Anti-Cancer Drugs, 3, pp. 155–162

Interaction between lymphokine activated killer cells generated in the presence of cisplatin/FK-565 and tumor cells: transmission electron microscopy analysis

Sunanda Basu and Ajit Sodhi[CA]
The authors are at the School of Biotechnology, Banaras Hindu University, Varanasi 221 005, India. Tel: 310093.

Lymphokine activated killer (LAK) cells generated in the presence of cisplatin/FK-565 along with interleukin-2 (IL-2) showed enhanced binding to tumor cells as compared with LAK cells generated in the presence of IL-2 alone. The LAK cells showed a reorientation of cellular organelles in the presence of tumor cells. LAK–tumor conjugate pairs showed vesicle-like structures in the extracellular pockets. The number of vesicles in the extracellular pockets was found to be increased in the case of LAK cells generated in the presence of cisplatin/FK-565 along with IL-2. The closer interaction between the LAK cell–target cell and the large number of vesicles in the extracellular space of conjugate pairs may explain the increased lysis of target cells by LAK cells generated in the presence of cisplatin/FK-565 along with IL-2 as compared with IL-2 alone.

Key words: Lymphokine activated killer cells, cisplatin, FK-565.

Introduction

Recent advances in adoptive immunotherapy have used cytotoxic lymphocytes with broad antitumor reactivity. Studies have shown that co-culture of normal lymphocytes with interleukin-2 (IL-2) induces the generation of broadly cytotoxic antitumor cells commonly termed LAK cells[1–3], and these cells have proven useful in the control of metastatic tumors in animal models and humans.[4–6] LAK cells are a heterogeneous population with a subset of lymphocyte effector cells responsible for tumor cytotoxicity. The mechanism by which LAK cells lyse their targets is not known. It is assumed that LAK cells kill tumor cells by direct cell–cell contact, using mechanisms similar to those proposed for cytotoxic T lymphocytes (CTL) and natural killer (NK) cells.[8–10]

In order to exert their cytotoxic activity, CTL and NK cells bind to the target, undergo an activation process leading to the release of lytic factors and, finally, establish suitable spatial conditions in order for these factors to reach the target and to exert their function.[11]

Cisplatin, a potent antitumor compound, has been used successfully in the treatment of tumors in mice[12] and human cancers.[13–16] Further, it has been shown to activate monocytes/macrophages and NK cells to a tumoricidal state.[17–21] FK-565, a heptanoyl tripeptide, is an immunoadjuvant. It activates human monocytes to a tumoricidal state,[22,23] and also enhances the production of IL-1 and superoxide anions[22,24] by murine macrophages.

We have previously reported that both cisplatin and FK-565 can up-regulate LAK activity.[25] In the present study, transmission electron microscopy (TEM) analysis of the interaction of LAK cells generated in the presence of IL-2 alone or along with cisplatin/FK-565 with tumor cells was carried out.

Materials and methods

Media and reagents

Fetal calf serum (FCS) was purchased from Sera Lab (UK). It was heat inactivated in a water bath at 56°C for 30 min. Synthetic heptanoyl tripeptide (FK-565; hepttanoyl - Y - D - Glu - (L) - meso - α,ε - A_2pm-(L)-D-AlaOH) and recombinant human IL-2 (Takeda Pharmaceutical, Japan) of specific activity 3.5×10^4 U/mg, as assayed on murine NKC3 cells,

Supported by financial assistance from CSIR.

[CA] Corresponding Author

was a kind gift from Dr Saburo Sone (University of Tokushima, Japan). Ficol-Hypaque was purchased from Nyegaard, Norway. Medium RPMI 1640 and Hank's balanced salt solution (HBSS) were purchased from Sigma Chemical Co. (St Louis, MO).

Target cell

Raji cell (human Burkitt lymphoma) line was maintained as a suspension culture in RPMI 1640 with 10% heat inactivated FCS, penicillin (100 U/ml), streptomycin (100 μg/ml) and gentamycin (20 μg/ml), referred to as complete medium, at 37°C in a humidified atmosphere of 5% CO_2 in air. The cell line was obtained from the National Tissue Culture Facility, Pune, India, and subcultured every 3–4 days.

Generation of LAK cells

Heparinized blood was obtained from normal healthy donors, diluted with an equal volume of HBSS and layered over lymphoprep as described by Boyum.[26] It was then centrifuged at 18°C for 30 min at 400 × *g*. Peripheral blood mononuclear cells (PBMCs) collected from interface were washed twice with HBSS. PBMCs (2×10^6 cells/ml) were cultured in complete medium alone or medium containing IL-2 (2.5 U/ml) alone or along with cisplatin (5 μg/ml) or FK-565 (5 μg/ml) and incubated for 4 days at 37°C in a CO_2 incubator.

LAK–tumor cell conjugate assay

The technique described by Bonavida *et al.*[27] was employed with minor modifications. In brief, LAK cells and Raji target cells, both at a concentration of 4×10^6/ml, in complete medium were equiliberated separately for 30 min at 30°C. Then, 100 μl of effector (E) cells and 200 μl of target (T) cells were incubated together (E:T ratio of 1:2) for 10 min at 30°C and centrifuged for 5 min at 200 × *g*. Part of the supernatant was removed with a pasteur pipette. The tubes were placed in a water bath at 39°C and 25 μl of melted agarose (Sigma type VII; 2% in RPMI 1640 without serum at 39°C) was added. The pellet was gently resuspended and 25 μl was added to a slide, covered with an object glass and sealed with paraffin. Conjugate-forming cells (CFCs) on this slide were counted immediately after preparation. The percentage of CFCs was defined as the proportion of LAK cells binding to Raji target cells and was scored by counting at least 400 mononuclear cells. The absolute number was obtained by multiplying the percentage of CFCs with the number of LAK cells.

LAK-Raji cells co-cultures for TEM

LAK cells and Raji cells were pelleted at 250 × *g* at room temperature for 5 min at an E:T ratio of 20:1. The pelleted cells were prefixed with 2% glutaraldehyde (0.1 M cacodylate buffer) and postfixed with an aqueous solution of 1% OsO_4. The specimens were then dehydrated in an alcohol series and embedded in epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined by TEM.

Results

PBMCs treated with IL-2 (2.5 U/ml) for 4 days showed an enhanced binding to the tumor target as compared with untreated PBMCs or PBMCs treated with cisplatin (5 μg/ml) or FK-565 (5 μg/ml) alone. The binding capacity of effector cells to target cells was significantly enhanced when LAK cells were generated in the presence of cisplatin (5 μg/ml)/FK-565 (5 μg/ml) along with IL-2 (2.5 U/ml) (Figure 1).

Figure 1. Morphology of PBMC [L] and tumor cell [T]. No interaction between the PBMC and the tumor cell can be seen.

Under TEM, the LAK-Raji cells conjugates were found to be very similar to CTL- or NK–target cell conjugates.[28-31] Contact between the LAK and target cells was established by penetrating pseudopodia. In general the pseudopodia were free from organelles other than a few ribosomes. The effector cell nucleus was seen at a position distal to the point of contact and organelles oriented towards the target cell. Ultrastructurally the target cell showed extended microvilli which were swollen and partly dissolved. The extensions were given out only from the surfaces in close apposition to LAK cell. Few vesicles could be seen on the surfaces of or in the spaces between the LAK–target cell conjugates. The injured target cell showed swelling of mitochondria and extensive vacuolization (Figure 2). PBMCs and Raji cells did not show any such modification and retained their normal morphology when co-cultured (Figure 1).

Figure 2. LAK cell [L] interacting with a tumor cell [T]. Close interaction between the LAK and tumor cell by microvilli extension is visible. The LAK cell shows reorientation of cellular organelles. The injured target cell is characterized by swelling of organelles and vacoulization.

Figure 3. Tight binding between LAK–CP cell [L] and Raji cell [T] by close plasma membrane apposition (arrow) is visible. The arrow head points to the vesicles filling the extracellular pockets.

Figure 4. Close interaction between LAK–CP cell [L] and tumor cell [T] by cytoplasmic bridges (arrow).

Figure 5. Interaction between LAK–CP cell [L] and tumor cell [T]. The injured target cell shows degenerated mitochondria [m]. The intercellular space is filled with cytoplasmic extension from LAK cell, making contact with the tumor cell surface (arrow).

In the case of LAK cells generated in the presence of IL-2 together with cisplatin (LAK–CP), the LAK-tumor cell conjugates showed closer apposition of plasma membranes (Figure 3). LAK cells could be seen binding very tightly to tumor cells with numerous plasma membrane interdigitations (Figure 4). Between the effector and target cells, small extracellular pockets were formed that contained membrane fragments and a large number of vesicles (Figure 5). The injured target cells showed swelling and extensive damage to mitochrondria (Figure 6). Figure 7 shows an extensively damaged target cell. In addition to vacuolization, some myelin sheath-like structure could also be seen in the target cell.

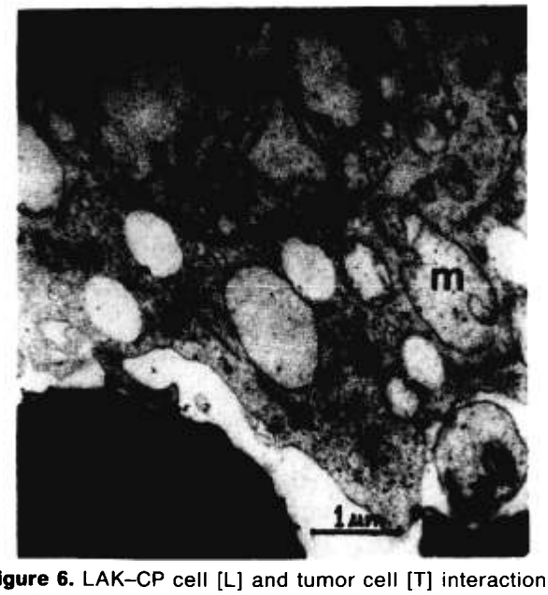

Figure 6. LAK–CP cell [L] and tumor cell [T] interaction. The injured target cell is characterized by extensive damage to the mitochondria.

LAK cells generated in the presence of FK-565 together with IL-2 (LAK–FK-565) showed a similar interaction with target cells. The effector LAK cells formed very tight plasma membrane bonds with target cells (Figures 8 and 9). There was a reorientation of cytoplasmic organelles in the LAK cells interacting with tumor cells—the nucleus at the trailing end and the other cellular organelles oriented towards the tumor cell (Figure 8). Numerous interdigitations were formed between the plasma membrane of effector and target cells, and small extracellular pockets filled with numerous vesicle-like structures (Figure 10). Figure 11 shows numerous cytoplasmic extensions between the effector–target cells with their plasmalemmae showing close and tight contacts.

Discussion

The LAK effector cells are pleiomorphic, undergoing a characteristic rearrangement upon binding with target cell. The basic effective mechanism of

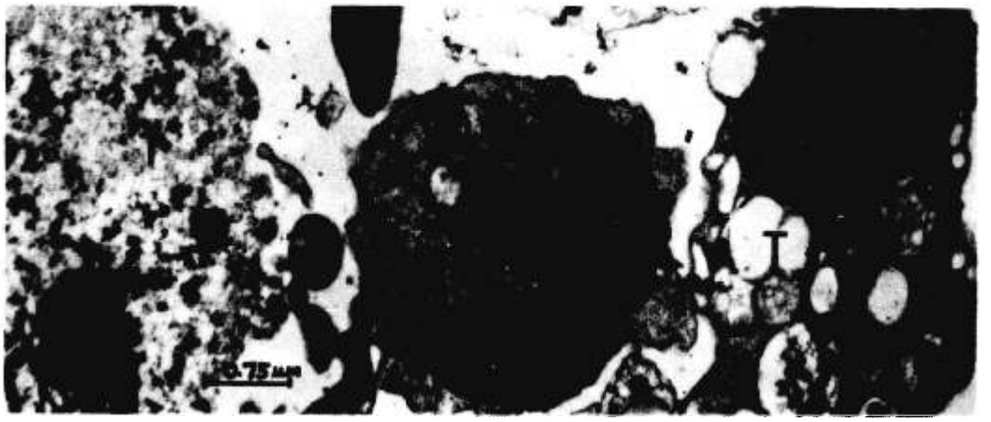

Figure 7. LAK–CP cell [L] seen interacting with tumor cell [T]. The target cell shows a myelin sheath-like structure [my] and highly degenerative morphology.

killing of tumor cells by LAK cells generated in the presence of IL-2 alone was similar to those generated in the presence of IL-2 plus CP/FK-565, except in finer details.

In the case of killing of tumor cells by LAK cells generated in the presence of IL-2 alone, the target cells gave out extensive microvilli interdigitating with microvilli of LAK cells (Figure 2). Further, swelling of mitochondria and formation of large vacuoles were most noticeable in target cells (Figure 2). These observations suggested that target cell killing was not only due to cell surface interactions but involves the whole cell simultaneously. Recent

Figure 8. LAK–FK-565 cell [L] interaction with tumor cell [T]. Note the plasma membrane bonds formed between the cells. The LAK cell shows reorientation of organelle.

Figure 9. Higher magnification of enclosed area shown in Figure 8. Note the close plasma membrane apposition.

Figure 10. Numerous interdigitations formed between the LAK–FK-565 cell [L] and the tumor cell [T]. The small pockets are filled with numerous vesicle-like structures.

Figure 11. LAK–FK-565 cell [L] and tumor cell interaction [T]. Note the numerous cytoplasmic extensions between the effector and target cell, with their plasmalemmae showing close and tight contact.

evidence suggests that the target cell may participate actively in the mechanism leading to its own lysis.[32] Further, there is evidence for the involvement of the cytoskeletal elements and surface structures of the target cell.[33] It is conceivable, therefore, that the signal from LAK cells is transmitted across the target cell and that the switch to sudden death is manipulated deep inside the cell, as suggested in the case of CTLs.[34]

In the case of LAK cells generated in the presence of IL-2 plus CP/FK-565, the LAK cell–target cell conjugates showed close proximity of their plasmalemmae. There was a very close apposition of plasma membranes and the extracellular spaces were filled with numerous vesicles (Figures 3–5 and 10–11). A common CTL and NK cell cytotoxicity mechanism has been proposed based on the local exocytosis of lethal substances by the effector cell into the target cell.[35] Cytolysin and perforins derived from cytolytic granules have been implicated in cell mediated cytotoxicity by CTL and NK cells. These molecules form pores on the target cell. As proposed by Hook *et al.*[36], the large number of vesicles seen in the extracellular pockets between LAK cells and target cells probably contain granules having a pore forming protein similar to that of CTL effector cell.[37]

The present study suggests that target cell killing could be due to massive plasma membrane blebbing, condensing heterochromatin and swelling of mitochondria, which are the general features of apoptotic cell death. Apoptotic cell death, also known as programmed cell death, has been suggested in the killing of tumor cells by NK cells and CTLs.[38,28] The mechanism of killing Raji cells by effector LAK cells is not clear, though it may possibly involve lymphotoxins and various other agents that may enter the target cell through the pores in plasma membranes and trigger the apoptotic death in target cells. Moreover, it has recently been suggested that changes in the target cell lysosomes are induced by cytotoxic T cells. Lysosomes would act as 'suicide capsules' causing target cell lysis from the inside.[39]

The exact mechanism of killing of tumor cells by LAK cells is not yet known. However, it is essential that LAK effector cells must physically bind to tumor cells in order to kill. Indeed, in the present study it was observed that treatment of PBMCs with IL-2 along with cisplatin/FK-565 enhances their binding to tumor cells as compared with LAK cells generated in the presence of IL-2 alone. This may be a possible mechanism involved in the up-regulation of LAK activity by cisplatin FK-565.

Conclusions

In conclusion our results show that generation of LAK cells in the presence of cisplatin/FK-565 together with IL-2 enhances their binding capacity to tumor cell as compared with LAK cells generated in the presence of IL-2 alone. Further, LAK and tumor cells interact by giving out interdigitating microvilli-like processes which are devoid of cellular organelles but contain a few ribosomes. The LAK cells also show a reorientation of cellular organelles in the presence of tumor cells. There are a few vesicle-like structures in the extracellular pockets formed between the conjugate pairs. These may contain the pore forming elements, as in CTL and NK cells. LAK cells generated in the presence of cisplatin/FK-565 plus IL-2 showed a closer interaction with target cells and a large number of vesicles in the extracellular space of the conjugate pair, which may explain the increased lysis of target cells by LAK cells generated in the presence of cisplatin/FK-565 plus IL-2 as compared with IL-2 alone.

Acknowledgements

We are grateful to Saburo Sone, MD, Third Department of Internal Medicine, University of Tokushima, Japan and to Fujisawa Pharmacuetical Company, Osaka, Japan for providing IL-2 and FK-565. We are also grateful to Defence Science Centre, DRDO, New Delhi, India for providing transmission electron microscope facilities. S.B. is a CSIR fellow.

References

1. Grimm EA, Mazumdar A, Zhang HZ, *et al.* Lymphokine activated killer cell phenomenon. I. Lysis of natural killer resistant fresh solid tumor cells by interleukin-2 activated autologous human peripheral blood lymphocytes. *J Exp Med* 1982; **155**: 1823–41.
2. Grimm EA, Ramsey KM, Mazumdar A, *et al.* Lymphokine activated killer cell phenomenon. II. The precursor cells are serologically distinct from peripheral T lymphocytes memory CTL and NK cells. *J Exp Med* 1983; **157**: 884–97.
3. Grimm EA, Robb RJ, Roth JA, *et al.* Lymphokine activated killer cell phenomenon. III. Evidence that IL-2 is sufficient for direct activation of peripheral blood lymphocytes into lymphokine activated killer cells. *J Exp Med* 1983; **158**: 1356–61.
4. Kedar E, Weiss DW. The *in vitro* generation of effector lymphocytes and their employment in tumor immunotherapy. *Adv Cancer Res* 1983; **38**: 171–287.
5. Rosenberg SA. Adoptive immunotherapy of cancer using lymphokine activated killer cells and interleukin-2. In: Devita VT, Hellman S, Rosenberg SA, eds. *Important Advances in Oncology*, New York: JP Lippincott 1986: 55–91.
6. Salup RR, Wiltrout RH. Adjuvant immunotherapy of established murine renal cancer by interleukin-2 stimulated cytotoxic lymphocytes. *Cancer Res* 1986; **46**: 3358–62.
7. Ferrini S, Miescher S, Zocchi MR, *et al.* Phenotypic and functional characterization of recombinant IL-2 induced activated killer cells: analysis at the population and clonal levels. *J Immunol* 1987; **138**: 1297.
8. Henkart PA, Yue CC, Yang J, *et al.* Cytolytic and biochemical properties of cytoplasmic granules of murine lymphokine-activated killer cells. *J Immunol* 1986; **137**: 2611–7.
9. Young JD-E, Liu C-C, Persechini PM, *et al.* Perforin dependent and independent pathways of cytotoxicity mediated by lymphocytes. *Immunol Rev* 1989; **103**: 61.
10. Wright SC, Wilbur SM, Bonavida B. Studies on the mechanism of natural-killer cell mediated cytotoxicity. VI. Characterization of human, rat and murine natural killer cytotoxic factors. *Natl Immunol Cell Growth Regul* 1985; **4**: 202.
11. Arancia G, Malorni W, Iosi F, *et al.* An SEM analysis of the interaction between lymphokine activated killer cells and tumor targets. *Int J Cancer* 1989; **4**: s58–s61.
12. Onoda JM, Jacobs JR, Taylor JD, *et al.* Cisplatin and nifedipine: synergistic cytotoxicity against murine solid tumors and their metastasis. *Cancer Lett* 1986; **35**: 181–9.
13. Shinkai T, Saino N, Tominago K, *et al.* Comparison of redisine plus cisplatin or redisine plus mitomycin in the treatment of advanced non small lung cancer. *Cancer Treat Rep* 1985; **69**: 945–51.
14. Hosokawa T, Ito H, Tanaka T, *et al.* Phase (II) trial with cisplatin in ovarian malignancies. *J Jap Soc Cancer Therap* 1982; **17**: 31–40.
15. Volge SE, Lenner H, Kaplan HB, *et al.* Mitomycin, methotrexate, bleomycin and *cis* DDP II in the chemotherapy of advanced squamous cancer of head and neck. *Cancer* 1982; **50**: 6–9.
16. Yap HY, Salem P, Hortobagyi CN, *et al.* Phase II study of *cis* dichlorodiammine platinum II. *Cancer Treat Rep* 1978; **62**: 405–11.
17. Sodhi A, Gupta P. Increased release of hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) by murine macrophages *in vitro* after cisplatin treatment. *Int J Immunopharmacol* 1986; **8**: 709–14.
18. Sodhi A, Singh SM. Release of cytolytic factor(s) by murine macrophages *in vitro* on treatment with cisplatin. *Int J Immunopharmacol* 1986; **8**: 701–07.
19. Sodhi A, Pai K, Singh RK, *et al. In vitro* activation of human NK cells and monocytes with cisplatin. *Int J Immunopharmacol* 1990; **12**: 893–8.
20. Kleinerman ES, Zwelling LA, Muchmore AV. Enhancement of naturally occuring human spontaneous monocyte mediated cytotoxicity by *cis*-dichlorodiammine platinum (II). *Cancer Res* 1980; **40**: 3099–102.
21. Sodhi A, Pai K, Singh SM, *et al.* Activation of human NK cells and monocytes with cisplatin *in vitro*. *Int J Immunopharmacol* 1990; **12**: 893–8.
22. Inamura N, Sone S, Okubo A, *et al.* Tumor cytotoxicity of human monocyte membrane bound IL-1 a induced by synergistic actions of interferon-Y and synthetic acyltripeptide FK-565. *Cancer Immunol Immunotherap* 1989; **28**: 1641–170.

23. Talmadge JE, Lenz B, Schneider M, *et al.* Immunomodulatory properties of FK-565 in mice. *Cancer Immunol Immunotherap* 1989; **28**: 93–100.
24. Watanabe Y, Tawara S, Mine Y, *et al.* Immunoactive peptides FK-156 and FK-565 activation of mouse macrophages. *J Antibiot Res (Tokyo)* 1985; **38**: 1781.
25. Basu S, Sodhi A, Singh SM, *et al.* Upregulation of IL-2 induced lymphokine activated killer (LAK) activity by FK-565 and cisplatin. *Immunol Lett* 1991; **27**: 199–204.
26. Boyum A. Isolation of mononuclear cells and granulocytes from human blood. *Scand J Lab Invest* 1968; **21(Suppl 97)**: 77–98.
27. Bonavida B, Bradley TP, Grimm EA. Frequency determination of killer cells by single cell assay in cell mediated cytotoxic assay. *Methods Enzymol* 1983; **93**: 270.
28. Sanderson CJ, Glauert AM. The mechanism of T cell mediated cytotoxicity. V. Morphological studies by electron microscopy. *Proc R Soc London (Biol)* 1977; **198**: 315–323.
29. Bykovskaja SN, Rytenko AN, Rausenbach MO, *et al.* Ultrastructural alteration of cytotoxic T lymphocytes following their interaction with target cells. I. Hypertrophy and change in orientation of golgi apparatus. *Cell Immunol* 1978; **40**: 164–174.
30. Bykovskaja SN, Rytenko AN, Rausenbach MO, *et al.* Ultrastructural alteration of cytotoxic T lymphocytes following their interaction with target cells. II. Morphogenesis of secretory granules and intracellular vacuoles. *Cell Immunol* 1978; **40**: 175–185.
31. Frey T, Petty HR, McConnell HM. Electron microscopic study of natural killer cell–tumor cell conjugates. *Proc Natl Acad Sci USA* 1982; **79**: 5317–21.
32. Ucker DS. Cytotoxic T lymphocytes and glucocorticoids activate an endogenous suicide process in target cells. *Nature* 1987; **327**: 62–4.
33. Malorni W, Sirianni MC, Fiorentim C, *et al.* Cytoskeletal changes in NK-K-562 pairs. In: Grignam F, Marlelli MF, Mason DY, eds. *Genotypic, phenotypic and functional aspects of heamatopoiesis.* New York: Raven Press 1987: 255–9.
34. Matter A. Microcinematographic and electron microscopic analysis of target cell lysis induced by cytotoxic T lymphocytes. *Immunology* 1979; **36**: 179–90.
35. Henkart PA. Mechanism of lymphocyte-mediated cytotoxicity. *Ann Rev Immunol* 1985; **3**: 31–58.
36. Hook GR, Greenwood MA, Barba D, *et al.* Morphology of interleukin-2 stimulated human peripheral blood mononuclear effector cell killing glioma-derived tumor cells *in vitro*. *J Natl Cancer Inst* 1988; **80**: 171–7.
37. Henkart PA, Yue CC, Yang J, *et al.* Cytolytic and biochemical properties of cytoplasmic granules of murine lymphokine activated killer cells. *J Immunol* 1986; **137**: 2611–617.
38. Bishop CJ, Whiting VA. The role of natural killer cells in the intravascular death of intravenously injected murine tumor cells. *Br J Cancer* 1983; **48**: 441–4.
39. Pitsillides AA, Taylor PM, Bitensky L, *et al.* Rapid changes in target cell lysosomes induced by cytotoxic T cell: indication of target suicide? *Eur J Immunol* 1988; **18**: 1203–8.

(Received 2 October 1991; revised version received 24 December 1991; accepted 2 January 1992)