Morphological Changes in Transplanted Solid Ehrlich Tumor of Mice after Transfer of Stimulated Spleen Cells

Ivan Raichev, Raina Borissova

Department of Pathology, Pleven University School of Medicine, Pleven 5800, Bulgaria

Spleen cells from mice bearing solid Ehrlich tumor stimulated in vitro with Ehrlich-tumor extract or with PHA, IL-2 and tumor extract were transferred i.v. in mice-bearers of small transplanted Ehrlich tumor (Group I) and in tumor-free mice, followed by tumor transplantation (Group II). Morphological findings for antitumor effect were found in tumors of 42.5% of experimental mice. Four criteria were established in this respect, including evidence for lymphocytic cytotoxity. Production of a factor inhibiting Ehrlich ascites-cell migration was found in a migration-inhibition assay from the stimulated spleen cells and spleen cells obtained from mice of Group I and II. The indirect data suggested that the transferred stimulated spleen cells (lymphocytes) propagated in lymphoid organs, particularly in the spleen of mice. Then T-cells, especially tumor-specific cytotoxic lymphocytes, migrated by circulation into the periphery of the tumor nodule and influenced on tumor cells.
ACTA MEDICA NAGASAKIENSIA 48 : 107−116, 2003

Key Words: *solid Ehrlich tumor, mitotic index, apoptotic bodies, lymphocytic cytotoxicity, inhibitory factor*

Introduction

It has been shown that Ehrlich tumor can grow in almost any mouse strain in an ascitic or solid tumor, across histocompatibility barriers[1,2], as it lacks H-2 antigens[3]. On the other hand, Ehrlich cells possess antigens recognized as foreign in mice, which provoke an immune response after s.c. but not after i.p. inoculation of the living tumor cells[4]. Subiza et al.[2] reported that a nonspecific suppressor cell population (splenic natural suppressor cells) had been developed in the spleen of mice bearing solid Ehrlich tumor.

The transplanted solid Ehrlich tumor in mice proved to be an appropriate model for investigation of the effect of adoptively transferred sensitized spleen cells. It is important for immunotherapy against the neoplasm to detect morphological findings connected with antitumor effect of transferred spleen cells stimulated with immunogenic Ehrlich-tumor extract.

The purpose of this study was to determine the morphological evidence suggesting for antitumor effect against transplanted solid Ehrlich tumors in mice in which spleen cells, obtained from Ehrlich-tumor bearing mice and stimulated with Ehrlich extract alone, or in combination with PHA and IL-2 were transferred.

Material and Methods

Animals

Inbred male C57BL/6 mice, 10-12 weeks old, were obtained from the production colonies of the Bulgarian Academy of Science and were used in experiments according to the Institutional Guidelines of Pleven University School of Medicine.

Tumors and tumor-bearing mice

Solid Ehrlich tumor was established by s.c. inoculation of Ehrlich ascites tumor cells (2×10^7) in the back of mice, and was maintained by serial s.c. transplantation of small pieces of tumor in mice. Animals carrying solid transplanted Ehrlich tumor of the 3rd to 8th passage generations were used. Ehrlich ascites tumor was maintained by serial i.p. transplantation (1×10^6 cells) every 10 days in intact tumor-free mice.

Spleen cell suspensions

The spleen cell suspensions were prepared in RPMI 1640 (GIBCO) supplemented with 5% FCS, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). The aseptically removed spleens from mice-bearers of growing tumors were pressed between

Address Correspondence: Ivan Todorov Raichev, M.D. D.Sc. Liulin, 605 Street, bl.603, apt. 35, 1336 Sofia, Bulgaria.
TEL: 359 2 27 47 98
E-mail: iven_ro@yahoo.com

two sterile slides, and, after filtration, the spleen cells were freed of erythrocytes by treatment with Tris-buffered 0.83% ammonium chloride (pH 7.2). The suspensions were incubated in plastic flasks at 37℃ for 35 min., and then were adjusted to a final concentration of 4×10^6 cells/ml. A T-lymphocytes- rich fraction was obtained by passing spleen cell suspensions through a nylon wool column.

Preparation of an Ehrlich-tumor extract and stimulation of the spleen cells with it

Tumor extract was obtained by using an extraction procedure as it was described previously[5,6]. Briefly, ten milliliters of cold 3 M KCL in PBS (pH 7.2) was added to every 1 g of tissue of solid Ehrlich tumor for 16 hr. The mixture was centrifuged at 40,000 x g for 1 hr at 4℃. Further centrifugation of the mixture at 18,000 x g was also done. The supernatants were dialyzed against PBS, and the protein content was adjusted to 0.30-0.40 mg protein/ml. Samples of 0.4 ml of spleen cell suspension, each containing 8×10^5 cells $(2 \times 10^6/ml)$ were mixed with 80 μl of Ehrlich extract and incubated for 24 hr at 37℃ in a 5% CO_2 atmosphere. The mixture in approximately 1/10 of the total number of samples were centrifuged at 18,000 x g for 20 min at 4℃. The supernatants were decanted and then prepared for examination of migration inhibition activity as was described previously[5]. The remaining samples were centrifuged at 400 x g for 10 min to preserve viability of the cells. The supernatants were decanted, passed through 0.45 μ m filters and investigated for migration inhibition, too. The spleen cells were harvested, washed and suspended in HBSS.

Complex stimulation of spleen cells was carried out in 24 flat-bottom well plates (Linbro, Flow Laboratories). Spleen cell suspensions containing 8×10^5 cells in 0.4 ml of each well were supplemented with PHA (1% v/v); 0.5 ml of the medium was added and incubated for 48 hr at 37 ℃ in a humid atmosphere of 5% CO_2. After the incubation with PHA a supernatant containing T-cell growth factor (IL-2), 50% v/v, was added, approximately 10 U IL-2 in each well with 8×10^5 cells. This supernatant was obtained from spleen cells of exbreeder mice $(1 \times 10^6$ cells/ml) stimulated with PHA (2 μg/ml) for 48 hr, and then depleted of PHA using chicken erythrocytes[7]. After 4 days of culture the procedure was continued by adding the extract of solid Ehrlich tumor of 80 μl to wells for 24 hr. The supernatants were decanted and after filtration were assayed for migration inhibition. The cells were harvested, washed in HBSS and prepared for inoculation in mice.

Assessment of migration inhibition

Samples of 0.2 ml of Ehrlich ascites tumor cells (2 x 10^7/ml), suspended in RPMI 1640 supplemented with 5% fetal calf serum and antibiotics were mixed with 0.1 ml of the supernatants of spleen cells stimulated as was indicated above, or not stimulated. The mixtures were incubated for 2 hr at 37℃ in an atmosphere of 5% CO_2. Capillary tubes sealed at one end were filled with Ehrlich cells, and centrifuged at 250 x g. The portions with sedimented cells were cut off, placed in chambers filled with medium and incubated at 37℃ for 24 hr. The areas of Ehrlich-cell migration were projected and measured by planimetry. The migration index (MgI) was calculated according to the following formula:

$$MgI = \frac{\text{Average area of migration of Ehrlich cells incubated with the supernatant}}{\text{Average area of migration of Ehrlich cells incubated without the supernatant}}$$

Fraction of responding (positive) cultures (FrRCs) was calculated as number of responding cultures per total number of cultures. Cultures were designated as responding (positive) when the supernatants obtained from them show an inhibition effect (IE) against Ehrlich-cell migration (MgI<0.80). For example, FrRCs is 0.8 (8/10) in the presence of 8 responding (positive) cultures).

Inoculation of spleen cells in mice and formation of experimental groups

The stimulated spleen cells from tumor-bearing mice were concentrated in HBSS at 20-25 x 10^6 cells/ml. Seventy animals were divided in three groups. In Group I the stimulated spleen cells were transferred by one or two i.v. injections through the tail vein in mice, bearing a small Ehrlich tumor, and in Group II in tumor-free mice in which the transplantation of tumor was accomplished 7-8 days after the inoculation. Twenty mice were in each group. According the preliminary experiments, the mice of Group I and II were treated predominantly with 35-40 x 10^6 stimulated spleen cells; ten mice-with 50-55 x 10^6 cells. Previously, doses of 20-25 x 10^6 cells were used, too. The mice of Group I were sacrificed by cervical dislocation after a mean term of 22.40±3.09 days of stimulated spleen cell transfer (34.86±4.13 days of tumor transplantation), and these of Group II- 34.45±4.62 days after spleen cell transfer (27.85±4.15 days of transplantation). Additionally, ten tumor-bearing mice treated with 35-40 x 10^6 stimulated spleen cells were killed within three to six days after the cell transfer.

As controls (Group III) were used 20 tumor-bearing mice untreated by transfer of stimulated spleen cells.

Histological examination

Small tissue slices were fixed in 10% neutral formalin (pH 7.0) for 24 hr and embedded in paraffin. Deparaffinized sections 4 μm thick were stained by using the following procedures: hematoxylin and eosin (H&E), v. Gieson, Feulgen for DNA, methylgreen pyronin for RNA, PAS (Hotchkiss). The mitotic count was performed on H&E stained sections in the most mitotically active areas[8]. High-power fields, limited by restrictive screen at 200 μm x 200 μm, were investigated by a Carl Zeiss microscope (Germany). Mitotic index (MI) was calculated as the number of mitoses per number of counted tumor cells x 100. The apoptosis, which is a mode of cell death, was established by the apoptotic cells and bodies. They were counted, in tumor slices stained with H&E and Feulgen. High-power fields, limited as is shown above, were randomly selected. The apoptotic index (AI) was assessed as a number of apoptotic cells and bodies per number of counted tumor cells x 100. Two thousand tumor cells were counted for determination the mitotic and the apoptotic index.

The reactive changes in the T- and B-cell regions of spleens and lymph nodes of the mice were graded in three degrees (I-III) according to their size, cell activity and cell transformation.

Staining of cells in the smears and electron microscopy

Smears from sediments were stained by the Giemsa method and by methylgreen pyronin for RNA. For electron microscopic examination pieces of tumor were fixed with 1.6% glutaraldehyde in 0.1 M phosphate buffered saline (pH 7.4) at 4℃ for 2 hr and with 2.5% for 1 hr, postfixed in 1% osmium tetroxide, dehydrated with ethanol and embedded in Durcupan (Fluka AG). Ultrathin sections were stained with uranyl acetate and lead citrate, and were investigated with an electron microscope Tesla B-500.

Statistics

Statistical analysis was performed by using the Student's t test, chi-square test and by determing of the correlation coefficient. The significance of difference between the compared groups was defined with the Mann-Whitney U test. P<0.05 was considered to be significant for the two-tailed test.

Results

Morphological changes in the transplanted solid Ehrlich tumors suggestive for antitumor effect

In mice bearers of solid Ehrlich tumor treated with stimulated spleen cells we described morphological changes related to the suppression of growth and extension of tumor, death of tumor cells (apoptosis and necrosis) and tumor regression. On the basis of these morphological changes we described four criteria for antitumor effect (a.e.) against the transplanted tumor of mice treated with stimulated spleen cells as follows: 1) Evidence of a small tumor nodule without extension in surrounding tissues usually with central zone of necrosis, and preserved tumor cells peripherally showing decrease of their proliferation and an activity of apoptosis. 2) Infiltration of the periphery of tumors with lymphocytes and a few plasmocytes which was complete, continuous, engaging entire circumference of tumor nodule, or incomplete. 3) Invasion of immune lymphocytes among tumor cells with histological and ultrastructural data for lymphocytic cytotoxity. 4) Formation of a fibrous capsule in the periphery of tumor between the cells of infiltrate.

Morphological findings in the transplanted solid Ehrlich tumor, suggesting for a.e., were found in 42.5% of mice of Group I and II treated by transfer of stimulated spleen cells (7 from 20 in Group I and 10 from 20 in Group II). In mice with such an effect small tumor nodule was found to be as big as grain lentil or pea, one was larger (5 x 6 mm). Histologically, tumor nodules were limited. Usually, around the area of necrosis tumor tissue was detected, sized between 250 μm and 600 μm wide, and rarely - 800 μm. It was composed mainly of mononuclear cells with pale nuclei, but in some tumors numerous cells with hyperchromatic nuclei were found, as well as giant mono- and multinuclear cells in the proximity of necrosis. Infiltrate rich in lymphocytes and mixed with a few plasmocytes and macrophages was established in the periphery of the tumor (Fig. 1), and partially invaded tumor tissue. This infiltrate was mainly continuous (complete) or, more rarely, incomplete engaging the half or two thirds of the circumference of the tumor nodule. Small blood vessels, often augmented in number, were found in this area (Fig. 2). Tumor cells in apoptosis and apoptotic bodies were observed among the invading lymphocytes and between preserved tumor cells (Fig. 3). Focuses of necrosis were detected in proximity to the lymphocytic infiltrate. The fibrous capsule formed in the periphery of tumor was mainly complete i.e. in the entire circumference of tumor, or incomplete. Evidence of proliferation of
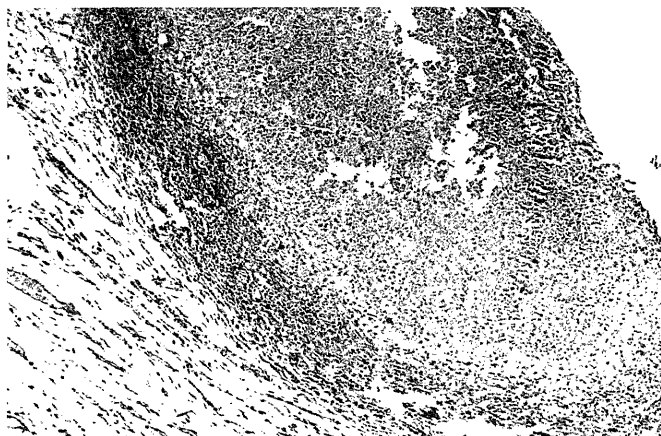
Figure 1. Small limited tumor nodule with continuous lymphocytic infiltrate in its periphery. (H&E, x 50). Ehrlich tumor in a mouse of Group I with findings for a.e.
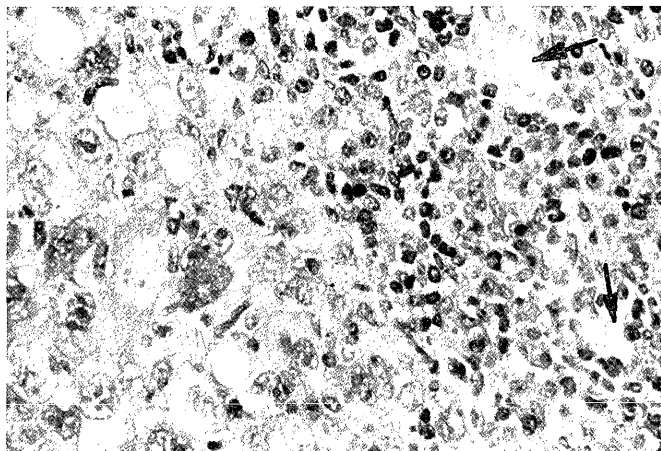

Figure 2. Cell infiltrate rich in lymphocytes in the periphery of tumor nodule. Small blood vessels (arrows) appeared among the infiltrate. (H&E, x 100). Ehrlich tumor in a mouse of Group II with findings for a.e.
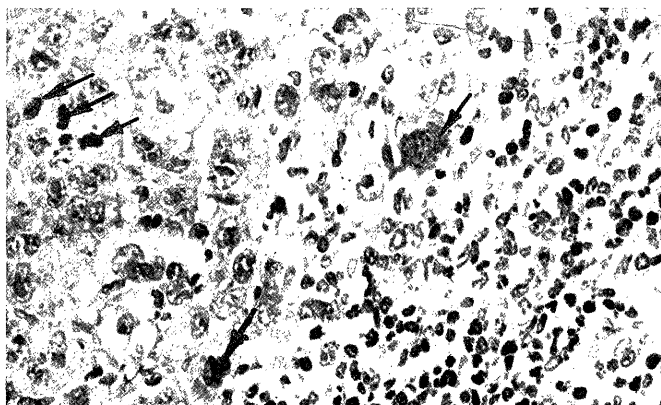

Figure 3. Lymphocytes from peripheral infiltrate partially invading tumor tissue. Apoptotic cells with compaction of nuclear chromatin and apoptotic bodies are observed (arrows). (H&E, x 100). Ehrlich tumor in a mouse of Group I with findings for a.e.

fibroblasts and formation of collagen fibers and their condensation among the infiltrate of lymphocytes were found, including by the ultrastructural examination.

Four mice of Group II did not show a development of a tumor after the transplantation. In one mouse of Group I, bearing a small tumor and also treated with stimulated spleen cells, thirty days after transplantation the tumor had completely regressed.

The data for a.e. in the transplanted tumor showed almost identical rate of cases among the mice treated with doses of 35 x 10^6 to 55 x 10^6 stimulated spleen cells. The previously examined doses of 20-25 x 10^6 proved to be with a low effectivity.

Morphological changes in the transplanted solid Ehrlich tumors without findings for antitumor effect

In these mice (13 in Group I and 10 in Group II), also treated with stimulated spleen cells like the mice with effect in the transplanted tumor and survived for the same periods, tumors were detected with the following sizes: 0.5 x 1-1.5 cm, 1.5 x 2.5 cm. Histologically, the tumors were found to infiltrate the adjacent tissues. Peripheral cell infiltrate rich in lymphocytes occupying as a tight strip from one third to half of the circumference of the tumor nodule was found in less than the half of the cases, but without evidence for lymphocytic cytotoxicity. An incomplete capsule was detected in a few tumors.

Morphological changes in the transplanted solid Ehrlich tumors in untreated mice

The tumor-bearing mice untreated by transfer of stimulated spleen cells (Group III, controls) killed in the identical term of survival after transplantation as in mice of Group I and II showed tumors sized 1 x 1.5 cm, 1.5 x 2.5 cm and rarely 3 x 4 cm. Histologically, the tumor displayed a solid growth pattern and infiltrated fibrotic, adipose and muscle tissue. Fields of necrosis were found among the growing tumor tissue and in central region of tumor. Scanty infiltrate of lymphocytes together with formation of a partial capsule was detected in the periphery of the tumor only in two cases, but no morphological evidence for lymphocytic cytotoxicity was found.

Mitosis and apoptosis

Mitotic rate and apoptosis of tumor cells at the time of killing of the mice, expressed by mean value of MI and AI, are shown in Table 1. The mean MI in solid

Table 1. Mitotic rate and apoptosis in transplanted solid Ehrlich tumors of mice

Groups	Mitotic Index (MI)		Apoptotic Index (AI)	
	Tumors with findings for antitumor effect (1)	Tumors without findings for antitumor effect (2)	Tumors with findings for antitumor effect (3)	Tumors without findings for antitumor effect (4)
I*	0.99±0.25	2.39±0.41	5.70±0.32	4.39±0.43
II**	1.22±0.29	2.45±0.27	4.06±0.72	4.18±0.43
III***		2.48±0.44		4.47±0.38

*Mice bearing a small Ehrlich tumor in which stimulated spleen cells were transferred.
**Mice previously treated by transfer of stimulated spleen cells and then transplanted with tumor.
***Tumor-bearing mice untreated by transfer of stimulated spleen cells (controls). Values are mean ± SE.

Ehrlich tumor of Group I and II with findings for a.e., column 1 in Table 1, was lower than the mean MI in tumors without finding for effect of the same groups, column 2 in Table 1, and in controls (Group III); P<0.001. Concerning apoptosis, the mean AI in tumors of all groups (I, II and III) exceeded the mean MI; P<0.001. The significance of the differences between the compared groups was determined by the Mann-Whitney U test, too (P<0.05). In tumors of Group I and II with findings for a.e. a few mitoses in one field (16 x 40) were counted, however there were fields without any mitoses. In tumors of Group I, II without findings for a.e. and Group III centers of proliferation with 8-14 mitoses in the high power field were found, mainly in the periphery of the tumor. The number of pathologic mitoses was high, at rate of 80.65% of all mitoses. Apoptotic cells and numerous apoptotic bodies were detected everywhere in tumor tissue. Apoptotic cells were found to decrease in size with condensation of the eosinophilic cytoplasm, compaction and margination of nuclear chromatin. Small apoptotic bodies were ingested by tumor cells. Between the cells, spherical globules (4-5 μm), basophilic, or acidophilic containing basophilic nuclear fragments, as well as large globules at the size 7-10 μm, were observed. Around apoptotic bodies, often a clear space (halo) was seen.

Electron microscopic changes

In tumor cells, oval, elongated or irregular in shape nuclei was observed, with blocks of condensed chromatin. In the cytoplasm, mitochondria, increased in number, rough endoplasmic reticulum appeared as small and tubule-like cisternae, ribosomes and

secondary lysosomes were detected (Fig. 4). Other cells showed enlarged nuclei with prevailing euchromatin and nucleoli with fibrilar centers. Multiple free ribosomes were found in the cytoplasm. These cells were characterized as actively synthesizing cells, particularly in protein synthesis (Fig. 5). Cells with exposed ultrastructural features were found in the preserved tumor tissue in small tumors of Group I and II with findings for a.e., and in tumor without such findings of the same two groups and of Group III.

Severe degenerative and necrotic changes in tumor cells akin to the irreversible hypoxic injuries were detected in all examined groups. Tumor cells in apoptosis in all groups were characterized by condensation and margination of nuclear chromatin, often
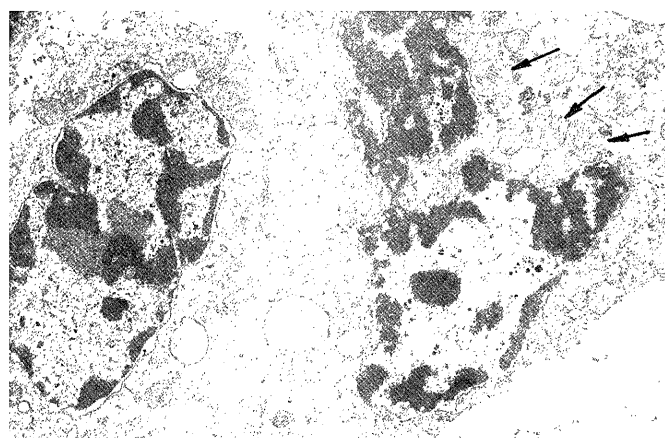

Figure 4. Electron micrograph of two tumor cells, one is binuclear. Heterochromatin in the nuclei is shown as irregular blocks. Mitochondria (arrows) are increased in number. Original magnification x 3, 500. Ehrlich tumor in a mouse of Group II without findings for a.e.
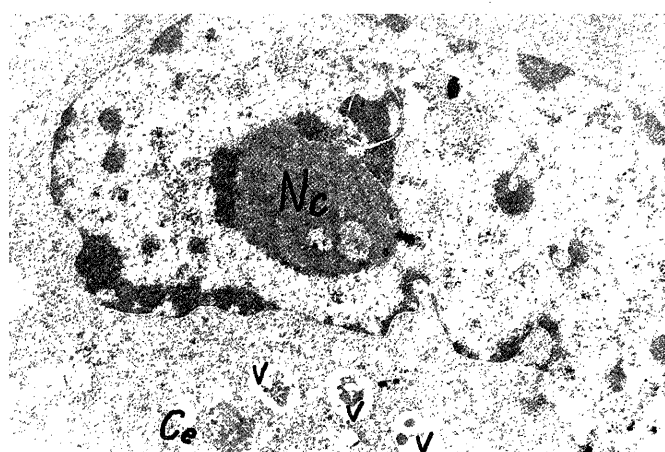

Figure 5. Electron micrograph of tumor cell with prevailing euchromatin in the large nucleus and big nucleolus (Nc). Free ribosomes, vacuoles (V) containing residual bodies and two centrioles (Ce) are seen in the cytoplasm. Original magnification x 8,000. Ehrlich tumor in a mouse of Group II with findings for a.e.
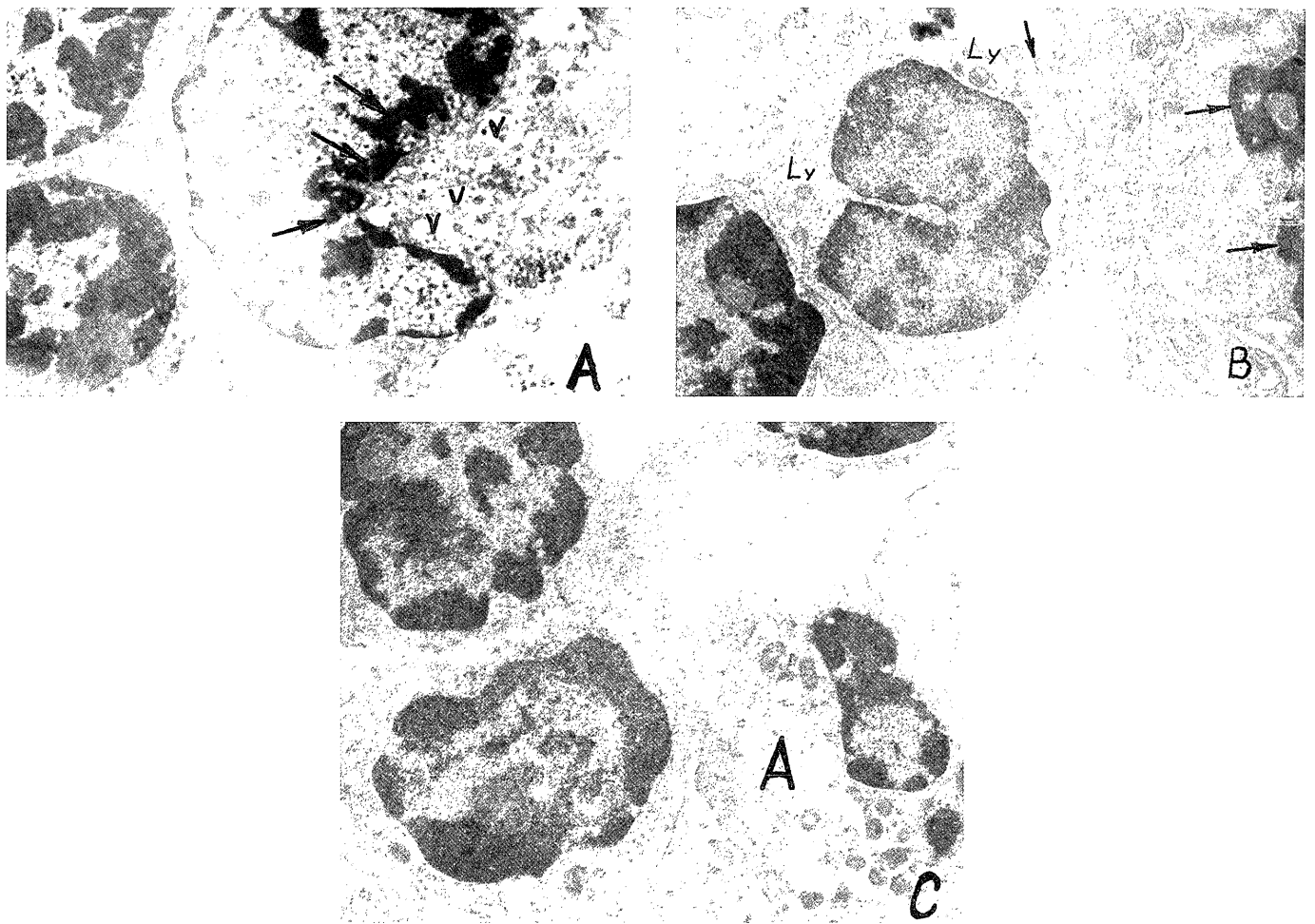
Figure 6. Ultrastructural findings for lymphocytic cytotoxicity: A. Two small lymphocytes close to a tumor cell in early apoptosis showing margination and condensation of nuclear chromatin (arrows). Vacuoles (V) in the cytoplasm containing residual material. B. Small lymphocytes with primary lysosomes (Ly), one displaying membrane cell-to-cell contact (arrow) with tumor cell in apoptosis, revealing condensation of nuclear chromatin (arrows) and cytoplasm. C. Small lymphocyte contacts with cell in advanced apoptosis (A). Original magnifications: in A x 6,000, in B and C x 4,000. Tumor nodule in a mouse of Group I with findings for a.e.

forming crescent profile or its compaction as a dense masses with irregular outline, and condensation of organelles of the cytoplasm. Apoptotic bodies containing masses of condensed chromatin and sometimes cytoplasmic organelles were seen.

Small lymphocytes were revealed in the peripheral cell infiltrate in tumor nodules of Group I and II with findings for a.e., as well as lymphocytes in activation. Immunoblasts (stimulated lymphocytes) were also found. Evidence for lymphocytic cytotoxicity was detected by electron microscopy (Group I and II with findings for a.e.). Small lymphocytes were disposed in immediate proximity to apoptotic cells and bodies (Fig. 6 A). Cell-to cell contact of lymphocytes with tumor cells in apoptosis was found, too (Fig. 6 B, C). The contacting lymphocytes contained in the cytoplasm primary lysosomes increased in number and

grouped. Infiltration by lymphocytes in tumor nodule with the evidence for lymphocytic cytotoxicity was observed in some mice 6 days after transfer of stimulated spleen cells but not in mice three to five days after this transfer. In tumors of Group I and II with findings for a.e., small lymphocytes were detected in close proximity to small areas of necrosis.

Changes in the reactive structures of the spleen and lymph nodes

In the spleen of the tumor-bearing mice untreated by transfer of stimulated spleen cells (controls), particularly in the periarterial (T) zone and the B zone of the Malpighian bodies lack of reaction, or rarely weakly expressed hyperplasia was observed. In some lymph nodes in the vicinity of tumors, the

paracortical areas were reacted - 450 μ m in transversal size (grade I). Plasmocytes in the lower paracortex and in the medullary cords were 25-30% of the total number of cells (grade I, PL1). In more of the mice of Group I and II with findings for a.e., reactive changes of mixed type, engaging T and B zones, were found in the spleens; T_1B_1 and in one - T_2B_1. Isolated reactive changes in T zones (T_1) and in B zones (B_1) or lack of such changes were observed in some cases, too. In T_1B_1, periarterial (T) zones were 250-400 μ m of transversal dimension, in T_2B_1- 450-550 μ m, and B_1 zones (germinal centers) - 250-300 μm. Small lymphocytes and scanty immunoblasts were detected in T zones. Centroblasts, centrocytes and macrophages ingested apoptotic bodies were found in the germinal centers. The mantle zone was well expressed. In the cords of the red pulp lymphocytes, macrophages, plasmocytes and megakaryocytes were revealed. The presence of the plasmocytes in the pulp was graded as 0 and grade I ($Pl_{0,1}$) - 10-20% of plasmocytes.

The examined tumor-draining lymph nodes in Group I and II with finding for a.e., situated nearby, close to the tumor, or in contact with it, displayed reactive changes mainly of the type T_1B_1 with paracortex 400-450 μm and germinal centers 250-300 μm. Plasmatization in the medullary cords was of grade 0 and I ($Pl_{0,1}$), and of grade II (Pl_2) - 51-55% plasmocytes. Isolated T_1 hyperplasia was also established. Reactive changes of type T_1B_1, Pl1 in lymph nodes occurred in areas more distant from the tumor i.e. in the axillary regions were detected, too. The paracortex was populated with lymphocytes, a few immunoblasts and interdigitating cells. Post-capillary venules previously with flattened endothelial cells, grade 1 in accordance with Syrjänen[9] or with cuboidal endothelium (grade 2) were found. Medullary sinuses contained lymphocytes and macrophages. In mice without development of a tumor after transplantation, including the case with complete tumor regression, reactive changes of type T_1B_1 were established in the spleens. Lymph nodes found in the areas of transplantation showed reactive changes of type T_1B_1, Pl1 and of type T_2 with paracortex 750 μ m in one case. In the active paracortex, post-capillary venules of grade 2 were revealed.

Mice of Group I and II without findings for a.e. demonstrated changes in the spleen mainly of type T_1B_1, $Pl_{0,1}$, but in others a reaction was absent. Lymph nodes situated near the tumor displayed changes of T_1B_1, T_1 and of T_2 (paracortex 720 μ m) in one mouse; $Pl_{0,1}$, rarely Pl_2. In the paracortex small lymphocytes with various density, a few immunoblasts, interdigitating and histiocytic cells, and post-capillary venules of grade 1 and 2 were discovered.

Inhibition effect of the culture supernatants obtained from spleen cells of mice bearing solid Ehrlich tumor

The supernatants of spleen cells from mice bearing solid Ehrlich tumor non-stimulated with cancer extract did not show an IE against migration of Ehrlich ascites tumor cells; mean MgI>0.80. On the contrary, the supernatants decanted from the samples and the wells inhibited Ehrlich-cell migration significantly, when the spleen cells were stimulated with Ehrlich extract, or with PHA, IL-2 and Ehrlich extract at almost equal results received for the two ways of stimulation (Table 2); P<0.001. IE was also appeared when spleen cells were enriched with T lymphocytes and then stimulated with Ehrlich extract; MgI was 0.64 ± 0.03. According to the data of FrRCs, a correlation between the IE showing production of a factor inhibiting Ehrlich-cell migration from the spleen cells of Ehrlich tumor-bearing mice, and the stimulation of these cells were found; correlation coefficient (r) =0.61, 0.60, 0.57.

Our preliminary experiments showed that the tumor extract, which could be found in a little amount in the supernatant containing inhibitory factor (IF), did not inhibit itself the migration of Ehrlich ascites cells in vitro. Identical results were received for the extract of the transplanted sarcoma induced in mice by 20-methylcholanthrene[5].

Spleen cells obtained from mice treated by transfer of stimulated cells showed evidence of production of IF without to be stimulated with Ehrlich-tumor extract in vitro. P<0.05 for Group I, and P<0.001 for Group II by comparison with the IE of the supernatants from unstimulated spleen cells (Table 2). Production of this factor was not found in all treated

Table 2. Inhibition of Ehrlich-cell migration by culture supernatants of spleen cells from mice

	MgI of culture supernatants of spleen cells from		
Ehrlich-tumor bearing mice	Spleen cells were not stimulated	Spleen cells were stimulated with	
		Ehrlich extract	PHA, IL-2 and Ehrlich extract
	0.84±0.05	0.61±0.02	0.62±0.04
Mice treated by transfer of stimulated spleen cells			
Group I*	0.75±0.08		
Group II**	0.68±0.07		

*Mice bearing a small Ehrlich tumor in which stimulated spleen cells were transferred.
**Mice previously treated by transfer of stimulated spleen cells and then transplanted with tumor. Values are mean ± SE.

mice (MgI- 0.63-0.83) and was not connected with the presence of findings for a.e. in the transplanted tumors.

In the smears obtained from suspension of spleen cells, stimulated with PHA, IL-2 and Ehrlich-tumor extract, the following proportion of lymphoid cells were determined: small lymphocytes (6-7 μ m) - 68.1 $\pm$ 1.51%, middle-sized lymphocytes (8-10 μ m) - 20.52 $\pm$ 0.45% and large lymphoid cells (blasts, immunoblasts) with pyroninophilic cytoplasm (18-25 μ m) - 10.88 $\pm$ 0.24%. The rate of the lymphoid cells in the smears of spleen cells from intact mice was as followed: small lymphocytes - 89.20 $\pm$ 1.06%, middle-sized lymphocytes - 8.65 $\pm$ 1.12% and large pyroninophilic cells 1.99 $\pm$ 0.32%. Besides the lymphoid cells, a few plasmocytes and macrophages were found in the smears, because the suspensions were preliminarily incubated in plastic flasks.

Discussion

Previous investigations have shown that the regression of localized and disseminated experimental tumors can be effectuated by adoptive transfer of specifically sensitized lymphoid cells[10, 11, 12, 13]. In the present study an adoptive transfer of spleen cells (lymphocytes) stimulated with Ehrlich-tumor extract or with PHA, IL-2 and tumor extract in mice-bearers of small transplanted Ehrlich tumor (Group I) or in tumor-free mice with following transplantation (Group II) was accomplished. The stimulation of spleen cells by Ehrlich extract was in agreement with the report that Ehrlich murine tumor may be considered clearly immunogenic[2].

Accentuating on the morphological changes in the transplanted solid Ehrlich tumors determined by mechanisms leading to blockage of growth and extension of tumor nodule, we set four criteria for a.e. These changes were interpreted together with the data for production of IF from the stimulated spleen cells. It was found the decrease of the proliferative activity in small tumor nodules of Group I and II with findings for a.e. in which mean MI was lower than this in tumors of the same groups (I and II) but without findings for a.e. and of Group III (Table 1). In this respect, it could be speculated on the effect of some cytokines: IFN γ, TNF α / β, TGF β produced by cells of the infiltrate in the periphery of tumor, and probably by tumor cells (TGF β) as antiproliferative agents for tumor cells[14]. TGF beta precursors were detected in Ehrlich ascites cells and in ascitic fluid[15].

Our results showed an activity of apoptosis, which in Groups I, II, and III at the time of killing of the

mice, exceeded the mitotic activity of tumor cells. According to data from literature, an influence of regulatory genes (oncogenes and oncosuppressor genes) as c-myc, bcl-2, p53 and ras could be suggested on this issue[16]. The connection of the local hypoxia with apoptosis was considered, too. Graeber et al[17] has reported that in transplanted tumor highly apoptotic regions strongly correlate with hypoxic regions expressing wild type p53.

The cell infiltrate rich in lymphocytes in the periphery of tumor nodules showed an evidence for lymphocytic cytotoxicity. Based on the ultrastructural findings, it was assumed that T-lymphocytes with cytotoxic activity, expressing as was shown elsewhere Lyt-2 phenotype[12], killed tumor cells by inducing apoptosis, particularly through cell-to-cell contact. Apoptosis induced by attack of lymphotoxin (TNF β), secreted from stimulated T-lymphocytes 18), may be suggested in case of presence of the apoptotic bodies in the proximity of lymphocytes but without cell-to-cell contact with tumor cells. Focuses and small areas of tumor-cell necrosis nearby the lymphocytic infiltrate in tumors in Group I and II with findings for a.e. were likely connected with the effect of TNF α / β[18, 19]. The proliferation of fibroblasts among the lymphocytes of the infiltrate with formation of collagen fibers, and in this way - a fibrotic capsule, was probably determined by stimulation of production of cytokines- FGFs, TGF β[20, 21], released from the cells of the infiltrate. Morphological changes for a.e. in transplanted solid Ehrlich tumors were also obtained in exbreeder ICR mice treated with stimulated spleen cells (our unpublished data).

The stimulated spleen cells (lymphocytes) from Ehrlich-tumor bearing mice were immunologically active as they produced IF in the supernatants, suppressing the migration of Ehrlich ascites cells. The IF was produced by T-lymphocytes, as the spleen cells enriched with T-cells accomplished it. Previously, a production of IF was found from spleen cells of mice immunized with killed sarcoma cells and from erythrocyte rosette-forming cells, separated from peripheral blood mononuclear cells, in patients with gastric and breast cancer, when the cells were stimulated with tumor extract. Active fractions were isolated from crude supernatants in the range of molecular weight between 14 000 and 5 800 daltons and lower than 5 800[5, 6]. In the present study, there were data that the stimulation of spleen cells with PHA, IL-2 and Ehrlich extract caused an activation, partial blast-transformation and proliferation of T-lymphocytes. The low concentration of IL-2 didn't favor generation of LAK cells[22].

Our unpublished data showed that a factor with cytotoxic effect in vitro[23] against Ehrlich ascites cells were produced in the supernatants of spleen cells from mice in which stimulated spleen cells were transferred. When these supernatants were added to the culture of Ehrlich cells, the rate of viable cells decreased, as compared to the rate when supernatants from unstimulated spleen cells of tumor-bearing mice were used.

Indirect data from our investigations suggested that the lymphocytes in tumor nodule showing antitumor effect derived, probably, from the transferred splenocytes, containing T-lymphocytes including in activation and blast-transformation. The stimulated spleen cells (lymphocytes) transferred in mice of Group I and II penetrated by normal traffic of recirculation in their lymphoid organs (spleen and lymph nodes), as was shown elsewhere[13], where the lymphocytes propagated by clonal proliferation and generated immune reactivity in the hosts. In our study it was demonstrated by the production of IF (positive MgIs) in the supernatants of spleen cells of the immunized mice, mostly from Group II. Then the lymphocytes invaded the tumor nodule through small blood vessels of the microcirculatory bed, mainly in its periphery, and cytotoxic lymphocytes influenced on tumor cells.

Histologically, lymphoid infiltrate in the periphery of a tumor nodule, containing active lymphocytes was detected six days after transfer of stimulated spleen cells and endured in mice of Group I and II with findings for a.e. in all time of survival. The lymphocytes of the cell infiltrate in tumor increased in number through a blast-transformation. The participation of NK cells in killing of tumor cells was minimal as in the tumor of control mice no findings for lymphocytic cytotoxity were revealed.

When, in mice treated by transfer of stimulated spleen cells both the production of IF and findings for lymphocytic cytotoxity in tumor were missing, a deficient propagation and clonal expansion of the immune cells (lymphocytes) in the lymphoid organs could be assumed. Previously, we have shown that the IF was produced from peripheral blood CD4+T-cells[6]. In the present experiments, the production of IF (positive MgIs) attested to the activity of helper T-cells (L3T4+) both in the transferred cells and in the spleen cells of the host. The subset of helper T-lymphocytes producing IF could be suppressed in the spleens of mice. In this respect, a development of nonspecific suppressor cell population in the spleen of C57BL/6 mice bearing solid Ehrlich tumor has been reported[2]. On the other hand, the possibility for clonal proliferation of lymphocytes with cytotoxic function in the lymphoid

organs could be preserved.

Concerning the changes in the spleen, it was suggested that the activation of the reactive structures (T and B zones) in mice of Group I and II with findings for a.e. could be related to the propagation of immune lymphocytes. The reactive changes in lymph nodes (Group I and II with findings for a.e.) in the vicinity of tumor nodule or close to it were connected with the drainage of tumor antigens which was assumed also for the control tumor-bearing mice (Group III). Infiltration and propagation of the transferred stimulated splenic lymphocytes in lymph nodes particularly in these disposed distantly from tumor could be considered, too. The lack of development of an Ehrlich-tumor after the transplantation and very rarely its complete regression in mice treated by stimulated spleen cells, together with the reactive changes in the spleens and lymph nodes, might be connected with the availability of antitumor immunity in the hosts despite the possible objection in this respect. It should be noticed that the counterparts of tumor-bearing mice untreated by transfer of stimulated spleen cells (Group III, controls) showed large tumors developed in the same terms of living.

In conclusion, we described morphological changes in the transplanted solid Ehrlich tumor, which were accepted as morphological criteria for a.e. These changes were determined by a transfer in tumor-bearing mice of stimulated spleen cells containing immunologically active lymphoid cells, producing IF in the supernatants. It was suggested that the transferred cells rich in lymphocytes, including such in activation and blast-transformation, propagated by clonal proliferation in the lymphoid organs, particularly in the spleens, in mice of Group I and II, and provoked immune reactivity established by the production of IF. T-lymphocytes migrated by the circulation in tumor nodules mainly in their periphery, and cytotoxic lymphocytes induced apoptosis on tumor cells.

References:

1) Carry RJ, Prescott DM, Ogilvie GK: Resistance to Ehrlich ascites tumor in a strain of dystrophic mice. *Cancer Res* 39: 2139-2140, 1979
2) Subiza JL, Viñuela JE, Rodrigez R, Gill J, Figueredo MA, De La Concha EG: Development of splenic natural suppressor (NS) cells in Ehrlich tumor-bearing mice. *Int J Cancer* 44: 307-314, 1989
3) Chen L, Watkins JF: Evidence against the presence of H2 histocompatibility antigens in Ehrlich ascites tumor cells. *Nature (Lond)* 225: 734-735, 1970
4) Olinescu A, Babes S, Pitur I: Immune reactions in Ehrlich ascites tumor bearing mice. *Arch Roum Path Exp Microbiol* 39: 249-257, 1980

5) Raichev I, Dotchevska R: Production of a factor inhibiting tumor cell migration by spleen cells from tumor-bearing mice. *Jpn J Med Sci Biol* 41: 49-56, 1988

6) Raichev IT, Borissova R: Production of a factor inhibiting tumor-cell migration in patients with gastric and breast cancer. *Jpn J Med Sci Biol* 47: 265-280, 1994

7) Alvarez J, Rodriguez J, Lopez-Botet M, Silva A, de Landazuri MD: Removal of PHA supernatants containing T-cell growth factor. *J Immunol Methods* 40: 289-296, 1981

8) Quinn CM, Wright NA: The clinical assessment of proliferation and growth in human tumors: Evaluation of methods and applications as prognostic variables. *J Pathol* 160: 93-102, 1990

9) Syrjänen KJ: The lymph nodes. Reaction to experimental and human tumors. *Exp Path Suppl* 8 (Bolck F, Holle G, Mohr U eds.; VEB Gustav Fischer Verlag, Jena) pp. 123, 1982

10) Fernandez- Cruz E, Woda BA, Feldman JD: Elimination of syngeneic sarcomas in rats by a subset of T lymphocytes. *J Exp Med* 152: 823-841, 1980

11) Eberlein TJ, Rosenstein M, Rosenberg SA: Regression of a disseminated syngeneic solid tumor by systemic transfer of lymphoid cells expanded in Interleukin 2. *J Exp Med* 156: 385-397, 1982

12) Shu S, Chou T, Rosenberg SA: Generation from tumor-bearing mice of lymphocytes with in vivo therapeutic efficacy. *J Immunol* 139: 295-304, 1987

13) Kjaergaard J, Shu S: Tumor infiltration by adoptively transferred T cells is independent of immunologic specificity but requires down-regulation of L-selectin expression. *J Immunol* 163: 751-759, 1999

14) Meager A. Cytokines (Prentice Hall Advanced Reference Series, Englewood Cliffs, New Jersey) pp.291, 1991

15) Segura JA, Barbero LG, Marguez J: Early tumor effect on splenic Th lymphocytes in mice. FEBS Lett 444:1-6, 1997

16) Wyllie AH: Apoptosis (The 1992 Frank Rose memorial lecture). *Br J Cancer* 67: 205-208, 1993

17) Graeber TG, Osmanian C, Jacks T et al: Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumors. *Nature* 379: 88-91, 1996

18) Beyaert R, Fiers W. Tumor necrosis factor and lymphotoxin. In *Cytokines* (Mire-Sluis AR, Thorpe R eds.; Academic Press, San Diego, London, New York, Tokyo) pp. 335-360, 1998

19) Zhang M, Tracey KJ: Tumor necrosis factor. In The *Cytokine Handbook* Third Edition (Thomson AW ed.; Academic Press, San Diego, London, New York, Tokyo) pp. 517-548, 1998

20) Cotran RS, Kumar V, Robbins SL. Neoplasia. In *Robbins Pathologic Basis of Disease* 5th Edition (Schnoen FJ managing ed.; Saunders Company, Philadelphia, London, Sydney, Tokyo) pp. 241-303, 1994

21) Derinck R, Choy L: Transforming growth factor-β and its receptors. In *The Cytokine Handbook* Third Edition (Thomson AW ed.; Academic Press, San Diego, London, New York, Tokyo) pp. 593-636, 1998

22) Chou T, Chang AE, Shu S: Generation of therapeutic T lymphocytes from tumor-bearing mice by in vitro sensitization. Culture requirements and characterization of immunologic specificity. *J Immunol* 140: 2453-2461, 1988

23) Eardley DD, Shen FW, Gershon RK, Ruddle NH: Lymphotoxin production by subsets of T cells. *J Immunol* 124: 1199-1202, 1980