

Growing Tumors Induce Hypersensitivity to Endotoxin and Tumor Necrosis Factor

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Lewis lung carcinoma and EMT6 sarcoma growing as solid tumors in mice caused a gradual increase in the susceptibility of the animals to lethal toxicity of endotoxins (lipopolysaccharides [LPS]). By day 15 following inoculation of the tumors, the 50% lethal dose of LPS, which in normal mice was approximately 400 µg, decreased to 2 µg for the sarcoma-bearing mice and 0.1 µg for the carcinoma-bearing mice. This sensitization to endotoxin was paralleled by a high sensitization to tumor necrosis factor (TNF). Human recombinant TNF given to normal mice was lethal at about 500 µg. It was lethal for 50% of the animals bearing EMT6 or Lewis lung carcinoma tumors in amounts of 4 and 0.01 µg, respectively, on day 15 following tumor inoculation. The sensitization of tumor-bearing animals to LPS and TNF was paralleled by marked granulocytosis.

Endotoxins (lipopolysaccharides [LPS]) isolated from gram-negative bacteria elicit a number of biological perturbations which can lead to lethal shock in experimental animals (27). Mice are relatively resistant to endotoxin, and within a given strain, sex, and age they exhibit a fairly homogeneous response to the lethal effects of LPS. This normal level of sensitivity to LPS can be enhanced by different treatments of the animals with various chemical agents or microorganisms. Treatment by adrenalectomy (15) or with actinomycin D (8), lead acetate (24), or D-galactosamine (12) sensitized the animals to very low amounts of LPS. Similarly, bacteria or bacterial constituents like *Mycobacterium bovis* BCG (26), *Propionibacterium acnes* (27), live or killed *Coxiella burnetii* (23), or muramyl-dipeptide (22) or infections with gram-negative bacteria (11) enhanced reactivity to endotoxin, which was in some cases accompanied by splenomegaly and hepatomegaly.

Tumor necrosis factor (TNF) is an endogenous mediator elicited by LPS in animals with an activated reticulo-endothelial system (9). This factor is produced by activated mononuclear phagocytes and causes tumor necrosis (17). The genes coding for TNF have been isolated and expressed by molecular cloning, allowing the preparation of large quantities of recombinant TNF and the determination of its amino acid sequence (18, 21). Human TNF showed 80% homology with murine TNF, and its biological activity did not seem to be species specific.

Besides tumor necrotic activity, TNF exhibits a number of biological effects (10). Galactosamine-treated mice were sensitized to TNF, which showed that the lethal activity of endotoxin is mediated by TNF (15a). It has not been possible to separate the beneficial antitumoral effect of TNF from all of the other endotoxic effects (16). In the present report, we demonstrate that two malignant tumors growing in mice rendered the animals very sensitive to low doses of LPS or TNF.

MATERIALS AND METHODS

Animals. Female C57BL/6 and BALB/c mice (6 to 10 weeks old) were obtained from the breeding stock of the Max Planck Institute.

Tumors. Lewis Lung (3LL) carcinoma was propagated and maintained in vivo by intramuscular injection of 5×10^5 3LL cells in the legs of C57BL/6 mice. EMT6 sarcoma was propagated in BALB/c mice by subcutaneous injection of 2×10^5 cells in the interscapular region. Tumor growth was assessed by measuring the diameters of the tumors with calipers in two perpendicular directions, and tumoral cross-section was calculated as previously described (3).

EL4 lymphosarcoma and a methyl cholanthrene-induced fibrosarcoma (MethA) were propagated intraperitoneally in ascites form in C57BL/6 and BALB/c mice, respectively. In this study, EL4 and MethA were used as solid tumors obtained by intracutaneous inoculation of washed peritoneal exudative cells (10^5) in the corresponding mice.

Lethal toxicity. At different times after tumor inoculation, groups of six mice received different amounts of LPS or TNF intraperitoneally in 0.2 ml of pyrogen-free phosphate-buffered-saline. Deaths were recorded during 48 h, and 50% lethal doses (LD₅₀s) were calculated by probit analysis (25).

Enzyme determination. Sorbitol dehydrogenase activity in heparinized plasma was measured kinetically (7).

LPS. LPS from *Salmonella abortus-equi* (S form) and *S. minnesota* R595 (Re) were isolated from bacteria by the phenol-water (28) and phenol-chloroform-petroleum ether methods (14), respectively. They were electro-dialyzed and converted to the uniform triethylammonium salt as previously described (13).

TNF. Human recombinant TNF was supplied generously by BASF, Ludwigshafen, Federal Republic of Germany.

RESULTS

Hypersensitivity to LPS induced by EMT6 sarcoma in mice. After subcutaneous transplantation of syngeneic EMT6 cells in BALB/c mice, the resulting solid sarcoma grew exponentially for 3 weeks (Fig. 1). A marked dose-dependent regression of the tumor mass with hemorrhagic necrosis of the

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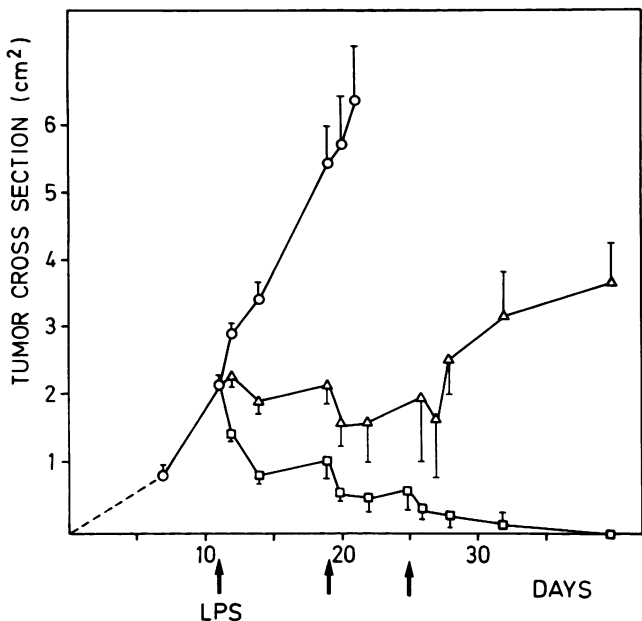


FIG. 1. Antitumoral effect of *S. abortus-equi* LPS on EMT6 sarcoma. EMT6 sarcoma was inoculated into BALB/c mice as described in Materials and Methods. LPS was injected intraperitoneally on days 11, 19, and 25 (arrows) after tumor inoculation. Symbols: O, control mice; Δ, mice injected with 2 μg of LPS. Means and standard errors of groups of six animals are presented.

center part was seen after intraperitoneal injection of *S. abortus-equi* LPS in the mice. The regression could be elicited between days 7 and 20 after tumor inoculation, and it was additive after repeated injections of LPS. We observed that tumor-bearing mice died after receiving doses of LPS normally nontoxic to healthy animals, suggesting hypersensitivity.

BALB/c mice bearing EMT6 sarcomas of increasing size were tested for their sensitivity to LPS or TNF at different times after tumor inoculation. The lethal dose of LPS for normal mice was approximately 300 μg (Table 1). By comparison, the LD₅₀ for tumor-bearing mice was reduced to 100 μg 3 days after tumor inoculation and 2 μg at day 15. The LD₅₀ then remained at this level until the animals died, between days 22 and 30 (Fig. 2). The above increase in sensitivity to LPS was paralleled by an increase in sensitivity

TABLE 1. Hypersensitivity of Lewis lung carcinoma and EMT6 sarcoma-tumor-bearing mice to LPS or recombinant TNF

Mouse ^a	LD ₅₀ (μg/mouse) of:	
	LPS	TNF
Normal C57BL/6	400	300
C57BL/6 with carcinoma (15-day tumor)	0.1	0.01
Normal BALB/c	300	300
BALB/c with sarcoma (15-day tumor)	2	4

^a Female C57BL/6 and BALB/c mice were inoculated, respectively, with 3LL cells intramuscularly or EMT6 cells subcutaneously. Fifteen days later, the animals were challenged intraperitoneally with increasing doses of recombinant TNF or *S. abortus-equi* LPS. Each group was formed of six animals receiving increasing doses of the test compound. Mortality was recorded during 48 h.

to TNF. The LD₅₀ in normal BALB/c mice was approximately 300 μg of TNF. It was reduced to 4 μg of TNF 2 weeks after inoculation of EMT6 sarcoma (Table 1).

The growth of EMT6 sarcoma in mice caused marked splenomegaly (a threefold increase in weight at day 20) and leukocytosis. The number of leukocytes increased with tumor growth, and they occurred mainly in the granulocyte fraction (Fig. 3). Granulocytosis paralleled the progressively increased sensitivity to endotoxin and TNF. Sorbitol dehydrogenase activity measured in the plasma of tumor-bearing mice was not affected by tumor growth, suggesting that the liver was not directly involved in the hypersensitivity of tumor-bearing animals.

Lewis lung carcinoma grew as a solid intramuscular tumor which could be monitored from day 5 on. Metastatic lesions could be counted as nodules on day 19, causing animal death shortly thereafter. Changes in the sensitivity of the mice to LPS or TNF, tested as described in Materials and Methods, are shown in Table 1 and Fig. 2. The LD₅₀ of *S. abortus-equi* LPS was 400 μg in healthy C57BL/6 mice. After inoculation of Lewis lung carcinoma, the LD₅₀ of LPS decreased progressively to 50 μg after 3 days, 10 μg after 7 days, 1 μg after 10 days, 0.1 μg after 15 days, and 0.03 μg after 20 days (Fig. 2). This high degree of sensitization in the presence of growing tumors was accompanied by strong granulocytosis and splenomegaly (three to five times the normal weight at day 15). The increase in blood leukocytes was primarily seen in the granulocyte population, which increased up to 15-fold the normal value (Fig. 3). The liver was apparently unaffected, as shown by normal sorbitol dehydrogenase activity in blood serum throughout tumor development. On day 15, the lethal toxicity of S-form LPS (LD₅₀, 0.01 to 0.1 μg) was higher than that of Re-form LPS (LD₅₀, 0.1 to 1.0 μg).

C57BL/6 mice bearing Lewis lung carcinoma were also very sensitive to the lethal effects of very low doses of

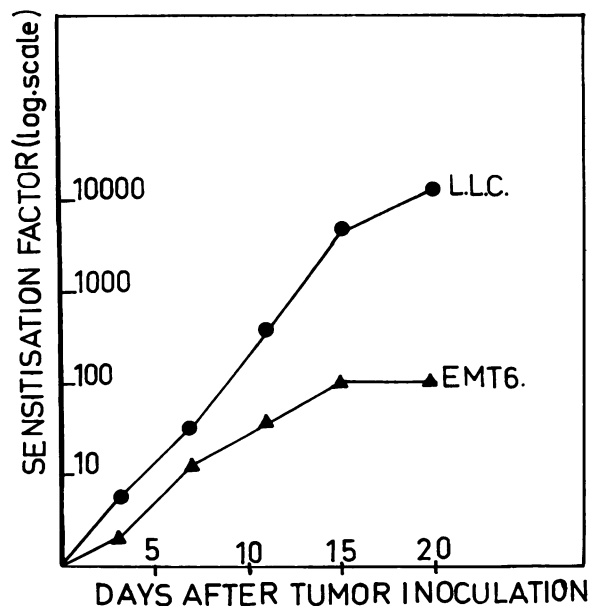


FIG. 2. Sensitization of tumor-bearing mice to LPS. Mice bearing Lewis lung carcinoma (L.L.C.; ●) or EMT6 sarcoma (Δ) were injected intraperitoneally with increasing doses of LPS. Lethal toxicity was recorded during 48 h. The LD₅₀ was expressed in micrograms of LPS per mouse for each time point and then expressed as increased sensitivity compared with the value in healthy animals.

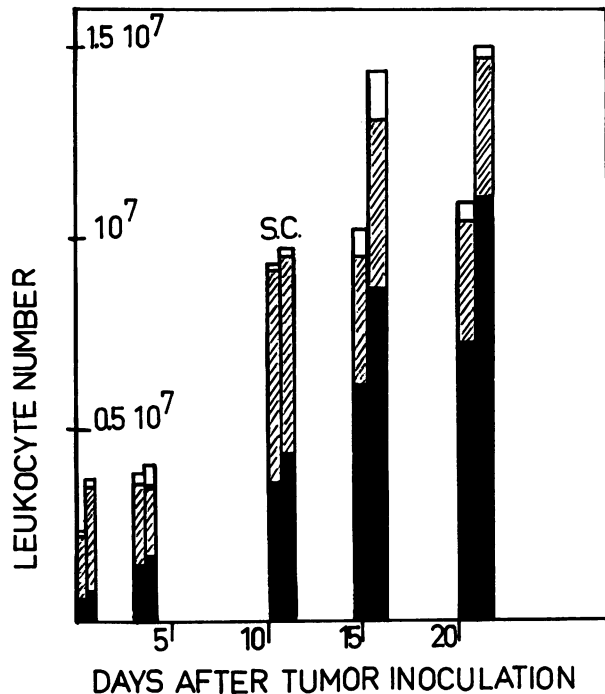


FIG. 3. Leukocytosis in tumor-bearing mice. Blood samples from BALB/c mice bearing EMT6 sarcoma (S; left histogram) and from C57BL/6 mice with Lewis lung carcinoma (C; right histogram) were collected by the retro-orbital technique at various time after tumor inoculation. Samples were diluted in Turk solution for total leukocyte counting or colored with May Grünwald and Giemsa solutions to differentiate granulocyte (■), lymphocyte (▨), and monocyte (□) populations. Means of groups of three animals are presented.

human recombinant TNF. The LD_{50} of TNF decreased from approximately 300 μ g in healthy animals to less than 0.1 μ g in mice bearing 15-day-old carcinoma. The degrees of sensitization to TNF and LPS were comparable (Table 1).

Sensitivity of EL4 and MethA tumor-bearing mice to LPS. Unlike the above tumors, an EL4 lymphosarcoma and a MethA fibrosarcoma growing in C57BL/6 and BALB/c mice, respectively, showed no sensitizing effects to the lethal toxicity of endotoxin or TNF. This shows that the property of sensitization to endotoxin is not shared by all tumors.

DISCUSSION

It was reported long ago that endotoxin can cause regression of some solid tumors by hemorrhagic necrosis. The biological mechanisms involved in the antitumor activity of LPS are still not fully understood. The rapid hemorrhagic necrosis can be explained by the vascular disturbance occurring within the tumor (hyperemia) and by the release of mediators by activated mononuclear phagocytes: mainly TNF (9), but also interleukin 1, the granulocyte-macrophage differentiation factor, arachidonic acid metabolites, O_2^- radicals, and arginase released by macrophages (1). Tumor regression is also probably dependent on the generation of specific antitumor immunity mediated by sensitized T cells and activated macrophages (6, 20).

LPS or its biologically active endogenous mediators such as TNF cannot presently be used as such in cancer immunotherapy because of their intrinsic toxicity despite the development of endotoxin derivatives of lower toxicity (1,

22). The present results indicate a marked hypersensitivity of tumor-bearing mice to the lethal effects of very low doses of endotoxin or TNF. The ratio between a toxic dose and an effective antitumor dose might, therefore, be very small and prevent any beneficial effect. The high mortality caused by gram-negative bacterial infections in cancer patients is likely related to increased sensitivity to endotoxin.

The correlation between increased granulocytosis and increased sensitivity to endotoxin that we observed might be relevant since granulocytosis was very often seen in other hypersensitivity models. Animals bearing tumors, as well as humans with cancer, display an activated reticuloendothelial system (4). These activated macrophages and granulocytes could give the host the capacity to reject or delay the growth of syngeneic tumors (4, 6, 19). They can also induce a preparative state for generalized Schwartzman reaction or shock, endotoxin serving to precipitate the reaction (2). If this concept is valid, the tumor necrosis seen after LPS treatment could be explained as a local Schwartzman reaction (the presence of a tumor corresponding to the first injection), whereas hypersensitivity would reflect a generalized Schwartzman reaction. The accumulation of activated macrophages and granulocytes in specific tissues or blood of cancerous animals would lead, after endotoxin challenge, to release by these cells of active substances (e.g., TNF for macrophages) mediating endotoxic activities and shock.

TNF, which was originally described as the key endogenous mediator causing necrosis of solid tumors, is also a key mediator of endotoxin effects of LPS. Studies of carcinoma- and sarcoma-bearing mice have shown that endotoxin and TNF are identical in causing lethal effects in tumor-bearing mice. Mice with LL carcinoma became hypersensitive to minute amounts of TNF or LPS, the sensitization factor of several thousand being very similar for both preparations.

Increased toxicity of endotoxin for tumor-bearing mice has already been described by Berendt et al. (5), concordant with the generation of systemic macrophage activation. Our data extend this observation to TNF and to other solid tumors. The exact mechanisms involved are still unknown. Some tumors and not others might prime the organism for release of TNF or increase its endogenous level. Mediators other than TNF could also be involved, which could be released by the activated reticuloendothelial system or by the tumors themselves.

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