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Ranopterins-Amphibia Skin Pteridines Displaying Hematopoietic, Immunomodulatory, and Macrophageal Proliferative Biological Activities*

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Introduction

Unconjugated pteridines-xanthopterin, isoxanthopterin, leukopterin, etc., are found in high concentrations as pigments in insects, amphibia, reptiles and fish (1-7). As early as 1929, A. I. Hadjioloff described for the first time dermal structures (layers) and bright pigment cells with proper fluorescence in the amphibian and fish skin (see also refs. 8-12) which later led to the discovery of their pteridines content (13-19). Efforts have simultaneously been aimed at the morphological localization of these luminescent phenomena, their physico-chemical exploration and biochemical identification together with the precise definition of the luminophores, their isolation, purification, and assessment of their possible biological activities and feasible therapeutic application (20-26). In the cases of fluorescent substances isolated and purified from amphibia and frog skin-ranopterins (20, 21, 26)-as well as these isolated from the skin of fish-ichtiopterins (12, 13)-the results of 60 years Bulgarian school studies were presented in a monograph of A. I. Hadjioloff and E. Zvetkova (27).

Pteridines-containing dermal pigment cells - chromatophores in amphibian (Rana ridibunda and Rana agilis) skin: cytochemical, ultrastructural and histo-autoradiographical characteristics

The morphological and functional changes in the

frog and toad skin pigmentation are determined by the presence of the dermal color pigment cells-chromatophores and by their cellular interactions (8, 20, 28, 29). Depending on the color of their cytoplasmic pigments, the chromatophores belong to several groups (Hadjioloff's classification; ref. 8), namely: black (melanin-containing)-melanophores; white and brown (guanine-containing)-guanophores; blue, yellow and red (pteridines-containing)-cyanophores, xanthophores and erythrophores. By means of light- and electron microscopy the following types of chromatophores were distinguished in the amphibian derma (30-41): melanophores with specific melanin-containing cytoplasmic differentiations-melanosomes; guanophores with cytoplasmic reflecting plates-at the places of the extracted guanine crystals and pteridines-containing with which properties this review deals briefly.

The data from our ultrastructural investigations on the dermal pteridine-containing chromatophores in *Rana ridibunda* and *Rana agilis* skin (40) demonstrated the presence of specific cytoplasmic differentiation-pterinosomes. These specific organelles were first described in toad skin by Matsumoto *et al.* (37) as round or oval pigment granules sized from 0.5 μ to 1 μ and consisting of different numbers of concentric electron-dense elementary lamellae which, in their turn, consist of elementary granules. These cytoplasmic differentiations organelles which vary in size in the by us examined two species-*Rana ridibunda* and *Rana agilis* - are also different in the electron density of their elementary lamellae: from faintly outlined to highly osmiophillic ones which frequently form an electron-dense center or more

*This paper is dedicated on the memory of my teacher Acad. Assen Ivanov Hadjioloff and my brother Inj. Ivan Borissov Zvetkov

Figure 1. Electron microscopic picture of chromatophores in the derma of *Rana esculenta*: guanophore (G); light coloured pigment cell with specific pteridines-containing cytoplasmic structures-pterinosomes (Pt) composed of concentric lamellae; M-mitochondria; L-lipid drops (Hitachi electron microscope, $\times 15,000$).

compact periphery (Fig. 1). There are also entirely electron-dense (osmiophilic) pterinosomes with unclear lamellar structure some of which are similar to melanosomes (Fig. 2). We assumed that the differentiation of pterinosomes begins from unclearly outlined elementary lamellae, passes through more strongly osmiophilic ones to fully differentiated organelles with greater electron density and unclearly outlined lamellar structure. Varying in size mitochondria, glycogen granules, lipid droplets with medium electron density and fine granularity (carotinosomes) and a smooth endoplasmic reticulum were also soon established in the cytoplasm of dermal bright colored chromatophores in the two amphibia species examined (Figs. 1, 2). The well developed smooth endoplasmic reticulum in these

Figure 2. Electronogram of guanophore (G) with extracted guanine crystals in the cytoplasm; red pigment cells-erythrophores (E) with cytoplasmic lamellar structures, pterinosomes (Pt) and melanophore (M) with melanosomes (M) in the cytoplasm. One could see melanosome-like structures (m) in the cytoplasm of erythrophores (Hitachi electron microscope, $\times 6,500$).

cells which frequently forms vesicles, is probably responsible for the glycogen and carotinoid synthesis in the dermal xanthophores, cyanophores and erythrophores (29, 37, 41).

The guanine-containing cytoplasmic differentiations (crystal-like formations) in the guanophores were denominated by certain authors as iridosomes (29, 30, 31, 37, 41). In this cell type mitochondria are also well presented and localized between the guanine crystals, in the so-called by us "interstitial cytoplasm". The last two classes of chromatophores-containing pteridines and guanine - are interesting from the biochemical point of view by means of isolation and purification of biologically and pharmacologically active substances which might be found in their specific cytoplasmic differentiations organelles-pterinosomes and iridosomes. From this point of view the histophysiology of amphibian skin fluorescent layers and pigment cells is of great interest and some histo-autoradiographic studies on the genesis of their pigments were carried out (27, 42-45). By means of the histo-autoradiographic method the incorporation of biogenic amines and their precursors in the *Rana ridibunda* skin pigment cells was investigated. Measuring the dynamics of incorporation of ^3H -labelled tyrosine and dopamine in the skin melanophores and melanocytes, only tyrosine (L-3.5- ^3H tyrosine; specific activity 42Ci/mM, Amersham, Great Britain) and tryptophan (L-G- ^3H tryptophan; specific activity 8.8 Ci/mM, Amersham) incorporation could be detected in the bright-colored pigment cells-containing guanine in their cytoplasmic organelles-iridosomes and chromatophores containing pteridines in their pterinosomes. The results obtained by us are probably related to certain common stages in the synthesis of melanine and other color pigments, including pteridines in the chromatophores of lower vertebrates and are in support of our ultrastructural data about the simultaneous presence of pterinosomes and melanosomes in some pigment cells of amphibian dermis (40). We supposed that the intense incorporation of labelled tryptophan in amphibia skin chromatophores could be a sensitive biological marker of their pteridines biosynthesis, which is related to the intracellularly-induced tryptophan degradation (46).

Isolation and purification of ranopterins from the dermal chromatophores of amphibia (Rana ridibunda)

Skins of *Rana ridibunda* were homogenized in 50% methanol solution (in a Yoring Blender). The supernatant was distilled under vacuum until the separation of the methanol and condensed to 1/10

of the initial volume. Pteridines were purified by gel chromatography on Sephadex G1, on a column 40 mm/600 mm (realized by A. Dikov with the aid of W. Pfeleiderer; the method of pteridines purification was described by Hadjioloff et al., ref. 43; and by Shivatcheva, Hadjioloff and Dikov, ref. 47). After lyophilization of the effluent four pteridine fractions were identified by their chemical reactivity and ultraviolet absorption spectra: the fractions with a maximal fluorescence at 254 nm. were collected and concentrated in a rotation vacuum evaporator at 35°C (47). The highly concentrated material was applied to plaques for preparative thin layer chromatography, covered with cellulose and separated in a butanol:water:ammonia system. The material from many plaques was collected and the pterin fractions were extracted with 30% solution of acetic acid. The following four fractions were identified: isoxanthopterin, neopterin, pterin-6-carboxylic acid (Pt-6-COOH) and biopterin. First attempts in this direction in Bulgaria were made by Hadjioloff and Atsev (20) and Atzev and Hadjioloff (21) who isolated in 1968 only one pteridine derivative from the skin of *Rana ridibunda*. An analogous substance (ichthyopterine) was synthesized by Tschesche and Korte in 1951 (48); the blue fluorescence of ichthyopterine-containing chromatophores in the skin of fish was examined by fluorescent microscopy earlier by Hadjioloff and Kostev (12). Two of ranopterin fractions obtained by Dikov et al. (43, 47) - neopterin and Pt-6-COOH - were tested for their biological activities by us in the last decade.

On the biological activities of ranopterins: in vitro and in vivo studies

The first isolated ranopterin fraction identified by Atsev and Hadjioloff (21), and given to experimental rats, influenced proliferation and differentiation processes in bone marrow and other hematopoietic organs: accelerated the development of the youngest hemoblastic cells and delayed their differentiation. A rapidly emerging proliferative reaction, with appearance of activated reticular cells and lymphoplasmocytes (plasmoblasts, proplasmocytes), was established in lymph nodes of experimental mice after the exogenous pteridine/ranopterin treatment *in vivo* and *in vitro*, by some fractions of ranopterins isolated by A. Dikov (43, 47). Pteridine fractions neopterin and Pt-6-COOH isolated from the chromatophores of *Rana ridibunda* skin were tested *in vitro* and *in vivo* in different experimental models (49-55). The results obtained demonstrated their hematopoietic, immunomodulatory and bone marrow macrophage/stro-

mal cells-proliferative activities.

The hematopoietic activity of the ranopterin fraction (Pt-6-COOH) was tested *in vitro* in conditions of murine bone marrow agar cultures (49, 50). Upon testing the pteridine fraction has been added to the agar cultures medium at doses of 10, 25, 50 and 100 µg/ml. The cell types present in the colonies and clusters were cytologically and cytochemically characterized after *in situ* staining (in agar cultures) for DNP, RNP and basic proteins (56). The results show a well expressed macrophageal-granulocyte colony-stimulating activity (MG-CSA) of the exogenously added ranopterin fraction Pt-6-COOH (Fig. 3). As to the induction of myeloid cell proliferation the maximum effect was established upon Pt-6-COOH application at a dose of 25-50 µg/ml culture medium. The MG-CSA of Pt-6-COOH in mouse bone marrow agar cultures is probably due to the effect of this biologically active substance on the proliferation and differentiation of the early bone marrow progenitors from the macrophage/granulocyte series. The problem whether the molecular and cellular mechanisms of the *in vitro* hematopoietic effects of Pt-6-COOH are similar to the ones of hematopoietic growth factors is suitable for further investigations. It is possible that exogenously

Figure 3. A: Macrophageal (M-) colony; B: macrophageal/granulocyte (MG-) colony; in murine bone marrow agar cultures stimulated by Pt-6-COOH (Staining in agar, ref. 56; $\times 1,250$ -immersion).

added Pt-6-COOH penetrates through the cellular and nuclear membranes influencing differentiation of granulocytes/macrophages from their bone marrow progenitors (49, 50, 57). In our previous studies we also established a presence of MG-CSA in the supernatants from mouse long-term bone marrow cultures exogenously treated with two ranopterine fractions Pt-6-COOH and neopterin (54).

In the neopterin treated (at doses 20-50 µg/ml) liquid short- and long-term mouse bone marrow cultures, a shift in the cellular composition was observed as early as the 3rd day of cultivation (51). The cytological (cytocentrifugal/cytospin) preparations prevailed small- and medium-sized, peroxidase positive- and phagocytically inactive macrophages. In the cytoplasm of these small immature bone marrow macrophages one can see a positive reaction for basic (fast green positive-) cytoplasmic proteins as well as strongly expressed NOS-activity (51, 52, and Zvetkova *et al.*, unpublished).

In the stromal layers of neopterin-treated long-term mouse bone marrow cultures one could see hematopoietic islets among stromal cells stained on the plastic. Our results obtained on the bone marrow stromal macrophages proliferation under the exogenous *in vitro* treatment by ranopterine neopterin could serve as a subject for further discussions and may be of practical importance, having in mind the role of bone marrow stromal cells in the entire hematopoiesis. In this respect it is interesting to note that in murine erythroleukaemia cells tetrahydrobiopterin synthesis has been demonstrated to be functionally connected with cell proliferation (58). The pioneers in this field of investigations, Jacobson and Simpson (59), first described the possible use of pteridines in the elucidation of antipernicious anemia factor.

Using small doses of ranopterine neopterin in our *in vitro* model system (exogenously added neopterin, at doses 10-25 mg/ml medium, to the liquid mouse bone marrow cultures) we observed from 96 h to day 7 of cultivation that bone marrow stromal cells-stromal macrophages and fibroblast-like cells- considerably enhanced their adhesiveness, forming abundant cytoplasmic protrusions and synthesizing extracellular matrix, probably containing fibronectin and/or other cell adhesion molecules (Fig. 4; E. Zvetkova, E. Katzarova, Sv. Petkova, unpublished). The formation of cytoplasmic protrusions and the production of extracellular matrix with high adhesiveness by bone marrow stromal cells may be interpreted as one sign of bone marrow stromal activation (51) which is probably of importance in some pathological cases of enhanced or reduced marrow

Figure 4. Bone marrow stromal cells stained in the bottom of the flask in the long term bone marrow cultures, treated *in vitro* by 25 µg/ml ranopterine-neopterin (A, B, D) and by 50 µg/ml (C): Ma-macrophages differing in size and degree of cell activation (A-C) and with apoptotic nuclei (C-arrowheads); intercellular (FN-) matrix (A-arrows); fibroblast-like cell with a long cytoplasmic dentrit-like uropode (D - arrow). Staining by the method of Zvetkova and Zvetkov, (ref. 99; $\times 1,250$ -immersion).

hematopoietic functions as well as in cases of bone marrow transplantation (60). In this line of thoughts and having in mind ours and others experimental results (60, 61) confirming that fibronectin and other molecules of high adhesiveness in the bone marrow matrix produce better blood cell differentiation we suggest that low doses of exogenously applied ranopterine neopterin could be suitable for improving the adhesive and motility properties not only of the normal hematopoietic cells but probably of the leukemic ones (E. Zvetkova, E. Katzarova, Sv. Petkova, unpublished). We also obtained that exogenously added ranopterine neopterin at higher doses (50-100 µg/ml culture medium) could act as an apoptosis-inducing factor (E. Zvetkova, unpublished).

In the literature there are other interesting facts concerning cell adhesion phenomena and mononuclear cells expression of surface adhesion molecules such as MAC-1, an active integrin, involved in triggering the neutrophils adhesion to endothelial cells (62). On the one hand, the higher expression of adhesion molecules on lymphocytes and monocytes in various diseases is relevant in the activated immune

mechanisms leading to organ damage; on the other hand, the expression/overexpression of some surface adhesion molecules is inducible by inflammatory cytokines like interleukin-1 and tumor necrosis factor-(TNF)- α mainly secreted by the monocyte/macrophage system producing also large amounts neopterin as a sensitive indicator of activated cell-mediated immune response (63, 64). Instead of the fact that in some cases of chronic immune activation the concentrations of neopterin were not related to the adhesion molecules levels (64) the probable involvement of immunocompetent cells in the production of both neopterin and adhesion molecules could be further discussed in the light of data that TNF- α synergistically amplifies macrophages for neopterin secretion (46) and that like neopterin, the soluble receptors for TNF are parameters of cell-mediated

immune activation (65-67).

The activation of the adhesion phenomena has been also observed locally in the amphibian (*Rana esculenta*) skin-e.g. in the first described by Hadjioloff (8) and so-called "fibronectin pathways" (68) for pigment cell migration in the dermal tract of the frog skin. These vertical tracts (Fig. 5) connected the dermal stratum spongiosum with subcutaneous tissue (see also refs. 22-25, 27). The fibronectin tracts in the frog skin contained fibronectin-outlined flattened pigmentary cells and fibroblasts (68). In the organ/tissue cultures, both pigmentary cells and fibroblasts disappeared from the vertical dermal fibronectin-tracts when antiserum to fibronectin was added to the culture medium. The data of Deneffe *et al.* (68) suggested that the vertical tracts in the frog skin are involved in the migration of both cell

Figure 5. Dorsal (left panel) and ventral (right panel) skin of *Rana ridibunda*: Fibronectin (FN-) tracts-arrows; one can see melanophores-in the dorsal skin; bright pigment cells-chromatophores and guanophores (arrowheads) are well presented in the ventral skin (staining by hematoxyline-eosine; $\times 300$).

types. In such cases one could speculate about the probable role of chromatophores and their pteridines content in the cellular mechanisms of fibronectin production. We supposed (in the light of our recent experimental data) that the fibronectin-outlined fibroblasts in the amphibian fibronectin dermal tracts could be stimulated for their fibronectin production by pteridines-dispersed as pterinosomes from the pteridines-containing dermal chromatophores in the extracellular space-between neighbouring fibroblasts. Moreover, Denefle *et al.* (68) also observed dispersed melanosomes from the cytoplasm of elongated melanophores. The melanophore migration observed by Iwata *et al.*, (69) in the scales of the fish medaka showed the same pattern. All these results are in good agreement with some recent data in the literature concerning the neopterin-induced intercellular adhesion molecule-1 expression in rat alveolar epithelial cells L2 (70). On the basis of these results of ours obtained *in vitro* and concerning the stimulatory effects of some ranopterin fractions - Pt-6-COOH and neopterin - on the entire bone marrow hematopoiesis (macrophage/granulocyte and stromal cell lines including) our studies in this direction have been continued as experiments *in vivo*.

On the putative in vivo influence of ranopterin neopterin

We have shown as early as 1968 that a pteridine derivative with high biological activity was isolated from the skin of *Rana ridibunda* and applied *in vivo* on the experimental mice (21). It was concluded that this pteridine preparation probably accelerate the processes of cell proliferation and differentiation of some cell lines in the hematopoietic system. Jacobson and Simpson (59) also identified the fluorescent substance isolated from the wings of butterflies as leucopterin and established the changes in the hematopoiesis under the biological action of this pteridine fraction. Busnel *et al.* (71) investigated the high biological activity of isoxantopterin-oxycarbonic acid, in the scales of fish. Hadjioloff *et al.* (26) examined the cellular lymph nodes responses after *in vivo* administration of some ranopterin fractions and showed the rapid increase in the values of the lymphoplasmacytes as an indication for immunogenic action of the ranopterins.

We have investigated the *in vitro* (51) and *in vivo* (52) influences of the exogenous ranopterin fraction-neopterin-on the lymphoproliferative, immunogenic and mitogenic lymphocyte responses in mice. It was found previously that in the liquid mouse long-term bone marrow cultures, exogenously treated by ranopterin neopterin, the one of the stim-

ulated cell population was this of the small marrow lymphocytes-with intensively stained cytoplasmic RNP-granules (51). These results of ours could be discussed in the light of other data (26, 47) demonstrating a pronounced cell proliferation in the so-called B-zones of the mouse lymph nodes (with a high number of plasmacytes)-after *in vitro* and *in vivo* treatment with some ranopterin fractions.

Our recent data strongly suggest that the *in vivo* (i. p.) application of the ranopterin fraction-neopterin-in mice (at a dose of 25 µg per animal) induced lymphocyte proliferation in the spleen and increased responsibility of spleen lymphocytes to the T-cell mitogens (52). Flow cytometric analysis on the peripheral blood lymphocytes was also performed in the same experimental conditions using mice CD3+T lymphocytes marker (conjugated with Phycoerythrin) and FITC-conjugated marker for the surface IgG on B cells. The results obtained from the flow cytometric analysis show that the *in vivo* neopterin treatment of mice induced an increase of the whole blood CD3+T lymphocytes, simultaneously with expressed lymphocytosis in the peripheral blood of neopterin-treated mice. In the blood smears of neopterin-treated animals as well as in their spleen histological preparations and electronograms one can see a high number of lymphoblasts (probably T-immunoblasts; Fig. 6). Thus a significant stimulatory effects of the exogenous ranopterin neopterin on the cell-mediated immune activation (spleen T-lymphocytes responsibility to the T-cell mitogens including) suggest that this ranopterin fraction could be examined as a source of new preparations in states of immunodeficiency and/or immunosuppression. On the other hand, it is generally accepted that macrophages synthesize neopterin as a result of stimulation by cytokines derived from activated T-cells, such as interferon-g (57, 72, 73). Though the biological function of neopterin remains unclear, elevation of its contents in body fluids have been reported in patients with various immune-related diseases, as well as in patients with viral infections such as HIV (63, 74-80). In turn, interferon-g stimulates neopterin production in human monocytes/macrophages, and a neopterin concentration in body fluids is a sensitive indicator of the activation of cell-mediated immunity (63, 72, 81).

In all cases of neopterin-induced T-cell activation as well as in the cases of bone marrow and stromal cells stimulation-under the *in vitro* and *in vivo* influence of exogeneous treatment by ranopterins (Pt-6-COOH, neopterin) the fact could be generally considered that pteridines derivatives and especially neopterin shows some structural similarity to gua-

Figure 6. Neopterin-induced lymphoproliferation in the spleen of mice with the prevalence of euchromatin in blast cells nuclei, active nucleoli and polyribosomes in the cytoplasm (Opton electron microscope; $\times 65,000$).

nosine triphosphate (GTP) (57, 82). In parallel, interferon- γ activated macrophages secrete neopterin and 7,8-dihydroneopterin (72, 83, 84) synthesized from GTP, which is in a first step enzymatically cleaved by the GTP-cyclohydrolase I to 7,8-dihydroneopterin triphosphate (85). We supposed (49) that exogenously added pteridines/ranopterins to the cell cultures, as a small molecules, generally penetrated through the cell and nuclear membranes thus affecting the cellular/nuclear metabolism including cellular proliferation and differentiation in insufficiently clarified biological ways-probably after an increase of the intracellular GTP concentrations (51, 53, 57). By contrast, colchicine and colchicine derivatives (colcemide) are able to inhibit T-cell activation within the cellular immune response as well as interferon- γ and neopterin production in human peripheral blood mononuclear cells (86). Recently we have shown the strongest activation of nitric oxide synthase (NOS) established in a population of large and medium-sized mouse peritoneal macrophages and a pronounced NOS activity in one population of medium-sized and small mouse bone marrow macrophages (Fig. 7), under the *in vivo* influence of the ranopterins fraction neopterin-applied at the same experimental conditions as described in our previous models (52). We concluded that the enhanced NOS macrophageal activity under the *in vivo* action of exogenously applied ranopterins neopterin could be due to both: directly to pterin (tetrahydrobiopterin-BH4) requirement of murine macrophage NOS in

Figure 7. Nitric oxide synthase (NOS) activity in the cytoplasm of murine peritoneal macrophages-activated after *in vivo* treatment by the ranopterins-neopterin (A, B); NOS-positive small- and medium-sized bone marrow macrophages following intravital treatment by neopterin. (Hope and Vincent method, 1989, in our modification; $\times 1,250$ -Immersion).

a concentration-dependent fashion and/or indirectly-through the stimulation of the synthesis of cytokines (cytokine-stimulating pteridine biosynthesis in human monocytes/macrophages; ref. 7). Schobersberger *et al.* (87) showed that the pteridine compound neopterin is a stimulus for inducible NOS (iNOS) gene expression in rat vascular smooth muscle cells *in vitro*. The authors speculated that in this way (induction of iNOS) neopterin enhances the macrophage-induced extracellular toxicity and this might be of relevance for situations associated with excessive release of cytokines, neopterin and nitric oxide (NO), as is observed in septic shock. The mechanisms whereby neopterin stimulates *in vitro* iNOS gene expression and influenced/activated NO synthesis remained unclear but it is conceivable (88) that neopterin exhibits these effects through activation of transcription factor nuclear factor- κ B (NF- κ B). Hoffmann *et al.* (88) concluded that neopterin activates the translocation of NF- κ B subunits to the nucleus by modulating the intracellular redox state.

These data correlate with the findings of other authors that neopterin-derivatives produced from activated macrophages have a similar impact on NF- κ B (89). Induction of programmed cell death by these substances (90, 91) was also most likely due to the stimulation of iNOS gene expression and NO synthesis (52, 92-94) and thus macrophage-derived neopterin is potentially involved in the basic pathological processes such as atherogenesis (94). It is also interesting fact that *in vivo*, neopterin and proinflammatory cytokines interferon- γ and TNF- α , are closely correlated concerning their synthesis, secretion and biological effects as well as their proapoptotic behavior (46, 90, 91, 94-96). Moreover, data of Hoffmann *et al.* (94) revealed a dose-dependent effect of neopterin on TNF- α gene expression.

Recent data suggesting that the pteridines neopterin and 7,8-dihydroneopterin as well as the proinflammatory cytokines like TNF- α and interferon- γ are common events in situations associated with an increased cellular immune system and macrophageal activity (52, 53, 65, 67, 74, 77, 81) gave us ideas to study the potential protective effect of exogenously applied ranopterin fraction neopterin on the experimentally induced Graffi tumors in hamsters (55). The results pointed out the best protective effect of the ranopterin neopterin against the neoplasia development and tumor progression in cases when experimental tumor-bearing animals were neopterin-pretreated (a single neopterin application, 21-42 days before the tumor implantation at a dose 30 μ g per animal). The precise mechanisms by which the neopterin pretreatment could modify tumor growth are probably indirect-by activation/pre-activation of the host cell-mediated immunity (52, 53): one population of small cytotoxic T-lymphocytes with RNP-granules in their cytoplasm appeared in a high percent in the peripheral blood smears of pre-treated by neopterin tumor-bearing animals (Fig. 8). In previous studies (97, 98) we have demonstrated that this small cytotoxic T-lymphocytes population directly participates in the cytotoxic/immunogenic antitumor mechanisms in cancer patients as well as in preneoplasias (Fig. 8C, D). In all these cases one can see the loss of cytoplasmic RNP from the cytoplasm of small cytotoxic lymphocytes in cytological and/or peripheral blood smears of cancer patients and tumor-bearing animals which gave us ideas about probable direct participation of small T-lymphocytes cytoplasmic RNP in the direct tumor cells cytotoxicity (Fig. 8A, B; E. Zvetkova, G. Kostov, I. Zvetkov, I. Valkov, unpublished). Yet we supposed that the potent antitumor effector T-cells could be CD8, also responsible for the direct lysis of SIV-

Figure 8. A: Cancer cells (arrowheads) and small lymphocytes (small arrowheads) interrelationships: small lymphocytes forming rosette-like structure around one tumor cell; B: cancer cells (arrows), small lymphocytes (arrowheads) and their relationships on the level of cytoplasmic RNP (*) which are unstained in places of cell to cell contacts (a possible influence of integrins or other biologically active substances on the cell membranes permeability could be discussed-Zvetkova *et al.*, unpublished). Cytological smears, stained by the method of Zvetkova and Zvetkov, ref. 99 ($\times 600$); C: small lymphocytes from the peripheral blood of tumor-bearing animals, with reduction of cytoplasmic RNP, by clasmatoses (arrowheads); blood smears stained by the same method ($\times 600$); D: electronograms of cancer-patient lymphocytes with abundant ribosomes-containing clasmatoses (arrows) at the cytoplasm periphery (Opton electron microscope, $\times 6,000$).

infected cells (inhibiting SIV-virus replication through the release of soluble cellular antiviral factors; ref. 80). As it is demonstrated in Fig. 8A and B, it is possible that T-lymphocytes/tumor cells interactions

in cancer patients and tumor-bearing animals could be also mediated by soluble antitumor factor(s) released from the T-cells cytoplasmic RNP, moreover that the released of CD8 cell-derived antiviral/suppressive factor(s) are more common, yet unidentified, and/or active on different immunodeficiency viruses (80) (why not on these involved in the pathogenesis of some tumors and leukaemias; refs. 55, 88). In addition, a direct antitumor action of the neopterin-activated *in vivo* and NO-producing mononuclear (monocyte/macrophage) populations/subpopulations could be also discussed (51-53) as a possible cellular mechanism of protection against tumor growth and development.

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