

CLOINED MOUSE CELLS WITH NATURAL KILLER FUNCTION
AND CLOINED SUPPRESSOR T CELLS EXPRESS
ULTRASTRUCTURAL AND BIOCHEMICAL FEATURES
NOT SHARED BY CLOINED INDUCER T CELLS*

BY ANN M. DVORAK, STEPHEN J. GALLI, JAMES A. MARCUM,‡
GARY NABEL, HAROUT DER SIMONIAN, JEROLD GOLDIN,
RITA A. MONAHAN, KATHRYN PYNE, HARVEY CANTOR,
ROBERT D. ROSENBERG, AND HAROLD F. DVORAK

From the Departments of Pathology, Medicine, and Cell Biology, Harvard Medical School; Departments of Pathology, Medicine, and the Charles A. Dana Research Institute, Beth Israel Hospital; the Sidney Farber Cancer Institute, Boston, Massachusetts 02215; and Whitaker College of Health Science, Technology and Management, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

We have previously described cloned populations of mouse leukocytes specialized to perform different immunologic activities (1-9). According to their function and surface glycoprotein expression, individual clones represent antigen-specific suppressor T cells (2, 3) inducer T cells (4, 5), or mast cells (5, 6). Another clone (7-9) mediates natural killer (NK)¹ cell activity and antibody-dependent cell-mediated cytotoxicity (ADCC) and also expresses plasma membrane receptors (Fc_cR) that bind IgE with high affinity. These clones are phenotypically stable and can be expanded to >10⁸ cells in vitro. In addition to facilitating studies of leukocyte function, these cloned lines constitute a unique resource for examining the morphology and biochemistry of leukocyte subsets.

In this report, we define a constellation of ultrastructural, cytochemical, and biochemical features expressed by both a cloned line with NK activity (Cl.Ly-1⁻2⁻NK-1⁺/11) and cloned suppressor T cells (Cl.Ly-2⁺3⁺/4), notably osmiophilic, chondroitin sulfate-rich cytoplasmic granules similar to those of mouse basophils (8, 10, 11). Cloned inducer T cells (Cl.Ly-1⁺2⁻/9) lack cytoplasmic granules and also differ from the other clones in ability to take up serotonin and in ultrastructural localization of nonspecific esterase activity. We suggest that the features defined here, when considered together with patterns of surface glycoprotein expression and immunologic activity, may help elucidate questions of leukocyte ontogeny and function.

* Supported by U. S. Public Health Service grants CA 28834, AI 16925, GM 07753, AI 12184, and HL 28625.

‡ Supported by American Heart Association Fellowship 13-420-801.

¹ Abbreviations used in this paper: ADCC, antibody-dependent cell-mediated cytotoxicity; ANAE, alpha naphthyl acetate esterase; Con A, concanavalin A; Con A-CM, modified Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal calf serum, 5 × 10⁻⁵ M 2-mercaptoethanol, and 2 mM glutamine (DME-FCS) supplemented with 20% (vol/vol) DME-FCS conditioned by Con A-activated BALB/c spleen cells; CTL, cytolytic T lymphocyte; Fc_cR, plasma membrane receptors for IgE; 5HT, 5-hydroxytryptamine-[1,2³H-(N)] binoxalate; HMEM-FCS, Hanks' minimal essential medium supplemented with PIPES buffer (470 mg/liter) and 10% fetal calf serum; LGL, large granular lymphocyte; NK, natural killer; OCUB, osmium-collidine uranyl en bloc; OPF, osmium-potassium ferrocyanide; TEM, transmission electron microscopy.

Materials and Methods

Cells

MOUSE BONE MARROW CELLS. Adult (12-16 wk) C57BL/6 mice were killed by cervical dislocation, and hematopoietic bone marrow was flushed from the femurs with phosphate-buffered saline containing bovine serum albumin (0.1 mg/ml). Nucleated marrow cells were fixed for electron microscopy immediately (see below) or after overnight culture in medium supplemented with supernatants of concanavalin A (Con A)-stimulated splenocytes (10).

CLONED CELLS. The glycoprotein phenotype, Fc_εR expression, and function of cloned mouse cells with NK activity (7-9), cloned Ly-1⁻2⁺3⁺ suppressor T cells (2, 3), cloned Ly-1⁺2⁻ inducer T cells (4), and cloned mast cells (5, 6) have been described in detail and are summarized in Table I.

For the experiments reported here, Cl.Ly-1⁻2⁻NK-1⁺/11, Cl.Ly-2⁺3⁺/4, and Cl.Ly-1⁺2⁻/9 were maintained at 37°C in a humidified atmosphere of 5% CO₂-95% air in modified Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal calf serum, 5 × 10⁻⁵ M 2-mercaptoethanol, and 2 mM glutamine (DME-FCS) supplemented with 20% (vol/vol) DME-FCS conditioned by Con A-activated BALB/c spleen cells (Con A-CM).

Transmission Electron Microscopy (TEM), Autoradiography, and Cytochemistry. The fixation and processing of cells for TEM has been described in detail (12, 13). Briefly, cells were fixed for 1 h at room temperature in a sevenfold volume excess of 1% paraformaldehyde, 1.25% glutaral-

TABLE I
Comparison of Function and Surface Structures of Cloned Cells

| Functional designation | Cells | | | |
|--|---|---|---|----------------|
| | Cl.Ly-1 ⁻ 2 ⁻ NK-1 ⁺ /11* 11* | Cl.Ly-2 ⁺ 3 ⁺ /4‡ 4‡ | Cl.Ly-1 ⁺ 2 ⁻ /9§ 9§ | Cl.MC/9 9 |
| | NK cells | Suppressor T cell | Inducer T cell | Mast cell |
| Function | | | | |
| Phagocytosis of IgG-coated RBC | - | - | - | - |
| Lysis of YAC-1 | + | - | - | - |
| ADCC | + | - | - | - |
| Antigen-specific suppressive activity | - | + | - | - |
| Activation of Ig secretion by B cells | - | - | + | - |
| Stimulation of granulocyte-macrophage colony formation and proliferation of cloned T cells and cloned mast cells | - | - | + | - |
| Surface Structure | | | | |
| Ig | - | - | - | - |
| Thy-1 | + | + | + | - |
| Ly-1 | - | ± | + | - |
| Ly-2 | - | + | - | - |
| Ly-3 | - | + | - | - |
| Ly-5 | + | + | + | + |
| NK-1 | + | - | - | - |
| Qat-5 | + | - | - | + |
| Fc _ε R | + | - | - | + |

* Refs. 2, 4, 7, 8.

‡ Refs. 2-4.

§ Refs. 2, 4, 8.

|| Refs. 5, 6, 8.

dehydrate, 0.025% CaCl_2 in 0.1 M sodium cacodylate buffer, pH 7.4, and were centrifuged through soft 2% Difco agar (Difco Laboratories, Detroit, MI) in a microfuge. Agar-embedded pellets were postfixed for 2 h in 2% collidine-buffered osmium tetroxide at 20°C, dehydrated in a graded series of alcohols, and embedded in a propylene oxide-Epon sequence. Some specimens were stained en bloc with uranyl acetate before dehydration (OCUB technique). For demonstration of glycogen, other samples were postfixed for 2 h in 2% aqueous osmium tetroxide and 1.5% potassium ferrocyanide in 0.1 M sodium phosphate buffer, pH 6.0 (OPF technique), before dehydration and embedding as described above.

For autoradiography, $^{35}\text{SO}_4$ -labeled cells (see below) were fixed and processed by the OPF technique as above. Thin Epon sections were cut with an LKB 5 ultratome (LKB Instruments, Inc., Rockville, MD), placed on copper grids, covered with Ilford L-4 emulsion (Polysciences, Inc., Warrington, PA), exposed for up to 4 mo under desiccated conditions at 4°C, developed with Microdol X (Eastman Kodak Co., Rochester, NY), and stained lightly with lead citrate. Sections were examined in a Philips 400 electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ).

Cells fixed for 1 h were examined for peroxidase activity as previously described (13). For demonstration of alpha naphthyl acetate esterase (ANAE) activity, cells were fixed for 15 min, then processed as previously described (14, 15).

Labeling of Cells with $^{35}\text{SO}_4$. Cloned cells (5×10^6) were labeled overnight (12–18 h) with 1 mCi sodium [^{35}S]sulfate (NEX-041; New England Nuclear, Boston, MA) at 37°C in 5 ml Con A-CM prepared with DME lacking unlabeled SO_4 . After labeling, cells were washed three times in DME, an aliquot was taken for TEM autoradiographs, and the remainder was stored at -70°C for biochemical analysis.

Glycosaminoglycans are long-chain polysaccharides consisting of repeating disaccharide units. In mast cell granules, glycosaminoglycans are linked to a protein core (16, 17) to form proteoglycans. The ^{35}S -labeled glycosaminoglycans of cloned cells were characterized by DEAE chromatography and sequential hydrolysis with highly purified mucopolysaccharidases of established substrate specificities. Thawed aliquots of frozen ^{35}S -labeled cells were heated in boiling water for 5 min to inactivate endogenous degradative enzymes (18), and then exhaustively digested with pronase P, which solubilized >90% of incorporated [^{35}S]sulfate. Glycosaminoglycans were chromatographed on DEAE-Sephadex A-25 (0.8- × 6.9-cm column) previously equilibrated with 0.15 M NaCl, 0.01 M Tris, pH 7.5. Fractions were eluted in 0.5-ml volumes with a two-stage linear gradient of NaCl (0.15–2.0 M to 2.0–4.0 M) (19). Glycosaminoglycans were then incubated with specific mucopolysaccharidases and the products were chromatographed on Sephadex G-25 (0.6- × 24.5-cm column) equilibrated with 0.15 M NaCl, 0.01 M Tris, pH 7.5. The flow rate was 8 ml/h and the fraction volume was 0.3 ml.

Chondroitin AC and ABC lyases (Miles Laboratories, Inc., Elkhart, IN) (20) and heparinase purified from *Flavobacterium* (21) were used according to published methods (20, 21). National Institutes of Health glycosaminoglycan reference standards were generously provided by J. A. Cifonelli, University of Chicago, Chicago, IL. Nonlabeled glycosaminoglycans and degradation products were detected spectrophotometrically by the carbazole method of Bitter and Muir (22) with glucuronolactone as the standard.

Uptake of [^3H]5-hydroxytryptamine. We determined whether cloned cells could concentrate 5-hydroxytryptamine from the external medium by using a modification of the method of Mazingue et al. (23). Cells were removed from Con A-CM by centrifugation, washed once in Hanks' minimal essential medium supplemented with piperazine-N-N'-bis(2-ethane sulfonic acid) buffer (470 mg/liter) and 10% fetal calf serum (HMEM-FCS), and then resuspended in HMEM-FCS. Cells (generally $1-2 \times 10^5$ in 200 μl) were prewarmed to 37°C and added to 1.5-ml polypropylene tubes containing 2 μCi of [^3H]5-hydroxytryptamine-[1,2- ^3H -(N)] binoxalate (5 Ci/mmol; New England Nuclear) (5HT) in 50 μl HMEM-FCS at 37°C. Incubation was carried out at 37°C. To determine nonspecific uptake of 5HT, aliquots of cells and [^3H]5HT were incubated in HMEM-FCS at 4°C. After 1 h at 37 or 4°C, the cells were washed three times at 4°C with 1 ml HMEM-FCS, the pellets were resuspended in Aquasol (New England Nuclear), and cell-associated ^3H disintegrations per minute (dpm) were measured in a liquid scintillation counter. Specific uptake of [^3H]5HT was calculated according to the formula: specific uptake dpm = dpm at 37°C – dpm at 4°C.

Results

Ultrastructure and Cytochemistry. Cl.Ly-1⁻2⁻NK-1⁺/11 was 1 of 10 clones with NK function, all of which contained prominent cytoplasmic granules by light microscopy. Cl.Ly-2⁺3⁺/4 was one of two cloned cytotoxic suppressor T lymphocytes that contained cytoplasmic granules. As previously reported (8), the morphology of Cl.Ly-1⁻2⁻NK-1⁺/11 cells (Fig. 1) was similar to that of basophilic myelocytes. The cells had an abundant cytoplasm and an eccentrically located, round, reniform, or bilobed nucleus with a prominent nucleolus. The chromatin was dispersed, with occasional

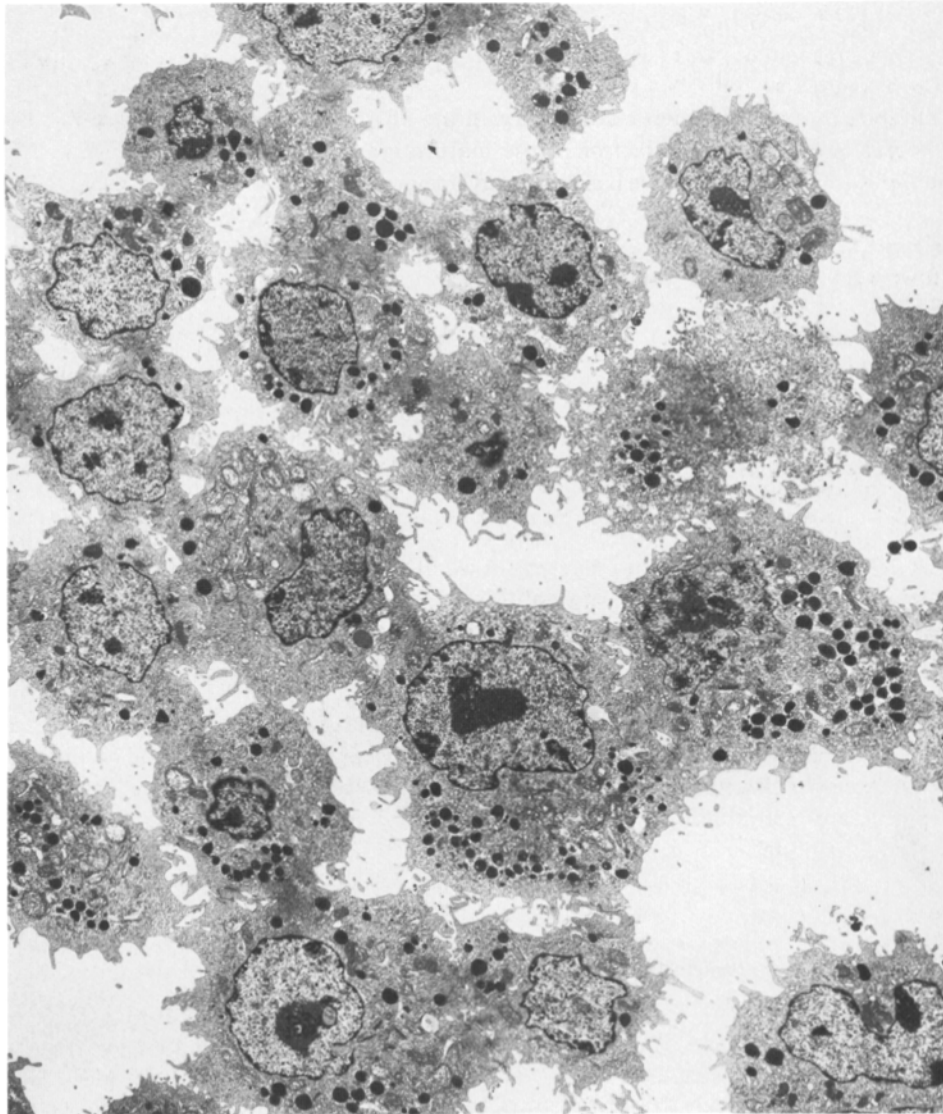


FIG. 1. Low-magnification electron micrograph of Cl.Ly-1⁻2⁻NK-1⁺/11. The cells contain numerous, large, electron-dense cytoplasmic granules. The lobular nuclei have dispersed chromatin and contain large nucleoli. The surfaces display irregular blunt processes. 2,000X.

aggregates beneath the nuclear membrane. The cell surface displayed irregular, broad, pointed, or blunt processes. The most striking ultrastructural feature was numerous, large electron-dense cytoplasmic granules. The cytoplasm also contained abundant, rough endoplasmic reticulum, often with dilated cisternae (Fig. 2). Routine processing for TEM (Fig. 2A) rendered the cytoplasmic granule matrix uniformly electron dense. Processing by the OPF technique revealed abundant cytoplasmic deposits of particulate glycogen (Fig. 2B).

Lymphocytes, monocytes/macrophages, basophils, and other granulocytes express distinctive and different ultrastructural patterns of nonspecific ANAE activity (14, 15). Cl.Ly-1⁻2⁻NK-1⁺/11 cells had ANAE reactivity on the cell surface, a reaction that was particularly intense on more immature cells (Fig. 3A). Cl.Ly-1⁻2⁻NK-1⁺/11 also displayed strong ANAE reactivity within cytoplasmic vesicles and in a vesicle-rich zone immediately beneath the membrane of immature granules (Fig. 3A). By contrast, homogeneously electron dense mature granules were ANAE negative. Although the granules of mouse basophilic myelocytes (Fig. 3B) resemble those of Cl.Ly-1⁻2⁻NK-1⁺/11 by routine TEM, mouse basophils account for <0.3% of nucleated cells in C57BL/6 bone marrow (10), and their ANAE reactivity has not been defined. However, among guinea pig leukocytes, the pattern of reactivity expressed by Cl.Ly-1⁻2⁻NK-1⁺/11 was demonstrated only in basophilic myelocytes (14; A. M. Dvorak, unpublished data). Cl.Ly-1⁻2⁻NK-1⁺/11 displayed neither the multiple cytoplasmic vesicle clusters noted in macrophages (15) nor the single cytoplasmic vesicle cluster typical of guinea pig and human lymphocytes (15). Cl.Ly-1⁻2⁻NK-1⁺/11 lacked peroxidase by ultrastructural cytochemistry. The ultrastructural features and ANAE reactivity of Cl.Ly-1⁻2⁻NK-1⁺/11 cytoplasmic granules are shown at a higher power in Fig. 4.

The ultrastructure of Cl.Ly-2⁺3⁺/4 (Fig. 5) was very similar to that of Cl.Ly-1⁻2⁻NK-1⁺/11 and basophilic myelocytes (Fig. 6). Cl.Ly-2⁺3⁺/4 expressed a pattern of ANAE reactivity similar to that of Cl.Ly-1⁻2⁻NK-1⁺/11, contained cytoplasmic deposits of particulate glycogen, and lacked peroxidase activity. Like Cl.Ly-1⁻2⁻NK-1⁺/11, Cl.Ly-2⁺3⁺/4 incorporated ³⁵SO₄ into its mature and immature cytoplasmic granules (Fig. 5B).

To determine whether the synthesis of basophil-like granules was a property of all murine lymphocyte clones, we examined two representative mouse inducer T lymphocyte clones. Inducer T cells (Fig. 7) lacked cytoplasmic granules and glycogen deposits, and contained ANAE-positive cytoplasmic vesicle clusters similar to those reported in peripheral blood lymphocytes of other mammalian species (15). Cl.Ly-1⁺2⁻/9 cells also had numerous ANAE-positive, non-membrane-bound cytoplasmic lipid bodies.

Sulfated Glycosaminoglycan Synthesis

CELLULAR LOCALIZATION OF INCORPORATED [³⁵S]SULFATE. TEM autoradiography localized silver grains to the cytoplasmic granules of both Ly-1⁻2⁻NK-1⁺/11 and Ly-2⁺3⁺/4 (Fig. 5B). The majority of cells were labeled; the most intense labeling was associated with cells exhibiting the greatest degree of cytoplasmic granule maturation. By contrast, Cl.Ly-1⁺2⁻/9 cells incorporated little or no ³⁵SO₄.

CHARACTERIZATION OF [³⁵S]GLYCOSAMINOGLYCANS. Assessment of the relative charge of the Cl.Ly-1⁻2⁻NK-1⁺/11 [³⁵S]glycosaminoglycans, as determined by chro-

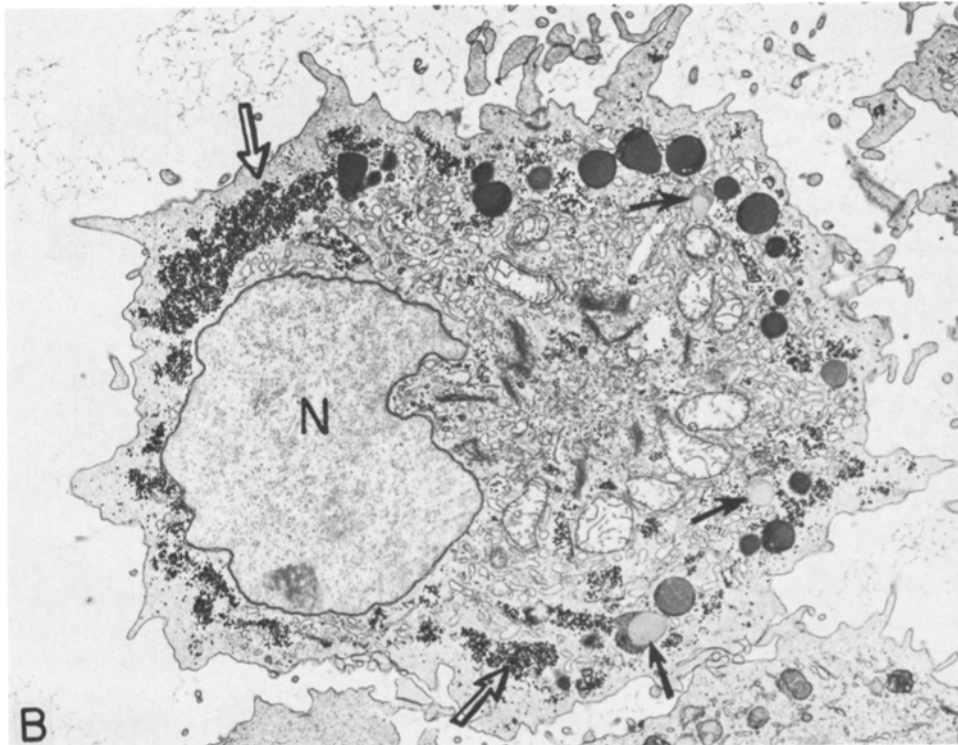
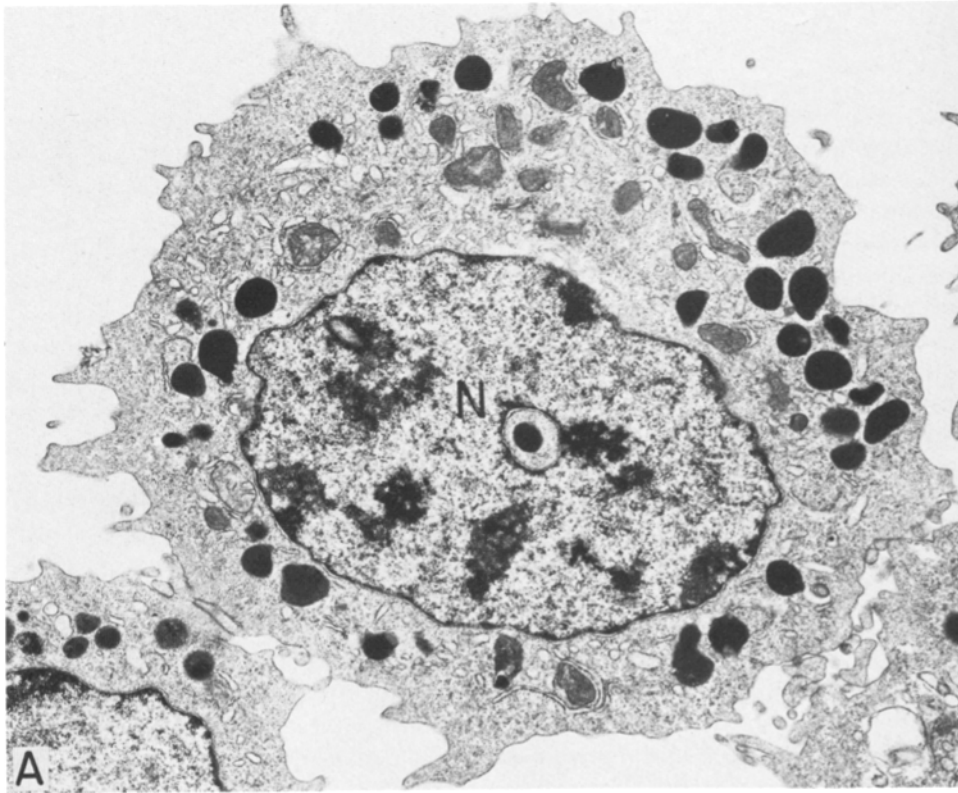


FIG. 2. Cl.Ly-1⁻2⁻NK-1⁺/11 cells after processing by the OCUB (A) or OPF (B) techniques. In A, the typical round cytoplasmic granules appear uniformly dense. The cytoplasm contains mitochondria and dilated rough endoplasmic reticulum (RER). In B, the lobular nucleus (N) is located eccentrically adjacent to the central Golgi area. The cytoplasmic granules appear moderately dense to nearly lucent (arrows), and contain a more dense outer rim. Large amounts of glycogen are present (open arrows). The RER is abundant and expanded. (A) 7,500X. (B) 6,000X.

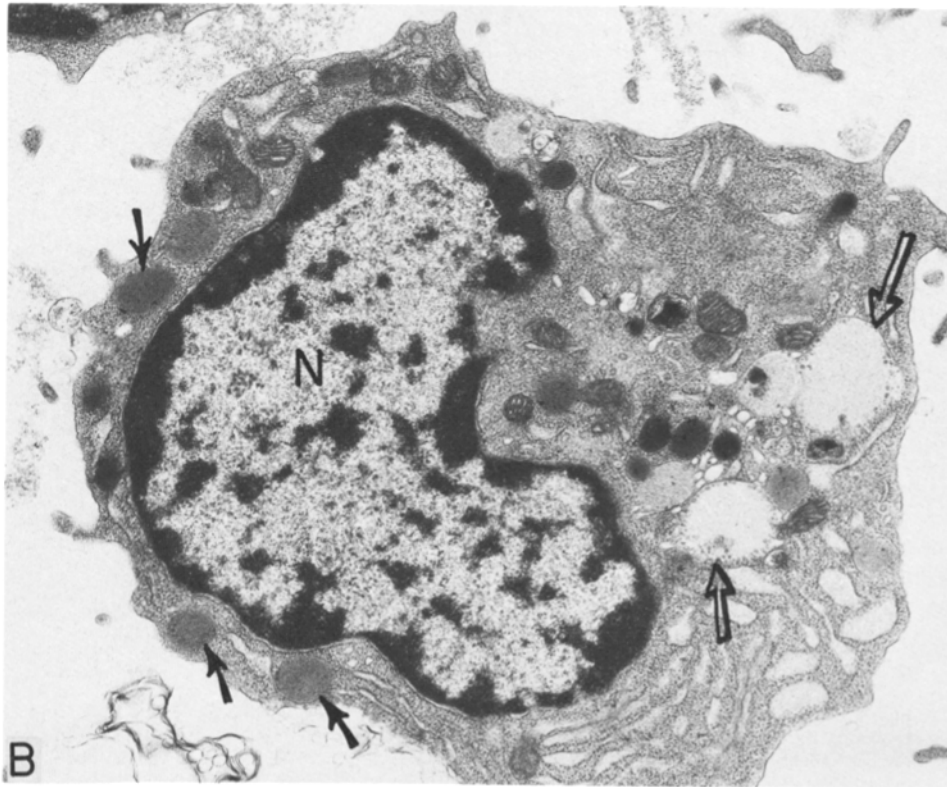
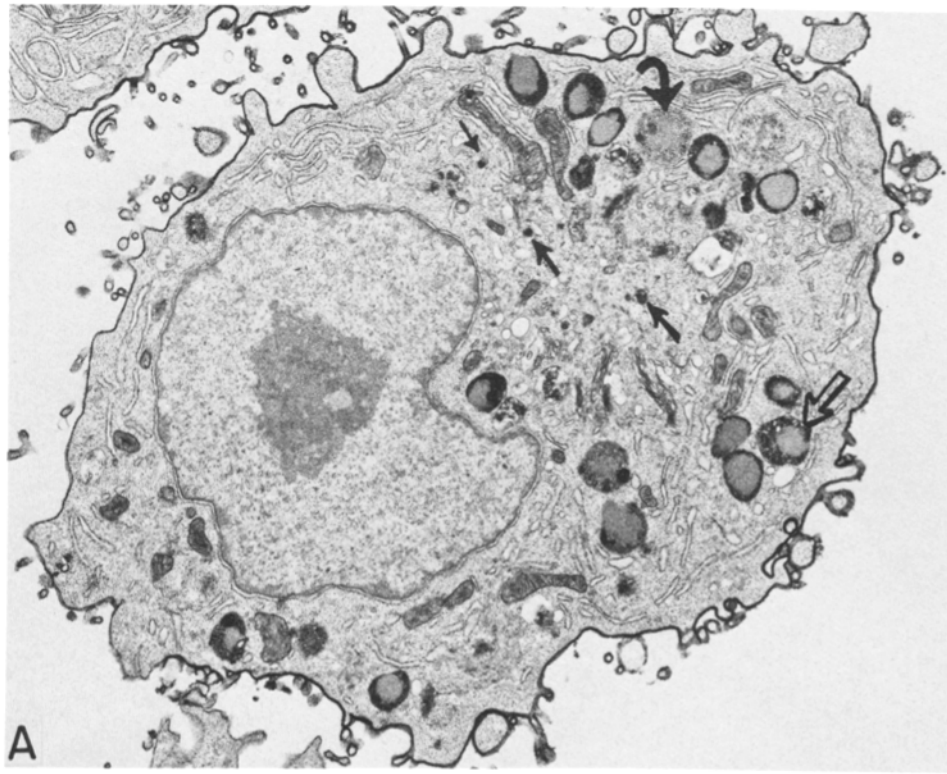


FIG. 3. Cl.Ly-1⁻²NK-1⁺/11 cell processed to demonstrate ANAE. Very immature granules filled with electron-lucent vesicles (curved arrow) are ANAE negative. More mature granules display strong ANAE reactivity in a vesicle-rich zone immediately beneath the granule membrane (open arrow). Mature granules (not shown) appear ANAE negative and lack vesicles. ANAE-positive cytoplasmic vesicles appear in the Golgi area (arrows). The plasma membrane also exhibits ANAE reactivity. (B) A basophilic myelocyte from C57BL/6 bone marrow after OCUB processing. The nuclear configuration and the immature (open arrows) granules resemble those of the Cl.Ly-1⁻²NK-1⁺/11 cell in A. More mature cytoplasmic granules (arrows) are also present. (A) 6,000 \times . (B) 16,000 \times .

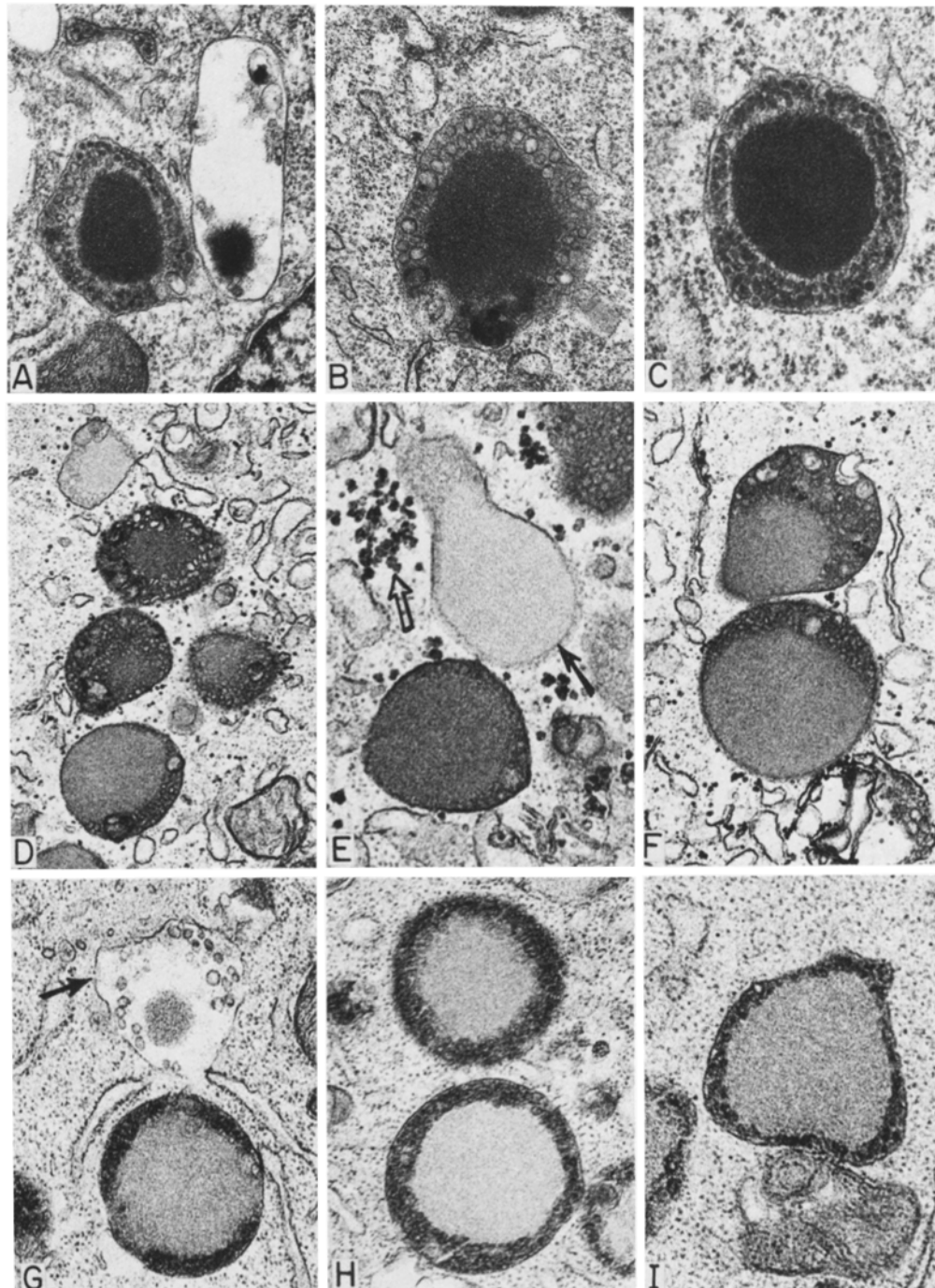


FIG. 4. High-magnification micrographs of the immature granules of Cl.Ly-1⁻²NK-1⁺/11 cells processed by three different techniques. (A-C) By routine TEM (OCUB), the central granule matrix appears homogeneously dense without discernible substructure. A rim of vesicles appears beneath the granule membrane. OPF processing (D-F) renders the central granule matrix less dense and the outer, vesicle-containing area more dense. Some granules have markedly reduced density (arrow). Glycogen particles can be visualized and are often located close to granules (open arrow). The ANAE technique (G-I) demonstrates no enzyme activity in the granule matrix but intense activity in the vesicle-rich zone beneath the cell membrane. One immature granule is entirely negative (arrow). (A) 26,500 \times . (B) 36,500 \times . (C) 32,000 \times . (D) 21,000 \times . (E) 23,500 \times . (F) 33,000 \times . (G) 23,000 \times . (H) 32,500 \times . (I) 25,500 \times .

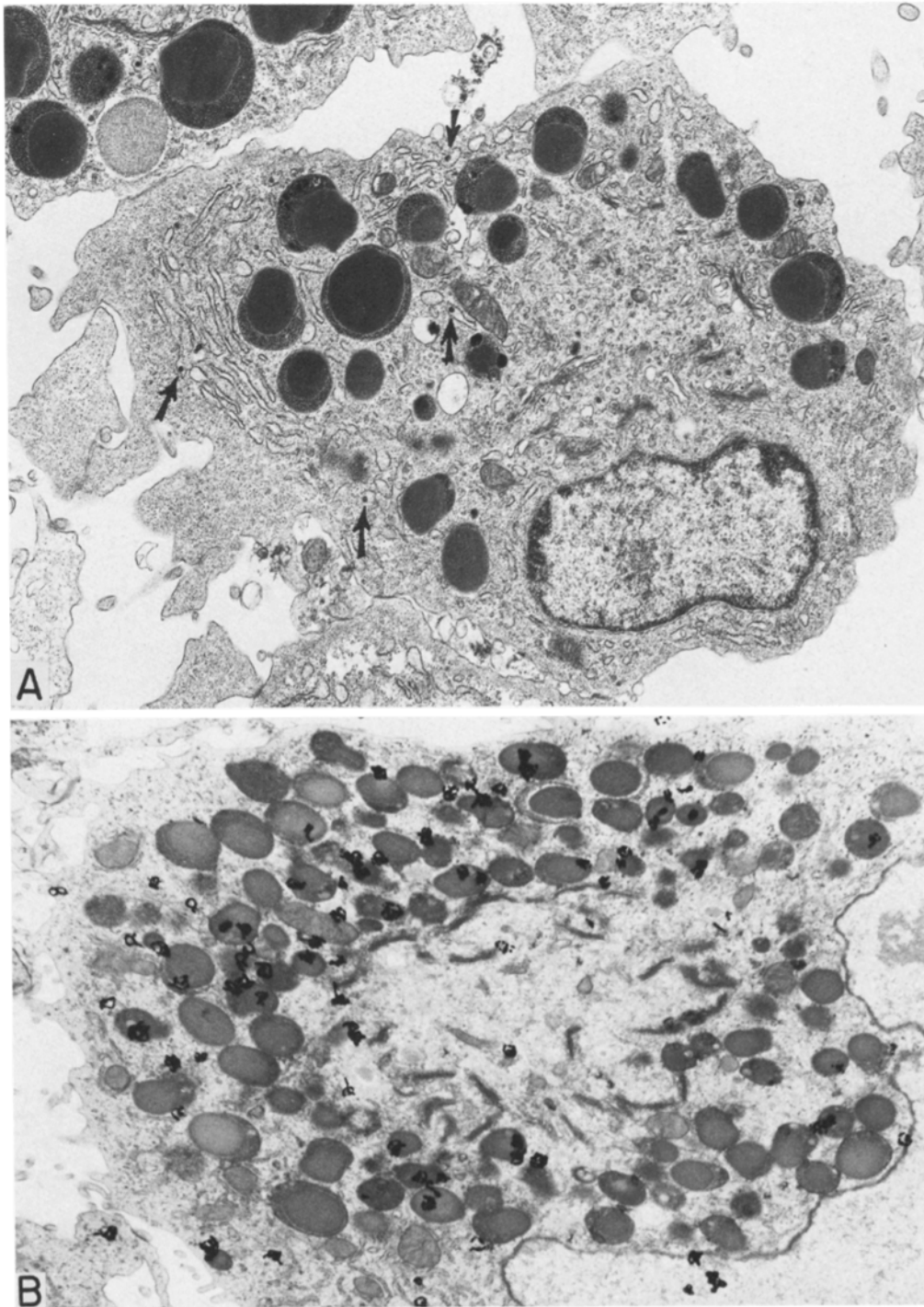


FIG. 5. Cl.Ly-2*3⁺/4 cell with an eccentrically located nucleus and numerous, predominantly immature cytoplasmic granules surrounding the Golgi region. There are many strands of RER, some containing C-type virus particles (small arrows). The cell surface displays broad irregular projections. (B) Autoradiograph of Cl.Ly-2*3⁺/4 cell after labeling with ³⁵SO₄, demonstrating incorporation of ³⁵S into cytoplasmic granules. (A) 10,500X. (B) 11,000X.

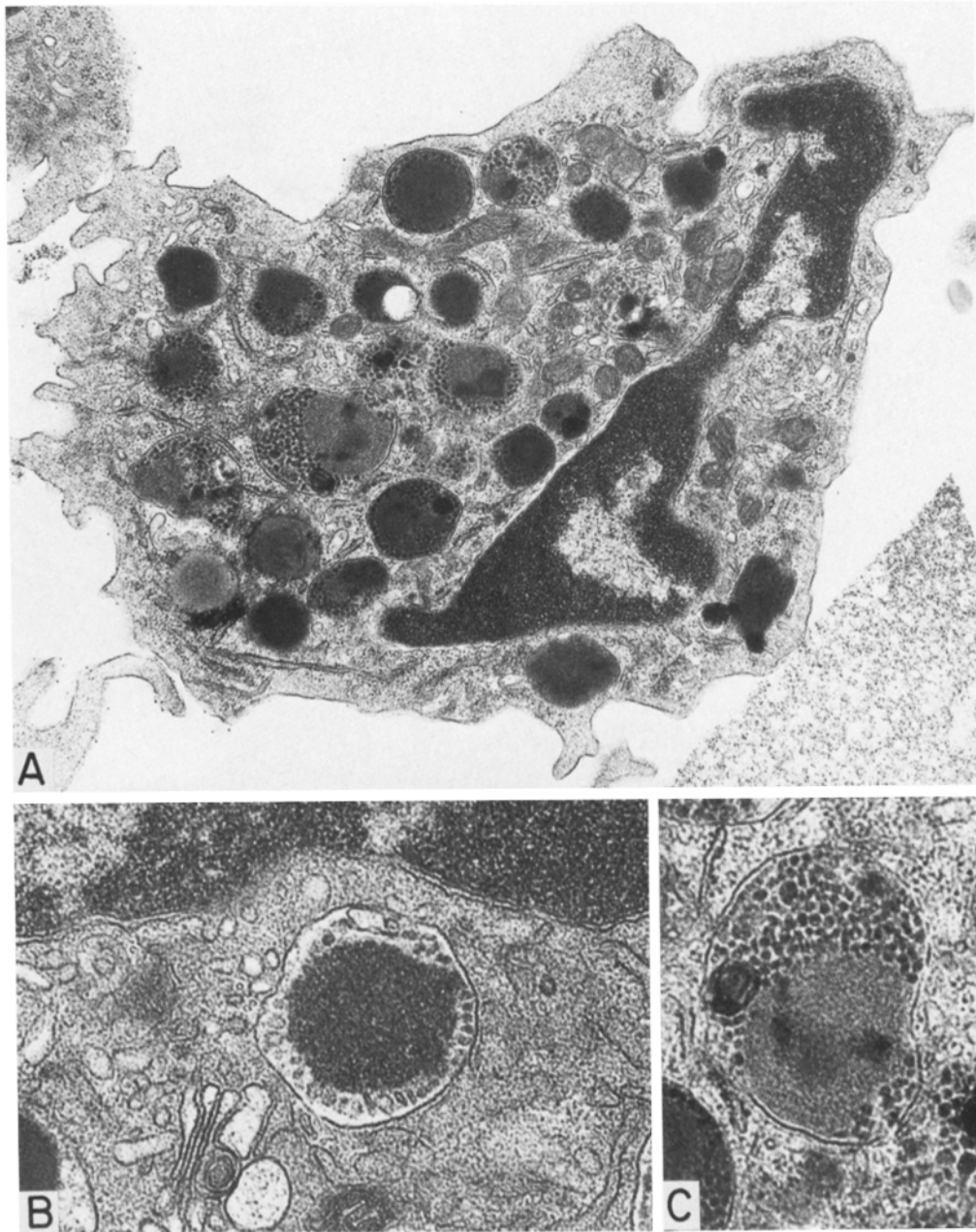


FIG. 6. (A) Late basophilic myelocyte from a short-term culture of C57BL/6 bone marrow. The nucleus has dense chromatin aggregates. The cytoplasm contains granules in varying stages of maturity (B, C) that closely resemble those of Cl.Ly-1⁻2⁻NK-1⁺/11 and Cl.Ly-2⁺3⁺/4. (A) 16,000 \times . (B) 38,000 \times . (C) 34,000 \times .

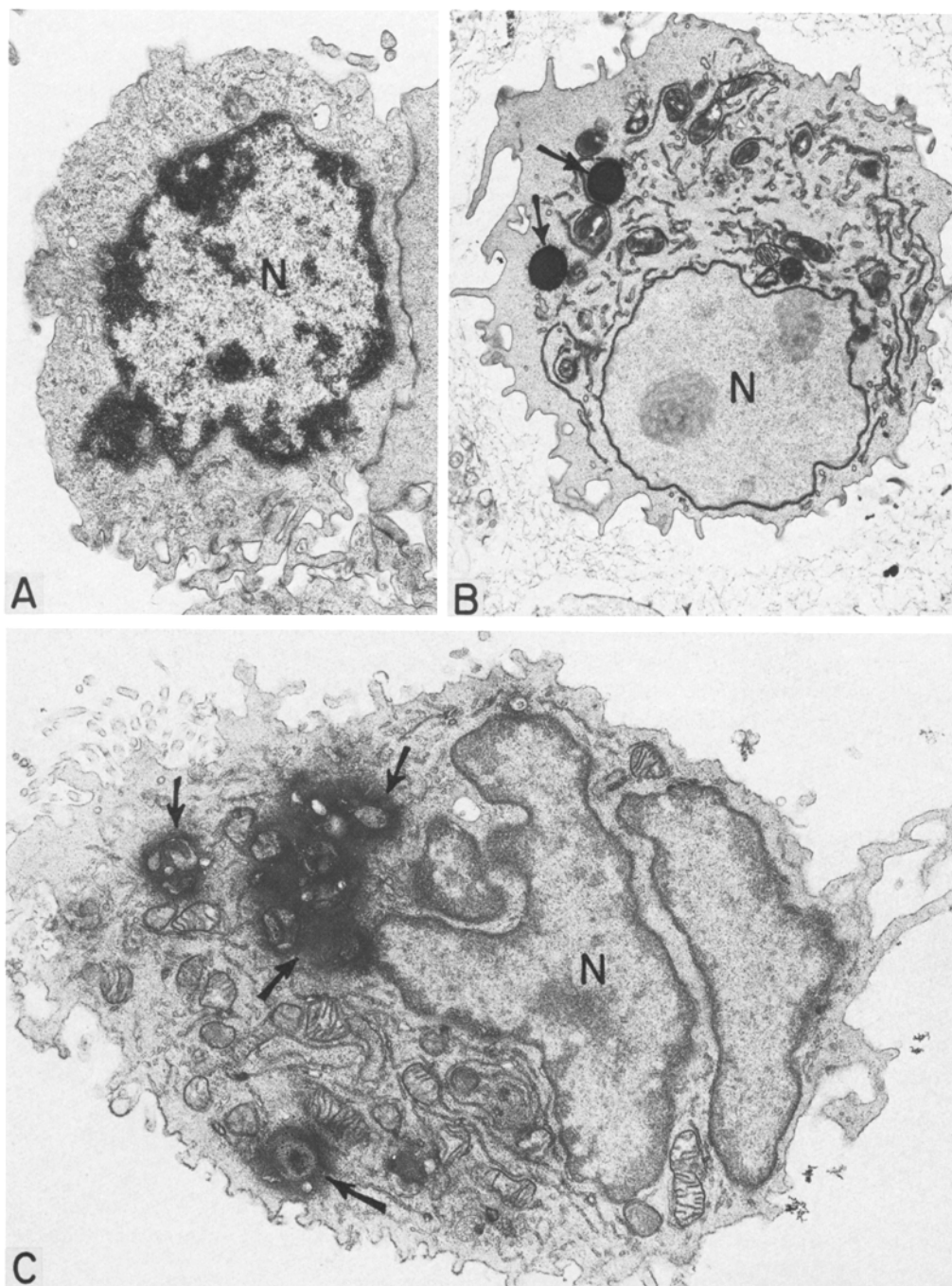


FIG. 7. Cl.Ly-1⁺2⁻/9 inducer T cells. (A) Routine processing (OCUB) demonstrating lack of cytoplasmic granules. (B) OPF processing demonstrating no cytoplasmic glycogen. The very dense structures (arrows) are lipid bodies which, unlike cytoplasmic granules, lack limiting membranes. (C) ANAE-positive cytoplasmic vesicle clusters resembling those described in peripheral blood lymphocytes (15). (A) 12,500 \times . (B) 7,500 \times . (C) 13,000 \times .

matography on DEAE cellulose, revealed a macromolecular peak which appeared coincident with the chondroitin sulfate reference standard (Fig. 8). Like the chondroitin sulfate reference standard, these ^{35}S macromolecules were susceptible to complete hydrolysis to disaccharides by chondroitin ABC or AC lyase, but were insensitive to heparinase (Fig. 8), a degradation pattern like that of chondroitin sulfates A and/or C without detectable heparin or chondroitin sulfate B (dermatan sulfate). Cl.Ly-2⁺3⁺/4 [^{35}S]glycosaminoglycans also eluted from DEAE cellulose coincidentally with the chondroitin sulfate reference standard (Fig. 9). Like Cl.Ly-1⁻2⁻NK-1⁺/11 ^{35}S macromolecules, Cl.Ly-2⁺3⁺/4 [^{35}S]glycosaminoglycans were resistant to degradation by heparinase (Fig. 9). However, unlike Cl.Ly-1⁻2⁻NK-1⁺/11, only 60% of Cl.Ly-2⁺3⁺/4 [^{35}S]glycosaminoglycans resembled chondroitin sulfates A and/or C, as determined by their degradation to disaccharides by chondroitin AC lyase. The chondroitin AC lyase-resistant material was hydrolyzed to disaccharides by chondroitin ABC lyase, evidence that it represented chondroitin sulfate B (dermatan sulfate).

Uptake of [^3H]5HT. The active uptake of 5HT at low ambient concentrations has been reported for certain neurons (24, 25), platelets (26), and mast cells (23, 27). Particularly high rates of 5HT uptake and storage are observed in platelets (26) and mast cells (27) whose cytoplasmic granules contain abundant, homogeneous, osmiophilic matrix material by ultrastructure. Because the matrix material of Cl.Ly-1⁻2⁻NK-1⁺/11 and Cl.Ly-2⁺3⁺/4 granules resembled by ultrastructure that of rabbit platelet (26) or murine mast cell (5, 6, 27) granules rich in 5HT, we evaluated whether these clones could concentrate [^3H]5HT from the external medium. Cl.Ly-1⁻2⁻NK-1⁺/11 and Cl.Ly-2⁺3⁺/4 cells incorporated similar amounts of [^3H]5HT as cloned

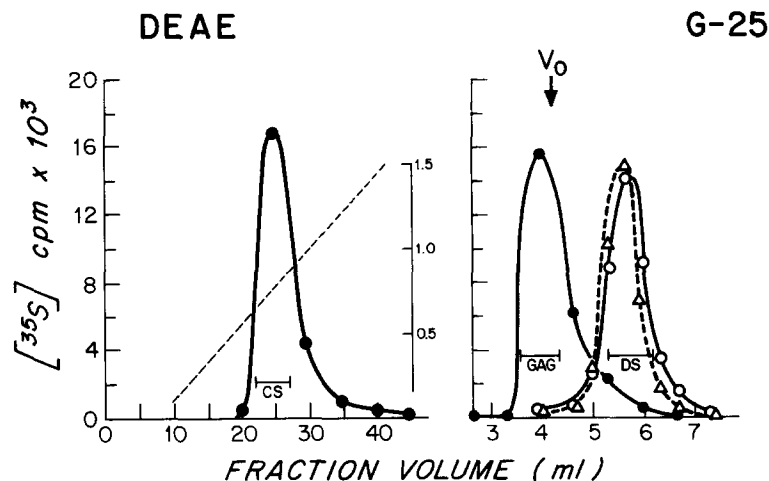


FIG. 8. DEAE-Sephadex A-25 column (0.8 × 6.9 cm) chromatography (left) of pronase-digested Cl.Ly-1⁻2⁻NK-1⁺/11 cells labeled with [^{35}S]sulfate for 18 h (see text). NaCl (---). The bar indicates the elution pattern of the chondroitin sulfate reference standard. Sephadex G-25 column (0.6 × 4.5 cm) chromatography (right) of aliquots of pronase-digested ^{35}S -labeled Cl.Ly-1⁻2⁻NK-1⁺/11 cells after treatment with purified heparinase (Hase; ●) or chondroitin ABC (ABCCase; ○) or AC (ACCase; △) lyase. Bars indicate the elution patterns of native glycosaminoglycan standards (GAG) and their disaccharide degradation products (DS). The Cl.Ly-1⁻2⁻NK-1⁺/11 macromolecules were degraded to disaccharides by chondroitin ABC or AC lyase, but were unaffected by heparinase.

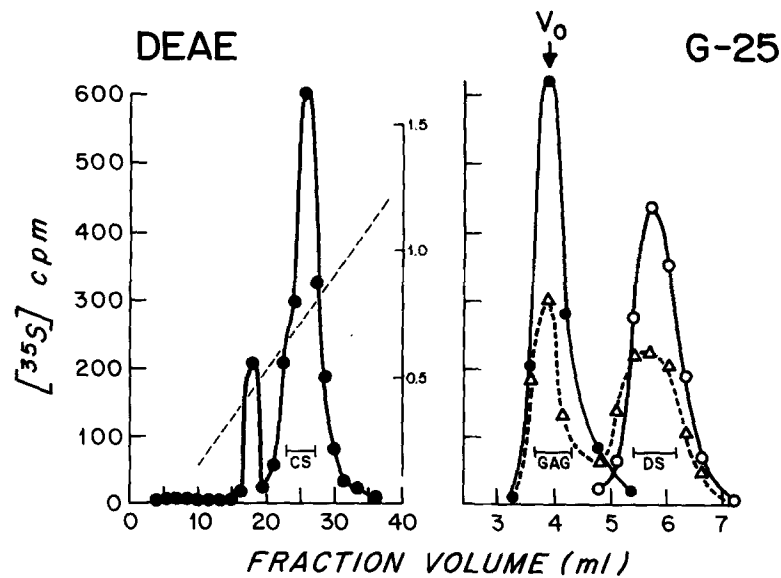


FIG. 9. DEAE-Sephadex A-25 column (0.8×6.8 cm) chromatography (left) of pronase-digested Cl.Ly- $2^{+3^{+}}/4$ cells labeled with [35 S]sulfate (\bullet) for 18 h (see text). NaCl (---). The bar indicates the elution pattern of the chondroitin sulfate or dermatan sulfate reference standards. Sephadex G-25 column (0.6×24.5 cm) chromatography (right) of aliquots of pronase-digested 35 S-labeled Cl.Ly- $1^{-2^{-}}\text{NK-}1^{+}/11$ cells after treatment with heparinase (Hase; \bullet) or chondroitin ABC (ABCCase; \circ) or AC (ACase; Δ) lyase. Bars indicate the elution patterns of native glycosaminoglycans (GAG) and their disaccharide degradation products (DS). The Cl.Ly- $2^{+3^{+}}/4$ macromolecules were unaffected by heparinase, which indicated the absence of heparin. They were partially degraded by chondroitin AC lyase, consistent with the presence of chondroitin sulfates A and C. The chondroitin AC lyase resistant macromolecules were degraded to disaccharides by chondroitin ABC lyase, suggesting that this material represented dermatan sulfate (chondroitin sulfate B).

TABLE II
Serotonin ($^3\text{H-5HT}$) Uptake by Cloned Cells*

| Cell | Functional designation | Specific dpm/ 10^5 cells |
|---------------------------------------|------------------------|----------------------------|
| Cl.MC/9 | Mast cell | $7,306 \pm 1,205$ |
| Cl.Ly- $1^{-2^{-}}\text{NK-}1^{+}/11$ | NK cell | $6,570 \pm 2,102$ |
| Cl.Ly- $2^{+3^{+}}/4$ | Suppressor T cell | $7,005 \pm 580$ |
| Cl.Ly- $1^{+2^{-}}/9$ | Inducer T cell | $701 \pm 222\ddagger$ |

* Cells were incubated with [^3H]5HT for 1 h at either 37 or 4°C (see Materials and Methods). Specific uptake was calculated according to the formula: specific uptake = dpm at 37°C - dpm at 4°C. Results represent SEM of four to six determinations.
 $\ddagger P < 0.005$ when compared with each of the other values by the Newman-Keuls multiple sample comparison test. All other comparisons were not significant ($P > 0.05$).

immature mast cells (Table II). By contrast, cloned inducer T cells, which were devoid of osmiophilic cytoplasmic granules, incorporated little [^3H]5HT.

Discussion

We have examined the ultrastructure and certain cytochemical and biochemical properties of mouse cloned cell lines that express NK lysis (Cl.Ly- $1^{-2^{-}}\text{NK-}1^{+}/11$),

antigen-specific suppressor T cell activity (Cl.Ly-2⁺3⁺/4), or inducer T cell function (Cl.Ly-1⁺2⁻/9). Cl.Ly-1⁻2⁻NK-1⁺/11 and Cl.Ly-2⁺3⁺/4 appeared very similar by ultrastructure. Both contained numerous large cytoplasmic granules with a mixture of vesicular and homogeneous osmiophilic content. Granules such as these have previously been considered a unique characteristic of basophils: they have been described in the basophilic myelocytes of mice (10) and other mammalian species but not in other granulocytes, monocytes, or circulating lymphocytes (28). In both basophilic myelocytes and regenerating mature basophils (29), granule development involves the progressive accumulation of osmiophilic material within vesicle-rich cytoplasmic vacuoles, a process that ultimately renders the granules homogeneously electron dense. The synthesis of cytoplasmic granules by Cl.Ly-1⁻2⁻NK-1⁺/11 and Cl.Ly-2⁺3⁺/4 proceeds by a morphologically similar sequence. The granules of cloned murine mast cells also contain osmiophilic matrix material, but these granules differ from those of Cl.Ly-1⁻2⁻NK-1⁺/11 and Cl.Ly-2⁺3⁺/4 in the distribution of vesicles and pattern of accumulation of electron-dense matrix (6). In addition to prominent granules, Cl.Ly-1⁻2⁻NK-1⁺/11 and Cl.Ly-2⁺3⁺/4 contained abundant deposits of particulate cytoplasmic glycogen. Glycogen deposits occur in all granulocytes (28), but have not generally been observed by ultrastructure in lymphocytes or mast cells. Because the signals that control the development of cytoplasmic granules and deposition of glycogen have not been identified, we cannot predict under what circumstances these features would be displayed *in vivo*. Nevertheless, they do not represent obligatory consequences of the prolonged cultivation of murine lymphoid cells *in vitro*, since cloned inducer T cells lacked both of these characteristics. In addition, cytoplasmic granules similar by ultrastructure to those of our clones have recently been described in freshly isolated human (30, 31) and mouse (32) cells associated with high levels of NK activity, and in mouse leukocytes enriched for cytolytic T lymphocyte (CTL) function (33).

Analysis of nonspecific esterase activity in human (30, 34), rat (35), and mouse (36) leukocyte populations enriched for NK cell activity has produced conflicting results. This may reflect differences in species, variation in cytochemical technique, or problems related to the limited resolution afforded by light microscopic methods. Leukocyte nonspecific esterase activity can be more precisely localized with ultrastructural cytochemistry (14, 15, 37). When Cl.Ly-1⁻2⁻NK-1⁺/11 and Cl.Ly-2⁺3⁺/4 were analyzed they exhibited similar ultrastructural patterns of ANAE reactivity. In the least mature-appearing cells, reactivity was detected on the cell surface and in small cytoplasmic vesicles. More mature cells exhibited strong reactivity in the vesicle-rich zone immediately beneath the limiting membranes of developing granules but diminished cell surface activity. Fully developed, homogeneously dense granules expressed little or no ANAE reactivity. We have observed a similar pattern of ANAE reactivity during the maturation of guinea pig basophils (14; A. M. Dvorak, unpublished data), but not in lymphocytes, monocytes, macrophages, neutrophils, eosinophils (15), or cloned mast cells (S. J. Galli, R. A. Monahan, and A. M. Dvorak, unpublished data). In contrast to Cl.Ly-1⁻2⁻NK-1⁺/11 and Cl.Ly-2⁺3⁺/4, cloned inducer T cells expressed a cytoplasmic vesicle cluster pattern of ANAE similar to that of certain other lymphocytes or macrophages (15). Like uncloned lymphocytes (38) and guinea pig basophils (13, 28), all three clones lacked endogenous peroxidase activity.

The notion that certain murine cells with NK activity share features of Ly-1⁻2⁻3⁺ T cells is also supported by data from other laboratories. Brooks et al. (39) have recently reported five cloned murine cell lines with a spectrum of lytic activity similar to that of Cl.Ly-1⁻2⁻NK-1⁺/11. Like Cl.Ly-1⁻2⁻NK-1⁺/11 and Cl.Ly-2⁺3⁺/4, these clones were peroxidase negative, contained glycogen-like material by histochemistry, and expressed nonspecific esterase activity by light microscopy. All five clones expressed Thy-1, all of the four tested expressed Ly-5, and four of the five expressed the NK alloantigen. In addition, two of the five clones expressed the Lyt-2 glycoprotein, which led these authors to suggest that murine NK cells and CTL might belong to the same lineage. Kedar et al. (40) have also derived multiple clones with NK activity from mouse spleen cells. All of these clones expressed Thy-1 and T-200 (Ly-5), and several expressed Lyt-2 as well. Although these clones were not evaluated for Fc_εR, [³⁵S]glycosaminoglycan synthesis, or 5HT uptake (39, 40), those of Brooks et al. contained cytoplasmic granules very similar by ultrastructure to those of Cl.Ly-1⁻2⁻NK-1⁺/11 and Cl.Ly-2⁺3⁺/4 (A. M. Dvorak, C. G. Brooks, H. F. Dvorak, K. Pyne, C. Henney, and S. I. Galli, unpublished data). Similar granules have also been described in cloned cytolytic T cells (41, 42).

Despite their morphologic and cytochemical similarities, Cl.Ly-1⁻2⁻NK-1⁺/11 and Cl.Ly-2⁺3⁺/4 are not identical. These clones differ in surface glycoproteins and pattern of immunologic activity. Perhaps the most significant distinction between these clones is that Cl.Ly-2⁺3⁺/4 secretes ~2-3% of its internally labeled proteins; ~10% of this represents an ~70,000-mol wt polypeptide with antigen-specific suppressor activity *in vitro* (2). In contrast, Cl.Ly-1⁻2⁻NK-1⁺/11 secretes no detectable internally labeled protein (2). These clones also differ in content of sulfated glycosaminoglycans. The granules of Cl.Ly-2⁺3⁺/4 contained a mixture of chondroitins, including a substantial amount of chondroitin sulfate B, whereas Cl.Ly-1⁻2⁻NK-1⁺/11 chondroitins lacked this molecule. In contrast to our cloned cell lines, which have been analyzed in detail, the distribution of sulfated glycosaminoglycans among lymphocyte populations *in vivo* is poorly understood. Earlier work detected little ³⁵SO₄ incorporation by mouse thymic (43) or guinea pig peripheral blood (19) lymphocytes; however, these studies were performed with uncloned leukocytes containing undefined but probably minor numbers of NK cells or cytolytic/suppressor T cells. At the present time, guinea pig (19) and human basophils (44, 45) and guinea pig Kurloff cells (19, 46) represent the only circulating leukocytes known to contain high concentrations of granule-associated chondroitin sulfates. The role of these molecules in leukocyte function is unknown.

The precise relationship of Cl.Ly-1⁻2⁻NK-1⁺/11 and Cl.Ly-2⁺3⁺/4 to basophils is uncertain. The mouse basophil has only recently been identified by morphology (10, 47) and accounts for ≤0.3% of nucleated cells in C57BL/6 bone marrow. Methods for its purification have yet to be devised. As a result, the mouse basophil's surface glycoprotein phenotype, cytochemistry, and glycosaminoglycan content are unknown. Although the full extent of the similarities and differences among cytolytic/suppressor T cells, Fc_εR-bearing NK cells, and basophils thus remains to be defined, the available evidence suggests that these cells represent a spectrum of leukocyte populations with overlapping morphology and biochemistry. These cells also may express certain similarities in function. For example, CTL or NK lysis appears to require an intact secretory apparatus (33, 48-51) similar to that necessary for mast cell (and basophil)

degranulation (52). In addition, unlike earlier studies (53–55), a recent report suggests that genetic defects of lysosomal function impair CTL lysis as well as NK cell and granulocyte function (56). The clones discussed in this report will permit direct analysis of the role of cytoplasmic granules and other secretory structures (13) in NK lysis and suppressor T cell function.

Summary

We have examined the morphology, cytochemistry, and biochemistry of mouse leukocyte subsets by analyzing cloned leukocyte populations specialized to perform different immunologic functions. Cloned cells expressing high-affinity plasma membrane receptors for IgE and mediating natural killer (NK) lysis and cloned antigen-specific suppressor T cells contained prominent osmiophilic cytoplasmic granules similar by ultrastructure to those of mouse basophils. Both clones also incorporated $^{35}\text{SO}_4$ into granule-associated sulfated glycosaminoglycans, expressed a characteristic ultrastructural pattern of nonspecific esterase activity, incorporated exogenous [^3H]5-hydroxytryptamine, and contained cytoplasmic deposits of particulate glycogen. By contrast, cloned inducer T cells lacked cytoplasmic granules and glycogen, incorporated neither $^{35}\text{SO}_4$ nor [^3H]5-hydroxytryptamine, and differed from the other clones in pattern of nonspecific esterase activity. These findings establish that certain cloned cells with NK activity and cloned suppressor T cells express morphologic and biochemical characteristics heretofore associated with basophilic granulocytes. However, these clones differ in surface glycoprotein expression and immunologic function, and the full extent of the similarities and differences among these populations and basophils remains to be determined.

Note added in proof: We have examined the ultrastructure of a long term guinea pig cell line (colony K2; *J. Immunol.* 1981. **127**:616) with alloreactive cytolytic T cell activity (S. J. Galli, A. M. Dvorak, T. R. Malek, and E. M. Shevach, unpublished data). By electron microscopy, these cells represent lymphocytes with relatively abundant cytoplasm containing a few small, dense lysosomes. They do not resemble guinea pig basophils by morphology. This finding illustrates that the ultrastructure of functionally similar leukocytes maintained in vitro may vary considerably according to species.

Received for publication 23 August 1982 and in revised form 24 November 1982.

References

1. Nabel, G., M. Fresno, A. Chessman, and H. Cantor. 1981. Use of cloned populations of mouse lymphocytes to analyze cellular differentiation. *Cell.* **23**:19.
2. Fresno, M., G. Nabel, L. McVay-Boudreau, H. Furthmayer, and H. Cantor. 1981. Antigen-specific T lymphocyte clones. I. Characterization of a T lymphocyte clone expressing antigen-specific suppressive activity. *J. Exp. Med.* **153**:1246.
3. Fresno, M., L. McVay-Boudreau, G. Nabel, and H. Cantor. 1981. Antigen-specific T lymphocyte clones. II. Purification and biological characterization of an antigen-specific suppressive protein synthesized by cloned T cells. *J. Exp. Med.* **153**:1260.
4. Nabel, G., J. S. Greenberger, M. A. Sakakeeny, and H. Cantor. 1981. Multiple biologic activities of a cloned inducer T-cell population. *Proc. Natl. Acad. Sci. USA.* **78**:1157.
5. Nabel, G., S. J. Galli, A. M. Dvorak, H. F. Dvorak, and H. Cantor. 1981. Inducer T

- lymphocytes synthesize a factor that stimulates proliferation of cloned mast cells. *Nature (Lond.)*. **291**:332.
6. Galli, S. J., A. M. Dvorak, J. A. Marcum, T. Ishizaka, G. Nabel, H. Der Simonian, K. Pyne, J. M. Goldin, R. D. Rosenberg, H. Cantor, and H. F. Dvorak. 1982. Mast cell clones: a model for the analysis of cellular maturation. *J. Cell Biol.* **95**:435.
 7. Nabel, G., L. R. Bucalo, J. Allard, H. Wigzell, and H. Cantor. 1981. Multiple activities of a cloned line mediating natural killer function. *J. Exp. Med.* **153**:1582.
 8. Galli, S. J., A. M. Dvorak, T. Ishizaka, G. Nabel, H. Der Simonian, H. Cantor, and H. F. Dvorak. 1982. A cloned cell with NK function resembles basophils by ultrastructure and expresses IgE receptors. *Nature (Lond.)*. **298**:288.
 9. Nabel, G., J. Allard, and H. Cantor. 1982. A cloned cell line mediating natural killer function inhibits immunoglobulin secretion. *J. Exp. Med.* **156**:658.
 10. Dvorak, A. M., G. Nabel, K. Pyne, H. Cantor, H. F. Dvorak, and S. J. Galli. 1982. Ultrastructural identification of the mouse basophil. *Blood*. **59**:1279.
 11. Dvorak, A. M., S. J. Galli, G. Nabel, J. Marcum, R. A. Monahan, K. Pyne, H. Der Simonian, J. Goldin, H. Cantor, T. Ishizaka, and H. F. Dvorak. 1982. Similarities among a mouse clone mediating natural killer (NK) lysis, cloned Ly 1⁻2⁺3⁺ T lymphocyte and basophilic granulocytes. *Fed. Proc.* **41**:377. (Abstr.)
 12. Dvorak, A. M., S. J. Galli, E. Morgan, A. S. Galli, M. E. Hammond, and H. F. Dvorak. 1981. Anaphylactic degranulation of guinea pig basophilic leukocytes. I. Fusion of granule membranes and cytoplasmic vesicles: formation and resolution of degranulation sacs. *Lab. Invest.* **44**:174.
 13. Dvorak, A. M., M. E. Hammond, E. Morgan, N. S. Orenstein, S. J. Galli, and H. F. Dvorak. 1980. Evidence for a vesicular transport mechanism in guinea pig basophilic leukocytes. *Lab. Invest.* **42**:263.
 14. Monahan, R. A., N. S. Orenstein, S. J. Galli, H. F. Dvorak, and A. M. Dvorak. 1979. Cell surface esterase activity of guinea pig basophilic leukocytes. *J. Cell Biol.* **83**:302a.
 15. Monahan, R. A., H. F. Dvorak, and A. M. Dvorak. 1981. Ultrastructural localization of nonspecific esterase activity in guinea pig and human monocytes, macrophages and lymphocytes. *Blood*. **58**:1089.
 16. Yurt, R. W., R. W. Leid, Jr. K. F. Austen, and J. E. Silbert. 1977. Native heparin from rat peritoneal mast cells. *J. Biol. Chem.* **252**:518.
 17. Metcalfe, D. D., R. A. Lewis, J. E. Silbert, R. D. Rosenberg, S. I. Wasserman, and K. F. Austen. 1979. Isolation and characterization of heparin from human lung. *J. Clin. Invest.* **64**:1537.
 18. Ogren, S., and U. Lindahl. 1971. Degradation of heparin in mouse mastocytoma tissue. *Biochem. J.* **125**:1119.
 19. Orenstein, N. S., S. J. Galli, A. M. Dvorak, J. E. Silbert, and H. F. Dvorak. 1978. Sulfated glycosaminoglycans of guinea pig basophilic leukocytes. *J. Immunol.* **121**:586.
 20. Saito, H., T. Yamazata, and S. Suzuki. 1968. Enzymatic methods for the determination of small quantities of isomeric chondroitin sulfate. *J. Biol. Chem.* **243**:1536.
 21. Linker, A., and P. Hovingh. 1972. Heparinase and heparitinase from *Flavobacterium*. *Methods Enzymol.* **28**:902.
 22. Bitter, T., and H. M. Muir. 1962. A modified uronic acid carbazole reaction. *Anal. Biochem.* **4**:330.
 23. Mazingue, C., J.-P. Dessaint, and A. Capron. 1978. ³H Serotonin release: an improved method to measure mast cell degranulation. *J. Immunol. Methods.* **21**:65.
 24. Shaskan, E. G., and S. H. Snyder. 1970. Kinetics of serotonin accumulation into slices from rat brain: relationship to catecholamine uptake. *J. Pharmacol. Exp. Ther.* **175**:404.
 25. Gershon, M. D., and R. F. Altman. 1971. An analysis of the uptake of 5-hydroxytryptamine

- by the myenteric plexus of the small intestine of the guinea pig. *J. Pharmacol. Exp. Ther.* **179**:29.
26. Tranzer, J. P., M. Da Prada, and A. Pletscher. 1966. Ultrastructural localization of 5-hydroxytryptamine in blood platelets. *Nature (Lond.)*. **212**:1574.
 27. Nunez, E. A., and M. D. Gershon. 1973. Species differences in mast cells in the thyroid gland. *Endocrinology*. **92**:152.
 28. Dvorak, A. M. 1978. Biology and morphology of basophilic leukocytes. In *Immediate Hypersensitivity: Modern Concepts and Developments*. M. K. Bach, editor. Maral Dekker, New York. 369.
 29. Dvorak, A. M., S. J. Galli, E. Morgan, A. S. Galli, M. E. Hammond, and H. F. Dvorak. 1982. Anaphylactic degranulation of guinea pig leukocytes. II. Evidence for regranulation of mature basophils during recovery from degranulation *in vitro*. *Lab. Invest.* **46**:461.
 30. Grossi, C. E., A. Cadoni, A. Zicca, A. Leprini, and M. Ferrarini. 1982. Large granular lymphocytes in human peripheral blood: ultrastructural and cytochemical characterization of the granules. *Blood*. **59**:277.
 31. Carpen, O., I. Virtanen, and E. Saksela. 1982. Ultrastructure of human natural killer cells. Nature of the cytolytic contacts in relation to cellular secretion. *J. Immunol.* **128**:2691.
 32. Luini, W., D. Boraschi, S. Alberti, A. Aleotti, and A. Tagliabue. 1981. Morphological characterization of a cell population responsible for natural killer activity. *Immunology*. **43**:663.
 33. Bykovskaja, S. N., A. N. Rytenko, M. O. Rauschenbach, and A. F. Bykovsky. 1978. Ultrastructural alteration of cytolytic T lymphocytes following their interaction with target cells. II. Morphogenesis of secretory granules and intracellular vacuoles. *Cell Immunol.* **40**:175.
 34. Timonen, T., J. R. Ortaldo, and R. B. Herberman. 1981. Characteristics of human large granular lymphocytes and relationship to natural killer and K cells. *J. Exp. Med.* **153**:569.
 35. Reynolds, C. W., T. Timonen, and R. B. Herberman. 1981. Natural killer (NK) cell activity in the rat. I. Isolation and characterization of the effector cells. *J. Immunol.* **127**:282.
 36. Roder, J. C., R. Kiessling, P. Biberfeld, and B. Anderson. 1978. Target-effector interaction in the natural killer (NK) cell system. II. The isolation of NK cells and studies on the mechanism of killing. *J. Immunol.* **121**:2509.
 37. Bozdech, M. J., and D. F. Bainton. 1981. Identification of alpha-naphthyl butyrate esterase as a plasma membrane ectoenzyme of monocytes and as a discrete intracellular membrane-bounded organelle in lymphocytes. *J. Exp. Med.* **153**:182.
 38. Dvorak, A. M., R. A. Monahan, and G. R. Dickersin. 1981. Diagnostic electron microscopy. I. Hematology: differential diagnosis of acute lymphoblastic and acute myeloblastic leukemia. Use of ultrastructural peroxidase cytochemistry and routine electron microscopic technology. In *Pathology Annual*. S. C. Sommers and P. P. Rosen, editors. Appleton-Century-Crofts, New York. 101.
 39. Brooks, C. G., K. Kuribayashi, G. E. Sale, and C. S. Henney. 1982. Characterization of five cloned murine cell lines showing high cytolytic activity against YAC-1 cells. *J. Immunol.* **128**:2326.
 40. Kedar, E., B. L. Ikejiri, B. Srendi, B. Bonavida, and R. B. Herberman. 1982. Propagation of mouse cytotoxic clones with characteristics of natural killer (NK) cells. *Cell Immunol.* **69**:305.
 41. Hackett, C. J., K. Sullivan, and Y.-L. Lin. 1982. Ultrastructure of an influenza virus-specific cytotoxic T-cell clone and its interaction with P 815 and macrophage targets. *Cell Immunol.* **68**:276.
 42. Tyler, J. D., D. Steinmuller, S. J. Galli, and K. G. Waddick. Allospecific graft-versus-host lesions mediated in MHC-restricted fashion by cloned cytolytic T cells. *Transplant. Proc.* In press.

43. Clark, S. L. 1968. Incorporation of sulfate by the mouse thymus: its relation to secretion by medullary epithelial cells and to thymic lymphoporesis. *J. Exp. Med.* **128**:927.
44. Galli, S. J., N. S. Orenstein, P. J. Gill, J. E. Silbert, A. Dvorak, and H. F. Dvorak. 1979. Sulphated glycosaminoglycans synthesised by basophil-enriched human leukaemic granulocytes. In *The Mast Cell: Its Role in Health and Disease*. J. Pepys and A. M. Edwards, editors. Pitman Medical, Kent, England. 842.
45. Bland, C., S. Wasserman, and D. Metcalfe. 1982. Characterization of human leukemic basophil proteoglycans. *Fed. Proc.* **41**:822. (Abstr.)
46. Dean, M. F., and H. Muir. 1970. The characterization of a protein-polysaccharide isolated from Kurloff cells of the guinea pig. *Biochem. J.* **118**:783.
47. Urbina, C., C. Ortiz, and I. Hurtado. 1981. A new look at basophils in mice. *Int. Arch. Allergy Appl. Immunol.* **66**:158.
48. Roder, J. C., S. Argov, M. Klein, C. Pettersson, R. Kiessling, K. Andersson, and M. Hansson. 1980. Target-effector cell interaction in the natural killer cell system. V. Energy requirements, membrane integrity, and the possible role of lysosomal enzymes. *Immunology.* **40**:107.
49. Carpen, O., I. Virtanen, and E. Saksela. 1981. The cytotoxic activity of human natural killer cells requires an intact secretory apparatus. *Cell. Immunol.* **58**:97.
50. Bykovskaja, S. N., A. N. Rytenka, M. O. Rauschenbach, and A. F. Bykovsky. 1978. Ultrastructural alterations of cytolytic T lymphocytes following their interaction with target cells. I. Hypertrophy and change of orientation of the Golgi apparatus. *Cell. Immunol.* **40**:164.
51. Zagury, D., J. Bernard, P. Jeannesson, N. Thiernes, and J.-C. Cerottini. 1979. Studies on the mechanism of T cell-mediated lysis at single cell level. I. Kinetic analysis of lethal kits and target cell lysis in multicellular conjugates. *J. Immunol.* **123**:1604.
52. Quan, P.-C., T. Ishizaka, and B. R. Bloom. 1982. Studies on the mechanism of NK cell lysis. *J. Immunol.* **128**:1786.
53. Roder, J. C. 1979. The beige mutation in the mouse. I. A stem cell predetermined impairment in natural killer formation. *J. Immunol.* **123**:2168.
54. Roder, J., and A. Duwe. 1979. The beige mutation in the mouse selectively impairs natural killer function. *Nature (Lond.)*. **278**:451.
55. Roder, J. C., M.-L. Lohmann-Matthes, W. Domzig, and H. Wigzell. 1979. The beige mutation in the mouse. II. Selectivity of the natural killer (NK) cell defect. *J. Immunol.* **123**:2174.
56. Saxena, R., Q. B. Saxena, and W. H. Alder. 1982. Defective T-cell response in beige mutant mice. *Nature (Lond.)*. **295**:240.