The rate of penetration of cobalamins into the cell is evidently controlled with the aid of modulation of the number of TC-II membrane receptors in response to external stimuli. Regulation of this rate at the endocytosis level is unlikely, for internalization of the complex within a definite period of the cell cycle rises extremely rapidly, a characteristic feature of several cooperative processes acting in accordance with the "all or nothing" principle.

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RATE OF GROWTH OF HUMAN MELANOMA CELLS IMPLANTED INTO IMMUNOSUPPRESSED MICE IN A FIBRIN CLOT

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KEY WORDS: human melanoma; immunodepression; fibrin clot.

Xenografts of human tumors are the most adequate model with which to study human cancer. To evaluate the activity of therapeutic agents the method of implanting fragments of human tumors [2] or cells from culture, incorporated into a fibrin clot (FC) [7], beneath the renal capsule (subcapsular implantation, SCI) of mice is used. It is considered that comparing the dimensions of the tumor in control and experimental mice provides a basis for assessing the activity of such preparations, but infiltration of the tumor by the host's cells in immunocompetent mice may disturb the reliability of these measurements [3]. To prolong the viability of the implant, immunosuppression of the recipients can be used [4].

It is advisable to use tumors such as human melanoma BRO, which proliferate rapidly after implantation in nude [5] and immunosuppressed mice [1, 6]. This paper describes the use of FC with BRO cells obtained from culture in order to study growth of the human tumor in mice.

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EXPERIMENTAL METHOD

BRO cells and primary embryonic human fibroblasts were cultured in medium RPMI-1640 with 10% embryonic serum. FC were formed by the use of sterile bovine fibrinogen (20 mg/ml in Hanks' solution) and thrombin without plasminogen. The amount of thrombin was chosen experimentally, and was about 1 mg/ml, in order to induce the formation of a fibrin clot in not less than 1-2 min.

The cell suspensions were obtained by enzymic treatment of the cultures [6]. After a single centrifugation, the supernatant was drawn off as much as possible and the cell residue, containing (6-10) $\times$ 10^6 cells, was treated with a solution of fibrinogen, then mixed with thrombin solution as described in [4], and transferred in volumes of 2.5 μl into a plate with wells 10 μl in volume. The cells were incubated at 37°C for 5-7 min, after which the clots thus obtained were tested for quality under an inverted microscope, and those containing densely packed cells were chosen. The clots thus formed were transferred one by one by means of a spatula into wells in the plates filled with Eagle's medium without serum. The clots measured between 9 and 11 optical micrometer units, and each of them consisted of about 200,000 cells. The clots were kept at 4°C for 2-3 h before implantation.

(CBA $\times$ C57BL/6)F_1 female hybrid mice aged 2-4 months, bred at the "Stolbovaya" nursery, were used. For immunosuppression the animals were irradiated on a ^{137}Cs source (power 8.7 rad/sec) 24 h before transplantation of the tumors. The SCI operation was performed under sterile conditions under hexobarbital anesthesia. The capsule on the dorsal aspect of the left kidney was punctured along its longitudinal axis and retracted with the aid of a bent stilet, and the FC was inserted into the hole thus formed. After measurement of two mutually perpendicular diameters of the implant by means of an ocular micrometer (scale division equivalent to 0.1 mm), the kidney was returned to its original place and the wound closed in layers. Later, the mice were killed after certain intervals and the tumors were again measured. The volume of the tumor was calculated by the formula: length $\times$ breadth $\times$ 1/2 thickness. The results were subjected to statistical analysis by the U test. The material was studied histologically and cytologically by standard methods.

EXPERIMENTAL RESULTS

In the experiments of series I the duration of growth of the tumors (Fig. 1) and viability of the tumor cells were studied. On the 6th day the volumes of the tumor in the irradiated (5.5 Gy) mice were almost twice their volume in the intact animals ($p < 0.01$). By the 11th-14th day the volume of the tumors in the unirradiated mice had not increased, whereas in the irradiated mice they continued to grow until the 11th day, the tumor occupied a wide area, and its extensive "vertical" growth was evident. Histological study of the irradiated animals on the 11th day revealed a lymphohistiocytic reaction around the implant with moderate penetration by lymphocytes, macrophages, and fibroblasts into the tumor tissue.

In the experiments of series II the volume of the tumors was determined on the 6th and 8th days. On the 6th day, differences in volume of the tumors in the intact mice and in animals irradiated in doses of 5.5 or 6.5 Gy were present ($p < 0.05$). By the 8th day the volume of the tumors was reduced in the control animals, but considerably increased in the immunosuppressed mice (Fig. 2). The difference between the control and irradiated (5.5 or 6.5 Gy) groups of animals was highly significant ($p < 0.005$). There was no significant difference in the volumes of the tumors between mice irradiated in doses of 5.5 or 6.5 Gy.

After injection of embryonic fibroblasts incorporated into FC into the immunosuppressed (5.5 Gy) mice, only a twofold increase in the area of the implant was observed on the 8th day, and it remained flat. In the unirradiated mice a decrease in size of the implant was observed on the 8th day, or it was completely absorbed. After injection of tumor cells, proliferation of which was interrupted by irradiation in a dose of 15 Gy, into the immunosuppressed mice growth of the tumor was not observed on the 6th day, and on the 8th day the volume of the tumor was reduced to 0.3 of its initial value. The tumors under these circumstances appeared flat and tapering.

Histological study of the control animals revealed intensive infiltration of the connective tissue of the capsule and kidney around the graft by lymphocytes and macrophages as early as on the 6th day. In the tumor itself lymphocytes, macrophages, and fibroblasts were detected in very small numbers. Invasion of the tumor by small blood vessels was observed. Changes in the tumor tissue were expressed as partial loss of the syncytial connection between individual cells, and the appearance of solitary giant cells among the tumor cells. On the 8th day the host's cells predominated in the graft: connective-tissue cells including lymphocytes and macrophages. The tumor cells were present in separate foci, and evidence of cellular degradation was observed

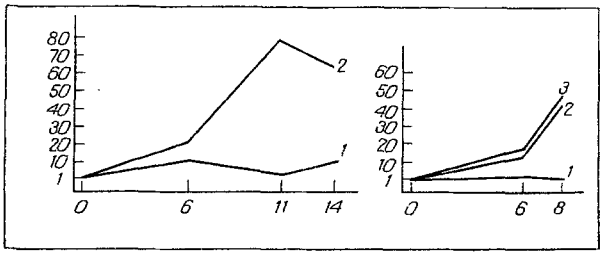

Fig. 1 Fig. 2

Fig. 1. Growth of BRO cells beneath renal capsule of intact (1) mice and mice irradiated with 5.5 Gy (2) for 14 days. Here and in Fig. 2: abscissa, days after implantation; ordinate, relative volume of tumor. Five mice were used in each group.

Fig. 2. Growth of BRO cells beneath renal capsule of intact mice (1) and mice irradiated with doses of 6.5 Gy (2) and 5.5 Gy (3) for 8 days.

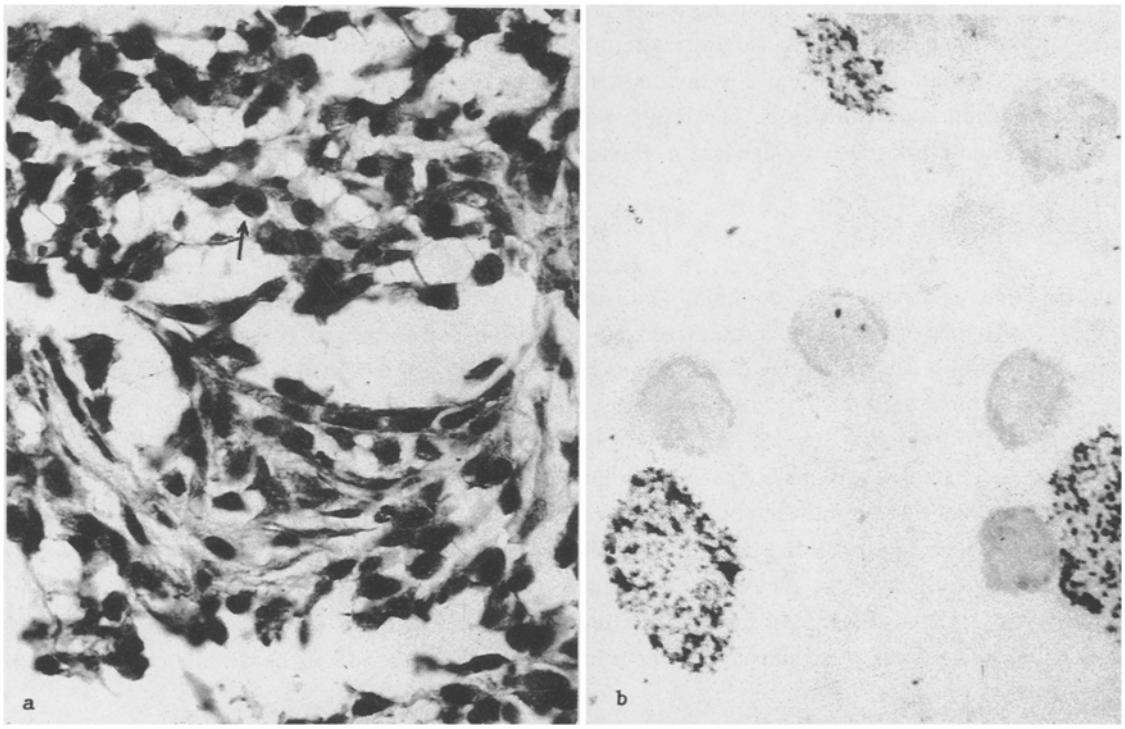

Fig. 3. BRO cells on 8th day after SCI in FC. Irradiation in a dose of 5.5 Gy. a) Morphological picture. Arrow indicates mitotically dividing tumor cell. Hematoxylin and eosin, 2000×; b) histoautoradiography. Accumulation of ^{3}H-thymidine in cells. 1200×.

(karyopycnosis, cytolysis). Accumulation of tumor cells was on a small scale, but the number of giant cells was increased compared with the 6th day of observation, and some of them were mitotically dividing cells.

In mice irradiated in a dose of 5.5 Gy many viable tumor cells, occupying almost the whole area of the graft, could be seen on the 6th and 8th days. The lymphocytic and interstitial reaction of the host's kidney was suppressed, but there were signs of commencing vascularization of the tumor. The dimensions of the tumor tissue were greater than in the control. On the 6th day (Fig. 3a) growth of the tumor cells was diffuse and no evidence of cellular degradation was present. As regards the capsule, rapid proliferation only of fibroblasts was observed, with penetration of solitary lymphocytes and histiocytes inside the tumor,

and the tissue of the graft consisted mainly of tumor cells, with solitary mitotic figures and blood vessels inside the tumor. Cytological study on the 6th and 8th days revealed many polymorphic malignant cells with large nuclei and multiple nucleoli. Figures of amitotic division, multinuclear cells, and a few lymphocytes were observed.

Accumulation of ^{3}H-thymidine, injected in a dose of 2.5 μCi/g 2 or 24 h (Fig. 3b) before sacrifice in the animals, was observed in the tumor cells on the 8th day after transplantation. This is evidence of continuing DNA synthesis in the tumor cells.

After intraperitoneal injection of (1.5-5) $\times$ 10^7 BRO cells in FC (three experiments, eight mice, irradiated in a dose of 6.5 Gy) the formation of easily palpable tumors was observed in all animals by the 5th-6th day. By the 10th-14th day, the tumors exhibited marked necrosis in the central part. The mass of the tumors without necrosis after injection of 1.5 $\times$ 10^6 cells varied from 70 to 94 mg, whereas after injection of (3-5) $\times$ 10^6 cells it varied from 140 to 230 mg. Passage of material from these tumors after intramuscular injection into irradiated (6.5 Gy) mice could not be repeated more than 3 times.

These results show that injection of BRO cells in FC by SCI into mice can be used as a method of studying growth of human tumors. A single moderate dose of irradiation (5.5 Gy) leads to an approximately 40-fold increase (Fig. 2) in volume of the tumor on the 8th day and prevents marked invasion of the host's cells inside the tumor. In experiments in [4], irradiation in a dose of 6.5 Gy or cyclophosphamide + daily injection of cyclosporin A was used for immunosuppression, and similar or even less growth of a human tumor was observed on the 9th-10th day.

Continuing the experiment until the 8th day is sufficient to study the action of antitumor preparations in the form of monotherapy and, possibly, combination therapy. The method described would appear to be promising for widespread practical studies of activity of various preparations relative to human tumors.

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