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# IMMUNOHISTOCHEMICAL AND ELECTRON-MICROSCOPIC IDENTIFICATION OF SEROTONIN, MELATONIN, AND β-ENDORPHIN IN GRANULES OF NATURAL KILLER CELLS

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The attention of immunologists, oncologists, and specialists in other disciplines is currently being drawn to natural killer (NK) cells, which are large granular lymphocytes, whose function is linked with high cytotoxicity against cells of various types and, in particular, tumor cells [10, 11, 14]. Despite many investigations of NK cells, the problem of their origin and mechanisms of the cytotoxic action of NK cells on target cells has not yet been solved. Investigations of the functional significance of a characteristic ultrastructural feature of these cells, namely the presence of electron-dense granules, distinguishing them from other types of lymphocytes, have been few in number [11]. The discovery of the nature of these cytoplasmic organelles is a key approach to the uncoding of the mechanism of the cytotoxic effect of NK cells. The solution to this problem raises many questions: how is the presence of granules linked with the cytotoxic properties of NK cells, what biologically active substances are synthesized in the granules, and how is their activity realized? During analysis of the literature, attention is drawn to an investigation into the use of Leu-7 antigen as a marker of NK cells. It has been shown that Leu-7 antigen reacts positively not only with NK cells, but also with the secretory granules of many cells of the APUD system [17, 18] and it correlates with a property so characteristic of apudocytes as argyrophilia [15].

These data suggest that peptide hormones and biogenic amines, which are usually produced by cells of the APUD system [4, 12, 16], may be synthesized in the cytoplasmic granules of NK cells.

The aim of this investigation was to identify biologically active substances in the granules of NK cells and to evaluate their participation in the realization of their cytotoxic action.

## EXPERIMENTAL METHOD

Peripheral blood lymphocytes from healthy blood donors and from the spleens of Wistar rats were used as the test objects. A lymphocyte suspension was obtained by centrifugation of the samples in a Ficoll–Verografin density gradient (d = 1.077 g/ml) by the method [10, 11]. Intact lymphocytes from separate donors and lymphocytes from different donors incubated together for 4 h at 37°C were studied. The aim of combined incubation was to provoke contact interaction between NK cells belonging to different donors. The material was stained with azure-eosin and by Grimelius' argyrophilic method. The immunohistochemical investigation was carried out by the immunoperoxidase method [13]. Antisera against serotonin and metatonin, prepared in the appropriate way, and antisera against β-endorphin, insulin, somatotrophic hormone (STH), chorionic gonadotrophin, ACTH, and C-peptide (from Amersham Corpora-

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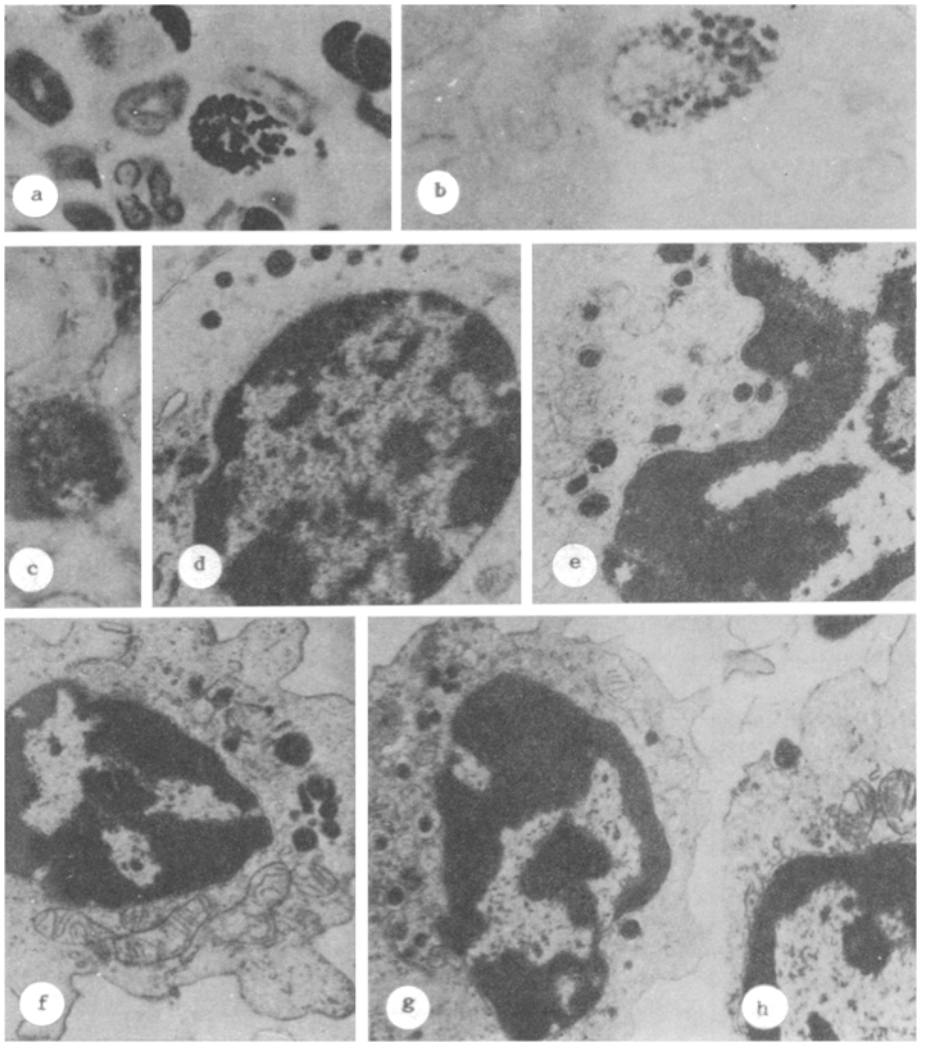


Fig. 1. Endocrine granules in NK cells. a) Large granular lymphocytes from rat spleen. Positive Grimelius' reaction. 600×; b) large granular lymphocyte from rat spleen. Positive reaction with antiserum against serotonin. Immunoperoxidase method. 900×; c) large granular lymphocytes from human blood. Positive reaction with antiserum against β-endorphin. Immunoperoxidase method. 900×; d) intact NK cell from human blood: numerous polymorphic granules of different sizes, lysosomes, and mitochondria. 15,800×; e) intact NK cell from human blood. Distinct thin membrane can be seen around electron-dense core of secretory granules. 20,000×; f) changes in surface of cell membrane and hyperplasia of mitochondria in NK cell during contact with a similar cell from another donor. 15,800×; g) many polymorphic secretory granules in NK cell from human blood on incubation together with NK cells from another donor. 18,000×; h) exocytosis of endocrine granule of NK cell during combined incubation of natural killer cells from different donors. 18,000×.

tion, England) were used. Material for electron-microscopic investigation was fixed in a mixture of 2.5% glutaraldehyde and 2% formaldehyde (1:2) in 0.1 M Sorensen's phosphate buffer for 1 h followed by postfixation for 1 h with 1% $OsO_4$ solution in the same buffer (pH 7.2). After treatment with a saturated solution of uranyl acetate in 70° ethanol the material was dehydrated in alcohols of increasing strength and embedded in Epon. Semithin sections were stained with toluidine blue, after which the block was sharpened to a point. Ultrathin sections were cut on the LKB-4800A Ultratome, stained with lead citrate by Reynolds' method, and studied in the JEM-100C electron microscope.

## EXPERIMENTAL RESULTS

Large granular lymphocytes, corresponding to the concept of the "NK cell," were found in fractions of human blood serum and rat spleen, obtained in a Ficoll–Verografin density gradient.

On staining with azure-eosin numerous azurophilic granules appeared in these cells, and in serial sections they reacted positively with silver nitrate applied by Grimelius' method (Fig. 1a). These cells exhibited positive immunoreactive properties relative to antisera against serotonin, melatonin, and β-endorphin (Fig. 1b, c). Immunohistochemical reactions with antisera against insulin, STH, ACTH, chorionic gonadotrophin, and C-peptide were negative.

Electron-microscopic investigation revealed polymorphic electron-dense secretory granules (circular, bean-shaped, dumbell-shaped) of different sizes with a distinctly outlined membrane in the cytoplasm of the large granular lymphocytes (Fig. 1d, e). Besides them, a well-developed endoplasmic reticulum, lamellar complex, numerous ribosomes, mitochondria, and lysosomes also were present in the cytoplasm of the NK cells (Fig. 1d-h). During combined incubation of lymphocytes from different donors for 4 h, the large granular lymphocytes were in a state of well-marked mutual contact, as shown by changes in the surface of the cell membrane (Fig. 1f, g). In some cases exocytosis of the contents of the granules outside the lymphocyte could be recorded (Fig. 1h).

These results show for the first time that the secretory granules of NK cells contain highly active hormonal substances – serotonin, melatonin, and β-endorphin, with marked ability to regulate the rates of cell proliferation [1, 3, 6, 7, 19]. Considering that these substances give rise to certain general regulatory effects [2, 4, 5, 8, 9], their direct participation can be suggested in autoregulatory mechanisms of the cytotoxic action of NK cells.

The positive Grimelius' reaction, which is characteristic of cells of the APUD system, and the identification of several hormones in the granules of NK cells suggest that, in principle, besides serotonin, melatonin, and β-endorphin, NK cells may also contain an unknown peptide with marked cytolytic properties, and further research is in progress in order to shed light on this problem.

The discovery that hormone synthesis may perhaps take place in the secretory granules of natural killer cells thus opens up fundamentally new prospects for the study of the mechanisms of the cytotoxic action of NK cells.

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# ULTRASTRUCTURAL CHANGES IN THE SKIN AFTER ELECTROCUTION

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The conclusion that death has been due to electrocution is something which the investigator has always to bear in mind. At the microscopic level a distinctive combination of morphological changes has been described in the skin: swelling of the stratum corneum with the formation of empty spaces of different sizes, stretching of the cell nuclei of the stratum basale and stratum spinosum resembling a whirlpool, with the formation of curious shapes in the form of brushes and fishtails, swelling of the collagen fibers with some degree of basophilia, a response of the blood vessels, etc. [1-3, 7]. Investigations of the skin with scanning and transmission electron microscopes have shown partial conglomeration and conglutination of the tonofilaments, which lie parallel to the longitudinal axis of the stretched cell nuclei, injuries of the cell and nuclear membranes, rarefaction of the cytoplasm, precipitation of nuclear and cytoplasmic material on fragments of the nuclear membrane, destruction of mitochrondria, separation of the basement membrane from the underlying connective tissue, and other changes [4-6, 8]. Meanwhile dependence of the morphological changes in the skin on the voltage of the electric current, the character of contact, its duration, and other factors, which is also of great forensic-medical importance, has not been adequately studied.

Ultrastructural changes developing as a result of electrocution were studied in experiments in which different voltages were used.

## EXPERIMENTAL METHOD

Experiments were carried out on 20 male black and white guinea pigs weighing 250-300 g. Injuries were produced with a stabilized alternating electric current of 380 and 220 V with a frequency of 50 Hz, applied through iron electrodes with an area of 1 $cm^2$ for 10 sec. The electrodes were securely fixed to the animals' footpads (dry contact), under hexobarbital anesthesia. The animals died immediately after electrocution. Material for electron-microscopic study was fixed in glutaraldehyde and processed by the usual method. Pieces of skin for histologic investigation were fixed in formalin, embedded in paraffin wax, and stained with hematoxylin and eosin, with picrofuchsine, by Mallory's and Cason's metods, with toluidine blue, by Perls' method, and by the PAS reaction.

## EXPERIMENTAL RESULTS

After the action of an electric current of 380 V on the skin a definite combination of morphological changes was discovered. Macroscopically injuries could be seen on the skin of the animals' limbs immediately beneath the electrodes, measuring from 0.1 × 0.1 to 0.5 × 0.5 cm, firm to the touch, with a depressed whitish gray base and raised edges. Sometimes partial charring of the skin was observed, in the form of brownish black deposits. As a result of the uneven surface of the skin of the feed the shape of the injuries did not always correspond exactly to the configuration and dimensions of the electrode. Microscopically, homogenization and compaction of the keratin scales were observed in the epidermis, tears of different sizes and shapes were formed in the center of the lesions, and sometimes zigzag tears shaped like flashes of lightning were seen (Fig. 1a). The changes were most marked in cells of the stratum basale and stratum spinosum. Their nuclei became hyperchromic

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