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Infiltration and Lysis of Tumour Cell Aggregates by Adherent Interleukin-2-Activated Natural Killer Cells Is Distinct from Specific Cytolysis

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Abstract

Adoptively transferred activated natural killer (A-NK) cells infiltrate tumours in vivo. Two in vitro B16-F10 melanoma tumour models were used to study with fluorescence and electron microscopy the infiltration of adherent interleukin 2 (IL-2) A-NK cells: (1) substratum-bound sessile microtumours (MTs), and (2) three-dimensional cell growth on macroporous gelatinous microcarriers (Cultispheres®). From 2 h and on increasing numbers of A-NK cells infiltrated the MTs regularly surrounded by a widened intercellular space. An IL-2-dependent disintegration of MTs began at 6–8 h resulting in a release of vital and dead cells. A-NK cell invasion into Cultispheres effectively displaced the melanoma cells from the highly convoluted substratum. Thus, A-NK cell infiltration had a protease-like effect on the tumour cell aggregates which might have a bearing on the interpretation of their cytolytic effect on target cells. Ultrastructural evidence was not obtained of specific A-NK/target conjugate formation or of granule-mediated target cell destruction in either model tumour.

Key Words

Interleukin 2
Natural killer cells, adherent
Killer cells, lymphokine-activated
B16 melanoma
Tumour infiltration
Cytotoxicity
Fluorescence
Electron microscopy
Cell culture

Introduction

In recent years much interest has been focused on the antitumour effect of adoptively transferred lymphokine-activated killer (LAK) cells. The subpopulation of LAK cells that grows adherent to the plastic of the culture vessel (adherent activated natural killer, A-NK, cells) has attracted particular attention due to an enhanced cytolytic potential in vitro [1], a high capacity to infiltrate [2–5] and reduce [6] intravital tumours, and a clinical

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immunotherapeutic potential [7]. There are several interpretations as to how these cells exert their action on intravital tumours, one of which is that the A-NK cells must establish a direct contact with the target cells before their cytolytic machinery is activated. Thus, in vivo the assumed A-NK cell cytotoxic interaction with the target cell is the last step in a sequence of events that comprises circulation of the adoptively transferred cells with the blood stream, their homing in tumour or tumour-close microvessels, and emigration out of the microvasculature. Then migration through the interstitium must follow leading to the true infiltration into the tumour mass whereupon a close contact with the malignant cells could finally be achieved [2–4].

In an attempt to further elucidate the morphology of the isolated infiltration process we have applied light and electron microscopy on two in vitro B16 tumour models co-cultured with murine A-NK cells during continuous interleukin 2 (IL-2) stimulation. It was confirmed that A-NK cells were able to invade artificial tumour cell aggregates but somewhat unexpectedly little information was gained on the anticipated cytolytic process since the dominant effect of A-NK cells was that the tumour cell masses disintegrated through the release of mainly vital cells.

Materials and Methods

A-NK Cells

Male C57/BL6 mice were used. A-NK cells were derived from splenic lymphocytes, basically according to Gunji et al. [1]. Spleens from young animals (body weight 12–15 g) were homogenized. After lysis of erythrocytes cell suspensions were transferred to tissue culture flasks (Falcon) and grown in a humidified atmosphere (5% CO_2 in air) at 37°C in A-NK medium: RPMI 1640 with the addition of 10% fetal calf serum (FCS), 2 m*M* glutamine, 50 m*M* mercaptoethanol, 10 ml/l nonessential amino acids (GIBCO), 0.8 g/l streptomycin, 1.6×10^5 U/l penicillin and 1,000 Cetus U/ml recombinant human IL-2 (a generous gift from EuroCetus). Two days after seeding the flasks were decanted. Cells remaining in suspension were collected and resuspended in half the volume of used, conditioned medium. An equal volume of fresh medium was added + 1,000 U/ml IL-2 and the cells were further grown in new flasks. At 48-hour intervals thereafter adherent cells were rinsed thoroughly with warmed RPMI 1640 in order to remove nonadherent cells. Again half the volume of conditioned medium together with an equal volume of fresh A-NK medium + IL-2 were added to the adherent cells. A-NK cells used in the present report were harvested on days 5–8 after splenectomy. They were released from the plastic by treatment with 0.02% EDTA in phosphate-buffered saline (PBS), washed in RPMI 1640, and resuspended in the above medium with/without IL-2 to give 50,000 or 100,000 A-NK cells/cm^2 culture dish surface area when transferred to the model tumours.

As assessed repeatedly with flow cytometry this A-NK cell culture routine results in preparations highly enriched for cells with an NK phenotype, i.e. 95–99% asialo-GM_1+ cells, ≈90% NK1.1+ cells, ≈5% CD8+ cells; 1–3% are immunopositive for CD4, MAC-1 and TCR.

In vitro Model Tumours

Tumour Cells. B16-F10 cells (a murine melanoma cell line of C57/BL6 origin) were cultured (37°C, 5% CO_2) as monolayers in RPMI 1640 supplemented with 10% heat-inactivated FCS, 10 m*M* Hepes buffer, 0.8 g/l streptomycin and 1.6×10^5 U/l penicillin (tumour cell medium). Cell cultures were passaged as required to maintain the cultures in log growth phase. Cells were detached by exposure to 0.02% EDTA for 2 min and washed twice in RPMI 1640. Cell viability was always >95% as judged by the trypan blue dye exclusion test.

Sessile B16-F10 Microtumours. This model tumour type used for incubation with A-NK cells forms spontaneously upon prolonged culture under standard conditions. Cultures on 16-mm 4- or 24-well plates (Falcon) or on the membranes of 6.5-mm Transwell® (Costar) inserts were used, the former for light microscopy and the latter for electron-microscopic preparation. After confluence numerous aggregates of malignant cells rise from the culture surface with a rather regular distribution. The aggregates expand further as smooth-surfaced hills and ridges or in a mushroom-like manner with a cap and a broad shaft. They retain their anchorage to the culture surface for 2–3 weeks, hence the designation sessile microtumours (MTs). In the present study co-incubations with A-NK cells were

made within 1 week of MT growth. The height and width of MTs were then about 10 cells.

Microcarrier-Grown B16-F10 Cells. Microcarriers (Cultispheres G®, Hyclone) were prepared for culture according to the manufacturer's instructions. Dry microcarrier beads (5 mg) were hydrated in RPMI 1640 and 1 ml B16 cells (10^6 cells/ml in RPMI 1640) was added. The density of the mixture was adjusted by adding Percoll (Pharmacia, Sweden) to a final concentration of 30% v/v. The medium density kept the cells and the beads in suspension avoiding that cells preferably attached to the plastic. After incubation for 90 min with agitation every 20 min the microcarriers with attached cells were washed by sedimentation in RPMI 1640 and resuspended in tumour cell medium for further culture. With this seeding density the cavities and surface of the Cultispheres were completely covered with B16 cells 3 days later and they were used for co-incubation with A-NK cells at this stage. About 50 microcarriers were transferred to 16-mm wells and 50,000 or 100,000 A-NK cells/cm^2 culture dish surface area were added and allowed to interact with the melanoma cells up to 48 h.

Fluorescence Light Microsopy

Calcein AM or Cell Tracker Green Bodipy® (Molecular Probes, Eugene, Oreg., USA) fluorescent probes were used for prelabelling of A-NK or melanoma cells, allowing discrimination of cell identities during examination of live co-cultures. Ethidium homodimer was added to some cultures to indicate devitalized cells (Live/Dead™ viability/cytotoxicity kit; Molecular Probes). Cultures were examined and photographed in a Nikon Diaphot inverted photomicroscope equipped with phase contrast and Hoffman modulation contrast optics. Epifluorescence observations were made with a standard fluorescein filter set.

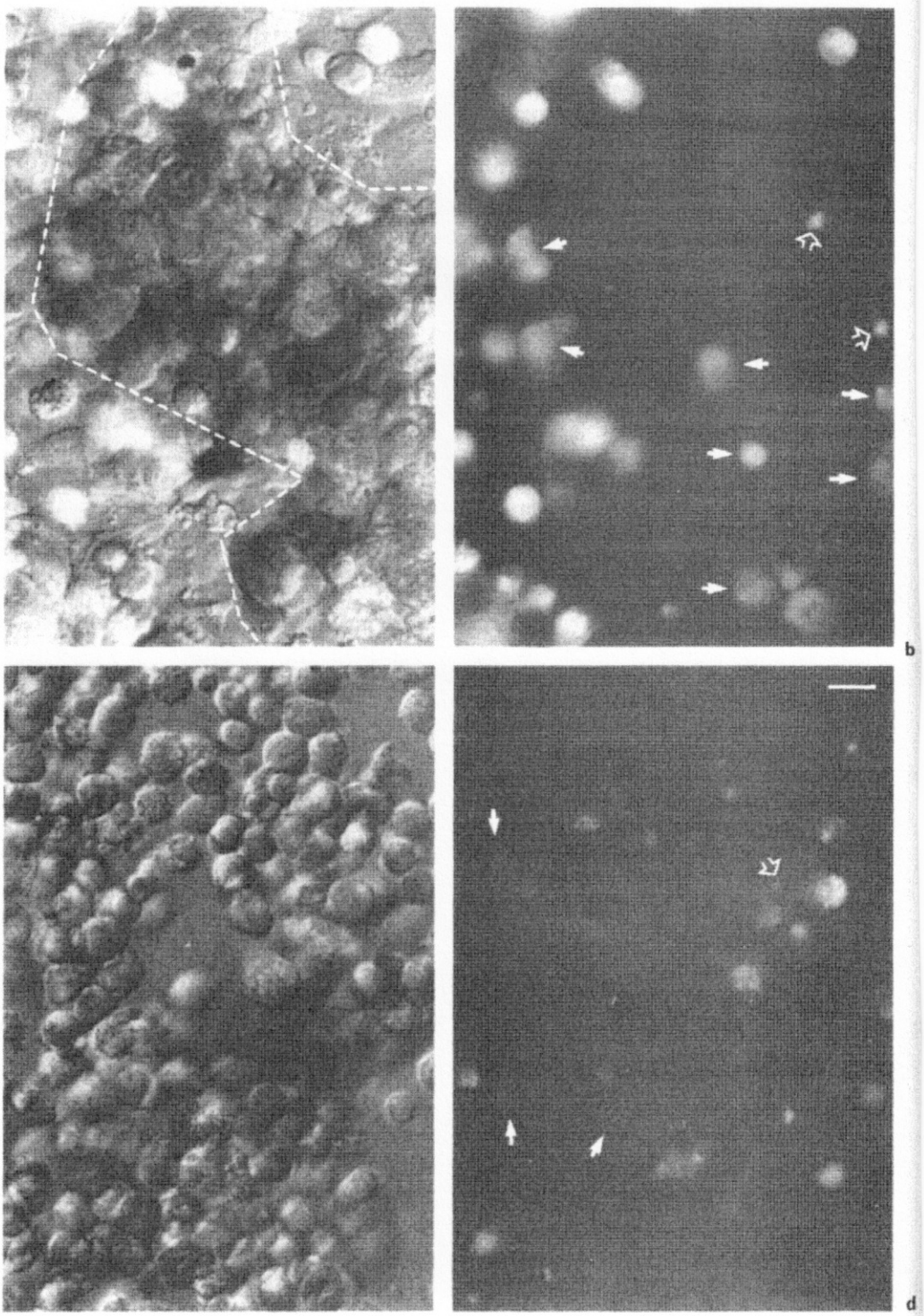
Electron Microscopy

Specimens were fixed with a 1:1 mixture of PBS and 2.5% glutaraldehyde + 2% paraformaldehyde in 0.05 *M* Na cacodylate. Postfixation was achieved with 0.5% OsO_4 in 0.1 *M* cacodylate followed by dehydration in ethanol and infiltration with Agar 100 resin. In MT specimens that were processed in the cell culture vessels ethanol was used as a solvent for the resin whereas propylene oxide was used for the suspended material (Cultispheres). After curing ultrathin sections were cut with a diamond knife in a Reichert Ultracut E microtome and examined in a Zeiss CEM 902 electron microscope after contrasting with uranyl acetate and lead citrate.

Results

A-NK Cell Infiltration into Sessile MTs

On melanoma/A-NK cell co-cultures with addition of IL-2, fluorescence microscopy revealed that Calcein AM-labelled A-NK cells initially were distributed over the B16 cultures at a seemingly random distribution but tended to accumulate in the valleys between MTs after 1 h in the incubator. From 2 h after addition, the A-NK cells were also observed within MT aggregates, first along the basolateral aspect of the MTs and at 4–6 h as isolated fluorescing spots within the MTs (fig. 1a, b) with a remaining cellular organization of the co-cultures. However, between 6 and 8 h a general breakdown of the MTs began to result in a release of individual cells or small cell groups from the aggregates into the culture medium. The initially organized cultures became increasingly fragile so that a slight mechanical disturbance caused a general stirring of the cells. Thus, from 24 to 26 h there were no remaining sessile MTs in the 16-mm wells with 100,000 A-NK cells added (fig. 1c, d). Cellular fragments appeared in large amounts in the medium. Ethidium homodimer added to the medium labelled nuclei (indicating cell death) of cells that were unstained by Calcein AM at an increasing frequency but quantitative estimates could not be done in the mixture of anchored and floating cells, aggregates and debris. In the absence of IL-2 the infiltrative events took place during the initial 6 h as in the stimulated cultures. Destruction of the MT topography did not follow, though, or was much less pronounced than when IL-2 was supplemented. MT cultures incubated for up to 48 h in fresh or conditioned A-NK cell medium with IL-2 did not undergo any changes. The sequence of events described above took place in a seemingly identical manner when A-NK cells that were unlabelled with fluorescent probes were used.


a
b
c
d

Electron microscopy confirmed and extended the light-microscopic observations. The MTs had an upper convex aspect where the B16 cells often had a flattened epitheloid appearance (fig. 2a). Along the basolateral aspects the intercellular organization of the melanoma cells could be less ordered with irregularly shaped cells in loose contact (fig. 2f). In the core of the MTs melanoma cells were generally irregularly polygonal on transection. The cells were closely apposed with a very narrow, often highly convoluted, intercellular space and thus formed a tightly organized cell mass (fig. 2a). MTs did not exhibit central necrosis as an indication of growth beyond the nutritive demands of the tumour cells.

Up to 2 h of co-incubation most A-NK cells associated with MTs were located along their sides and in clefts between basolateral melanoma cells. Figure 2d illustrates such an A-NK cell in obvious migration at 2 h immediately beneath superficial melanoma cells. Only few effector cells were encountered on the upper surface of the MTs but on rare instances single A-NK cells penetrated between surface-covering melanoma cells (fig. 2c). From 2 h, and more frequently at 4 and 6 h, A-NK cells were found in the central core of MTs, both along the culture vessel surface and completely surrounded by melanoma cells (fig. 2e, f). Occasionally microscopy gave the impression that one leading A-NK cell was followed by others from the entrance site at the lateral aspect of the MT. The intracellular organization of these intratumoural A-NK cells indicated that they were in a state of active migration (fig. 2d–f). Quite consistently, invading A-NK cells occupied widenings of the intercellular space (fig. 2d–f). This loosening of the intercellular organization became generalized throughout some MTs at 6–7 h (fig. 2b) and apparently preceded the general dissolution of the B16 cell aggregates that made it impossible to prepare specimens for electron microscopy of IL-2-stimulated cocultures from after 8 h.

Although the observation is stressed that infiltrating A-NK cells were surrounded by a widened extracellular space in the MTs, various close A-NK to B16 cell contacts were also observed. These ranged from pin-point contacts formed by cellular extensions to broad appositions. Signs of specific conjugate-forming adhesions, i.e. with extensive membrane interdigitation or flattened occluding junction-like areas surrounding closed intercellular cavities, were not recorded. Albeit frequently located close to the surface of A-NK cells, unambiguous evidence was not obtained that the specific (lytic) cytoplasmic dual-compartment granules were involved in exocytotic events with fusion to the plasmalemma and ejection of their contents.

Within MTs with A-NK cell infiltration, fixed at 6–8 h, some target cells were in obvious lysis. Lytic cells were scattered among

Fig. 1. Light micrographs of identical fields in a 16-mm cell culture well showing A-NK cell infiltration into a ridge-like MT after 6 h (**a, b**) and its dissolution after 28 h (**c, d**) of co-incubation. Combined Hoffman modulation contrast and epifluorescence imaging in **a** and **c**, fluorescence only in **b** and **d**. **d** Bar = 20 µm. **a** Focus is adjusted close to well surface, flattened cells on the plastic are therefore in focus whereas the bulk of the MT is out of focus. The contour of the MT is outlined with the dashed line. **b** A-NK cells were prelabelled with Calcein AM and exhibit green fluorescence. Cells indicated by filled arrows were identified to be located within the MT, the others are on the surface or beside the MT. Two labelled cells that were distinctly smaller (open arrows) than the vast majority of A-NK cells had also infiltrated the MT. **c** After 28 h the same area shows rounded cells released from the culture surface. **d** A-NK cells still fluoresced, ethidium homodimer was added and labelled some cell nuclei with red fluorescence (arrows), probably representing devitalized melanoma cells. One dead A-NK cell is included (open arrow, out of focus and thus faintly visible) that showed double labelling.

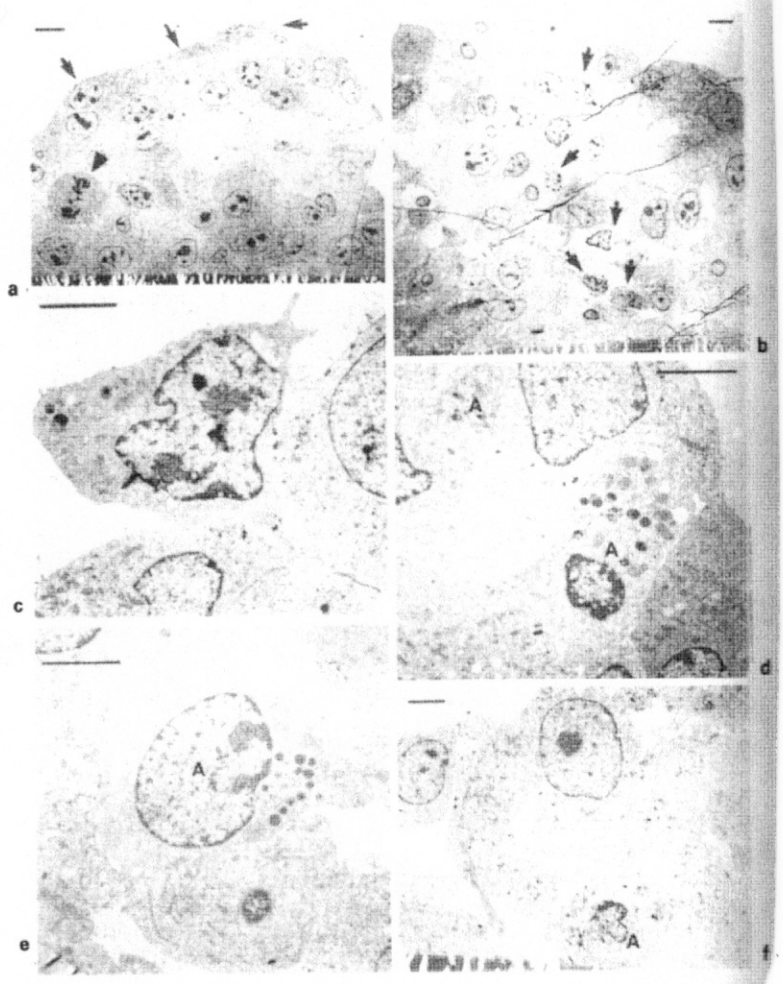


Fig. 2. Electron micrographs of MTs grown on Transwell filters and co-incubated with A-NK cells for 2–6 h. The ultrathin sections were colleted on support films which caused folding, seen as black lines in **a** and **b**. **a** Two hours' co-incubation; low power micrograph of MT that does not exhibit A-NK cell infiltration. Note tight contact between melanoma cells and the elongated shape of some surface-covering cells (arrows). One cell in mitosis is also shown (arrowhead). Bar = 10 μm. **b** Similar overview as in **a**, 6 h co-incubation. Observe the loosened intercellular organization as compared with the preceding micrograph. Five infiltrating A-NK cells are included (arrows). Bar = 10 μm. **c** A-NK cell penetrating between two melanoma cells on the upper convex aspect of an MT, 2 h co-incubation. Bar = 5 μm. **d** Two A-NK cells (A) immediately below the basolateral surface of MT after 2 h co-incubation. Note that the central A-NK cell is in migration with an organelle-free leading end below and organelles displaced to the rear end, i.e. upwards. Bar = 5 μm. **e** An A-NK cell (A) in locomotion after 6 h co-incubation within a widened extracellular space in the core of an MT and close to the Transwell membrane (lower left corner). Bar = 5 μm. **f** An AN-K (A) cell is apparently tunnelling between the Transwell membrane (below) and the bulk of MT (extends to the right) from the basolateral aspect. Bar = 5 μm.

preserved cells but examination of sections could not reveal any obvious co-location between lytic cells and A-NK cells. The morphological pattern of apoptotic melanoma cell death was occasionally recorded in control MT cultures as well as in A-NK cell-infiltrated cultures.

A-NK Cell Infiltration into Cultisphere-Cultured Melanoma Aggregates

Fluorescence microscopy of Calcein AM-prelabelled A-NK cells during co-incubation with melanoma cell-covered Cultispheres indicated that A-NK cells gained access to the interior of the macroporous carriers and some cells also adhered to the external aspect. When the melanoma cells were prelabelled instead it was evident that labelled cells were released into the medium at increasing numbers from the microcarriers during co-incubation with A-NK cells [data not shown].

In electron-microscopic sections it was seen that melanoma cells grew predominantly as monolayers on the gelatinous Cultisphere external surface and cavities. Large cavities could be filled with melanoma cells that thus formed aggregates of 4- to 5-cell diameters. Upon co-incubation for 2–6 h the preferred A-NK cell location was apparently between melanoma cells and the dense surface of the microcarriers (fig. 3a) giving the impression that the A-NK cells bulldozed their way into the Cultisphere at the expense of melanoma cell anchorage. A-NK cells situated on or between monolayered B16 cells were also encountered within larger cavity-filling clusters of target cells. The invasion of A-NK cells occurred regardless of IL-2 supplementation. After 24–48 h of cell co-incubation in the presence of IL-2 the microcarriers were almost totally devoid of intact melanoma cells while scattered A-NK cells remained adhering to the convoluted substratum (fig. 3b is ac-

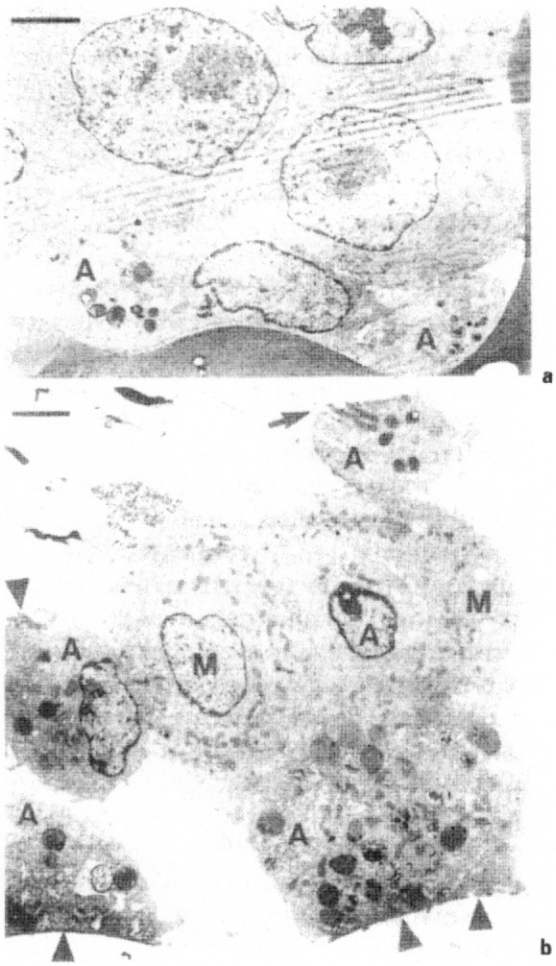


Fig. 3. Electron micrographs of A-NK cell co-incubations with melanoma cells growing on Cultispheres. **a** Two A-NK cells (A) are seen squeezed into a narrow space between cavity-filling melanoma cells and microcarrier surface (dark, smoothly curved material below and to the right) in the interior of a Cultisphere. Six hours' co-incubation. Bar = 5 μm. **b** Forty-eight hours' co-incubation; view of a cavity within the microcarrier. Such cavities were regularly filled with melanoma cells at the early observation times. A = A-NK cells; M = melanoma cells. Four A-NK cells occupy the microcarrier surface (arrow and arrowheads; note flattened adhering surfaces of upper and lower cells) and one A-NK cell is situated between remaining melanoma cells. Bar = 5 μm.

tually a rare exception with a few remaining melanoma cells surrounded by A-NK cells). This extensive clearing of melanoma cells did not develop in the absence of IL-2.

Discussion

The acute cytotoxic effector cell interaction with suspension target cells in vitro, as evidenced by for example the common ^{51}Cr release assay, is assumed to follow a simple sequence of events: effector-to-target conjugate formation, effector cell release of cytolytic substance, target cell lysis. The present descriptive analysis of effector:target cell interactions in a slightly more in vivo-like setting, i.e. with the tumour cells in three-dimensional growth, implicates that additional effects occur which from a morphological point of view rather overwhelmed cytolytic events per se. Thus, the main effects that were obviated were (1) infiltration of A-NK cells into the core of tumour cell aggregates, (2) loosening of the intercellular bonding of the tumour cells which seemed to begin around the infiltrating A-NK cells, and (3) release of tumour cells from neighbouring cells and substratum. In addition, some target cell lysis occurred in both co-incubation systems that were used. It seems justified to assume that the release of B16 cells from their anchorage to Cultispheres was due to the same mechanisms that caused the dissociation of MTs after A-NK cell infiltration.

A main conclusion of the present observations is that the disintegrative action of A-NK cells on tumour cell aggregates was distinct from directed cytolytic action in the classical sense, i.e. after conjugate formation with individual target cells followed by release of cytotoxic substances [8–11]. For example, it was observed that MTs were effectively broken down although the effector-to-target cell ratio within the aggregates was very low. We were not able to quantitate the infiltration by either fluorescence or electron microscopy. As a rough indication, though, it could be stated that after 6 h co-incubation at most 4–5 A-NK cell profiles were identified in electron microscopy transections of MTs containing 50–100 melanoma cell profiles. Since an effector-to-target ratio of 5:1 would be required to achieve a 20–30% melanoma cell lysis in a 4-hour ^{51}Cr release assay [unpubl. data] the above-indicated ratio between effectors and aggregated tumour cells could not be expected to result in more than a few isolated kills. Possibly, the scattered lytic cells that were observed had been damaged through such directed cytolysis. It is intriguing, though, that although numerous close A-NK to B16 cell contacts have been scrutinized we have not been able to obtain images that clearly suggest specific conjugate formation as described in the literature [12, 13] or cytolysis through exocytosis of specific dual-compartment granule contents [13]. Of course, electron microscopy sections of interacting cells in a three-dimensional tissue-like setting should be interpreted with caution, since it cannot be determined with certainty whether cell contacts reflect the migration of A-NK cells into the tumour cell mass or constitute specific conjugates during a cytotoxic event. Diverse ultrastructual observations such as interdigitation of plasmalemmata between effector and target cells [14], presence and/or re-orientation of intracellular granules [15], or the appearance of minute vesicles in the intercellular space [10, 16] would not be sufficient evidence for an exocytotic process leading to target cell damage. Against this background, and being aware of the possibility that our electron microscopy has failed to reveal the exocytotic cytolysis-inducing action of individual A-NK cells, we find it necessary to consider alternative mechanisms to explain the killing of B16 cells that

occurred besides the disintegration of microtumoural organization.

The present observations strongly indicated that the A-NK cells exerted mainly a tumour-lytic action that was effective over a distance of several cell diameters. The experiments could not define whether or not infiltrated A-NK cells alone induced these phenomena. Maybe the non-infiltrating cells also contributed to the total effect by release of material that dissociated the aggregates of malignant cells. This latter possibility is contradicted but not excluded by the observation that conditioned A-NK cell culture medium did not cause an obvious MT breakdown. Regardless of the acutal role of free A-NK cells in the co-incubations the results indicate that a significant step in the A-NK cell interaction with the tumour cell aggregates was undirected secretion of material with a proteolysis-like effect. From a phenomenological point of view the separation and release of malignant cells in our incubations were similar to the clearing of fibroblasts from monolayers after exposure to LAK cells [17]. These events included cytotoxic action and were paralleled by secretion of mucus-like material including chondroitin sulphate and perforin from the LAK cells [17, 18]. It has also been demonstrated that rat IL-2-activated A-NK cells release proteolytic enzymes belonging to the cytosolic proteasome complex which contribute to antitumour and cytolytic effects [19, 20]. Immunocytochemically these enzymes are also present in considerable amounts in the cytoplasm of mouse A-NK cells [21]. Moreover, human A-NK cells express ectopeptidases and produce collagenase [5]. It is conceivable, therefore, that the observed dissolution of tumour cell aggregates and scattered cytolytic events could be explained by the action of extracellular proteasome or other proteolytic enzymes from the A-NK cells.

To our knowledge, studies on in vitro interactions between multicellular tumour aggregates and cytotoxic effector cells have been performed only in human cell systems: glioma [14, 22–24], colorectal cancer [25, 26], SW948 adenocarcinoma [27], head and neck cancer and breast carcinoma [5, 28], and ovarian carcinoma [29]. It is interesting that Jääskeläinen et al. [24] pointed out that damage induced by infiltrating LAK cells extended beyond the effector-target contacts as in the present work. It was also demonstrated that the distribution of cell adhesion molecules within glioma spheroids exhibited gradient-like patterns and that the migration of killer cells could be inhibited by pre-incubation with appropriate antibodies against the adhesion pathways. It would thus be of obvious interest to elucidate the composition of the extracellular matrix in the present murine tumour models and the expression of adhesion molecules on the tumour and the A-NK cells. In a recent report based on agarose migration assays we have demonstrated that A-NK cells are chemoattracted by distant isolated MTs and that the composition of matrix macromolecules on the migration surface determines the A-NK cell migratory capacity [30]. Moreover, Vujanovic et al. [5] emphasized that human adherent A-NK cells had a superior infiltrating capacity into in vitro spheroids as well as into in vivo tumours as compared to a non-adherent A-NK cell population.

The present observations must of course be extrapolated with great caution to an intravital situation where adoptive immunotherapy is attempted on established tumours. If, however, similar interactions between A-NK cells and organized tumours take place in vivo as reported here it is conceivable that the tumorilytic effects reflect a mechanism that facilitates the A-NK cell infiltration. On the other hand, the question arises of whether the release of tumour cells from their attachment to

the surroundings could favour local or distant dissemination of tumour cells from the A-NK cell-infiltrated lesion. Such adverse side effects might occur even if a significant number of tumour cells are cytolytically destroyed. The validity of this reasoning cannot be evaluated at present but to the best of our knowledge the short- and long-term structural effects on tumour organization after challenges with varieties of LAK cells are not elucidated.

Acknowledgements

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