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Regression of Malignant Melanoma in a Dog by Local Injections of a Partially Purified Preparation Containing Human α-Lymphotoxin[1] (41345)

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Abstract. Partially purified lymphotoxin preparation containing α-lymphotoxin was investigated for antitumor effect. A 12-year-old male dog weighing 14 kg developed malignant melanoma in the mouth. The tumor measured 4.5 × 4.0 cm. α-Lymphotoxin-containing preparation obtained from human lymphocytes was injected into the tumor at 2- to 5-day intervals. The doses varied from 600 to 2100 units of lymphotoxin activity per injection. A total of 13,200 units of lymphotoxin activity was given over the 30-day period of treatment. The tumor size decreased by 30% during the first 3 days, accompanied by necrosis in the center, and disappeared completely by the 20th day. The remission lasted for 6 months. No untoward effects were seen except for an increase in serum alkaline phosphatase. A repeat biopsy from the tumor site was done 12 days after the completion of treatment which showed fibrosis and lymphocytic infiltration, but no tumor cells. It was suggested that partially purified human α-lymphotoxin preparation possessed antitumor effect across the species barrier. Since the preparation was crude, it is possible that activities other than lymphotoxin were responsible for the antitumor effect. This question can only be resolved when large quantities of pure preparations of lymphotoxin become available.

Lymphokines are liberated by lymphocytes upon activation by mitogens, antigens, and in mixed leukocyte cultures (1, 2). One such group of molecules had nonspecific cytotoxic effect on different target cells (3–6). These cytotoxic factors are designated as lymphotoxins. α-Lymphotoxin represents one of the stable molecules of the lymphotoxin group (7). A preparation containing lymphotoxin has been shown to have tumor inhibitory effect in leukemia (L1210) and melanoma (B16) in mice (8). We report complete regression of melanoma in a dog with a partially purified preparation containing human α-lymphotoxin.

Materials and Methods. *Lymphotoxin assay.* The lymphotoxin activity was measured by the method described by Spofford *et al.* using L929 cells as targets (9). The activity of the lymphotoxin was expressed in units which were the reciprocal of that dilution which caused 50% cytotoxicity on L929 cells in the tissue culture (10). An internal standard of lymphotoxin was used as reference in each assay.

Lymphotoxin production. Lymphocytes were obtained from patients with multiple sclerosis, after obtaining informed consent, who were undergoing lymphapheresis as an investigational procedure for therapy. The lymphapheresis was performed with the aid of continuous flow cell separator (Celltrifuge, Aminco, Silver Springs, Md.) or IBM cell separator (IBM, Princeton, N.J.) as described previously (11). This procedure yields $>1 \times 10^{10}$ lymphocytes with a lymphocyte concentration of approximately 75%. Lymphotoxin was produced according to the procedure described by Spofford *et al.* (9). To describe the procedure briefly: lymphocytes were suspended at a concentration of 5×10^6 cells/ml in Eagle's minimum essential medium with Hank's salts containing 0.3 g/liter L-glutamine, 2 g/liter sodium bicarbonate, 50 μg/ml gentamicin, and 5% inactivated human agamma serum. Phytohemagglutinin (Wellcome Lab), at a concentration of 20 μg/ml, was added to the cell suspension. The cells were incubated in 100- to 500-ml vol in flasks with gentle stirring at 37° in 5% CO_2 in air atmosphere for 5 days. At the end of incubation, the super-

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nate was obtained by centrifugation at 1000 *g* for 10 min. The supernate was partially purified by precipitation with 45% ammonium sulfate. This yielded a 7- to 10-fold increase in the specific activity of lymphotoxin. The final preparations had specific activities which ranged from 300 to 500 units/mg protein.

Treatment of the dog with melanoma. A 12-year-old male dog (Scottie), weighing 14 kg, developed a mass in his mouth over the right side of the body of the mandible near the angle. The tumor grew rapidly and surrounded the molar teeth. A biopsy of the mass was done and the microscopic examination of the tissue revealed a neoplastic proliferation of cells, which was forming clusters and large aggregates throughout the tissue. These cells had ovoid or round vesiculated nuclei with scant and poorly defined cytoplasm. Mitotic figures were encountered with high frequency. Irregular zones of tumor necrosis were noted. The covering epithelium was focally ulcerated and there was a mild secondary inflammatory infiltrate in the tissues. Granular golden brown pigment, typical of melanin, was found in the cytoplasmic boundaries of many cells. The histologic picture was consistent with melanoma in the mouth and carries poor prognosis (12). The tumor measured 4.5 × 4.0 cm with an ulcerated crater in the center which was the site of a tooth. The ulcer measured 2.5 × 1.0 cm. The partially purified lymphotoxin-containing preparation was injected directly into the tumor at multiple points at 2- to 5-day intervals. The dose of lymphotoxin activity ranged from 600 to 2100 units/injection (Table I). The dog received a total of 13,320 units of lymphotoxin in the material injected over a 30-day period. The tumor size decreased by 30% during the first 3 days, accompanied by sloughing of the tumor which started in the middle. The tumor regression continued and the mass disappeared completely by Day 20 with complete healing of the ulcer. No untoward effects of treatment were seen in the dog. Initial blood chemistry was not done. However, serum chemistry was tested on SMA 12/60 on the 15th day of treatment and on the 12th day after the final injection. Serum alkaline phosphatase increased from 1110 to 1850 mμ/ml. The rest of the chemistry failed to show significant change except for a drop in LDH from 1125 to 204 mμ/ml. Peripheral blood count on the 12th day after treatment was as follows: Hb 15.8 g, Hct 43%, total WBC 14,500 with a differential count of bands 13%, segs 40%, lymphocytes 44%, monocytes 2%, and eosinophils 1%. A repeat biopsy from the tumor site was done 12 days after the completion of treatment which showed fibrosis and lymphocytic infiltration but no tumor cells. Figures 1 and 2 show the histology of the tissue before and after treatment, respectively. The dog was checked monthly. At 6 months checkup, a small subcutaneous nodule was found on the right side of the ventral surface of the neck. It grew to a size of 8 × 9 cm in 3 weeks at which time the animal was euthanized at the request of the family. Autopsy revealed subcutaneous metastatic malignant melanoma in the neck. No tumor was found in other organs or other sites.

TABLE I. DOSAGE SCHEDULE

Day	α-Lymphotoxin injection	
	Volume (ml)	Units
1	7	2100
5	5	1500
7	4	1200
9	3.4	1020
12	4	1200
14	2	600
19	4	1200
21	3	900
23	2	600
28	3	1500
30	3	1500
		13,320

Note. α-Lymphotoxin was produced by incubating human lymphocytes in the presence of phytohemagglutinin for 5 days at 37° in 5% CO_2. The lymphotoxin was partially purified by precipitation with 45% ammonium sulfate. The precipitate was resuspended in 0.9 *M* saline and sterilized by filtration through 0.22-nm Milipore filters. The specific activity of lymphotoxin varied from 300 to 500 units/ml in the final preparations. It was tested and found to be sterile and free of pyrogens.

Discussion. Lymphotoxin has been shown to be toxic to cells of homogeneic,

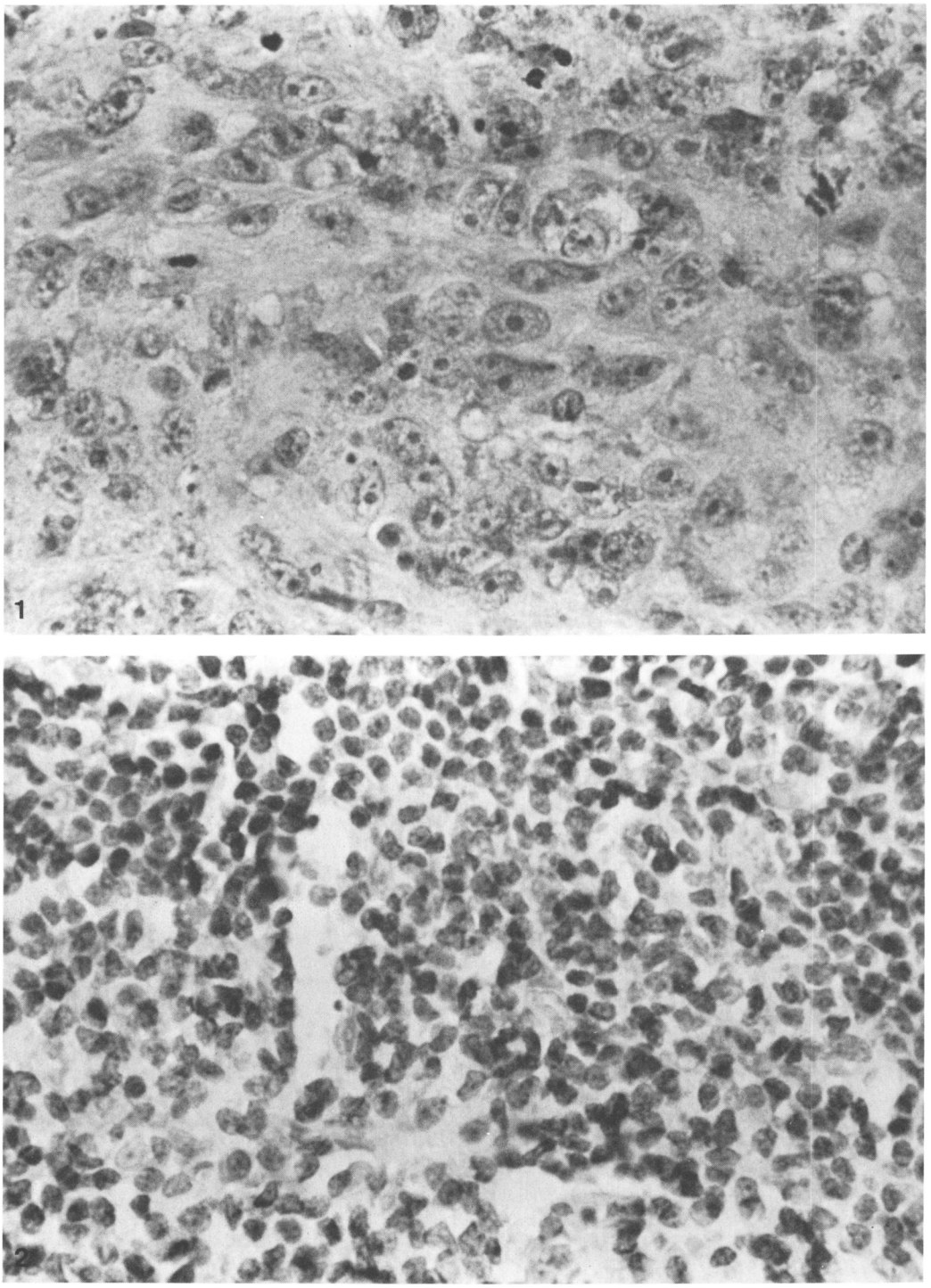

FIG. 1. Photomicrography of the tumor biopsy (melanoma) before treatment. ×1630.

FIG. 2. Photomicrography of the biopsy of tumor site 2 weeks following the lymphotoxin treatment. There was lymphocytic infiltration, fibrosis, and no tumor. ×1630.

allogeneic, and xenogeneic origin (5, 8, 12). A preparation containing lymphotoxin was also shown to possess antitumor activity in leukemia (L1210) and melanoma (B16) in mice (8). Complete regression of melanoma in the dog as described in the present report showed that a crude preparation of lymphotoxin obtained from human lymphocytes is active in the dog. This observation of lack of species specificity will be in keeping with the reported *in vitro* and *in vivo* effects of lymphotoxin in mice where human lymphotoxin preparation has been shown to be active (6, 7). Our lymphotoxin was partially purified and other activities in the preparation could have produced the antitumor effect. This question can only be resolved when large quantities of pure preparations of lymphotoxin become available. However, it can be suggested that an α-lymphotoxin-containing human preparation possessed antitumor activity in the dog and may be a suitable candidate for possible human trials.

The presence of lymphocytic infiltrate at the site of regressed tumor shows the involvement of cellular immunity in producing antitumor effect. This is in agreement with previous observations (13).

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