THE IMMUNOLOGICAL BASIS OF ENDOTOXIN-INDUCED TUMOR REGRESSION

Requirement for a Pre-Existing State of Concomitant Anti-Tumor Immunity*

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Evidence was presented in the preceding paper (1) to support the interpretation that, although parenteral administration of bacterial endotoxin causes hemorrhagic necrosis of most established, syngeneic murine tumors, it is only those tumors that are immunogenic enough to evoke the generation of T-cell-mediated immunity that subsequently go on to completely regress. It was suggested, therefore, that tumor regression, but not hemorrhagic necrosis, is mediated by an acquired population of specifically sensitized T cells.

However, a recent series of published papers (2–5) has implicated a host molecule(s), called tumor necrosis factor, in the mediation of the anti-tumor effects of endotoxin. The evidence in these same papers strongly suggests, moreover, that an activated macrophage system is an essential requirement for the endotoxin-induced liberation of this molecule, in that it is only released into the circulation of mice that have had their macrophage systems activated by Bacillus Calmette-Guérin (BCG),[1] *Corynebacterium parvum*, or other agents known to activate macrophages. It is apparent, however, that it has yet to be shown that tumor necrosis factor is liberated in tumor-bearing mice themselves. This would seem important to know, because if, indeed, tumor necrosis factor is the mediator of the anti-tumor effects of endotoxin, then it would follow that those tumors that regress in response to endotoxin therapy must be capable by themselves of priming the host to liberate tumor necrosis factor by virtue of their capacity for evoking the generation of an activated macrophage system. In support of this possibility, a recent publication from this laboratory shows (6) that subcutaneous growth of the SA-1 sarcoma, a tumor that predictably regresses completely in response to endotoxin therapy (1), causes the generation of a highly activated macrophage system, as evidenced by a greatly increased macrophage-mediated capacity for resisting experimental infection with bacterial parasites. It is possible, therefore, that an activated macrophage system is generated in response to growth of all endotoxin-susceptible tumors.

In addition, it is difficult to reconcile the evidence for a direct role for tumor necrosis factor in the mediation of endotoxin-induced tumor regression, with that which strongly suggests that tumor regression is dependent on the acquisition of T-cell-mediated immunity (1). Presumably, if tumor necrosis factor does play a role in tumor regression, it does so either by augmenting the generation of T-cell-mediated immunity by virtue of its reported immunoregulatory properties (5), or alternatively,

by creating conditions within the tumor, via hemorrhagic necrosis, that are conducive to the entry and functioning of effector T cells. The alternative explanation would require, however, that a potential immune effector mechanism must already exist before endotoxin is administered. The purpose of this paper is to show that such an effector mechanism already exists in the form of concomitant anti-tumor immunity. It will also show that it is only those tumors that evoke this form of anti-tumor immunity which are susceptible to endotoxin-induced regression.

Materials and Methods

The materials and methods employed were the same as those described in the preceding companion paper (1), except for additional procedures that were used to measure macrophage-mediated anti-bacterial resistance and concomitant anti-tumor immunity in tumor-bearing mice.

Anti-Bacterial Resistance. Changes against time in the level of macrophage-mediated, non-specific, anti-bacterial resistance in response to tumor growth were measured in terms of changes in the capacity of the host to resist a standard 10^5 intravenous challenge with the intracellular, bacterial parasite, *L. monocytogenes* (strain EGD). Anti-bacterial resistance was recorded as changes in $\log_{10}$ resistance which were obtained by subtracting the 48-h growth of the organism in the livers of tumor bearers from its 48 h growth in the livers of controls. A log phase culture of *L. monocytogenes* seeded from an infected mouse spleen, was grown in trypticase-soy broth, and stored in small aliquots at $-70°C$. Before each experiment an aliquot was quickly thawed and diluted in 0.9% sodium chloride solution for intravenous inoculation as described previously (7). Bacteria were enumerated by plating 10-fold serial dilutions of whole liver homogenates on trypticase-soy agar.

Concomitant Immunity. Concomitant immunity generated in response to growth of intradermal tumors was measured as the ability of the host to suppress the growth of 10^6 cells of the same tumors implanted in the right-hind footpad. Tumor growth was monitored by measuring increases in the dorsoventral thickness of the footpad with dial calipers as described before (6).

Results

Evidence That the Acquisition of Concomitant Immunity Precedes Endotoxin Susceptibility. A review of the literature (8) indicates that tumors must be above a certain critical size before they become susceptible to the anti-tumor effects of endotoxin. It is apparent, moreover, that with certain transplantable tumors, such as the Meth A fibrosarcoma (2), the period of endotoxin susceptibility is relatively brief; a characteristic that is undoubtedly responsible for the unpredictable results obtained with this tumor. Although there are a number of possible reasons for these findings, it is tempting, in view of the results presented in the preceding companion paper (1), to give them an immunological explanation. It is already known from published experiments (6), for instance, that the highly endotoxin-susceptible SA-1 sarcoma, after a certain stage of growth, evokes the generation of T-cell-mediated concomitant immunity. On the other hand, the results of unpublished pilot experiments show that concomitant immunity is not generated in response to growth of the endotoxin-resistant BP3 sarcoma. This information, plus the knowledge that endotoxin-induced regression is dependent on T-cell-mediated immunity (1), suggests the possibility that endotoxin-induced regression depends on the acquisition by the host of a state of concomitant anti-tumor immunity.

Testing this suggestion involved making concurrent measurements against time of tumor growth of changes in (*a*) susceptibility to endotoxin-induced regression, (*b*) concomitant resistance to growth of a standard subcutaneous tumor implant, and (*c*)
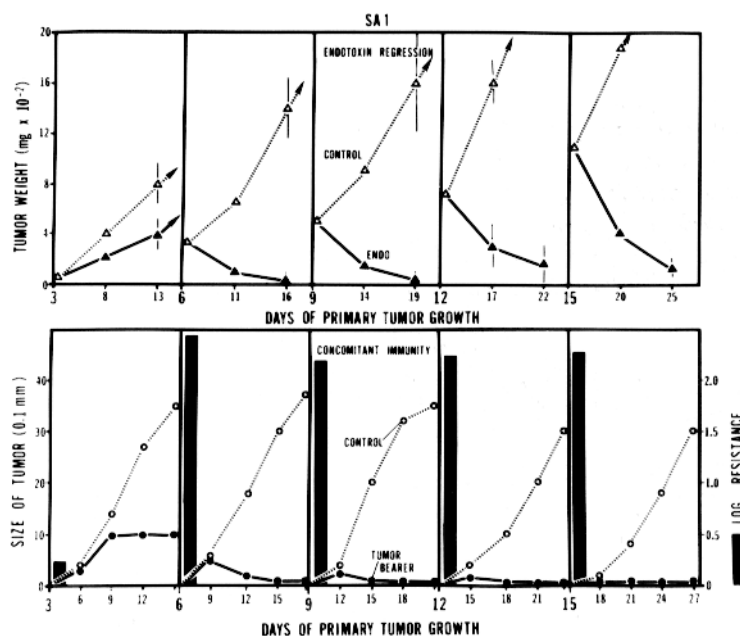
Fig. 1. Changes against time of intradermal SA-1 growth in: (a) susceptibility to endotoxin-induced regression (top graphs), (b) the level of concomitant immunity to growth of a standard 10^6 tumor cell challenge implanted subcutaneously in the right-hind footpad (bottom graphs), and (c) $\log_{10}$ protection expressed in the liver against a standard 10^5 intravenous challenge with L. monocytogenes (histobars). The larger numerals on the abscissa represent days of growth of the primary tumor. The small numbers refer to days of measurement of the in vivo assays. It can be seen that the SA-1 sarcoma was susceptible to endotoxin-induced regression at all times tested after day 3 of growth, even when the tumors had reached a very large size. Concomitant immunity was not immediately expressed against a challenge implant until 6 days growth of the primary tumor, and was sustained at a high level until the end of the experiment. Likewise, macrophage-mediated antibacterial resistance was not acquired until day 6 of tumor growth, and was sustained at a high level thereafter.

The results indicate that the SA-1 sarcoma did not become susceptible to endotoxin-induced regression until day 6 when concomitant immunity and macrophage activation reached high levels. Means of five mice per time interval.

systemic macrophage activation, as evidenced by changes in the level of macrophage-mediated anti-bacterial resistance. Making these concurrent measurements required the use of over 300 mice for each of the four tumors studied. The tumors studied were the same as in the preceding paper, and were the endotoxin-susceptible SA-1 and Meth A fibrosarcomas, and the endotoxin-resistant BP3 sarcoma and CaD_2 mammary carcinoma. The results in Figs. 1–4 are consistent with the hypothesis that endotoxin-induced regression requires the preceding generation of a state of concomitant anti-tumor immunity.

The SA-1 Sarcoma. It can be seen in Fig. 1 that a single intravenous injection of endotoxin given at any time after day 3 of SA-1 implantation resulted, in all cases, in hemorrhagic necrosis and substantial tumor regression. With 6 day and 9 day tumors, regression was complete, and was not followed by tumor regrowth in the 10 additional animals that were kept for visual inspection over a 60 day period. However, with the very large 12 and 15 day tumors, regression, although very substantial, was not complete in all animals. Of the additional animals kept for visual inspection, 4 out of
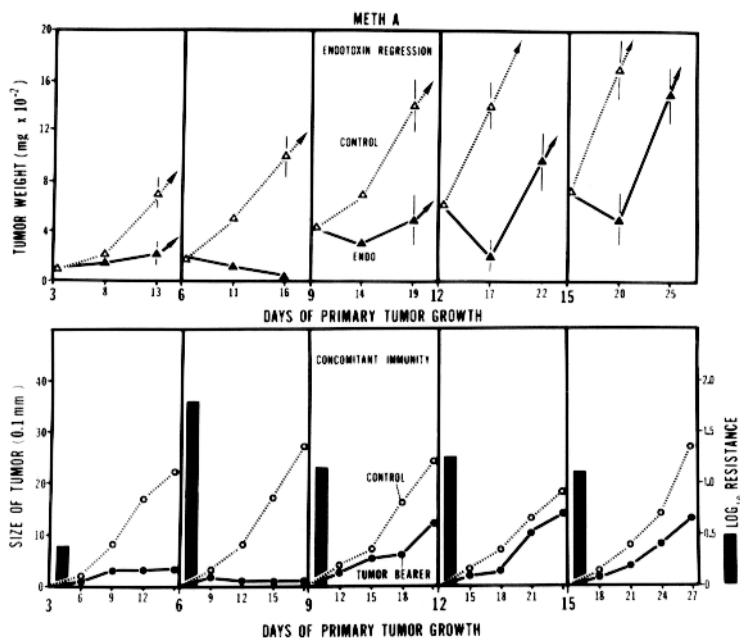
FIG. 2.   Same as for Fig. 1, but with the Meth A fibrosarcoma. This tumor only completely regressed in response to an injection of endotoxin given on day 6. It was also only on day 6 that a significant level of concomitant immunity was expressed against a tumor challenge implant, and that macrophage-mediated anti-bacterial resistance reached its highest level. Means of five mice per time interval.

10 of the 12 day tumors, and 6 out of 10 of the 15 day tumors soon regrew progressively from very thin rings of surviving tumor tissue.

As expected from a previous study (6), intradermal growth of the SA-1 sarcoma evoked the generation of a high and sustained level of concomitant anti-tumor immunity. It was not immediately expressed against a 10^6 challenge implant, however, until after 6 days of growth of the primary tumor. It will be noted in this connection that although growth of the 3 day challenge implant was significantly suppressed, it was not suppressed until after a 3-day delay. Presumably, therefore, rapid generation of concomitant immunity occurred between days 6 and 9.

Likewise, increased macrophage-mediated anti-bacterial resistance to an intravenous *Listeria* challenge apparently was generated between days 6 and 9 of tumor growth, and was sustained at a high level until the experiment was terminated. It can be seen that from day 6 of tumor growth on, the standard *Listeria* challenge grew about 2 logs less in the livers of tumor bearers than in the livers of control mice during a 48-h period. It should be realized that, since anti-bacterial resistance was measured 48 h after the challenge times show in Fig. 1, the 2 logs protection apparently present on day 6 may not have been fully acquired at this time.

Taken together, the results with the SA-1 sarcoma show that this tumor was exquisitely sensitive to endotoxin-induced regression, but only after the host had begun to generate concomitant immunity and an activated macrophage system.

The Meth A Fibrosarcoma.   The results with the Meth A fibrosarcoma are particularly interesting, because they show in Fig. 2 that this tumor was susceptible to endotoxin-
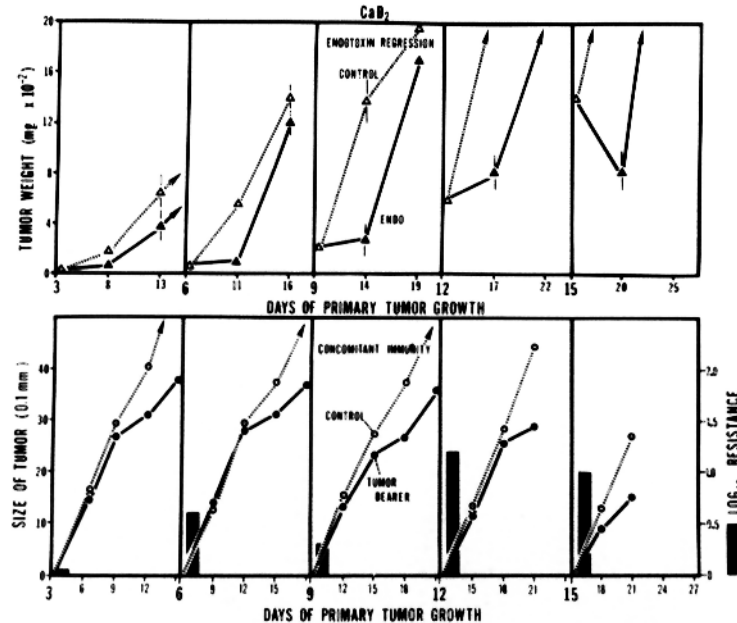
FIG. 3. Same as for Fig. 1, but with the CaD2 mammary carcinoma. This tumor was resistant to endotoxin-induced regression at all stages of growth. In addition, only a very low level of concomitant immunity was generated, although macrophage-mediated anti-bacterial resistance reached appreciable levels by day 2. Means of five mice per time interval.

induced regression for only a brief period of its growth. Only 6 day tumors completely regressed in response to intravenous administration of endotoxin. It was the combination of hemorrhagic necrosis and partial regression that was responsible for the initial drop in weight of older tumors shown in Fig. 2.

Concomitant immunity to a subcutaneous Meth A challenge implant was expressed at a significant level only on days 3 and 6 of growth of the primary tumor. The results indicate however, that there was a delay before growth of the 3 day challenge was suppressed. This means that peak immunity was not acquired until some time between days 6 and 9. Therefore, unlike concomitant immunity to the SA-1, concomitant immunity to the Meth A was not sustained at a level that was high enough to completely prevent growth of the standard challenge implant.

As with concomitant immunity, the highest level of macrophage-mediated anti-bacterial resistance was expressed against a bacterial challenge given on day 6 of tumor growth. Anti-bacterial resistance declined significantly by day 9, and remained at this reduced level until the experiment was terminated. Taken together, therefore, the results in Fig. 2 strongly suggest that the Meth A fibrosarcoma was susceptible to complete regression by endotoxin only during a relatively brief period when concomitant immunity and macrophage activation were at their highest levels.

The CaD2 Mammary Carcinoma and BP3 Sarcoma.   The results with these two tumors are described together, because they were both shown to be poorly immunogenic, and to be resistant to endotoxin-induced regression when tested at 7 days of growth (1). Indeed, it can be seen in Figs. 3 and 4 that both tumors failed to regress appreciably in response to an intravenous injection of endotoxin given at any stage during 15 days of growth. The apparent temporary cessation of growth which occurred in all cases
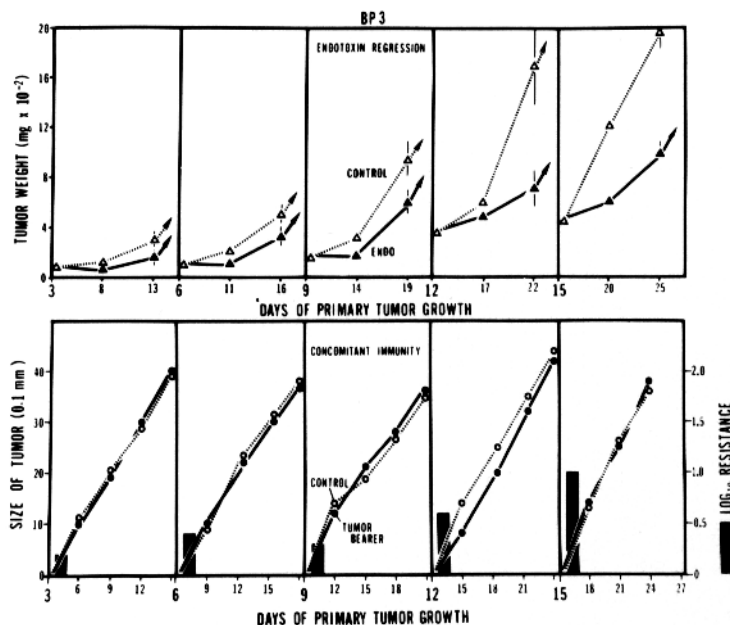
FIG. 4.   Same as for Fig. 1, but with the BP3 fibrosarcoma. Like the CaD2 tumor, this tumor was not susceptible to endotoxin-induced regression at any stage of its growth. Concomitant immunity to a challenge implant was not acquired, and increased anti-bacterial resistance was not generated at a significant level until the tumor became very large. Means of five mice per time interval.

after endotoxin administration, was caused by loss of the cores of the tumors because of central hemorrhagic necrosis, which was more extensive with increasing size of the tumors.

Concomitant resistance to growth of a standard subcutaneous challenge implant was not detected at any given time in mice bearing the BP3 sarcoma, and only low levels of resistance were measured in CaD2 bearers. However, the behavior of the CaD2 challenge implant was peculiar, in that it first grew substantially as in controls before showing a slower rate of growth.

Increased macrophage-mediated anti-bacterial resistance was generated in response to growth of each tumor, but did not reach appreciable levels until later stages of tumor growth. The results with these two tumors indicate, therefore, that lack of susceptibility to endotoxin-induced regression was associated with the absence of significant levels of concomitant anti-tumor immunity, rather than with the absence of an activated macrophage system.

Reduced Level of Concomitant Immunity in T-Cell-Deficient Mice.   It was shown in the preceding companion paper (1) that endotoxin injection failed to cause the regression of susceptible SA-1 and Meth A tumors growing in T-cell-deficient mice. It was established (6) in a previous publication, moreover, that T-cell-deficient mice bearing the SA-1 sarcoma also failed to generate concomitant immunity. It was the purpose of the following experiments, therefore, to determine whether T-cell deficiency also results in a failure of syngeneic mice to generate concomitant immunity in response to growth of the Meth A fibrosarcoma. If so, then it would represent additional evidence consistent with the hypothesis that the generation of concomitant immunity is a prerequisite for endotoxin-induced regression.
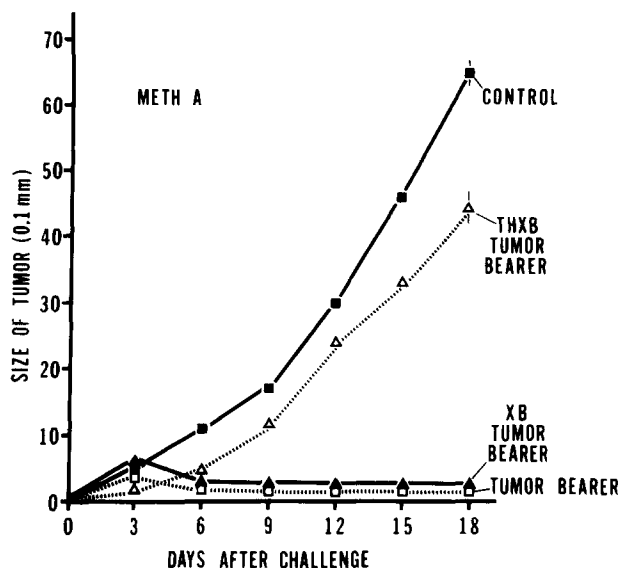
FIG. 5.   Evidence that concomitant immunity to the Meth A sarcoma was not generated by mice that were made T-cell-deficient by thymectomy and gamma-irradiation (THXB), and restored with bone marrow. Growth of 10^6 Meth A cell challenge implant was completely suppressed by mice bearing 6 day intradermal primary tumors, but was only slightly suppressed by THXB mice bearing 6 day tumors. Tumor-bearing, irradiated, bone marrow protected controls (XB) behaved like normal tumor-bearing mice.

The results in Fig. 5 support the hypothesis. They show that whereas 6 days of intradermal growth of the Meth A sarcoma resulted in a high enough level of concomitant immunity to completely suppress the growth of a 10^6 Meth A challenge implant, growth of the same challenge implant was only slightly suppressed in 6 day tumor-bearing mice that had been made T-cell deficient by thymectomy and gamma-irradiation.

The Specificity of Endotoxin-Induced Regression.   In the absence of direct evidence that sensitized T cells are the actual effectors of endotoxin-induced regression, and in view of the foregoing evidence that mice bearing endotoxin-susceptible tumors possess a possible nonspecific anti-tumor mechanism in the form of an activated macrophage system, it might still be argued that macrophages, and not sensitized T cells are the effectors of tumor regression. According to this explanation, the major purpose of sensitized T cells would be to mediate an adequate level of macrophage activation. If this were the case, however, then it might be expected that endotoxin, perhaps through events initiated by the action of tumor necrosis factor, would nonspecifically regress any nonimmunogenic tumor provided the host had acquired an activated macrophage system.

That this is almost certainly not the case is shown in Fig. 6 where it can be seen that simultaneous intradermal growth of the endotoxin-susceptible SA-1 sarcoma and the endotoxin-resistant BP3 sarcoma on the same semisyngeneic AB6F₁ host did not create conditions that enabled endotoxin to regress the BP3 sarcoma. Thus, when endotoxin was injected intravenously into these double tumor bearers, only the SA-1 tumor underwent regression. In other words, each tumor behaved as if it were growing alone on its own host. The highly activated macrophage system generated in response
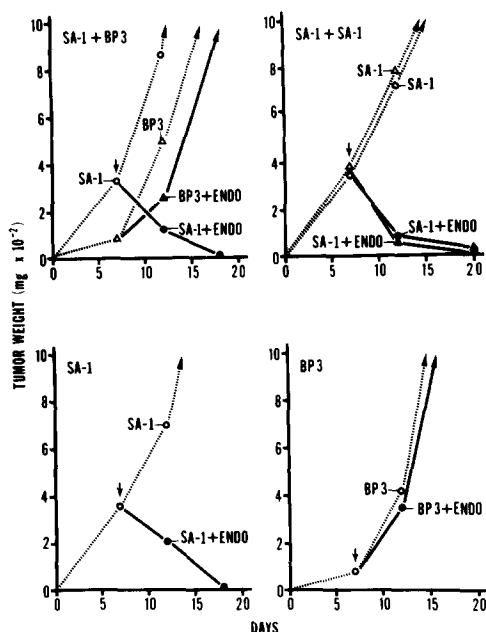
Fig. 6. The specificity of endotoxin-induced tumor regression is illustrated by the effect of endotoxin injection (arrows) on the endotoxin-susceptible SA-1 sarcoma and the endotoxin-resistant BP3 sarcoma both growing simultaneously on the same mouse. Only the SA-1 regressed, and each tumor behaved as though it were growing alone on its own host. Means of five mice per time interval.

to the SA-1 sarcoma, therefore, did not impart on the host the capacity to regress the BP3 tumor.

Discussion

The results of this paper and the preceding paper (1) are consistent with the hypothesis that endotoxin-induced regression of established, syngeneic, murine tumors depends on the preceding generation of a state of concomitant anti-tumor immunity which in the case of the SA-1 sarcoma is known to be T-cell-mediated (6). Of the four tumors investigated, only those two that were shown to be immunogenic as classically defined (1), and which could evoke a paradoxical state of concomitant anti-tumor immunity, regressed in response to endotoxin therapy. The results show, in addition, that a brief period of concomitant immunity generated in response to the Meth A sarcoma results in a correspondingly brief period of susceptibility to endotoxin-induced regression, hence, the reason for the unpredictable results obtained with this tumor. Hemorrhagic necrosis, on the other hand, occurred at all stages of growth of immunogenic as well as nonimmunogenic tumors, and was responsible for the apparent temporary cessation of tumor growth and temporary reduction in weight of nonsusceptible tumors.

These results point to the conclusion that endotoxin-induced regression is accomplished by the tumoricidal action of specifically-sensitized T cells, rather than by the action of a soluble molecular mediator, such as tumor necrosis factor (2). The finding that an injection of endotoxin given to mice bearing both an endotoxin-susceptible and an endotoxin-resistant tumor, resulted in regression of only the susceptible tumor

argues against the participation of a circulating factor that is selectively cytotoxic for neoplastic cells in general (5). It is possible, nevertheless, that such a factor is responsible for the mediation of hemorrhagic necrosis which was shown here to occur in the absence of T-cell-mediated anti-tumor immunity. Indeed, it seems highly likely that the destructive effects caused by hemorrhagic necrosis in the nutrient-starved core of the tumor are necessary for creating conditions that are conducive to the entry of an already acquired population of specifically-sensitized effector T cells. This could be achieved by the creation of a local inflammatory response inside the tumor, or by the elimination of a local immunosuppressor mechanism, or both. The possibility that hemorrhagic necrosis serves to augment T-cell-mediated immunity by causing the release of increased quantities of tumor specific antigens should also be considered.

In any case, the demonstration of an immunological basis of endotoxin-induced tumor regression means that endotoxin should be properly viewed as an immuno-therapeutic agent, possibly with a different mode of action than other immunotherapeutic agents, such as BCG and *Corynebacterium parvum*. The results with endotoxin indicate the need, moreover, for giving much more attention to the properties that tumors might need to possess for immunotherapeutic agents to give an anti-tumor effect. It is perhaps surprising in this regard that most published studies of tumor immunotherapy do not even take into account the antigenicity or immunogenicity of the tumors under investigation. The present study indicates that, so far as endotoxin therapy is concerned, tumor immunogenicity is perhaps the most important property to be considered. It should be brought to mind that a successful immunotherapeutic response should not only be evidenced by regression of the primary tumor, but also by the generation of a state of immunologic memory with the potential to suppress the growth of disseminated tumor cells that can later emerge as secondary tumors. It is apparent, however, that additional factors operate to determine the success of an immunotherapeutic modality. For example, the capacity of the tumor, under the pressure of host defenses, to generate cells that are resistant to native and acquired mechanisms of antitumor resistance is a distinct possibility (9–11). It is already known from this study, for instance, that endotoxin immunotherapy suffers from the same limitations as other forms of immunotherapy, in that endotoxin-induced complete regression of a primary SA-1 sarcoma is no guarantee that already seeded lymph node metastases will not eventually grow to kill the host.

Apart from a possible role in the liberation of tumor necrosis factor, the significance of the acquisition of a highly activated macrophage system to the antitumor effects of endotoxin is not known. According to purely in vitro evidence (12), it would provide an additional antitumor effector mechanism. It seems fairly clear, from this study, however, that the presence of these cells systemically is of little use, in the absence of an immune effector mechanism, in causing the regression of solid tumors. It is possible, nevertheless, that activated macrophages may be present in large enough numbers locally, or can be drawn from circulation to aid in the immunological destruction of immunogenic tumors. It should also be realized that macrophages undoubtedly play a major role in the resorption of dead tumor tissue which is measured as regression.

It can be pointed out in conclusion, that the present studies, like most published studies of immunotherapy, do not provide direct evidence that sensitized T-cells are the actual effectors of tumor regression in vivo. This would require the demonstration in endotoxin-treated, T-cell-deficient recipient mice, of tumor regression after intra-

venous infusion of specifically sensitized T cells from immune donors. This demonstration will appear in a forthcoming publication.

Summary

It was shown that of four syngeneic, murine tumors investigated, only those that evoked the generation of a state of concomitant anti-tumor immunity were susceptible to endotoxin-induced regression. Moreover, the temporal relationship between the generation of concomitant immunity and the onset of susceptibility to endotoxin-induced regression points to the likely possibility that tumor regression depends on the preceding acquisition of the specifically-sensitized, effector T cells that express concomitant immunity. It is suggested that endotoxin-induced hemorrhagic necrosis which invariably precedes tumor regression serves to create conditions inside the tumor that are conducive to the entry and the functioning of effector T cells. It is also suggested that tumor necrosis factor causes hemorrhagic necrosis rather than tumor regression.

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