Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 5317–5321, September 1982
Cell Biology

Electron microscopic study of natural killer cell–tumor cell conjugates

(cytotoxic mechanism/degranulation/lysosomal enzymes/immune surveillance)

TOM FREY*, HOWARD R. PETTY†, AND HARDEN M. MCCONNELL*‡

*Stauffer Laboratory for Physical Chemistry, Stanford University, Stanford, California 94305; and †Department of Biological Sciences, Wayne State University, Detroit, Michigan 48202

Contributed by Harden M. McConnell, June 4, 1982

ABSTRACT **Natural killer (NK) cells were obtained from C3H mouse spleens according to a modified version of the method of Kuribayashi *et al.* [Kuribayashi, K., Gillis, S., Dern, D. E. & Henney, C. S. (1981) *J. Immunol.* 126, 2321–2327]. These cells retain *in vitro* cytotoxicity against certain model tumor cell targets and appear homogeneous by morphological criteria. NK cells, YAC (tumor) cells, and NK cell–YAC cell conjugates have been examined with scanning (SEM) and transmission (TEM) electron microscopy. SEM experiments have shown that: (*i*) NK cells are large and possess various shapes in contrast to the YAC target cells which are smaller and round, (*ii*) YAC cells have uniformly distributed microvilli whereas the NK cell microvilli are most prominent in the area of effector-to-target contact, and (*iii*) in the absence of target cells, NK cell microvilli are found in a small number (usually 1–3) of cell surface locations. The region of NK cell–tumor cell contact has also been examined with TEM. The cells were stained with ruthenium red/OsO_4. The electron-dense ruthenium red/OsO_4 reaction product was consistently found in regions of close cell–cell contact, suggesting that carbohydrates were not completely cleared from areas of contact and that target and effector membranes do not fuse extensively. TEM observations indicate that NK cells have structurally unique granules. The granules are composed of at least two distinct compartments. The outer compartment contains the lysosome-associated enzymes acid phosphatase and inorganic trimetaphosphatase. No enzymatic activities have been found associated with the inner compartment. NK cells appear to degranulate when incubated with YAC cells. Under those circumstances, limited areas of the NK cytoplasm contain vacuole-like areas possessing granules and apparent granular debris. Degranulation appears to be involved in the cytotoxic function of NK cells.**

Natural killer (NK) cells have attracted considerable interest because of their possible roles in host resistance to infection and neoplasia (1). These cells can recognize and lyse tumorigenic cells without prior sensitization. This has led to the suggestion that these cells are involved in immune surveillance (2). Recent morphological evidence has associated NK cell activity with large granular lymphocytes in humans (3), rats (4), and mice (5). An extensive review has been published (6). Cytological studies of NK cells have been difficult due to the lack of pure cell populations or highly specific markers. The advent of new cloning and isolation procedures has accelerated research on this cell type (7, 8). NK cells selected and grown *in vitro* have been used in the electron microscopic study reported here.

MATERIALS AND METHODS

NK Cells. NK cells were prepared by a modification of the method of Kuribayashi *et al.* (8). Spleen cells from C3H mice were treated with anti-Thy-1.2 and rabbit complement (Cedarlane Laboratories, Hornby, ON) and plated at 5×10^6 viable cells per ml in spleen medium [Dulbecco's modified Eagle's medium with 10% fetal calf serum/2 mM glutamine/2 mM pyruvate/penicillin at 100 units/ml/streptomycin at 100 μg/ml/nonessential amino acids (GIBCO)/50 μM 2-mercaptoethanol] containing fibroblast interferon at 1,000 units/ml (Calbiochem). After 24 hr an equal volume of T-cell growth factor was added to the cultures. On day eight the viable cells were isolated and treated with a mixture of arsanilated anti-Lyt 1 and anti-Lyt 2 monoclonal antibodies (Becton Dickinson, Mountain View, CA), antiarsanilate antibodies, and complement. The cells were recultured in spleen medium and T-cell growth factor (1:1). When growth was established the parent cells were subcultured and the most lytic subculture was expanded. Samples were prepared on day 12 of culture, immediately before subculture and after expansion, and showed essentially identical characteristics.

T-Cell Growth Factor. The T-cell growth factor was prepared as a supernate of concanavalin A-stimulated spleen cells (9). A single cell suspension was prepared from spleens of DBA/2 retired breeders, diluted to 2×10^7 cells per ml in spleen medium containing concanavalin A at 20 μg/ml, and incubated for 2 hr at 37°C. The concanavalin A was then removed by several washes in balanced salt solution and the cells were resuspended at 2×10^7 cells per ml and incubated for 20 hr. Supernates were removed and used without further purification.

Cytotoxic Assay. Target cells were labeled by incubation with ^{51}Cr in the form of sodium chromate. Labeled targets (10^4 per 200-μl sample) were incubated with varying numbers of effectors for 4 hr and half of the supernate was counted. Total release was assessed by adding 1% Nonidet P-40 (Particle Data Laboratories, Elmhurst, IL). Spontaneous release was determined in wells with no effectors. The percentage of specific release is defined as: (cpm in sample − spontaneous release)/(total release − spontaneous release) × 100.

Scanning Electron Microscopy (SEM). The cell preparations were fixed in suspension with 1% glutaraldehyde in 0.1 M cacodylate buffer for 1 hr at room temperature. The cells were then washed in cacodylate buffer and postfixed for 1 hr in 1% OsO_4 in cacodylate buffer. The cells were dehydrated with a series of increasing concentration of ethanol in water. At least three changes in absolute ethanol were then made. The cells were critical-point dried from liquid CO_2 in baskets constructed from BEEM capsules as described (10). Solvent-resistant Nucleopore filters (Pleasanton, CA) with a pore size of 0.2 μm were attached

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: NK, natural killer; SEM, scanning electron microscopy; TEM, transmission electron microscopy; acid Pase, acid phosphatase; cP_3ase, inorganic trimetaphosphatase (cyclic triphosphatase).

‡To whom reprint requests should be addressed.

at one end. Cells were dispersed on specimen stubs coated with double-stick tape followed by sputter-coating with gold/palladium. The samples were examined with an ISI-40 scanning electron microscope.

Transmission Electron Microscopy (TEM). Cells were fixed for 1 hr with 1% glutaraldehyde in 0.1 M sodium cacodylate/HCl buffer (pH 7.4) containing 2% sucrose. The cells were then washed four times with cacodylate buffer containing 5% sucrose. In some cases the cells were cytochemically stained as described below. Cells were then postfixed with 1% OsO_4 in 0.1 M cacodylate buffer for 1 hr at room temperature. The samples were dehydrated in ethanol and embedded in Spurr's resin (11). The blocks were thin-sectioned and examined in a Hitachi HU-11E or Philips 301 electron microscope.

Cytochemical Procedures. Acid phosphatase (acid *P*ase) was stained by using modified Gormori's medium (12). The composition of the medium was: 13.9 mM Na β-glycerophosphate/1 mM $Pb(NO_3)_2$/0.05 M acetate buffer, pH 5.4/0.08% $CaCl_2$/5% sucrose. Inorganic trimetaphosphatase (cP_3ase) was stained with Berg's trimetaphosphatase medium as described by Doty *et al.* (13). These mixtures were filtered before use. The incubations were carried out at 37°C for 60 min with agitation. In some cases the cells were washed four times with buffer followed by treatment with 1% $(NH_4)_2S$ for 2 min. The cells were then washed four times with buffer.

The cell surfaces were stained with ruthenium red as de-

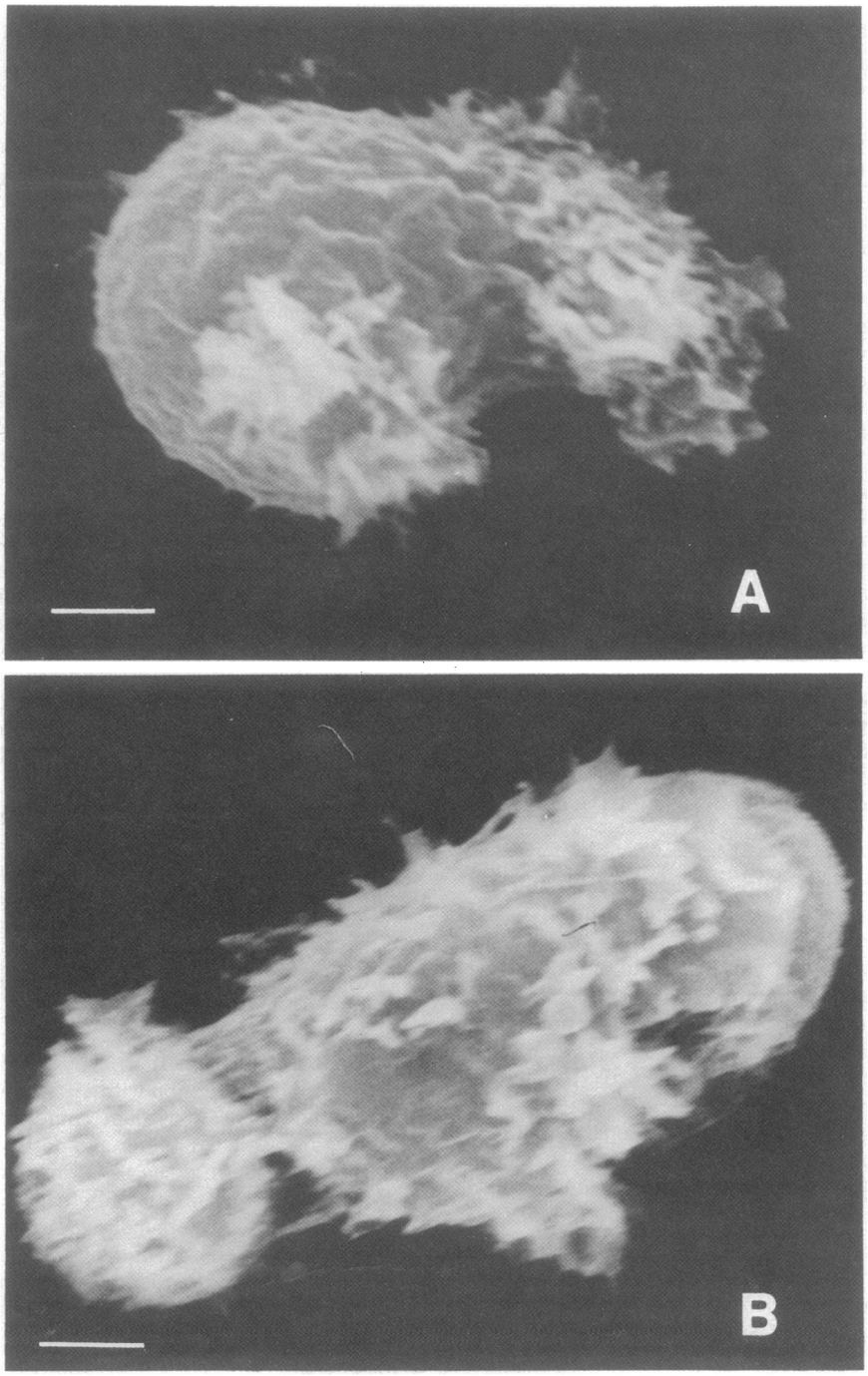

FIG. 1. (*A*) SEM of murine NK cell. The asymmetric morphology is characteristic of these cells. Bar = 2 μm. (*B*) SEM of a NK cell–YAC cell conjugate. The intercellular region possesses numerous microvilli. Bar = 2 μm.

scribed (14). Briefly, the glutaraldehyde-fixed cells were incubated in cacodylate buffer containing 0.1% ruthenium red for 30 min at room temperature. The cells were then pelleted and resuspended in buffer containing 0.1% ruthenium red and 1% OsO_4 for 1 hr at room temperature.

RESULTS

NK Cells. The cells used in these experiments are Thy-1^+, Lyt-1^-, and Lyt-2^- by second-step immunofluorescence and are large granular lymphocytes as judged by light and electron microscopy. Immediately after subculture they caused 64% specific lysis of YAC tumor targets at an effector-to-target ratio of 4.5:1. After expansion they were still capable of 15–20% lysis at 5:1 effector-to-target ratios. In all cases ≤3% lysis of P815 targets was observed. Overnight incubation of the NK cell cultures with fibroblast interferon at 1,000 units/ml decreases the number of NK cells required to kill a given number of target cells by a factor between two and four.

SEM. Glutaraldehyde fixation was carried out with cells in suspension. In Fig. 1*A* we show a representative micrograph of a NK cell. The particular cell shown above has a somewhat elongated and curved shape. Although round cells are sometimes found, the asymmetric morphology is typical of our NK cells. Microvilli tend to be associated with a small number of locations on the cell surface. The YAC tumor cell line has also been studied with SEM. These cells are substantially smaller than NK cells. They are round and possess uniformly distributed microvilli (data not shown). These morphological differences allow these cell types to be distinguished in SEM experiments.

NK cells and tumor cells were incubated together at 10^7 cells per ml for 15 min at 37°C followed by fixation. Conjugates were then examined with SEM. NK cell microvilli were observed in the area of YAC cell contact (Fig. 1*B*). In most cases numerous microvilli can be found between cells.

TEM. The structures of NK cells, YAC cells, and NK cell–YAC cell conjugates were examined by using thin-section TEM. The NK cell–tumor cell interface is rich in microvilli. However, the SEM does not have sufficient resolution to distinguish the tips of the microvilli from tumor cell surface structures. This interface was examined with TEM. The ruthenium red/OsO_4 method specifically stains cell surface carbohydrates

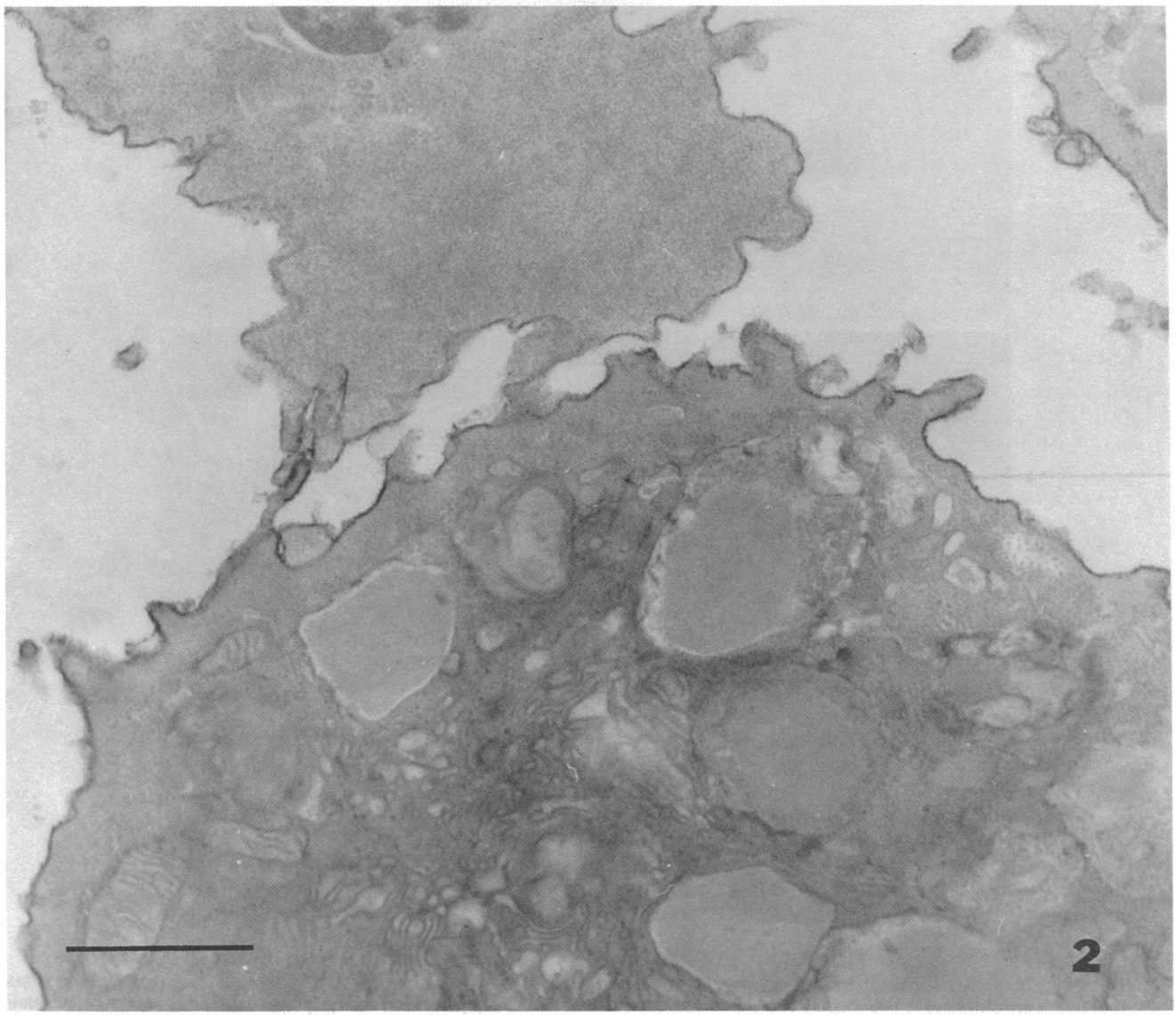

FIG. 2. TEM of NK cell–YAC cell conjugate stained with ruthenium red. The stain can be found in regions of close cell–cell contact. The numerous granules, mitochondria, and Golgi can be seen in this micrograph. Bar = 1 μm.

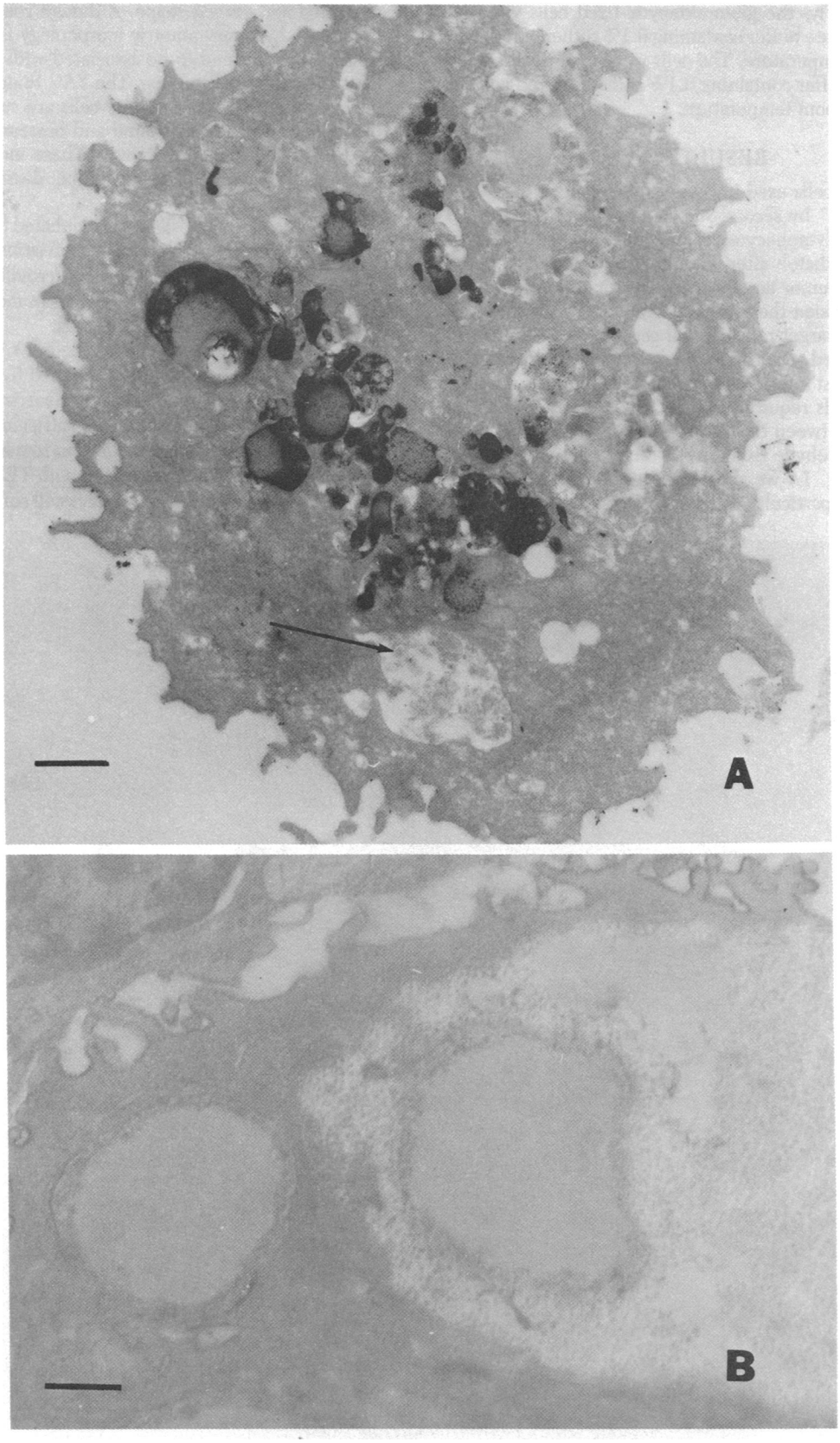

FIG. 3. (*A*) TEM of a degranulating NK cell (one vacuole containing granular debris is indicated by arrow). The cells were stained with cP_3ase. The NK granules are composed of two roughly concentric compartments. The outer compartment is the primary site of cP_3ase activity (extremely dark staining). Similar results have been obtained with acid *P*ase. Bar = 1 μm. (*B*) TEM of NK granules and a portion of an area of apparent degranulation. A granule and granular debris can be seen in the vacuole-like area. The ruthenium stain is seen only on the outer plasma membranes separating the NK cell and the YAC target (upper left). Bar = 0.5 μm.

(14). In Fig. 2 we show a NK cell–YAC cell conjugate stained with ruthenium red. The granules of the NK cell are evident. A YAC cell can be seen in close juxtaposition to the NK cell. The surfaces of both cells are lightly stained with ruthenium red. In areas of especially close contact, reaction product can be seen between the cell surfaces. This shows that carbohydrates are present in these areas.

The NK cells possess unique granules. The granules seen in these cells are virtually identical to those seen by Brooks *et al.* (15). These granules have two distinct, concentric compartments. The inner compartment has a moderate electron density. It remains largely undefined at this time. We have found that two lysosome-associated enzymes—acid *P*ase and cP_3ase—are present in the outer region of these granules. Fig. 3*A* shows a transmission electron micrograph of a NK cell stained for cP_3ase. The reaction product can be seen along the outer portion of the granules. This cell had been incubated with YAC cells. The cell is apparently in an early state of granule fusion with the vacuoles. A later stage of this process is shown in *B*. A granule and a region of apparent degranulation are shown. When NK cells are incubated in the absence of YAC cells, no vacuoles of this type are found (data not shown). Evidently vacuole formation and release of granules with their associated hydrolytic enzymes require the presence of tumor cells. These cells were stained with ruthenium red. The ruthenium red reaction product is noticeably absent in the vacuole-like regions of diminished electron density. This suggests that these regions are either not in direct communication with the plasma membrane or are devoid of carbohydrate moieties (or both).

DISCUSSION

In this report we have described several subcellular features of NK cells and conjugates of NK cells and YAC tumor cells. While the present manuscript was in preparation, Brooks *et al.* (15) reported NK cells very similar to those described here. They were prepared by essentially identical techniques. Brooks *et al.* find some Lyt-2^+ clones. Cells reported here are Thy-1^+ (although pretreated with anti-Thy-1) and Lyt-2^-. These cells do not strongly resemble those suggested to be NK cells found in less purified populations (see ref. 16, for example).

Certain subcellular features of the NK are modified when YAC targets are present. The formation of cytoplasmic vacuoles containing granules and debris in NK cells is observed only in the presence of tumor cell targets. This cytoplasmic response may be analogous to degranulation of other cell types. For example, mast cell activation is accompanied by the development of cytoplasmic vacuoles. These areas form a labyrinth structure that ultimately leads to the extracellular environment (17). Quan *et al.* (18) have demonstrated marked similarities beween NK-mediated lysis and histamine release by mast cells. The requirement for an intact secretory apparatus has been indicated for the lytic mechanism of human NK cells (19). Also, Neighbour and Huberman have reported that Sr^{2+}-induced loss of granules in human and mouse NK cells is correlated with a loss of cytotoxicity (20). Taken with the results presented here, these data strongly implicate degranulation in the NK cell's tumoricidal mechanism.

There are differences between the mast cell and NK cell systems. The mast cell contains many more granules than does the NK cell. The vacuole-like structures of the mast cell are in communication with the plasma membrane. As far as we can tell the vacuoles of the NK cell are not.

The NK cell granules are interesting structures. There are two distinct compartments within the granule. The granule matrix contains the enzymes cP_3ase and acid *P*ase. This is similar to the structure of eosinophil granules (21). The specific granules of the eosinophil contain two compartments. The enzymes acid *P*ase, arylsulfatase, and peroxidase are located in the matrix (outer compartment) of these granules (22, 23). The core or "internum" material is made of a crystalloid structure that contains a major basic protein (24). We have not observed crystallized material within the NK cell granules. The core of this granule is almost featureless. During degranulation the core of the granule remains in the vacuole for some time after the matrix has disappeared, indicating that dissolution is a slow process. The correlation between granule structure and function in tumor cell cytotoxicity is a critical point awaiting further investigation. The present work as well as recent work by others (19, 20) supports a direct involvement of NK cell granules in target cell killing. Studies of the enzymatic action of NK cell granules will be of interest in this connection. NK cell microvilli are involved in binding NK cells to their tumor targets. The limited number of locations of these microvilli on the surfaces of resting NK cells (absence of target cells) suggests specialization of these regions for target recognition.

There is much current interest in the use of reconstituted membranes to study recognition functions of cellular components of the immune system. The availability of morphological criteria for binding and triggering—such as those presented here—should facilitate such studies.

This work has been funded by a grant from the National Institutes of Health (AI 13587) to H.M.McC. and a grant from the Michigan Cancer Foundation to H.R.P. A portion of this study was conducted while H.R.P. was a Fellow in Cancer Research of the Damon Runyon–Walter Winchell Cancer Fund (DRG-396F).

1. Kiessling, R. & Haller, O. (1978) *Contemp. Top. Immunobiol.* **8**, 171–201.
2. Herberman, R. B. & Holden, T. H. (1978) *Adv. Cancer Res.* **27**, 305–377.
3. Timonen, T., Saksela, E., Ranki, A. & Häyry, P. (1979) *Cell. Immunol.* **48**, 133–148.
4. Herberman, R. B., Timonen, T., Reynolds, C. & Ortaldo, J. R. (1980) in *Natural Cell-Mediated Immunity Against Tumors*, ed. Herberman, R. B. (Academic, New York), pp. 89–104.
5. Luini, W., Boraschi, D., Alberti, S., Aleotti, A. & Tagliabue, A. (1981) *Immunology* **43**, 663–668.
6. Herberman, R. B., ed. (1980) *Natural Cell-Mediated Immunity Against Tumors* (Academic, New York).
7. Dennert, G. (1980) *Nature (London)* **287**, 47–49.
8. Kuribayashi, K., Gillis, S., Dern, D. E. & Henney, C. S. (1981) *J. Immunol.* **126**, 2321–2327.
9. Spiess, P. J. & Rosenberg, S. A. (1981) *J. Immunol. Methods* **42**, 213–222.
10. Petty, H. R. (1980) *Exp. Cell Res.* **128**, 439–454.
11. Spurr, A. R. (1969) *J. Ultrastruct. Res.* **26**, 31–43.
12. Bainton, D. F. & Golde, D. W. (1978) *J. Clin. Invest.* **61**, 1555–1569.
13. Doty, S. B., Smith, C. E., Hand, A. R. & Oliver, C. (1977) *J. Histochem. Cytochem.* **25**, 1381–1384.
14. Luft, J. H. (1971) *Anat. Rec.* **171**, 347–368.
15. Brooks, C. G., Kuribayashi, K., Sale, G. E. & Henney, C. S. (1982) *J. Immunol.* **128**, 2326–2335.
16. Shimamura, K., Habu, S., Fukui, H., Akatsuka, A., Okumura, K. & Tamaoki, N. (1982) *J. Natl. Cancer Inst.* **68**, 449–455.
17. Lagunoff, D. (1972) *J. Histochem. Cytochem.* **20**, 938–944.
18. Quan, P.-C., Ishizaka, T. & Bloom, B. (1982) *J. Immunol.* **128**, 1786–1791.
19. Carpen, O., Virtanen, I. & Saksela, E. (1981) *Cell. Immunol.* **58**, 97–106.
20. Neighbour, P. A. & Huberman, H. S. (1982) *J. Immunol.* **128**, 1236–1240.
21. Miller, F., DeHarven, E. & Palade, G. E. (1966) *J. Cell Biol.* **31**, 349–362.
22. Seeman, P. M. & Palade, G. E. (1967) *J. Cell Biol.* **34**, 745–756.
23. Bainton, D. F. & Farquhar, M. G. (1970) *J. Cell Biol.* **45**, 54–73.
24. Beeson, P. B. & Bass, D. A. (1977) *The Eosinophil* (Saunders, Philadelphia).