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Nerve Growth factor enhances precocious differentiation and numerical increase in mast cells in cultures of rat splenocytes

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SEZIONE III

(Botanica, zoologia, fisiologia e patologia)

Biologia. — *Nerve Growth factor enhances precocious differentiation and numerical increase in mast cells in cultures of rat splenocytes.* Nota di ARIELA BÖHM e LUIGI ALOE, presentata (*) dal Socio straniero R. LEVI MONTALCINI.

RIASSUNTO. — Si è utilizzato un sistema *in vitro* per indagare su alcuni aspetti dell'effetto stimolante esercitato da iniezioni sistemiche del Nerve Growth Factor (NGF) in ratti neonati sui mastociti riportato in un precedente lavoro del nostro gruppo. A questo scopo si sono coltivate in terreno semisolido cellule dissociate dalla milza di ratti neonati. La presenza nel mezzo di coltura di quantità fisiologiche di NGF (10 ng/ml) determina nel periodo di 2-3 settimane un aumento rilevante del numero di mastociti a paragone di quelli riscontrati in colture di controllo. I risultati ottenuti confermano ed estendono quelli riportati nel lavoro precedente e allo stesso tempo pongono le basi per analizzare in condizioni rigorose quanto controllabili il meccanismo di azione del NGF su questa linea cellulare appartenente, a differenza delle precedenti, al sistema immunitario.

INTRODUCTION

The Nerve growth factor (NGF) is a protein essential for the survival, differentiation and maintenance of sensory and sympathetic neurons [1-5], and for the differentiation [6, 7] and proliferation of adrenal chromaffin cells as well [8].

Moreover, when injected in newborn mice and rats, NGF produces a massive increase in the number and size of tissue mast cells [9].

Mast cells (MCs) are secretory cells which are a constant component of vertebrates' connective tissue. They are widely distributed around the small blood vessels, nerves and glandular ducts, and on mucosal and serosal cutaneus surfaces [10]. MCs have also been found in the central nervous system, particulary in the thalamic and hypothalamic regions where they contribute to the brain's histamine level [11-14]. They respond to mechanical, chemical and immunological stimuli by secreting a large number of biologically active substances such as histamine, serotonine, heparin, proteolitic enzymes, platelet-activating factor and prostaglandins [15, 16].

The origin and development of mast cells are not yet clearly understood. Extensive *in vitro* studies have shown that a subset of mast cells (mucosal mast

(*) Nella seduta dell'8 febbraio 1986.

cells) can be obtained by culturing cells from different sources such as bone marrow, spleen, peripheral blood or lymph nodes in the presence of conditioned media [17-21] or of a growth factor known as Interleukin 3 [22]. There is, however, no experimental evidence showing that *in vivo* injections of these media or factors evoke selective increases in mast cell number.

The above mentioned discovery that systemic injections of murine NGF *in vivo* result in a striking increase of maat cells in connective tissues suggested that the source of the cells might be sought by examining the effects of NGF on derivatives of hemopoietic organs such as bone marrow and the spleen of neonatal rats. To this aim we cultured rat spleen cells in the presence of physiological amounts of NGF. Although no other growth factors or conditioned media were added to the cultures, cells with structural and ultrastructural characteristics similar to those of fully differentiated mast cells were found in large number.

The present studies provide the first evidence of an *in vitro* effect of NGF enhancing the proliferation and/or differentiation of spleen cells toward the mast cell phenotype.

Materials and methods

Newborn Sprague Dawley NOS (Nossan, Correzzana, Italy) rats were used for all experiments. Spleen cells were prepared by gently teasing the spleen through a fine wire mesh onto a plastic dish and subsequently dissociating the cell clumps by repeated pipetting. 4-5 spleens were pooled for each experiment. Spleen cells were then cultured, in semisolid medium [23, 24], (1×10^6 cells/ml) in a 24 well tissue culture plate (0.5 ml/well), in Dulbecco Modified Eagle's Medium supplemented with 30% FCS, 10^{-4} M mercaptoethanol, 2 mM glutamine, antibiotics, and methylcellulose at a final concentration of 1%. 10 ng/ml of NGF was added to half of the cultures from the beginning, at four day intervals. The cultures were incubated in a highly humid atmosphere with 5% CO_2 at 37° C. Mouse 2.5 S NGF was purified from the mouse salivary glands as previously described [25].

After different periods of culture (8-20 days) colonies were counted and cells recovered from 20 wells per group. In some cases colonies were individually lifted and stained. Routinely, after three washings, cells were counted, cytospun and stained with May Grunwald-Giemsa (3-4 slides per group).

The criteria for mast cells identification were based on the presence of clearly visible round or oval nuclei and of cytoplasmic basophilic granules.

To determine the MCs percentage, at least ten MCs percent counts (globally 10^3 cells) were done in different areas and in different slides of the same group. The percentage of mast cells is expressed as mean value $\pm$ S.D.

Toluidine blue staining. Cells were cytospun on gelatine coated glass slides, fixed in alcoholic bouin, washed in 70% ethanol containing 10% ammonium

acetate and stained with 0.1% aqueous solution of toluidine blue, pH 5.0. Cells showing metachromasia were scored as mast cells.

Immunohistofluorescence. Cells were fixed in 4% paraformaldehyde in phosphateic buffer saline (PBS) pH 7.4, washed with the same buffer and incubated with monoclonal antibodies against serotonine for 2 hours at room temperature, then washed again in PBS and incubated for 1hr in fluoresceine isothiocynate (FITC)–conjugate antibodies (Miles) diluited 1:30. After a brief rinse, cells were covered with glycerine–PBS 1:1 and examined with a Leitz dialux fluorescence microscope.

Identification of surface receptor for IgE. Unfixed cells were incubated at 37 °C for 1hr with IgE, washed in PBS 3 times and then incubated again for 45′ with rabbit anti-mouse IgE. After several washings, samples were incubated with FITC-conjugated goat anti-rabbit IgG and examined with a Leitz dialux fluorescence microscope.

Electron-microscopic studies. Cultured cells were pelleted and fixed with 4% paraformaldehyde plus 1% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 for 2 hrs at room temperature. After washing in the same buffer, cells were post-fixed in 1% OsO_4 for 1 hr at room temperature. Samples were then dehydrated with ethanol and embedded in Epon 812. Semithin sections stained in 1% aqueous solution of toluidine blue were prepared for light microscopic localization of mast cells. Ultrathin sections stained with uranyl acetate and lead citrate were examined with a Philips 200 electron microscope.

Results

The rat splenic population at birth is rather heterogeneous but practically devoid of mast cells (Pl. I, fig. 1). When spleen cells are cultured in a semisolid medium (with or without NGF), colonies consisting of homogeneous populations of cells with round and smooth contours begin to appear. No mast cells are detectable in such colonies before day 7-8. After 9 days of culture, cells with basophilic granules appear in NGF^+ and NGF^- cultures.

Although these cells do not exhibit the characteristics of mature mast cells those grown in presence of NGF are in a more advanced stage of differentiation than the cells of the control cultures (Pl. I. fig. 2 *a* and 2 *b*). A May-Grunwald Giemsa-stained smear of cultured mast cells (18 days) is shown in Pl II, fig. 1. A comparison of mast cell percentages (fig. 1) in NGF treated and control cultures indicates the presence of more MCs in the former than in the latter. The difference is greatest after one week of culture and decreases, though is still very evident, in three–week–old NGF cultures.

No significative difference in cell number or in colony number is detectable between NGF^+ and NGF^- cultures. The number of viable cells recovered on day 16 is between 6.6×10^4 and 13.0×10^4 cells/well. The mean number

of colonies in both groups on the 10th day of culture (before confluency) is 53.4 $\pm$ 1.83 colonies/well (colony-forming efficiency about 0.01%).

When individual colonies are picked up after 10 days of culture and stained, two cell types are always found: mast cells and immature cells. We consider immature both blast cells and cells beginning to show signs of differentiation

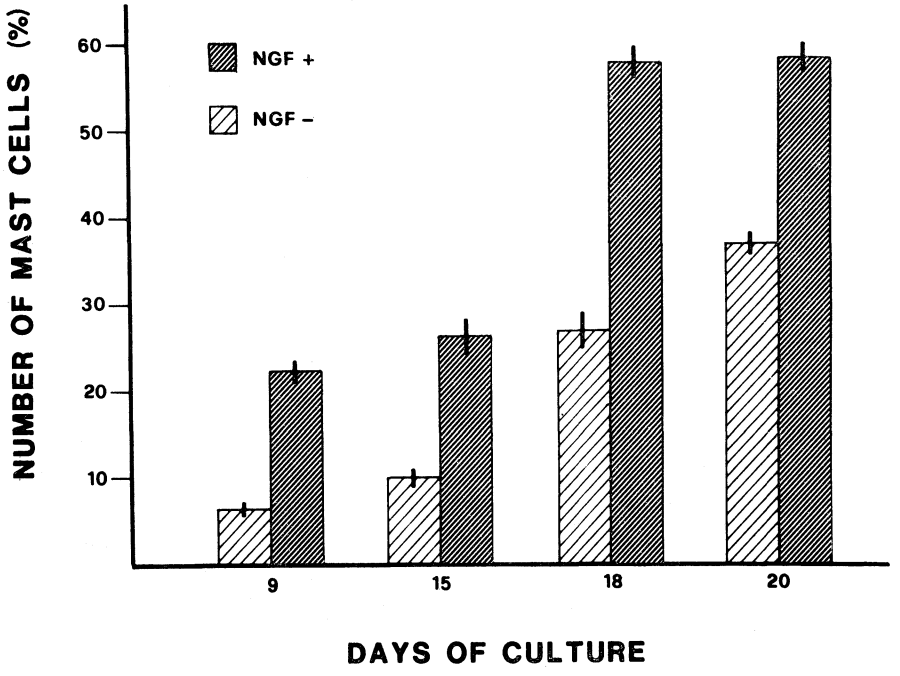

Fig. 4. - Histogram of mast cell percentage in NGF enriched cultures (dark bars) or in control NGF$^-$ cultures (light bars). Each point, referring to a single representative experiment, is presented as mean of ten percent counts $\pm$ S.D.

but insufficient morphological mark to permit identification of lineage. Macrophages or monocytes, however, are also present, while cells of other lineages are rarely observed. In no case pure mast cells colonies are scored.

In addition to May Grunwald-Giemsa staining the following techniques were used: Toluidine blue staining, immunohistofluorescence and electron microscopy. All provided additional unequivocable evidence that the cells analysed were mast cells. Criteria are summarized below.

Toluidine blue staining shows the typical metacrhomasia of cell granules. The presence of serotonine, characteristic of rat mast cells (Pl. II. fig. 2), as well as the presence of membrane IgE receptors (data not shown) are documented by indirect immunohistofluorescence studies.

Ultrastructural studies revealed that cells recovered after 16-20 days of culture have typical mast cell features. The cytoplasm is filled with large round-shaped electron-dense granules and the chromatin is more dense at the nucleus periphery than in the central core (Pl. III).

Discussion

The data presented demostrate that dissociated spleen cells of neonatal rats, when cultured for 2-3 weeks in presence of physiological amounts of NGF, acquire characteristics similar to those of fully differentiated mast cells [15]. These include: (i) presence of metachromatic granules in toluidine blue staining preparations and prominent dark cytoplasmatic granules after May Grunwald staining. (ii) presence of serotonine revealed by immunohistochemical techniques. (iii) demonstration of the presence of IgE receptors on the cell membrane. (iv) electron-dense granules of varying size and density throughout the whole cytoplasm.

In absence of NGF only a minority of cultured cells show features typical of mast cells, while the majority exhibit either the heterogeneous appearance of splenocytes or, more frequently, severe degenerative marks (when cells of NGF^+ or NGF^- cultures were maintained for long periods, viable cells are found only in the former (data not shown)).

The spleen, as is well known, is composed of erytrocytes, leucocytes, macrophages and precursors of these cell types [26]. NGF may be channelling the differentiation of one of these cell precursors or, alternatively, acting upon the pluripotent stem cell also present in the spleen.

After the early report by Ginsburg and Sachs [27] of the production of mast cells in thymus cell cultures, several authors described the existence of factors enhancing mast cell proliferation *in vitro* [17-20]. Although these studies have made a significant contribution to the elucidation of the properties of these factors [22, 28], no experimental evidence has been provided showing that such factors selectively stimulate mast cell increases *in vivo*. The present study, a previous one by our group [9], and those by Bruni *et al.* and by Sugiyama *et al.* [29, 30] demostrate that NGF exerts its effect *in vivo* as well as *in vitro*.

Since mast cells are endowed with an important role in immuno-defence mechanisms, our findings illustrate an entirely new property of NGF to enhance differentiation in a type of cell belonging to the immune system; they suggest that the search be extended to other putative target cells involved in immunological processes.

The mechanism of NGF's action on mast cells has still to be elucidated. Experiments aimed at exploring this and other possible NGF effects on cells of the immune system are currently under investigation in our laboratory.

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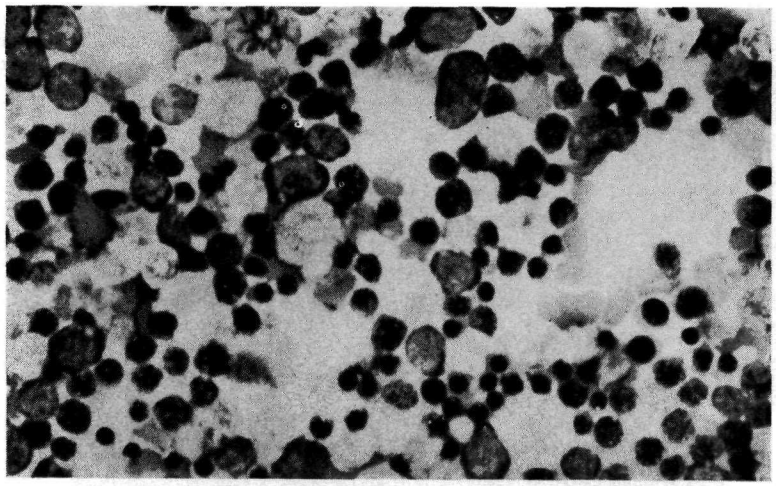


Fig. 1. – Photomicrograph of neonatal rat splenocytes stained with May Grunwald-Giemsa. Mast cells are extremely rare. Magnification × 470.

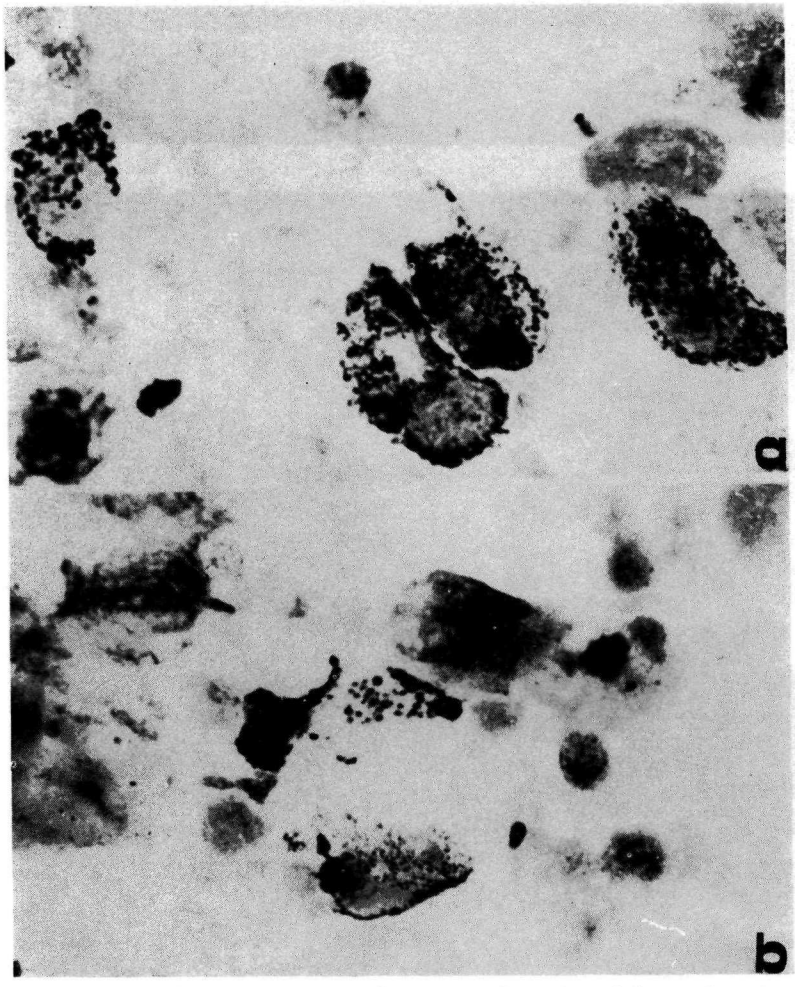


Fig. 2. – A cytocentrifuge preparation of spleen cells cultured for 9 days in presence of 10 ng/ml NGF (*a*) or without NGF (*b*). May Grunwald-Giemsa staining. The NGF-treated cells (*a*) have larger size and number of cytoplasmic granules than those of the control cultures (*b*). Magnification × 900.

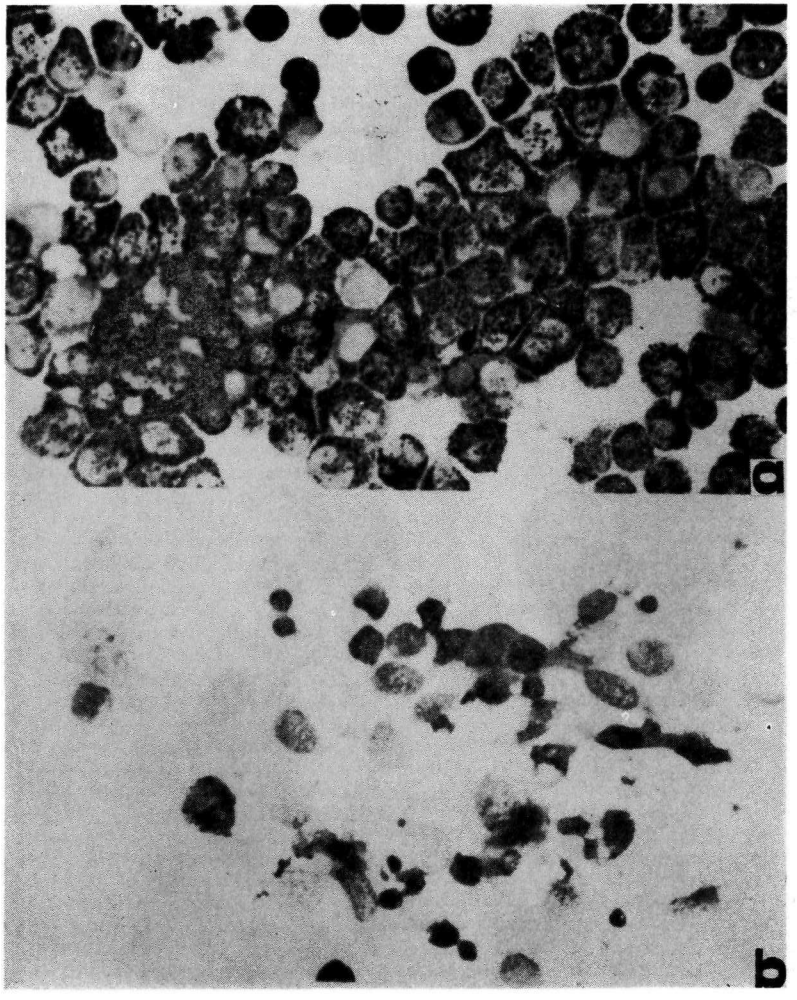


Fig. 1. – May Grunwald-Giemsa stain of a cytocentrifuge preparation of spleen cells cultured for 18 days with (*a*) or without (*b*) NGF. After 11 day of culture in semisolid medium the cells of both groups were transferred in liquid medium. Magnification × 470.

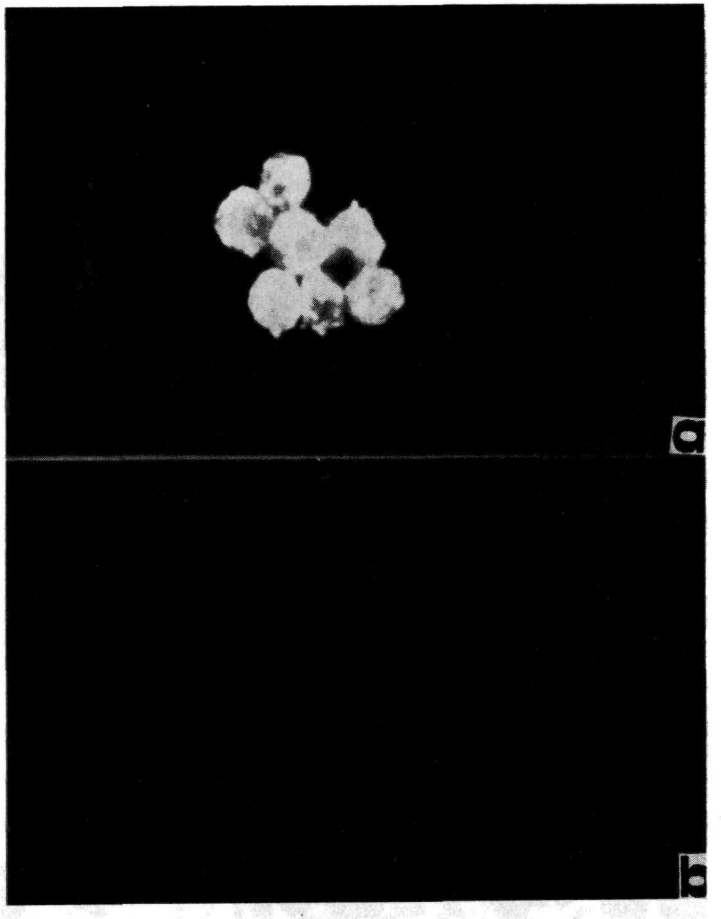
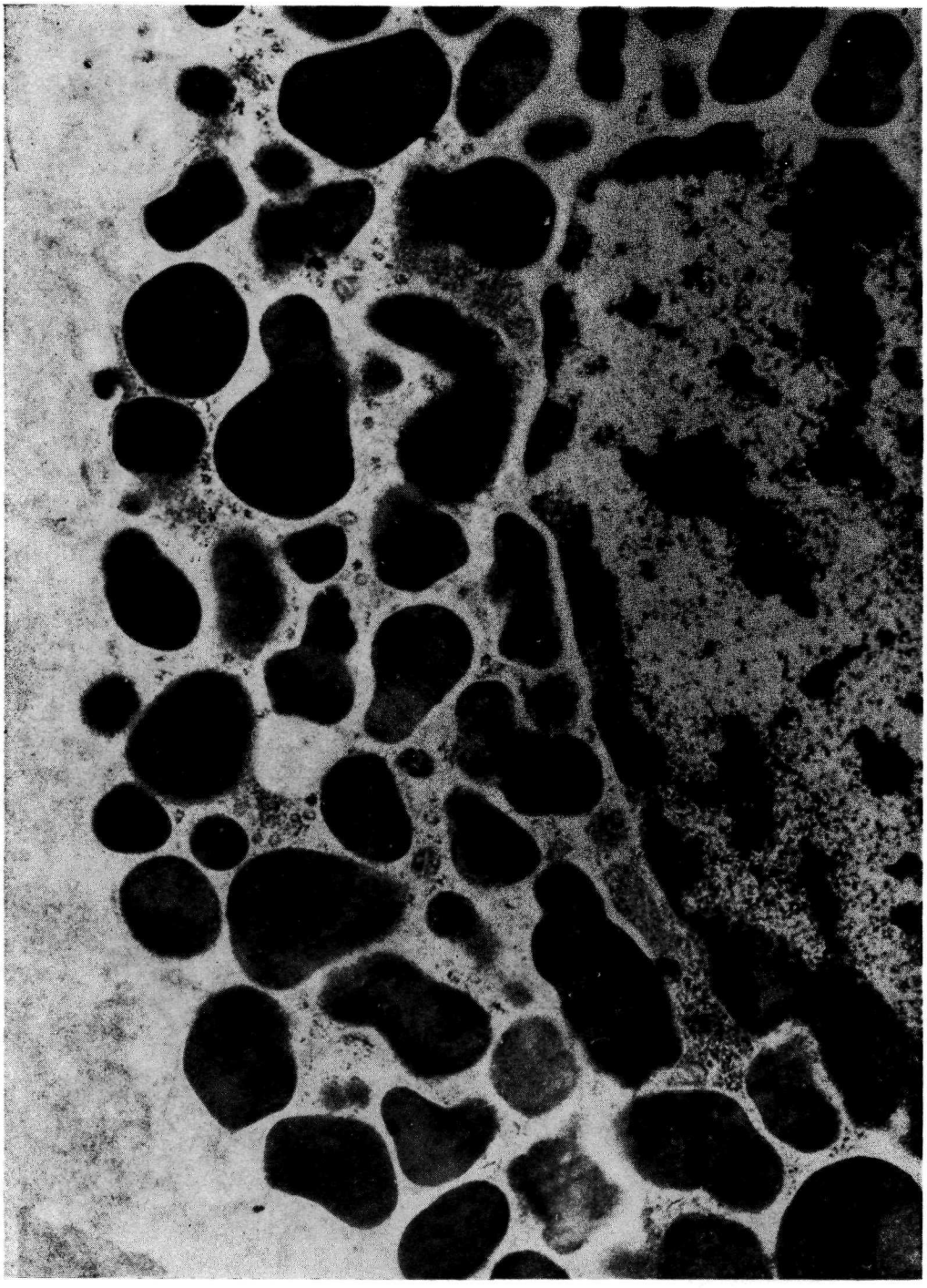


Fig. 2. – Photomicrographs of immunofluorescence preparations of cells from 16 days NGF^+ culture. Serotonine-containing granules are revealed after incubation with monoclonal antibodies against serotonine (*a*). As control cells of the same suspension were incubated with anti-serotonine previously adsorbed with an excess of antigen (*b*). Magnification × 470.



Electron micrograph of a part of the cytoplasmic and nuclear compartments of a mast cell from 18 days NGF^+ culture. Magnification × 20.500.