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Escherichia coli Endotoxin Effect on a Methylcholanthrene-Induced Sarcoma in the Hamster Cheekpouch[1] (39912)

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Introduction. Extensive observations of endotoxin effects on experimental tumors, notably by Shear and Perrault (1), have used animal model systems in which tumors were grown subcutaneously, treated with endotoxin, and the hemorrhagic response was studied. These experiments yielded much useful information, but by their nature did not permit very early observations on young tumors following endotoxin treatment, detailed study of surrounding vasculature, and repeated observations over a period of days or weeks of the same tumor. Because of these limitations, it was decided to determine if young transplantable tumors grown in the hamster cheekpouch could be used to study early endotoxin effects.

It has been shown that the cheekpouch of the golden hamster offers a unique site for experimentation with transplantable homologous and heterologous tumors (2, 3). The animal can be repeatedly anesthetized, the pouch everted, and the tumor observed directly without seriously upsetting a normal physiological condition. For these reasons, the golden hamster cheekpouch was investigated to determine its usefulness for studying endotoxin effect on young tumors. The results are reported here.

Materials and Methods. Tumor induction. Three male golden hamsters, *Mesocricetus auratus,* were injected subcutaneously in the thigh region with 10 mg of 20-methylcholanthrene in 0.25 ml of sesame oil. At 110 days postinjection, the animals had thigh tumors 5 to 8 mm in diameter. One of the tumors was removed using sterile technique, and a small piece was fixed in Bouin's fluid and stained with hematoxylin and eosin. The tumor was classified as a spindle-cell sarcoma. The remaining tumor tissue was minced, suspended in sterile phosphate buffer solution (PBS), and stirred for 10 min at 4°. The PBS was decanted, and 25 ml of 0.1% trypsin in Ca- and Mg-free PBS was added to the tissue. After an additional 10 min of stirring at 4°, the supernatant, containing cells, was centrifuged for 10 min at 1000 rpm in a Sorvall glc centrifuge at 4°. The pellet was gently dispersed and suspended in Medium 199 with 20% calf serum and incubated at 37° in 5% CO_2 in a Falcon 75-cm^2 culture flask. The culture was maintained in Medium 199 with 10% calf serum. This tumor line was used in all the experiments reported in this paper.

Transplantation and endotoxin treatment. Tumor cells, which had grown to confluency, were trypsinized in 0.1% trypsin in Ca- and Mg-free PBS. The suspension of cells was centrifuged for 10 min at 1000 rpm in a Sorvall glc centrifuge. The supernatant fluid was decanted and the pellet of cells was gently suspended in PBS. The number of cells per unit volume was determined and viability was established using the trypan blue dye exclusion test. The hamsters to be inoculated were anesthetized using 0.15 ml of 65 mg/ml pentobarbital per 100 g of body weight, intraperitoneally. The cheekpouches were everted by gently grasping the posterior wall of the pouch, pulling the pouch out, and pinning it to a styrofoam slab with bank pins. The tumor cell suspension (1×10^5 cells in 20 μl of PBS) was injected under the upper epithelial layer into the connective tissue at the tip of the pouch where it is thinnest. A 26-gauge needle and Hamilton[3] syringe were used for inoculating the tumor cells into the pouch. The pouch was then pushed back to its normal position. The animal usually recovered within 30 min. Normal hamster fetal fibroblasts cultured in the same man-

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[3] Hamilton 500-μl syringe obtained from VWR Scientific, P.O. Box 232, Boston, Massachusetts 02101.

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ner were transplanted as controls.

In some control experiments normal hamster kidney, liver, or placental tissue was minced and 1-mm^3 cubes were transplanted into the pouch. This technique has been described elsewhere (2).

Animals were treated with *Escherichia coli* endotoxin[4] by intraperitoneal injection of the toxin suspended in sterile physiological saline. The tumors were regularly observed by anesthetizing the animal and everting the pouch. The tumor was measured with the aid of an ocular micrometer, and its volume in cubic millimeters was determined using the formula for an ellipsoid.[5] All data were statistically analyzed for significance using a nonparametric method described by White (4).

Results. The first series of experiments was to determine an endotoxin dose at which the animal could survive in good condition for the duration of the 21-day experiment, yet exhibit endotoxin-induced damage to the cheekpouch tumor. The results are presented in Table I. One microgram of endotoxin per gram of body weight appeared to be a most effective dose for influencing the tumor growth rate and ensuring the survival of all treated animals. There was no significant different between 1 and 2 μg of endotoxin dose levels on tumors grown in the cheekpouch. In addition to the decreased growth rate seen in Table I, other changes occurred in and around the tumor within 4 hr post-treatment with endotoxin. A halo of hemorrhaging and capillary sprout rupture was evident peripheral to the tumor, and extended up to a maximum of 6 mm in diameter around a 2-mm-sized tumor nodule. The extent of halo hemorrhaging appearing within the short period following endotoxin treatment varied with tumor age. Halo hemorrhaging appeared in 4-day post-transplant tumors, but not in younger tumors. It reached its maximum intensity and distribution in 5-, 6-, 7-, and 8-day tumor transplants, and was not evident when older tumors were treated with endotoxin. Nine-day-old or older transplants, however, exhibited extensive hemorrhaging, but it was restricted to the tumor mass. Probably the reason that halo hemorrhaging is not seen in 9-day-old or older transplants is that the tumor becomes encapsulated. The connective tissue capsule may interfere with angiogenesis factor diffusion. About a quarter of the tumors which exhibited halo hemorrhaging also showed tumor necrosis between 5 and 10 days after endotoxin treatment (see Fig. 1).

Histological sections of endotoxin-treated 5-day-old cheekpouch tumors were prepared and stained with hematoxylin and eosin (see Fig. 2). It can be readily seen that disruption of the vascular pattern has occurred within 2 hr after endotoxin treatment. The damage occurs chiefly at the periphery, but not center, of the tumor nodule and consists of blood vessel rupture and red blood cell leakage into the tumor mass. Much more extensive damage has occurred by 4 hr post-endotoxin treatment. All parts of the tumor mass now show vascular damage, with extensive red blood cell leakage into the tumor. The tumor cells also appear pushed apart, suggesting that edema is present. Endotoxin clearly disrupts the vasculature within and peripheral to the tumor transplant, inhibits growth rate, and, in about a quarter of the tumors tested, causes necrosis and disappearance of the tumor.

Normal kidney, liver, and placental tissue from 10-day-pregnant golden hamsters was used as control transplants. A 1-mm^3 cube of allogeneic normal tissue was transplanted into the pouch. At Day 5 post-transplant, the animals were anesthetized and the transplants examined. Transplants appeared vascularized, but had not increased in size beyond 1 mm^3. The hamsters were treated with endotoxin and the transplants observed periodically beginning 24 hr later. The results are presented in Table II.

The results in Table II indicate that placental tissue elicits a response involving vascular tissue indistinguishable from tumor tissue. The vascular pattern of liver and kidney transplants, however, was relatively unaffected by endotoxin.

Autopsies on endotoxin-treated hamsters were performed at the time the experiments

[4] *E. coli* endotoxin 0111:B4, W (Difco Laboratories, Detroit, Mich.).

[5] Formula for volume of ellipsoid, $(4\pi/3)\ ABC/8$.

TABLE I. THE EFFECT OF ONE DOSE OF *E. coli* ENDOTOXIN (1 μg/g BODY WEIGHT) ADMINISTERED INTRAPERITONEALLY ON DAY 5 FOLLOWING IMPLANTATION WITH 10^5 TUMOR CELLS IN THE HAMSTER CHEEKPOUCH.[a]

Days after transplant	Number of control animals	Average control tumor size (mm^3)	Number of experimental animals	Average experimental tumor size (mm^3)	*P* value[b]
5	13	4.1	13	2.9	>0.05
9	12	14.0	12	1.3	<0.001
12	12	30.8	10	6.1	<0.001
16	12	113.5	8	9.1	<0.001
21	10	494.4	8	63.8	<0.01

[a] The results were analyzed using the nonparametric method described by White (4).
[b] *P* value of >0.05 was considered significant.

were terminated on Day 21. No obvious abnormalities were observed. Adrenal glands appeared normal, spleens were not enlarged, and no abnormal mesenteric vasculature was seen.

Hamster fetal fibroblasts were also used as a control. Cells (2×10^5) were suspended in 20 μl of PBS and transplanted into the cheekpouch. Five days later a 1-mm^3 nodule was present and endotoxin was administered. No visible differences between the vascular patterns of control fetal fibroblast or endotoxin-treated fetal fibroblast transplants could be seen.

Finally, a modified Takasugi–Klein microcytotoxic test technique (5) was used to determine if there was a direct toxic effect of endotoxin on the methylcholanthrene-induced tumor cells. Endotoxin was added to the microwells containing plated methylcholanthrene-induced tumor cells and incubated at 37° for 48 hr. The number of cells remaining in each well was counted. There appeared to be no significant difference between the number of plated control and treated tumor cells when 100 μg of *E. coli* endotoxin was added per milliliter of culture medium. It was concluded that endotoxin at dose levels exceeding *in vivo* effective levels did not directly influence the hamster tumor cells.

Discussion. The evidence presented in this paper supports earlier reports that endotoxin injures the vascular tissue in transplantable tumors (1). However, the hamster cheekpouch allows earlier and more frequent observations of the tumors and their surrounding vasculature and indicates that endotoxin damage to tumors occurs much earlier than has been reported previously by Shear and Perrault (1), in which only tumors 20 mm in diameter or larger exhibited damage.

There appears to be three distinct and possibly related effects of endotoxin on young tumors grown in the hamster cheekpouch. The first is extensive halo hemorrhaging, with vascular damage not only peripheral to the tumor, but also within the tumor. The second is inhibition of tumor growth rate. The third is necrosis, followed by disappearance of the tumor nodule in about a quarter of the cases.

Although an *in vivo* model system such as the hamster pouch is a complicated system, it may be possible to isolate and study certain responses following endotoxin treatment. For example, it appears likely that tumor angiogenesis factor (TAF) may play a role in producing blood vessels responsive to halo hemorrhaging (6–8). Endothelial cells stimulated to form new capillary sprouts by TAF may be especially sensitive to endotoxin and easily damaged. This idea is supported by the observation that placental transplants, after treatment with endotoxin, also exhibit extensive peripheral vascular damage. Trophoblasts are involved in establishing the maternal fetal vascular connection (9) and presumably have an ample supply of angiogenesis factor. Prevention of tumor growth by pretreatment with endotoxin 24 hr before tumor challenge has been reported (10, 11). This protection may be related to TAF production or damage to proliferating endothelial cells in the vicinity of the tumor transplant, thus preventing the establishment of a tumor vascular pattern.

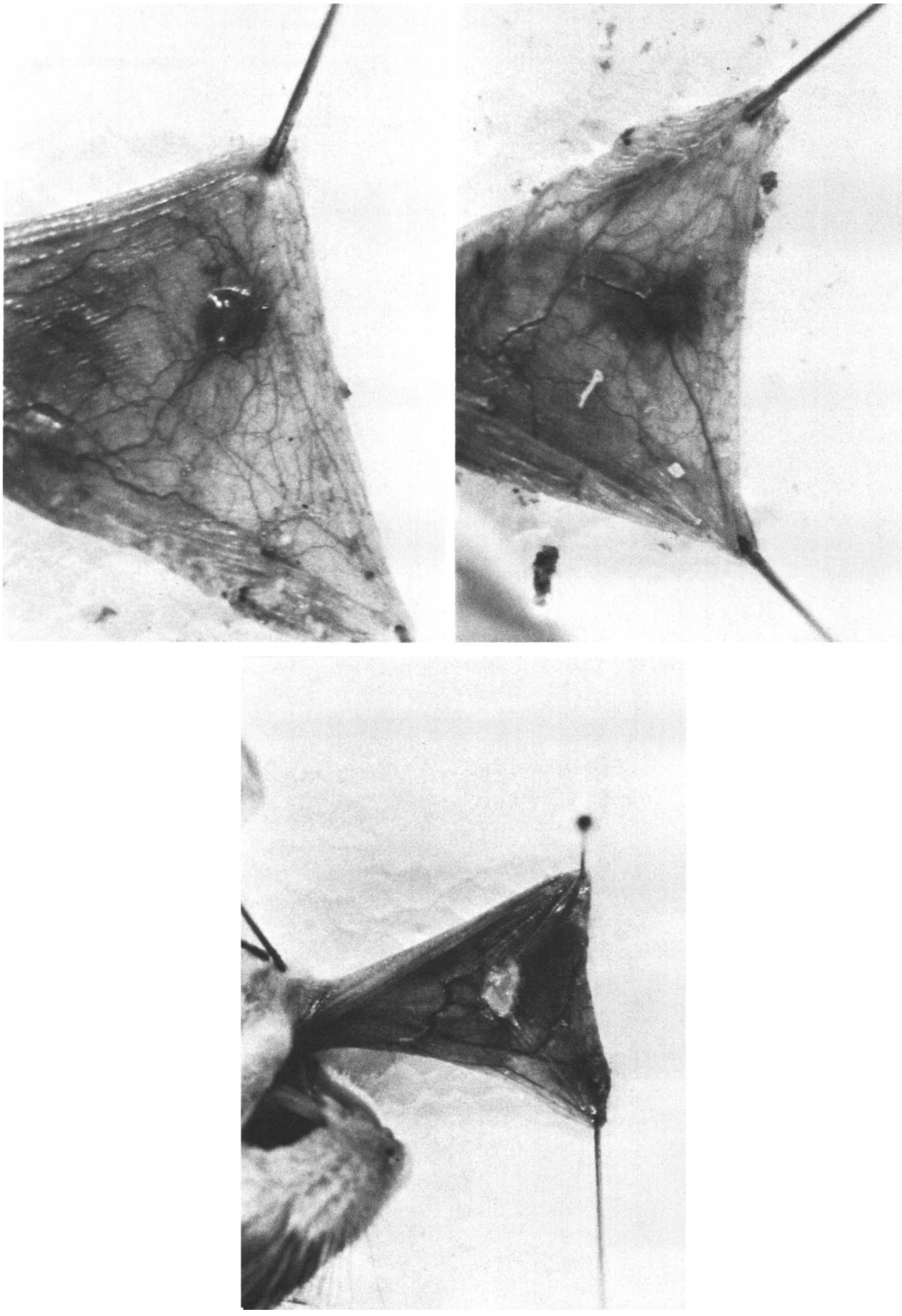

FIG. 1. Upper left: untreated cheekpouch tumor 5 days post-transplant. Upper right: tumor 5 days post-transplant, 4 hr after intraperitoneal injection of 1 μg of *E. coli* endotoxin/g body weight. Note halo hemorrhaging. Lower: same tumor 4 days after *E. coli* endotoxin treatment, showing necrosis in tumor area.

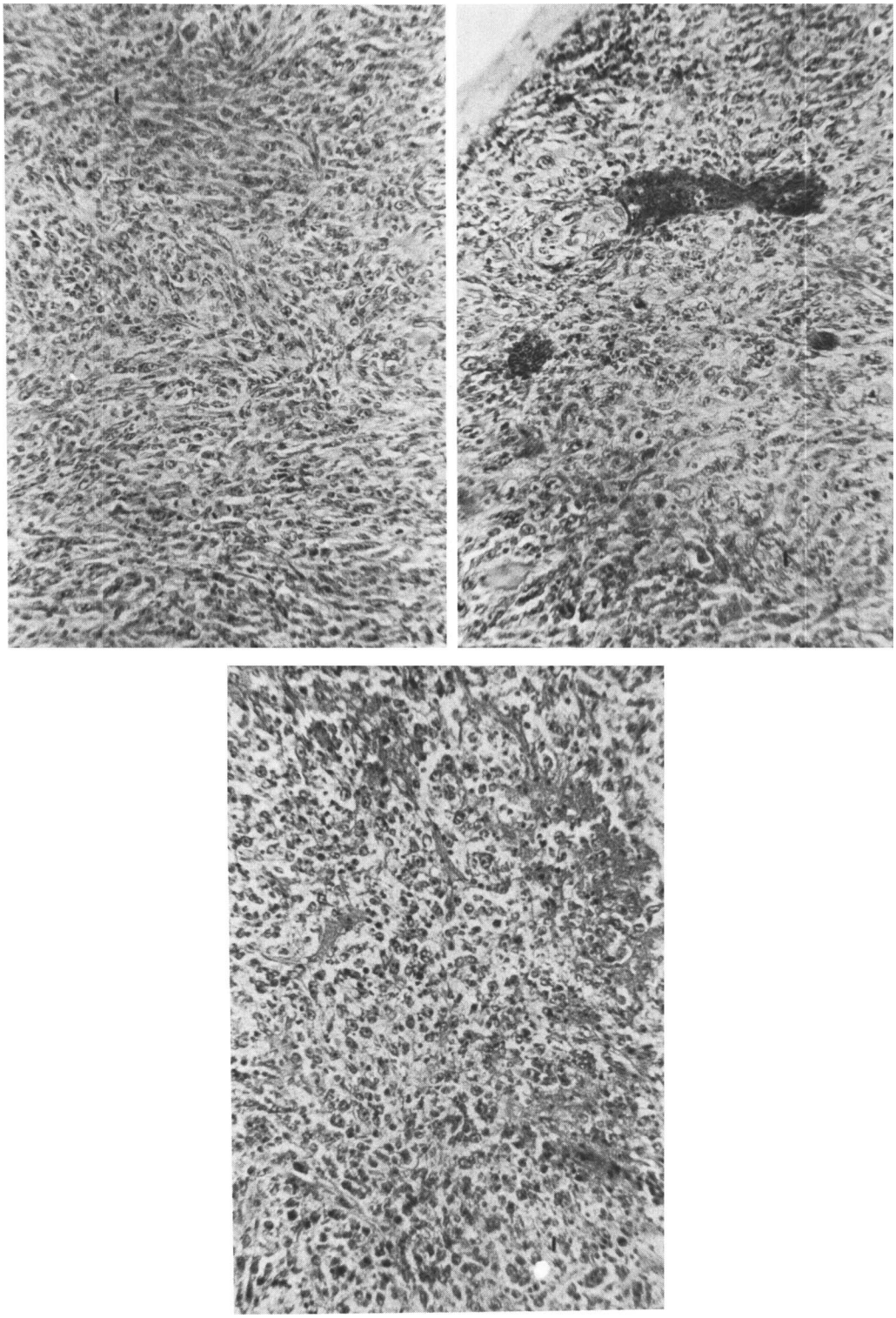

FIG. 2. Upper left: untreated cheekpouch tumor 5 days post-transplant. Upper right: tumor 5 days post-transplant, 2 hr after intraperitoneal injection of 1 μg of *E. coli* endotoxin/g body weight. Note blood vessel rupture and hemorrhaging. Lower: 4 hr after *E. coli* endotoxin treatment. Note increased hemorrhaging. Stained with hematoxylin and eosin. × 222.

TABLE II. THE EFFECT OF ONE DOSE OF *E. coli* ENDOTOXIN (1 μg/g BODY WEIGHT) ADMINISTERED INTRAPERITONEALLY ON DAY 5 FOLLOWING IMPLANTATION OF NORMAL TISSUE IN THE HAMSTER CHEEKPOUCH.

Tissue type	Days post-treatment	Size of transplant (mm^3)	Remarks
Kidney	1	1	Normal microcirculation
Liver	1	1	Normal microcirculation
Placental	1	1	Disrupted capillaries and halo hemorrhaging
Fetal fibroblasts	1	1	Normal microcirculation

Urbaschek *et al.* (12) have also reported that locally applied or intravenous injection of *E. coli* endotoxin causes pronounced effects on the microcirculation in the hamster cheekpouch. Our observations indicate that if *E. coli* endotoxin is injected intraperitoneally, the microcirculation is disrupted only in the immediate vicinity of the tumor, and this response appears to be dependent upon the age of the tumor transplant. Another parameter for investigation is the observation that endotoxin stimulates macrophages to produce a necrotizing factor. Subsequent necrosis of cheekpouch tumors following endotoxin treatment may involve necrotizing factor (13, 14), and if this is so, the cheekpouch model may be useful in further investigations of nonspecific immune responses to tumors.

Endotoxin severely injures the new blood vessels which are formed in response to the tumor implant and which are necessary for nourishment of the rapidly growing neoplastic tissue. Angiogenesis factor may play a major role in inducing the growth of new blood vessels (7). Since the vessels appear to be particularly vulnerable to endotoxin, the hamster cheekpouch may be a useful model to investigate possible relationships between tumor growth, vascular development, endotoxin injury, and tumor angiogenesis factor. We feel that knowledge of interactions of these factors may be of value in treating neoplasms.

Most attempts to destroy tumors have used agents which are designed to affect malignant tissue. The hamster cheekpouch, in which one can injure host structures and tissues which supply the parasitic tumor with its nourishment, may be a good method of studying the control of malignancy by interfering with host tissue components essential for the neoplasm's survival. Failure of endotoxin therapy in the past may be due to the lack of understanding some of these variables.

Summary. Golden hamsters, *Mesocricetus auratus,* were transplanted in their cheekpouches with 10^5 methylcholanthrene-induced sarcoma cells maintained in tissue culture. At varying times the animals were injected intraperitoneally with a single dose of *E. coli* endotoxin and the tumors were observed periodically for damage. Animals treated with endotoxin on Days 4 through 8 after tumor transplantation exhibited hemorrhaging adjacent to the tumor nodule, hemorrhaging within the tumor, decreased tumor growth rate, and, in a quarter of cases, necrosis of the tumor. Normal tissue transplants did not exhibit these effects. It appears that the hamster cheekpouch may be a useful model for the study of endotoxin effects on young tumors.

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