

THE IMMUNOLOGICAL BASIS OF ENDOTOXIN-INDUCED TUMOR REGRESSION

Requirement for T-Cell-Mediated Immunity*

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It is well documented (1-3) that parenteral injection of bacterial endotoxin can cause hemorrhagic necrosis of established experimental tumors. The anti-tumor effect is rapid, and is confined to the core of the tumor which darkens, separates, and eventually is sloughed off. However, in spite of the dramatic destruction of the center of the tumor, it is only in a small percentage of cases that hemorrhagic necrosis is followed by the complete regression of the ring of viable tumor tissue that remains. Therefore, in most cases, hemorrhagic necrosis does little more than cause an apparent temporary halt in tumor growth.

Investigations of the anti-tumor effect of endotoxin go back to the end of the last century when Coley and Bruns (4, 5) were describing spontaneous regressions of certain types of tumors in humans after acute bacterial infections, or after deliberate injections of bacterial toxins. However, in spite of an enthusiastic beginning, the use of mixed bacterial toxins in the treatment of cancer soon fell from favor because of inconsistent and unpredictable results. Indeed, we still are in no position to rationally predict which experimental tumors will undergo complete regression in response to endotoxin treatment. The knowledge that hemorrhagic necrosis is a common consequence of endotoxin treatment, whereas regression is relatively rare, would indicate, moreover, that tumor necrosis and tumor regression are mechanistically separate events. This possibility is strongly suggested by results of a published study (6) which shows that endotoxin-induced regression, but not hemorrhagic necrosis, is inhibited by treatment with anti-thymocyte serum. This result was interpreted as meaning that regression, but not hemorrhagic necrosis, is dependent on the expression of an acquired state of anti-tumor immunity.

The purpose of this paper is to provide additional evidence for hypothesizing that endotoxin-induced tumor regression, as distinct from hemorrhagic necrosis, is dependent on the expression of a state of T-cell-mediated anti-tumor immunity that is only generated in response to immunogenic tumors. The paper which follows will provide evidence to support the additional proposition that endotoxin-induced tumor regression will not occur until the tumor has grown to a large enough size to evoke the generation of an adequate level of concomitant anti-tumor immunity.

Materials and Methods

Mice. Breeding nuclei of A/J, BALB/c, and DBA/2 mice were purchased from The Jackson Laboratory, Bar Harbor, Maine, and used for the production of parental strains and F1 hybrids

* Supported by grant CA-16642 awarded by the National Cancer Institute, grant IM-155 from the American Cancer Society, and grant RR 05705 from the Division of Research Resources, National Institutes of Health.

‡ Recipient of Alan J. Hirschfield Fellowship from the Cancer Research Institute, Inc.

according to established breeding techniques. Breeding was performed under barrier sustained conditions. The F1 hybrids employed were AB6F1 (A × C57Bl/6)CB6F1 (BALB/c × C57Bl/6), and B6D2F1 (C57Bl/6 × DBA/2). Mice of either sex were employed when they were between 8 and 12 wk of age. C3H and AKR strains were used for producing anti-Thy-1.2 serum.

Tumors. The SA-1 spindle cell sarcoma syngeneic in A/J mice, the Meth A fibrosarcoma syngeneic in BALB/c, the CaD2 mammary carcinoma syngeneic in DBA/2, and the BP3 benzpyrene-induced fibrosarcoma syngeneic in C57Bl/6 were studied. Experiments with each tumor were performed with a single stock of biofrozen cells. In the case of the SA-1 and Meth A tumors, large numbers of cells were grown in ascites form in the peritoneal cavities of a large number of syngeneic mice. They were harvested after 7 days of growth in heparinized phosphate-buffered saline (PBS),¹ washed twice in PBS, and resuspended for biofreezing at 10⁷/ml in minimal essential medium (MEM) containing 20% fetal calf serum and 20% dimethyl sulfoxide. They were stored in small aliquots over liquid nitrogen. Before each experiment an aliquot was thawed, washed in PBS, and the cells grown intraperitoneally for two passages in semisyngeneic F1 hybrid mice before being harvested, washed, and resuspended at an appropriate concentration in PBS for initiating tumors.

The CaD2 carcinoma and BP3 fibrosarcoma were passaged subcutaneously by trocar. A single cell suspension of tumor cells was obtained by digesting finely diced pieces of tumor in PBS containing DNase, collagenase, and trypsin (Sigma Chemical, Co., St. Louis, Mo.) as previously described (7). These cells were biofrozen in aliquots as described above. Before each experiment an aliquot was thawed, washed in PBS, and used to seed in vitro cultures in large plastic tissue culture flasks containing Fisher's medium (Grand Island Biological, Grand Island, N.Y.) with 10% fetal calf serum, 100 U penicillin, 0.25 μg fungizone, and 100 μg streptomycin per ml. The cells were allowed to grow to the desired density at 37°C in an atmosphere of 5% CO₂ in air. They were detached from the bottom of the vessels by treatment with 0.2% EDTA and 2% trypsin in Ca⁺⁺- and Mg⁺⁺-free PBS, washed, and resuspended at an appropriate concentration in PBS for implantation into mice.

Tumor cells were always injected in a 0.05 ml vol of PBS with a 30 gauge needle. A great deal of care was taken with injections. Primary tumors were implanted intradermally on the ventral surface of the abdominal region, while challenge tumors were implanted subcutaneously in the right-hind footpad. The growth of primary tumors was monitored by measuring changes in tumor weight against time. The growth of challenge tumors was measured with dial calipers as increases in the dorsoventral thickness of the footpad as described previously (8).

Endotoxin. *Salmonella enteritidis* lipopolysaccharide B, lot no. 628857 from Difco Laboratories, Detroit, Mich. was used for all experiments. It was suspended in sterile PBS at a concentration of 1 mg/ml, aliquoted, and stored at -20°C.

T-Cell-Deficient Mice. Mice were rendered T-cell-deficient (THXB) as adults by thymectomy, followed 7 days later by lethal (900 rads) whole-body gamma-irradiation delivered from a cesium-137 irradiator at a midphantom dose rate of 35.5 rads/min. They were infused intravenously immediately after irradiation with 2 × 10⁶ syngeneic bone marrow cells and employed in experiments 4-6 wk later. Sham-thymectomized mice treated in the same way (XB) served as controls for the effect of irradiation.

Endotoxin-Induced Regression. Testing the susceptibility of tumors to endotoxin involved initiating primary intradermal tumors with 10⁶ or 2 × 10⁶ tumor cells, allowing the tumors to grow for 7 days, and then injecting the mice intravenously with 50 μg of endotoxin in a 0.2 ml vol. Large numbers of mice were used, and the populations sampled against time for changes in tumor weight. This involved excising and weighing the tumors from five mice at the time intervals indicated. In contrast to published results of others (6), it was not possible to use changes in tumor diameter to measure endotoxin-induced tumor regression, because the tumors regressed from the inside out.

Adoptive Transfer of Anti-Tumor Immunity. Spleen cells from mice whose tumors had regressed under the influence of endotoxin were examined for their ability to adoptively immunize normal syngeneic recipients against a tumor cell challenge. Spleens were taken 10 days after complete regression, and the cells harvested from finely diced pieces of spleen by passing the

¹ Abbreviations used in this paper: BCG, Bacillus Calmette-Guérin; MEM, minimal essential medium; PBS, phosphate-buffered saline; THXB, T-cell-deficient by thymectomy; XB, sham-thymectomized mice.

pieces through a 200-mesh stainless steel screen into PBS containing 1% heat-inactivated fetal calf serum according to procedures already described (9). The spleen cells were thoroughly washed and infused intravenously via a lateral tail vein into normal recipients that were challenged 1 h later in a hind footpad with cells of the immunizing tumor. The growth of the challenge implant was monitored against time with dial calipers.

Treatment with Anti-Thy 1.2 Serum. Anti-Thy-1.2 serum was produced by immunizing AKR mice with C3H thymocytes. The specificity of the antiserum was tested by absorption with brain tissue as described previously (10). Its ability to abrogate the capacity of spleen cells to transfer anti-tumor immunity was tested by treating the spleen cells at 5×10^7 /ml in a 1:5 dilution of it for 20 min at 4°C. This was followed by a 30-min incubation in agarose-absorbed guinea pig serum (11) at a dilution of 1:5 in PBS. The cells were then washed in PBS and resuspended for intravenous infusion.

Results

Susceptible and Nonsusceptible Tumors. The effect of a single intravenous injection of 50 μ g of endotoxin on the growth of four different syngeneic murine tumors initiated 7 days earlier is shown in Fig. 1. It can be seen that the SA-1 and Meth A tumors completely regressed in all cases, whereas growth of the CaD2 and BP3 tumors was hardly affected. Observation of mice for a period of 6 mo after tumor regression revealed that the overall frequency of reemergence of tumors was less than 10%. This conclusion is based on the results of several additional experiments, as well as on results obtained with groups of 50 mice in which tumor regression and regrowth were assessed visually. When regrowth of tumors did occur, it usually began within 10 days of complete regression. It should be pointed out, moreover, that although endotoxin caused complete regression of all primary SA-1 tumors, a small percentage of animals eventually succumbed to metastatic disease.

An additional observation was, that although endotoxin caused regression of only

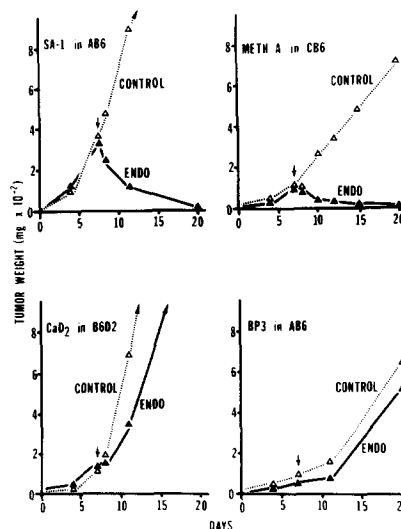


FIG. 1. The effect of intravenous administration of 50 μ g of endotoxin (arrows) on four different tumors initiated intradermally 7 days previously in semisyngeneic mice. All four tumors showed hemorrhagic necrosis, but only the SA-1 and Meth A sarcomas subsequently underwent complete regression. Means of five mice per time interval.

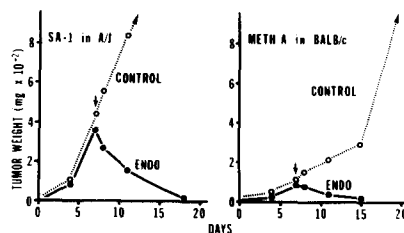


FIG. 2. Evidence that 7 day SA-1 and Meth A tumors showed the same pattern of endotoxin-induced regression in syngeneic parental strain mice as in semisyngeneic F1 hybrids. Means of five mice per time interval.

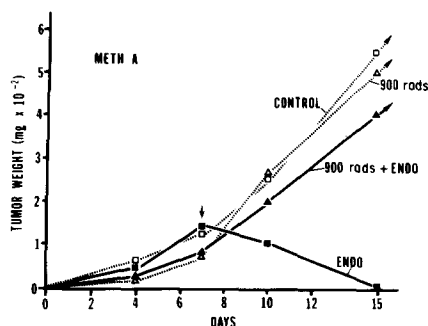


FIG. 3. Endotoxin administration (arrows) failed to cause regression of 7 day Meth A tumors in mice that were immunodepressed by 900 rads of gamma-irradiation given 24 h before tumor implantation. Means of five mice per time interval.

two of the four tumors tested, hemorrhagic necrosis occurred in all four of them, and was no greater in intensity in those tumors that underwent regression. In all cases, the core of the tumor began darkening within a few hours, and the hemorrhagic reaction increased in extensiveness over the next 48 h. It was the ring of viable tumor tissue that remained, however, which eventually regressed in the case of the SA-1 and Meth A tumors, but which grew progressively to kill the hosts in the case of the BP3 and CaD2 tumors.

Because most of the experiments to be reported in this paper were performed in F1 hybrid mice, it was necessary to show that the same results are obtained with syngeneic parental strains. That this was the case is shown in Fig. 2 where it can be seen that the pattern of endotoxin-induced regression of the SA-1 and Meth A tumors in syngeneic mice was similar to that measured in semisyngeneic F1 hybrids. The CaD2 and BP3 tumors did not regress in parental strains.

Effect of Gamma-Irradiation on Endotoxin-Induced Regression. Fig. 3 shows that exposure to 900 rads of whole-body gamma-irradiation 24 h before the intradermal inoculation of Meth A cells, completely abrogated the ability of mice to regress tumors in response to endotoxin therapy given on day 7 of tumor growth. In contrast, gamma-irradiation had little or no effect on the degree of hemorrhagic necrosis. The same result was obtained with the SA-1 sarcoma as evidenced by visual inspection. These findings show, therefore, that a radiosensitive host component plays a role in endotoxin-facilitated tumor regression, but not in hemorrhagic necrosis.

Failure of Regression to Occur in T-Cell-Deficient Mice. One interpretation of the

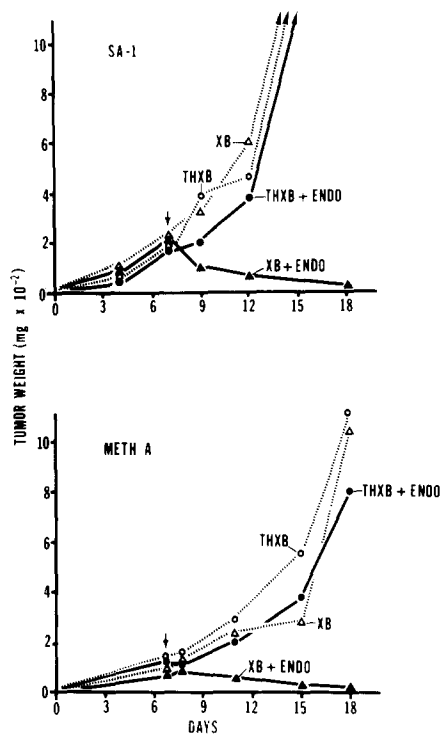


FIG. 4. Endotoxin injection (arrow) failed to cause regression of 7 day SA-1 and Meth A tumors in mice that were made T-cell deficient as adults by thymectomy and irradiation, and protected with bone marrow cells (THXB). Regression occurred in irradiated, bone marrow-protected controls (XB) in the same way as in normal controls (not shown). Means of five mice per time point.

preceding results is that endotoxin-induced tumor regression has an immunologic basis. It was predicted, therefore, that endotoxin would not cause regression of susceptible tumors growing on T-cell-deficient mice. Fig. 4 shows that this was indeed the case, in that endotoxin failed to cause the regression of 7 day SA-1 and Meth A tumors on mice made T-cell-deficient by thymectomy and gamma-irradiation, and protected with bone marrow cells (THXB). Here again, the degree of hemorrhagic necrosis in the core of the tumors was not affected by the deficiency of T cells. It seems clear, therefore, that hemorrhagic necrosis is not the cause by itself of tumor regression.

The Generation of T-Cell-Mediated Anti-Tumor Immunity after Endotoxin-Induced Regression. The foregoing results suggest that T lymphocytes are involved in endotoxin-induced tumor regression. It was expected, therefore, that endotoxin-induced tumor regression would be associated both with the generation of a state of immunity to tumor cell challenge, and with the possession of T cells capable of adoptively immunizing normal recipients systemically against a tumor cell challenge.

Fig. 5 shows that mice whose SA-1 or Meth A tumors had regressed under the influence of endotoxin were resistant to growth of an implant of cells of the same tumor given subcutaneously 10 days later. The results in Fig. 6 show, in addition, that the acquired resistance to tumor challenge was specific, in that mice whose Meth

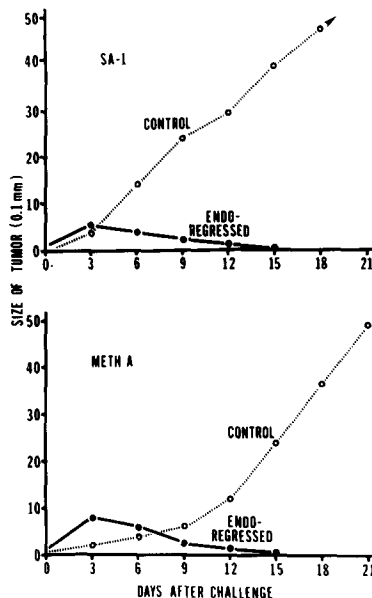


FIG. 5. Endotoxin-induced regression of 7 day intradermal SA-1 and Meth A tumors was followed by a state of resistance to growth of a 5×10^6 tumor cell challenge implant given subcutaneously in the right hind footpad. The mice were challenged 10 days after complete regression of the primary tumor. Means of five mice per time interval.

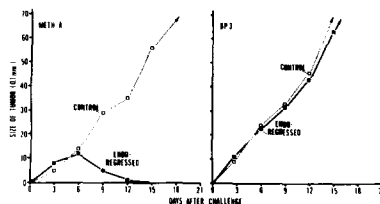


FIG. 6. Evidence that the anti-tumor immunity generated by mice whose 7 day Meth A tumors had regressed in response to endotoxin was specific. The strong resistant expressed against a 5×10^6 Meth A cell challenge implant was not expressed against 5×10^6 BP3 cells.

A tumors had regressed were not resistant to growth of 10^6 BP3 tumor cells. Another significant finding was that this state of anti-tumor immunity was long lived, as evidenced by resistance to a tumor challenge given 60 or 90 days after regression of the primary tumor (Fig. 7).

Evidence that this state of tumor-specific immunity was mediated by sensitized T cells is shown in Fig. 8 where it can be seen that it was associated with the presence of cells in the spleen which could adoptively immunize normal recipients against a tumor cell challenge, and that incubation of these spleen cells with anti-Thy-1.2 serum and complement completely ablated their protective capacity. The level of adoptive immunity shown was transferred with 1.5×10^8 spleen cells. It should be pointed out that in these experiments the adoptive immunity was systemically expressed, in that the spleen cells were infused intravenously and the tumor implant given subcutaneously 1 h later.

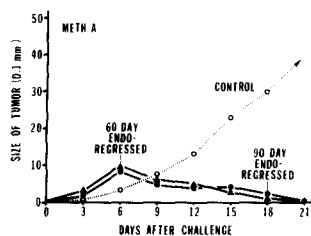


FIG. 7. Anti-tumor immunity generated after endotoxin-induced regression of the Meth A tumor was long-lived, as evidenced by resistance to growth of an implant of 5×10^5 Meth A cells given 60 or 90 days after regression of the primary tumor. Means of five mice per time interval.

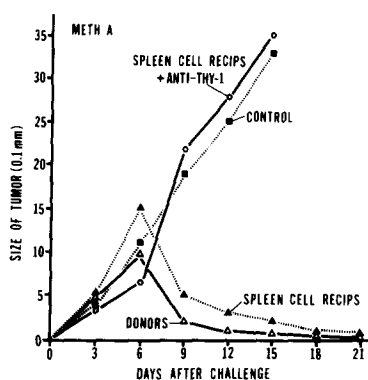


FIG. 8. Passive transfer of anti-tumor immunity (Meth A) to normal recipients with 1.5×10^8 spleen cells (1 spleen equivalent) from donor mice whose tumors had regressed 10 days previously in response to endotoxin treatment. The protective capacity of the spleen cells was completely ablated by incubating them in anti-Thy-1.2 serum and complement, but not by incubating them with normal AKR serum and complement (controls). An infusion of normal spleen cells is without effect (not shown). Means of five mice per time interval.

It will be noted that there was an initial period during which the tumor challenge implant apparently grew more in immunized and adoptively immunized mice than it did in controls. It seems quite likely that this represented a delayed-type hypersensitivity reaction to tumor associated antigens, and that an initial period of tumor growth was required to supply enough antigen to elicit the reaction.

The Tumors that Regress in Response to Endotoxin are Immunogenic as Classically Defined. It is logical to suggest on the basis of the foregoing results, that of the four tumors tested, it is only the two that regress under the influence of endotoxin which are immunogenic as classically defined. This prediction was tested by investigating the immunogenicity of all four tumors by the classical method of determining whether surgical removal of the primary tumor is followed by the acquisition of a state of immunity to a challenge implant.

Fig. 9 shows the results of challenging mice subcutaneously with 10^6 tumor cells 14 days after removal of their 7 day primary tumors. It can be seen that the only tumors that showed immunogenicity by this method were the SA-1 and Meth A sarcomas; those that completely regress in response to endotoxin therapy. The same result was obtained when the challenge implants were given 7 and 21 days after tumor excision.

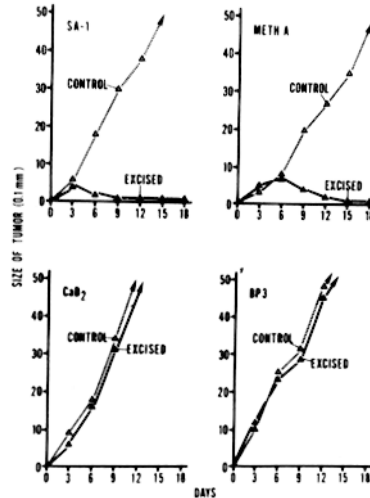


FIG. 9. Growth of a 10^6 subcutaneous challenge implant in control mice and in mice whose primary tumors were excised 14 days previously. Only the SA-1 and Meth A sarcomas proved to be immunogenic by this classical technique. Means of five mice per time interval.

Discussion

The results of this study are consistent with the hypothesis that endotoxin-induced regression of established murine tumors is immunologically mediated by an acquired population of sensitized T cells which is generated only in response to tumors that are immunogenic as classically defined. They show (a) that endotoxin will not induce regression of susceptible tumors in mice that have been immunodepressed by whole-body irradiation, or that have been made T-cell deficient by thymectomy and irradiation, (b) that regression is followed by specific resistance to growth of a tumor cell challenge, and with the presence of T cells that can passively transfer this resistance to normal recipients, and (c) that the only tumors that regress are those that are immunogenic as classically defined.

On the other hand, the dramatic phenomenon of hemorrhagic necrosis which invariably precedes tumor regression, is not significantly diminished by whole-body irradiation, or T-cell deficiency. Furthermore, it occurs with tumors that apparently are nonimmunogenic and which do not regress. It is reasonable to suggest, therefore, that endotoxin-induced hemorrhagic necrosis cannot be used as an indicator of the therapeutic effectiveness of endotoxin, even though it may well be a prerequisite for tumor regression to occur. It should be born in mind, moreover, that the cores of most established solid tumors are extensively necrotic to begin with. Consequently, endotoxin may do little more than speed up necrosis and make the core of the tumor and its immediate surroundings more visible. This would fit with the interpretations of Stetson (12) who showed that endotoxin treatment results in a profound, though temporary, reduction in the blood flow to solid tumors. Obviously, this would ensure the rapid destruction of the already undernourished central region of the tumor.

A contradiction to this explanation could exist, however, in the fairly recent series of publications of Carswell et al., Green et al., and Hoffman et al. (13-16) which describe the liberation into circulation of a host factor, referred to as tumor necrosis

factor, which follows the injection of endotoxin into *Bacillus Calmette-Guérin* (BCG)-infected, or *Corynebacterium parvum*-treated mice. These authors showed that infusion of serum containing tumor necrosis factor causes hemorrhagic necrosis and occasional regression of susceptible tumors in recipient mice, and that the same serum is cytotoxic for certain neoplastic and fetal cells in tissue culture. Indeed, it is mainly on the basis of the latter property that the authors suggest that tumor necrosis factor is the mediator of hemorrhagic necrosis and regression. However, most in vivo studies of tumor necrosis factor have concentrated almost exclusively on its ability to cause hemorrhagic necrosis, rather than on its ability to cause regression. It would be interesting, therefore, to determine whether tumor necrosis factor causes the regression of endotoxin-susceptible tumors in immunodepressed mice. If not, then it almost certainly is not the direct mediator of the most important antitumor effect of endotoxin: its capacity to cause a susceptible tumor to completely regress. It would seem important to determine, in addition, whether tumor necrosis factor, besides being liberated into the circulation of BCG- and *C. parvum*-treated mice, is also liberated into the circulation of tumor-bearing mice themselves.

In spite of the evidence presented here for the T-cell mediation of endotoxin-induced tumor regression, it is apparent from the literature that factors in addition to tumor immunogenicity are involved in determining whether a tumor will regress in response to endotoxin treatment. For example, tumor location and tumor size (17) have been shown to be important. The reason why tumor size is important will be discussed in the accompanying paper.

Summary

It was shown that although intravenous administration of bacterial endotoxin caused extensive hemorrhagic necrosis of four different syngeneic murine tumors, only two of these tumors subsequently underwent complete regression: the two that were shown to be immunogenic as classically defined. An immunologic basis for endotoxin-induced regression was further indicated by the additional findings that regression, but not hemorrhagic necrosis, of the two immunogenic tumors failed to occur in mice that were immunodepressed by whole-body gamma-irradiation, or that were made T-cell deficient by thymectomy and irradiation. That endotoxin-induced regression is T-cell mediated was suggested by the findings that tumor regression was followed by a state of long-lived immunity to a tumor cell challenge implant, and with the possession by the host of T cells that were capable of passively transferring this state of immunity to normal recipients.

It is concluded that although parenteral injection of endotoxin causes hemorrhagic necrosis of most solid murine tumors, it is only those tumors that are immunogenic enough to evoke the generation of T-cell-mediated immunity which subsequently go on to completely regress.

Received for publication 18 August 1978.

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