (Nunclon, Nunc, Denmark) for 1 h at 37° C. The NK-cells were isolated with the procedure of Timonen and Saksela (1980). PBMC depleted of adherent cells (peripheral blood lymphocytes, PBL) were centrifuged on discontinuous Percoll density gradients (Pharmacia Fine Chemicals, Sweden) using 100% Percoll diluted to 42.5%, 45%, 47.5%, 50% and 55%, which provided a range of densities from 1.053 to 1.077 g/ml. The PBL were placed on the least dense fraction and centrifuged at 400 g for 30 min at room temperature. After removing the six strata, cells from each fraction were collected, washed and processed for light microscopy, conjugation assays and SEM. Effector cells were selected to correspond to the low density gradients (1.060-1.063 g/ml).

Target cells

Cells of the K562 line were used as target cells. This line is derived from an erythroleukemia in blastic crisis, grown in RMPI 1640 medium (Flow Laboratories, U.K.) supplemented with 10% fetal bovine serum, 0.29 g/ml L-glutamine, 100 IU/ml penicillin and 10 μg/ml streptomycin.

Conjugation assay

1 x 10^6 effector cells were conjugated with 0.5 x 10^6 target cells (2:1 ratio) in a final volume of 1 ml. The suspension was centrifuged for 5 min at room temperature and 500 rpm, and the resulting pellet was disaggregated by repeated aspirations through a pasteur pipette. The suspension was incubated for 30 min and 60 min at 37° C with 5% pCO_2.

Light microscopy

Smears of effector cells and cell conjugates resuspended at 2.5 x 10^5 cells/ml were prepared in a cytocentrifuge (Cytospin, Shandon, U.K.) at 1000 rpm for 10 min. The slides were air-dried, fixed with methanol and stained with 10% Giemsa solution at pH 7.2. The different elements in each density layer were quantified.

Scanning electron microscopy

Isolated effector cells and effector/target cell conjugates were fixed overnight with 2.5% glutaraldehyde in 0.1M phosphate buffer, pH 7.2, at 4° C, then dropped onto poly-L-lysine (Sigma, USA)-coated coverslips. The cells were dehydrated in a rising series of acetones, critical-point dried and gold sputter-coated. The preparations were observed with a Philips 505 scanning electron microscope.

Results

Light microscope observations of effector cells from the 1.060-1.063 g/ml density gradient showed at least 60% LGL/NK elements. The same cells under SEM presented surface features characterized by microvilli and microridges (Fig. 1). Cells of the K562 NK-susceptible line had a mean diameter of 12 μm, a high nucleus/cytoplasm (N/C) ratio and a surface characterized by microvilli, filipodia and a few ridges (Fig. 2a).

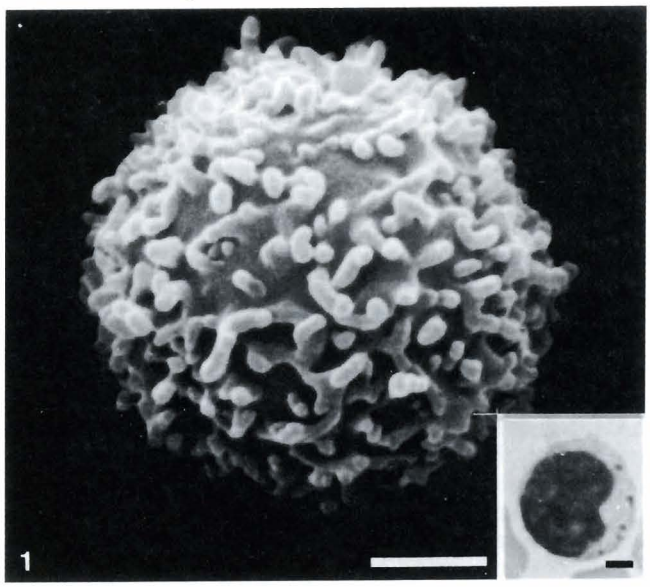

Fig. 1. Human NK/LGL-cell obtained by centrifugation on discontinuous Percoll gradients, showing the surface morphology of an NK/LGL-cell with microvilli and microridges. Scale bar = 1μm. Inset: Typical morphological features of a large granular lymphocyte: low nucleus/cytoplasm ratio, indented nucleus, azurophilic granules. Scale bar = 1 μm.

Effector/target cell conjugates were present after 30 minutes of incubation at 37° C. Most of the conjugates in our material consisted of a single effector cell with a single target cell, although occasionally several effector cells were bound to a single target cell. The effector cells in interaction with K562-cells exhibited the characteristic morphological features described above (Fig. 2a).

The zone of interaction was characterized, in early phases, by elongated, microvilli-like and/or filipodia-like cellular expansions (Fig. 2b) in the form of processes extending from the NK-cell to the target cell as well as from the target cell to the NK-cell. After 60 minutes of incubation at 37° C, the zone of interaction was larger (Fig. 3), and in many cases, the NK-cell was bound to the target cell by a bundle of elongated filipodia- and microvilli-like projections arising from one pole of the NK-cell. The surface of the target cell showed a pocket-like depression through which filipodia and microvilli from the effector cell appeared to enter (Fig. 4).

After this second phase of interaction the target cells

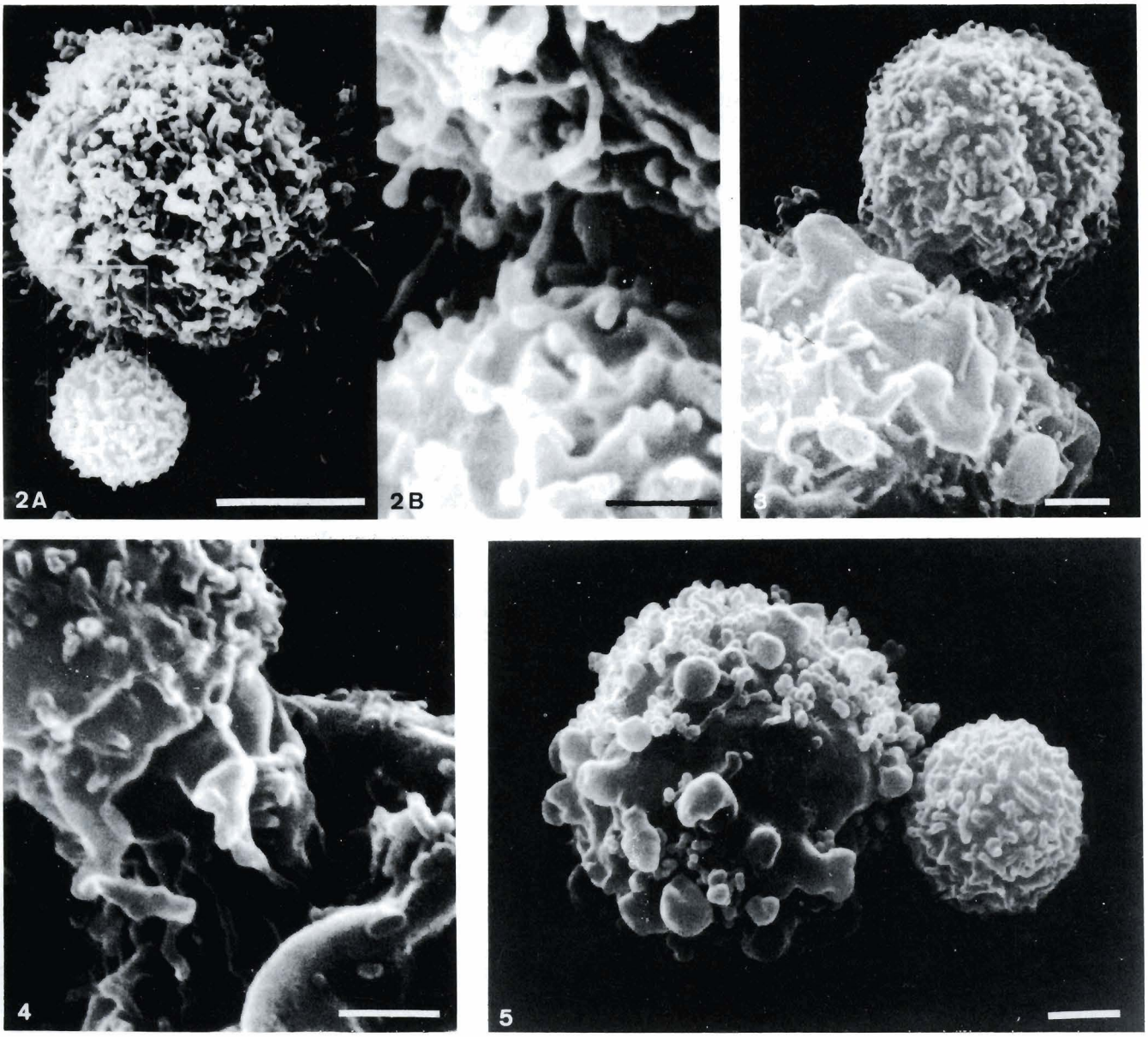

Fig. 2. Scanning electron microscope view of an NK/LGL- cell K562-cell conjugate after a 30 min incubation. **A.** SEM surface morphological features of the K562 target cell, showing numerous elongated microvilli. Scale bar = 5 μm. **B.** The same effector-target cell conjugate at higher magnification. The NK/LGL-cell is bound to the K562 target cell by microvilli-like projections. Scale bar = 2 μm.

Fig. 3. Extensive area of interaction between the NK/LGL effector cell and the K562 target cell after a 30 min incubation. Scale bar = 1 μm.

Fig. 4. NK/LGL effector cell coupled to a K562 target cell, after a 30 min incubation. The effector cell is polarized toward the latter, and the zone of interaction is characterized by long, compact protrusions which deform the surface of the target cell. Scale bar = 1 μm.

Fig. 5. NK/LGL-mediated cell cytolysis after a 60 min incubation. The K562 target cell shows characteristic changes on the surface membrane, including blebs and numerous pore-like lesions. Scale bar = 2 μm.

showed changes in the pattern of surface features. Under SEM, loss of microvilli was seen, together with large surface blebs and the appearance of numerous pore-like surface lesions. In this final stage the adhesive filipodia of the NK-cell were not clearly developed (Fig. 5).

Discussion

Human LGL-cells responsible for NK activity were isolated by density gradient fractionation in order to study their surface morphology and interaction/conjugation with NK-susceptible K562 target cells. Scanning electron microscopy was also used to investigate the pathogenetic mechanism leading to cell lysis by NK-cells.

As effector cells we used populations of low density peripheral blood lymphocytes enriched in LGL; the cell

responsible for human NK-cell activity (Timonen and Saksela, 1980; Trinchieri and Perussia, 1984). The density gradients used were those which have previously demonstrated the highest rates of NK activity (Phillips et al., 1983, 1984). By contrast, different SEM patterns have been described for NK-effector cells. Frey et al. (1982) observed large murine NK-cells of variable morphology, which bore microvilli on localized sites on the cell surface. Carpen et al. (1982) described isolated human NK-cells as presenting an intermediate microvillous surface similar to that seen on the lymphocytes remaining in the dense fractions, and clearly different from the surface features of monocytes and granulocytes, which showed ruffles and ridges respectively. Payne et al. (1985), who studied the surface features of human Fc-positive/LGL, alone and conjugated with K562 cells, noted a complex microvillous surface pattern of microvilli and microridges. Our observations are closer to those of this author.

Morphologically, two phases can be distinguished in the process of human NK-cell mediated cellular lysis. During the first phase recognition and binding take place, while the second phase involves post-binding events which lead to lysis of the target cell (Ortaldo et al., 1988). The first phase is a prerequisite for lysis mediated by immune cells, i.e. CTL, antibody-dependent cytotoxic cells (ADCC), lymphokine-activated cells (LAK) and macrophages (Carpen et al., 1982; Foa et al., 1988; Hook et al., 1988). The receptor responsible for the union between the NK effector and the target cell is still unknown; in contrast, CTLs are known to bind to target cells by a highly specific interaction involving the T-cell receptor (TCR) and MHC molecules (Meuer and Dienes, 1989). Human NK-cells are unable to recognize target cells through the TCR, nor does their mechanism of recognition seem to be mediated by MHC antigens (Perussia and Trinchieri, 1988). Different surface antigens and receptors (including CD2, CD16, LFA1 and transferrin receptor) may play a role in NK-cell-mediated recognition, although none of these molecules has been exclusively associated with this cell population (Ortaldo et al., 1988; Meuer and Dienes, 1988).

Different transmission electron microscope studies have shown that the physical process of interaction between effector and target cells occurs via active intercellular contacts consisting of cytoplasmic expansions (microvilli or filipodia-like formations), or through the formation of extensive zones of surface contact between the two cells (Sanderson and Glauert, 1979; Carpen et al., 1982; Hiserodt and Beals, 1985; Foa et al., 1988). Our SEM findings confirm the existence of filipodia- and microvilli-like membrane specializations in the zone of interaction, stretching in both directions between the effector and target cells. Extensive zones of contact were also seen with SEM. These membrane specializations represent a dynamic but relatively stable connection which may be involved in the initial phase of immune recognition (Roder et al., 1988). Foa et al. (1988) have demonstrated the exchange of membrane molecules to be linked to cellular recognition and interaction between the conjugated cells.

During the first phase of intercellular contact, the NK-cell is activated and programmed for the lethal hit which in turn irreversibly programs the target cell for cell death (Hiserodt et al., 1983a,b). Several authors, using light microscopy and TEM, have noted that this initial phase is characterized by the reorientation of the microtubule organizing centre, the Golgi apparatus and the electron dense granules toward the site of contact with the target cell (Carpen et al., 1981, 1982, 1983; Dennert, 1985). Our SEM observations showed polarized effector cells sending out complex expansions consisting of highly compact microvilli similar to those described by Hiserdot and Beals (1985). As noted by Heiskala et al. (1988), cellular polarization, including, among other phenomena, the appearance of a bundle of elongated microvilli-like projections, indicates that the activation and programming of lysis by NK-cells takes place. It therefore seems clear that there is, on a morphological level, a basic mechanism of interaction shared by different cytotoxic elements (CTL, NK, ADCC, LAK) and the target cell regardless of the molecular mechanism of recognition (Sanderson and Glauert, 1979; Carpen et al., 1982; Foa et al., 1988; Hook et al., 1988).

In the second phase of NK-cell-mediated cytolysis, secretion has been shown to occur duing lysis mediated by CTL-, K-, NK- and LAK-cells (Carpen et al., 1982; Neighbour et al., 1982; Berke, 1988; Henkart and Yue, 1988). The present SEM observations demonstrate pocket-like depressions on the surface of the target cell which match the polarized surface of the NK-cell. We assume that these are the sites where the effector cell secretes lytic factors and brings about the initial changes in the target cell membrane.

The mechanism responsible for the death of the target cell however has not yet been fully elucidated (Meuer and Dienes, 1989). Initially, a model similar to complement-mediated cytotoxicity was proposed. Complement-mediated cytotoxicity has been associated with the presence of pores surrounded by a superficial collarette on the membrane (Humphrey and Dourmashkin, 1969; Podack and Dennert, 1983). Similar observations were published by Dourmashkin et al. (1980), who studied erythrocytes lysed by human LGL in ADCC, cloned CTL- and cloned NK-cells. These results suggest a common mechanism shared by the complement and the lytic factors of the cytotoxic elements (Young and Cohn, 1986; Zalman et al., 1986). From a morphological point of view, complement-mediated cell death-necrosis is characterized by swelling of the cytoplasm and nucleus, disorganization of the organelles and cell membrane rupture (Bishop et al., 1987).

On the other hand, different authors have proven that cytotoxic cell mediated lysis is the result of a mechanism different from complement mediated lysis. In the former, target cell DNA is fragmented and broken down after interaction with the effector cell, and the target cell's nuclear membrane is damaged (Russell et al., 1980, 1982; Howell and Martz, 1988; Podack et al., 1988). How

the DNA becomes damaged following interaction has not been clearly established (Brahmi et al., 1989). Some studies with TEM have shown that DNA breakdown is associated with changes at the submicroscopic level (Russell et al., 1982); the term apoptosis has been used for such ultrastructural changes (Wyllie et al., 1980; Searle et al., 1982; Bishop and Whiting, 1984; Stacey et al., 1985; Bishop et al., 1987). Our findings demonstrate the appearance on the target cell surface of pore-like lesions similar to those described in association with complement-mediated cytotoxicity (Humphrey and Dourmashkin, 1969), and cell-mediated cytotoxicity (Dennert and Podack, 1983; Hiserodt and Beals, 1985). A progressively smoother cell surface and the appearance of blebs, as seen in our target cells, have also been well documented in previous SEM studies and characteristic features of apoptosis (Bishop and Whiting, 1984; Stacey et al., 1985; Bishop et al., 1987). It remains to be proven whether the NK-cell is able to bring about both complement-mediated-like lysis and apoptotic lysis in the course of its cytotoxic activity.

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