# SUBVERSION OF HOST DEFENSE MECHANISMS BY MALIGNANT TUMORS: AN ESTABLISHED TUMOR AS A PRIVILEGED SITE FOR BACTERIAL GROWTH*

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There is ample evidence to support the proposal that most, if not all, tumors possess tumor-specific antigens and are immunogenic to varying degrees. This proposal rests on two main lines of evidence. First, that a state of specific immunity to growth of a tumor cell challenge can be generated in a host pre-exposed to various immunizing regimens with tumor cells (reviewed in reference 1). Second, that a host bearing a progressive autochthonous or syngeneic tumor burden can, nevertheless, acquire a powerful mechanism of systemic immunity capable of eradicating a lethal challenge inoculum of cells of the same tumor line. This second type of immunity is referred to as concomitant immunity (2), and there is convincing evidence (3–5) that it is T-cell mediated. That this state of immunity represents a relatively common response to growing neoplasms is indicated by the numerous demonstrations (1, 6) of the presence in tumor-bearing humans and animals of lymphocytes that are specifically cytotoxic for tumor cells in vitro.

It was recently shown (5) that the generation of T-cell-mediated concomitant immunity to murine fibrosarcomas is associated with the concordant generation of an activated macrophage system. This was evidenced by a limited capacity of concomitantly immune mice for inhibiting the growth of antigenically unrelated tumors, but more convincingly by a striking enhancement in their capacity for destroying lethal challenge inocula of the bacterial parasite, *Listeria monocytogenes*. It was concluded, therefore, that the response to the tumor involved the generation of a population of sensitized T cells that mediated the activation of macrophages. In spite of the presence of these effector cells, however, the host nevertheless remained incapable of ridding itself of its primary tumor burden.

It is considered by many that this paradox can be satisfactorily explained on the basis of the action of antigen and antibody-dependent blocking factors (7, 8), which have been shown to be present in the serum of tumor bearers, and to be capable of neutralizing the cytotoxicity of sensitized lymphocytes for tumor cells in vitro. Although the evidence supporting a role for blocking factors is persuasive, it is well to realize that it is based almost exclusively upon studies that have been conducted in vitro. In fact, there is evidence (9) that could be interpreted as casting doubt on a role for blocking factors in vivo. The possibility remains, therefore, that other mechanisms may also operate to protect a progressive tumor from the anti-tumor immunity it evokes; mechanisms that would also need to account for the ability of the tumor to avoid destruction by the demonstrated (10–12) tumoricidal powers of activated macrophages.

Indeed, that tumors may possess mechanisms that subvert the anti-tumor activity of

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macrophages was recently revealed by the demonstration (13) that implantation of small numbers of murine neoplastic cells resulted in a rapid systemic suppression of macrophage function. This was measured as a strikingly reduced capacity of the host for expressing macrophage-mediated resistance to microbial infection, and as a state of suppressed native resistance to growth of a tumor cell implant. It was further shown (14) that the suppression of macrophage function was caused by a soluble factor that persisted in circulation long after the tumor-bearing host had counteracted its effects systemically by generating concomitant immunity and an activated macrophage system. It seemed reasonable to propose, therefore, that the factor was produced by the tumor itself, and that the host remained incapable of rejecting its primary tumor because it failed to counteract the suppressive effects of much higher concentrations of the factor in the tumor. The additional possibility arises, moreover, that besides suppressing the function of macrophages, the presence of this factor in the tumor might also be responsible for suppressing the expression of specific anti-tumor immunity by sensitized T cells.

The studies presented in this paper were designed to examine the hypothesis that an established primary tumor presents an environment that is not only antagonistic to the expression of anti-tumor immunity, but is also antagonistic to the expression of host defenses in general. They will show that a striking similarity exists between the capacity of a tumor-bearing animal to express T-cell-mediated concomitant anti-tumor immunity, and its capacity to express T-cell-mediated anti-bacterial immunity, in that while both can be efficiently expressed systemically, neither can be fully expressed within the growing tumor.

## Materials and Methods

*Animals.* Parental A/J, DBA/2J, C57BL/6J, and BALB/cJ mice were purchased from The Jackson Laboratory, Bar Harbor, Maine, and used to produce AB6F$_1$ (A/J × C57BL/6). CB6F$_1$ (BALB/c × C57BL/6)$_1$ and B6D2F$_1$ (C57BL/6 × DBA/2) hybrids. They were incorporated into experiments when they were 8–12 wk of age.

*Tumors.* The SA1 spindle cell sarcoma syngeneic in A/J, the Meth A fibrosarcoma syngeneic in BALB/c, and the P815 mastocytoma syngeneic in DBA/2 were studied. The experiments reported here were performed using a single biofrozen stock of cells of each tumor harvested in ascites from the peritoneal cavities of large numbers of syngeneic mice. Cells of each tumor were harvested in heparinized phosphate-buffered saline (PBS),[1] pooled, washed in PBS, and resuspended in MEM containing 20% fetal calf serum and 20% dimethyl sulfoxide. They were then biofrozen, and stored in liquid nitrogen until required. Before each experiment an aliquot was thawed, washed in PBS, and grown in the peritoneal cavity of an appropriate semisyngeneic host for 7 days. The cells were harvested and washed in PBS, examined for viability by trypan blue exclusion, and suspended at an appropriate concentration in PBS for initiating experimental tumors either subcutaneously or intraperitoneally.

Solid tumors were initiated by injecting tumor cells into the plantar surface of one of the hind foot pads in a vol of 0.05 ml with a 30 gauge needle. Enough tumor cells were implanted to give a palpable tumor that caused 1.0–1.8 mm dorsoventral foot pad swelling in 6 days. This required $10^5$ SA1 cells, and $2 \times 10^6$ cells each for the Meth A and mastocytoma. Growth of the tumors was monitored against time by measuring further increases in the dorsoventral thickness of the foot with dial calipers. Ascites tumors in all cases were initiated by infusing $5 \times 10^5$ cells into the peritoneal cavity. In all cases, experiments were performed with animals bearing 6-day tumors.

*Bacteria.* A log phase culture of *L. monocytogenes* (strain EGD) was grown in Trypticase-soy broth, and stored in aliquots at −70°C. For each experiment an aliquot was quickly thawed at 37°C and diluted in a standard fashion in 0.9% sodium chloride for intratumor, intravenous, intraperitoneal, or subcutaneous inoculation. Growth of the organism was followed against time in the

[1] *Abbreviation used in this paper:* PBS, phosphate-buffered saline.

liver, tumor-bearing foot, normal foot, and in the draining popliteal lymph node. This required plating 10-fold serial dilutions of tissue homogenates on Trypticase-soy agar. Livers and lymph nodes were homogenized in a glass tube with a loose fitting Teflon pestle; tumor-bearing and normal feet were homogenized with a high speed VirTis tissue grinder (VirTis Co., Inc., Gardiner, N. Y.). Preliminary experiments showed that it was possible by this method to recover all bacteria from normal and tumor-bearing foot pads 30 min after inoculation. To follow the growth of *Listeria* in the peritoneal cavity, the peritoneal contents were harvested in 2 ml of heparinized PBS and subjected for 5 s to sonication to release intracellular bacteria. In some experiments the number of cell-associated bacteria was distinguished from the number present extracellularly by centrifuging the peritoneal washings at 200 *g* for 10 min and enumerating the bacteria in the washed cell button and in the supernate.

*Tumor-Induced Anti-Bacterial Resistance.* Systemic resistance to *Listeria* was expressed as the $\log_{10}$ resistance index as described previously (5). It was calculated as the difference between the 48 h growth of a standard $2 \times 10^3$ intravenous *Listeria* inoculum in the livers of tumor-bearing mice and its 48-h growth in the livers of control mice.

*Irradiation.* Whole-body gamma-irradiation was performed in a cesium-137 irradiator at a midphantom dose rate of 35.5 rads/min.

## Results

*Suppressed Anti-Bacterial Resistance in Progressive Tumors.* The experiments reported in this section were designed to test the prediction that the conditions within a primary tumor which inhibit the expression of concomitant anti-tumor immunity would be reflected by a failure of the host to express anti-bacterial resistance in the same site. This was investigated by comparing the growth of a sublethal number of *Listeria* inoculated into an established 6-day foot pad tumor with the growth of the same number inoculated into the contralateral foot pad. It can be seen in Fig. 1 that growth of the organism in foot pads carrying the SA1 sarcoma, P815 mastocytoma, or Meth A fibrosarcoma progressed log linearly for 2 days, and then either continued at a slower rate, or plateaued. In contrast, *Listeria* was efficiently eliminated from the tumor-free contralateral foot pads at the same rate as from the foot pads of tumor-free control mice.

Whereas Fig. 1 refers to results obtained with semisyngeneic mice, Fig. 2 serves to show that the same results were obtained with syngeneic mice. Thus, it can be seen again that while A/J mice carrying the SA1 sarcoma were incapable of eliminating *Listeria* from their tumor-bearing foot pads they easily eliminated the organism from their contralateral foot pads.

Additional evidence to support the conclusion that tumor-bearing mice are capable of eliminating *Listeria* from any site except the tumor is presented in Fig. 3. This figure contrasts the growth of bacteria in the tumor with the growth of those organisms that were seeded from the tumor into the draining lymph node and the liver. It can be seen that while large numbers of bacteria persisted in the tumor, they were progressively eliminated during the same time from the lymph node and liver. From a wealth of studies (reviewed in reference 15) there is little doubt, therefore, that despite the persistence of bacteria in the tumor, the host both acquired and expressed anti-bacterial immunity systemically.

It can be seen from the results in Fig. 4, furthermore, that destruction of those bacteria that found their way from the tumor to the lymph node and liver probably cannot be attributed exclusively to the action of specific anti-bacterial immunity. That an additional mechanism of resistance must have contributed

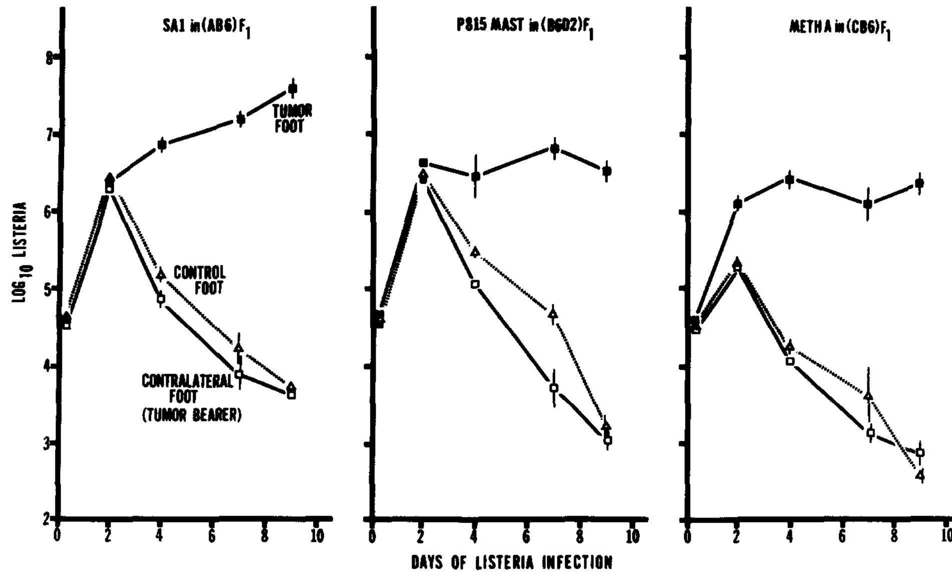


FIG. 1. Growth of 5 × $10^4$ *Listeria* inoculated into the tumor-bearing foot pad, the contralateral foot pad, or into the foot pad of a tumor-free control animal. The foot pad contained a 6 day SA1, mastocytoma, or Meth A tumor. In all cases *Listeria* grew rapidly for 2 days after which it was efficiently eliminated from the contralateral foot pad and control foot pad, but not from the tumor-bearing foot pad. Means of five mice ± SE.

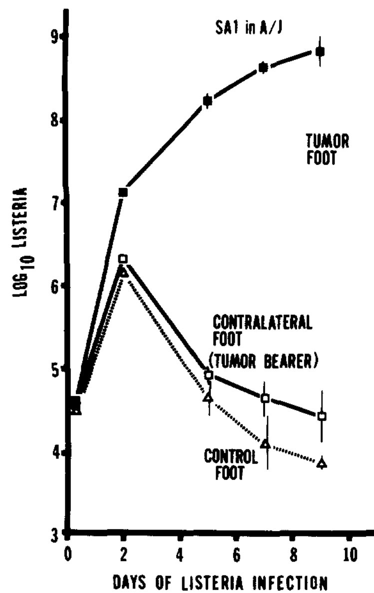


FIG. 2. Same result as Fig. 1 except that experiment was performed with SA1 in syngeneic mice. Means of five mice ± SE.

to the systemic destruction of microorganisms is evidenced by the faster rate of bacterial destruction in the lymph nodes and livers of tumor-bearing mice than in the livers and lymph nodes of tumor-free controls. This occurred, moreover, in spite of the continuous seeding of the lymph node and liver with organisms from

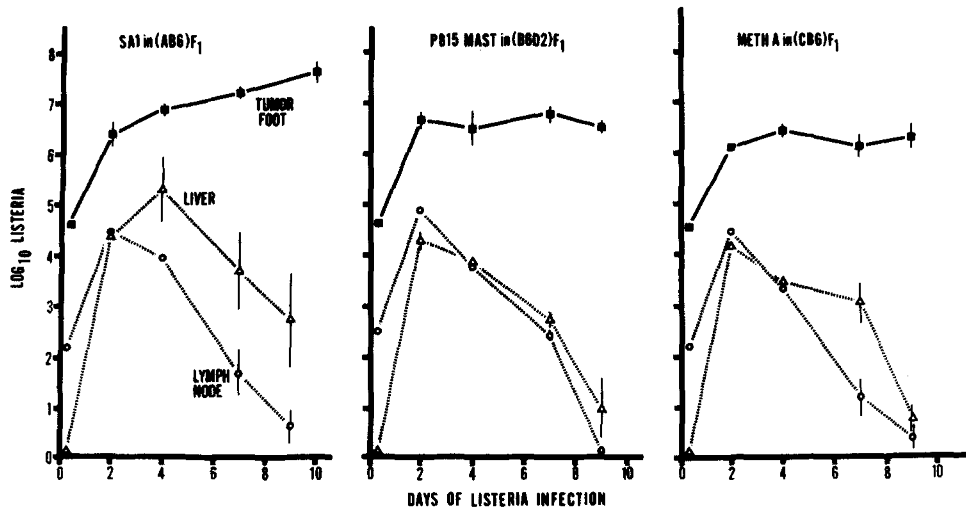


FIG. 3. Additional evidence that the tumor-bearing host can efficiently eliminate bacteria from any place but its tumor. Compared is the growth of *Listeria* in a 6 day SA1, Meth A, or mastocytoma foot pad tumor, with its growth in the draining lymph node and the liver. The organism was seeded into the lymph node and liver from the tumor. Means of five mice ± SE.

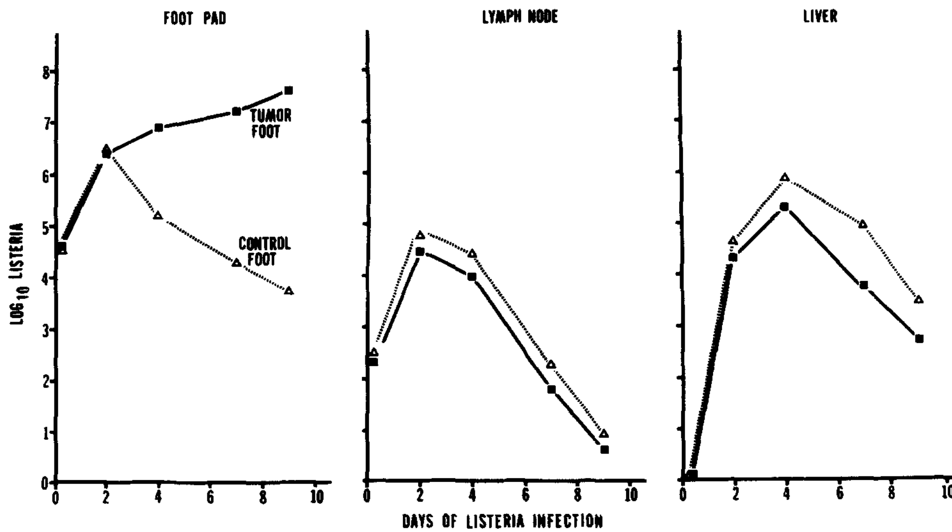


FIG. 4. Evidence that that tumor-bearing host has a greater capacity than a tumor-free host for inactivating *Listeria* systemically. *Listeria* was inoculated into the tumor-bearing foot pad of SA1 tumor bearers, and into the foot pad of tumor-free controls, and the growth of bacteria that were seeded into the lymph nodes and livers followed. Although approximately the same number of bacteria were initially seeded into the nodes and livers of both groups of mice, bacteria were more rapidly eliminated from the nodes and livers of tumor bearers. Means of five mice.

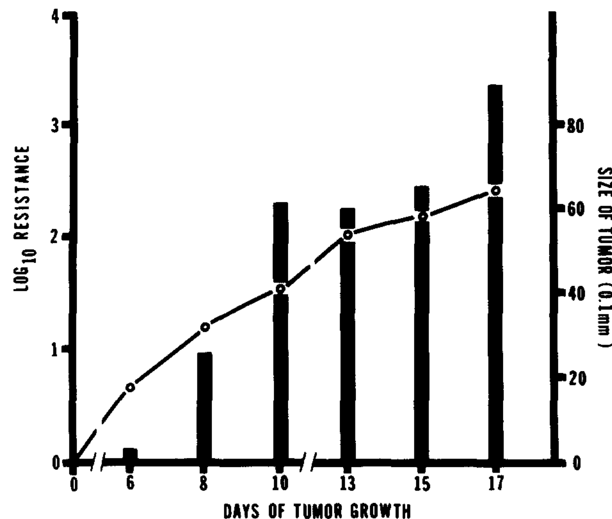


FIG. 5. Evidence that the response to the tumor itself results in the generation of anti-*Listeria* resistance. The right hind foot pads of mice were implanted with 5 × $10^5$ SA1 cells and their capacity to resist an intravenous (2 × $10^3$) challenge inoculum of *Listeria* was compared with this capacity in control mice on the days of tumor growth (line graph) indicated. Shown are differences ($\log_{10}$ resistance, bar graph) between the 48-h growth of the organism in the livers of tumor bearers and controls. The tumor bearers developed strikingly increased anti-bacterial resistance after day 6 of tumor growth. Means of five mice.

the large bacterial population in the tumor.

Indeed, it is known from previous publications (5, 13) that progressive growth of the tumors employed in this study, results, via the generation of concomitant immunity, in the activation of macrophages, and hence in an enhanced capacity of the host for rapidly inactivating an intravenous *Listeria* challenge inoculum. Direct evidence for this point is illustrated in Fig. 5 which measures changes against time of tumor growth, in the capacity of macrophages in the livers of tumor bearers to resist a standard 2 × $10^3$ *Listeria* inoculum given intravenously. It can be seen that the host's response to the tumor itself resulted in an enhanced capacity to nonspecifically destroy the bacterial challenge. By day 10 of tumor growth, for instance, tumor bearers showed two logs less growth of bacteria in their livers than controls. Since all of the foregoing results were based on the use of 6-day tumors, it is obvious that they were obtained with mice that had already begun to acquire an appreciable level of tumor-induced anti-microbial resistance based on the response to the tumor itself.

*Effect of Bacteria on Tumor Growth.* Fig. 6 compares the growth of a *Listeria*-infected foot pad tumor with the growth of a noninfected tumor. It can be seen that a large number of replicating bacteria inhibited progressive tumor growth. It was found, however, that inhibition of tumor growth was temporary, in that it always eventually resumed in the case of the SA1 and mastocytoma, and resumed in most cases with the Meth A (results not shown). It is significant, moreover, that on those very few occasions when infected Meth A tumors completely regressed, the bacteria were also completely eliminated from the foot

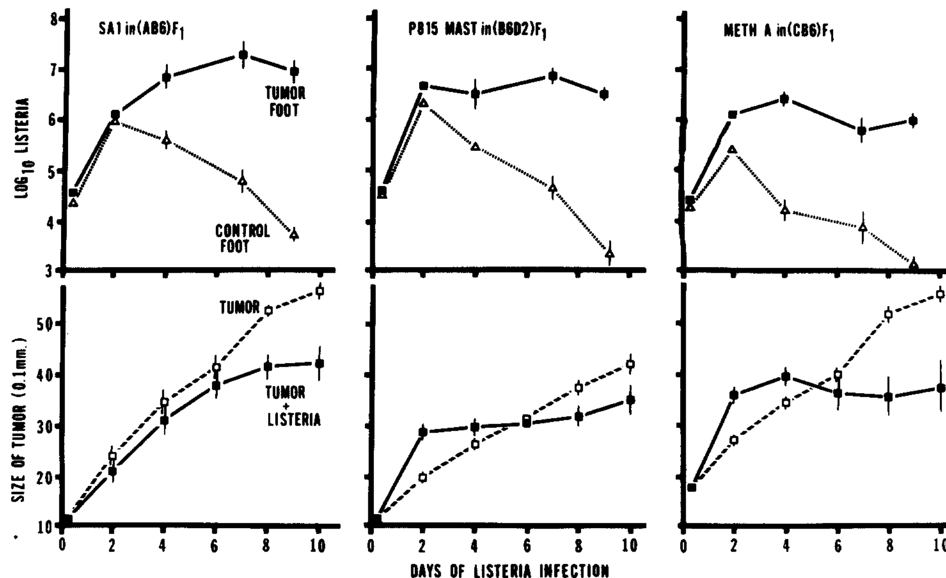


FIG. 6. Intratumor inoculation of *Listeria* resulted in suppression of growth of all three foot pad tumors. However, in all cases with the SA1 and mastocytoma tumors, and in practically all cases with the Meth A, tumor growth resumed at a normal rate by the 12th and 14th day (not shown). Means of five mice ± SE.

pad. The apparent initial increase in tumor growth that followed intratumor inoculation of *Listeria* in the mastocytoma and Meth A as shown in Fig. 6, was probably the result of an initial inflammatory response to the presence of bacteria.

*Evidence that Anti-Bacterial Resistance is Partially Expressed in Tumors.* It was demonstrated by the foregoing sections that a tumor-bearing host is incapable of completely eliminating *Listeria* from its primary tumor. Instead, at a time when the host was eliminating microorganisms from nonmalignant tissues with its acquired anti-bacterial defenses, bacteria either continued to grow in the tumor at a slower rate or their growth plateaued. The onset of the reduced rate of bacterial growth indicates, however, that the expression of anti-bacterial resistance was not completely blocked in the tumor, otherwise bacterial growth would have continued log linearly. That anti-bacterial resistance was partially expressed is indicated by the results in Fig. 7, which measured the effect of lethal whole-body gamma irradiation on bacterial growth in the SA1 sarcoma. It can be seen that whole-body irradiation resulted in the rapid resumption of log linear bacterial growth both in the tumor-bearing and the tumor-free foot pads. It follows, therefore, that irradiation abolished a radiosensitive component of anti-bacterial resistance that was being partially expressed in the tumor. Needless to say, mice so treated quickly succumbed to an overwhelming systemic bacterial infection.

*An Ascites Tumor as a Site of Suppressed Anti-Bacterial Resistance.* It could be argued that bacteria persist in a solid tumor because the architecture of the tumor presents physical or mechanical barriers to the entry of host effector cells. This possibility is made unlikely, however, in view of the results of

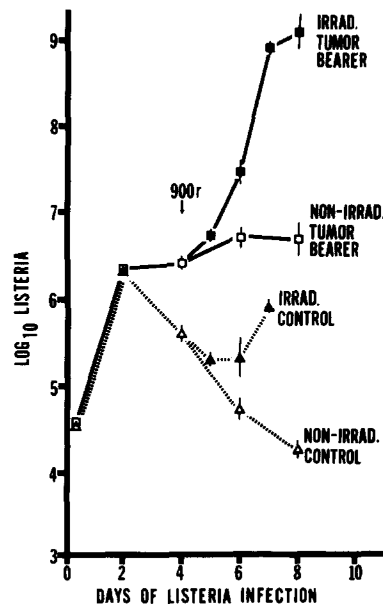


FIG. 7. Evidence that anti-bacterial resistance is partially expressed within a progressive tumor. Whole-body gamma irradiation resulted in resumption of rapid bacterial multiplication in the SA1-bearing and contralateral foot pads. This means that bacterial growth was being partially restricted in the tumor by a radiosensitive anti-bacterial defense mechanism. Means of five mice ± SE. Irrad., irradiated; r, rads.

experiments presented in Fig. 8 which compared the growth of a $10^4$ *Listeria* inoculum in the peritoneal cavities of animals bearing 6-day ascites tumors, with the growth of the same number inoculated into the peritoneal cavities of control mice. It can be seen that substantially more bacterial multiplication took place in the peritoneal cavities containing replicating cells of the SA1 sarcoma, mastocytoma, or Meth A fibrosarcoma. In fact, unlike a solid tumor where bacterial growth either slowed down or plateaued after 2 days, bacterial growth in ascites tumors continued log linearly, and soon resulted in death from a fulminating systemic infection. Preliminary experiments suggest that this was not the result of a systemically suppressed state of anti-bacterial resistance, but was caused instead by the escape of massive and overwhelming numbers of bacteria from the peritoneal cavity into the circulation.

*The Distribution of Bacteria in the Tumor.* It could be suggested that *Listeria* persists in a tumor because it is interiorized by tumor cells and thus made inaccessible to destruction by host effector cells. Indeed, Fig. 9 shows that when washings of infected ascites tumors were subjected to centrifugation, most bacteria were found to be associated with the cell button. An examination of the location of bacteria in ascites tumors revealed, however, that this was not the result of their association with tumor cells. On the contrary, the stained smears revealed that bacteria were cell associated because they were inside macrophages in very large numbers (Fig. 10). It was obvious, moreover, that most of the macrophages were so heavily parasitized that they were destined to die. Occasionally, polymorphonuclear leukocytes were also seen to be replete with bacteria.

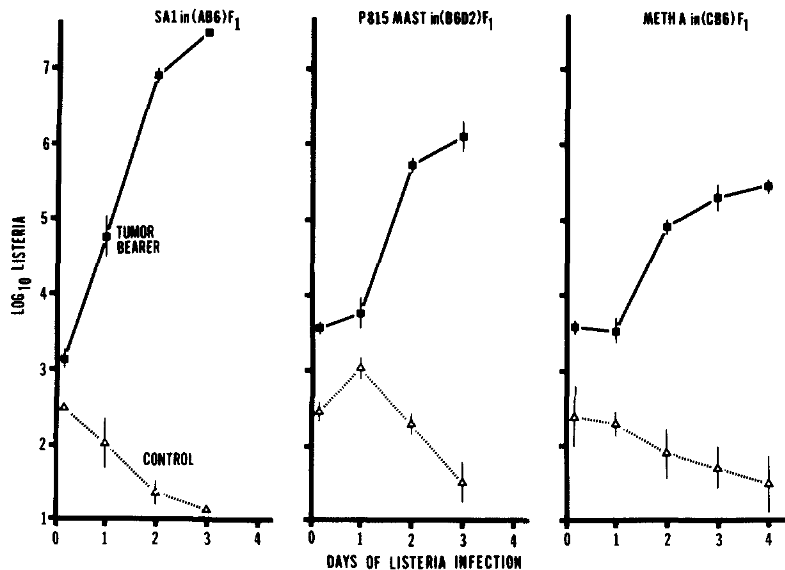


FIG. 8. Evidence that the host is incapable of expressing anti-bacterial resistance in an ascites tumor. Compared is the growth of a $10^4$ inoculum of *Listeria* in the peritoneal cavity of mice bearing 6-day peritoneal ascites tumors, with the growth of the same inoculum in the peritoneal cavities of normal mice. Tumor-bearing mice rapidly succumbed to an overwhelming systemic infection seeded from ascites tumors. Means of five mice ± SE.

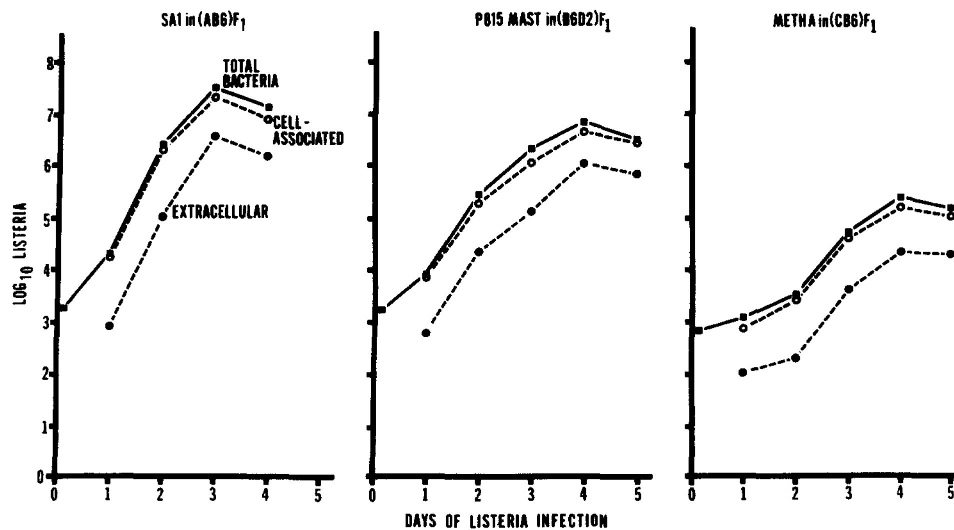


FIG. 9. Evidence that most bacteria in ascites tumors were associated with cells. Means of five mice.

Histologic examination of a solid foot pad tumor injected with *Listeria*, on the other hand, revealed that bacteria were distributed throughout the necrotic core as well as in the encompassing ring of living tumor cells and stroma. Clusters of microorganisms were seen in apparent association with cells, but it was not determined whether the cells involved were macrophages. Electron microscopy is therefore being currently employed to identify the cell types involved.

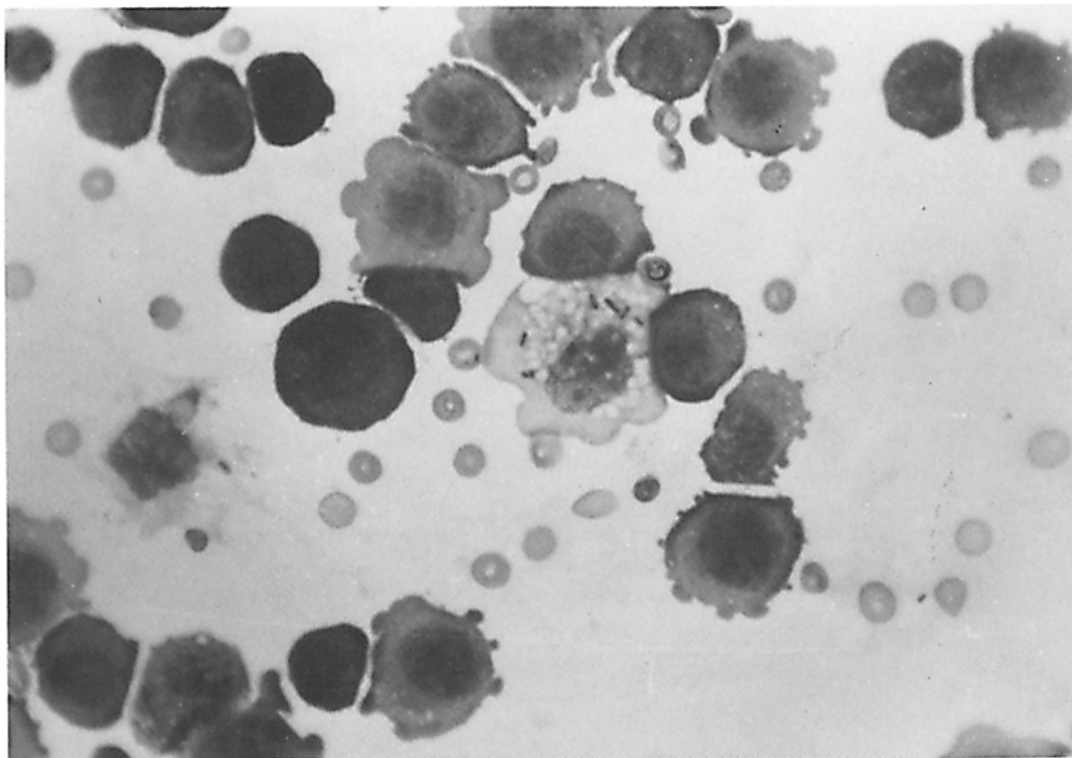

FIG. 10. Stained smear of a peritoneal SA1 ascites tumor showing the presence of *Listeria* inside macrophages, and its absence from the cytoplasm of tumor cells (× 1,025).

## Discussion

The results presented in this paper show that mice bearing any one of three syngeneic murine tumors, although capable of systemically generating and expressing acquired T-cell-mediated immunity to the bacterial parasite *L. monocytogenes* are, nevertheless, incapable of expressing this immunity within their primary tumors. It was demonstrated that whereas *Listeria* inoculated into a normal foot pad grew for 2 days and was then progressively eliminated by acquired anti-microbial immunity, the same number of bacteria inoculated into the contralateral foot pad bearing a 6 day established tumor either continued to grow at a slower rate, or failed to be eliminated. This occurred, moreover, in spite of the fact that tumor-bearing mice actually displayed a greater initial capacity for inactivating *Listeria* systemically than did normal mice, because of the generation of activated macrophages in response to the tumor itself. It was shown in a previous publication from this laboratory, furthermore, (5) that this tumor-induced state of macrophage activation is a consequence of the generation of T-cell-mediated concomitant anti-tumor immunity in response to progressive tumor growth.

It is well established that acquired immunity to *L. monocytogenes* is dependent on the generation of sensitized T cells which mediate the activation of macrophages that function to express immunity. That a macrophage system so

activated also gives the host the capacity to resist the growth of implanted syngeneic tumor cells is evidenced by the results of unpublished experiments in this laboratory which show that 6-day *Listeria*-infected mice can prevent the growth of threshold implants of tumor cells, as well as retard the growth of larger implants. There is little doubt that fully functional populations of mediator T cells and effector macrophages were generated in response to *Listeria* infection in tumor-bearing mice; otherwise, *Listeria* would not have been inactivated systemically. Direct evidence for this statement, moreover, will appear in a forthcoming publication. It follows, therefore, that the reason why *Listeria* was not eliminated from the tumor was because the mediator and effector cells of anti-bacterial immunity were either prevented from entering the tumor in adequate numbers, or their functions were suppressed after they entered.

Evidence that tumor cells secrete pharmacologically active molecules that suppress the function of macrophages and prevent their emigration into sites of tissue disturbance is rapidly accumulating. It was recently shown (13) in this laboratory, for instance, that subcutaneous implantation of cells of the same tumors employed in the present study, rapidly resulted in the liberation of a small molecular weight factor into the circulation which severely suppressed native and acquired macrophage-mediated resistance to bacterial infection. The effects of this factor, however, were eventually counteracted systemically by the generation of concomitant immunity and an activated macrophage system (14). However, because the serum of these mice continued to contain enough of the factor to suppress anti-bacterial resistance in normal recipients, it was suggested that the source of this factor was the tumor itself, and that it was present there in suppressive concentrations. Whether the presence of such a factor was responsible for the failure of mice to express anti-bacterial resistance as reported here has yet to be determined. This appears a likely possibility, however, when considered in conjunction with recent publications which show that murine tumor cells contain a small molecular weight factor that both inhibits macrophage chemotaxis in vitro (16), and suppresses the rate of emigration of these cells into inflammatory exudates (17). Indeed, there is now relatively convincing evidence that tumor-bearing animals (18–20) and tumor-bearing humans (21, 22) have an impaired capacity to mount inflammatory responses in general. Obviously, this evidence must be considered in any proposal that attempts to explain the failure of a *Listeria* immune host to express its anti-bacterial immunity in its primary tumor. Indeed, the finding that the host's inability to fully express this immunity against bacteria in a peritoneal ascites tumor was associated with the presence in the peritoneal cavity of only a small number of macrophages, most of which were heavily parasitized, strongly suggests that an adequate number of effector macrophages was prevented from emigrating into the tumor.

The results of this paper serve to show that there is a striking similarity between the ability of a tumor-bearing animal to express acquired concomitant anti-tumor immunity and its ability to express acquired anti-microbial immunity. Thus, while both types of immunity can be efficiently expressed systemically, neither can be expressed in the primary tumor. It can be suggested on the basis of these results, therefore, that there is probably more to an explanation of

the paradox of concomitant anti-tumor immunity than the action of serum-blocking factors. According to in vitro evidence, blocking factors in the form of tumor antigens, antibody, or antigen-antibody complexes, suppress specific anti-tumor immunity by neutralizing the antigen recognition sites of specifically sensitized, cytotoxic lymphocytes. The evidence presented here shows, however, that not only are conditions in the tumor antagonistic to the expression of specific anti-tumor immunity, but that they are also antagonistic to the expression of T-cell-mediated anti-bacterial immunity. It seems reasonable to suggest, therefore, that the reason why anti-tumor immunity is not expressed against a progressive primary tumor is because the tumor synthesizes molecules that pharmacologically suppress the function of host effector cells.

Even so, it is important to point out that in contradiction to the results presented here, intratumor inoculation of *Listeria* has actually been employed to regress murine tumors (23, 24). The contradiction, however, is more apparent than real, because it is only very small tumors that regress after *Listeria* inoculation. In fact, after tumors reach a certain critical size they not only fail to regress after intratumor inoculation with *Listeria,* but also after inoculation with BCG (25). It will be recalled, however, that tumor growth has been shown to temporarily stop after intratumor injection of *Listeria*. It is apparent, therefore, that partial anti-tumor therapy was in fact achieved. This is perhaps not surprising because during this time the host was also capable of partially expressing anti-microbial immunity in the tumors. Indeed, the published evidence strongly suggests (26) that the regression of tumors by intralesional inoculation of BCG requires that an immune response to this organism be generated and focussed in the tumor bed. Determining the nature of the mechanism that prevents the host from fully expressing its anti-tumor defenses against a progressive tumor is obviously important for a rational approach to the immunotherapy of cancer. It is significant in this regard, therefore, that in those few cases in this study where intratumor inoculation resulted in complete regression of Meth A foot pad tumors, *Listeria* was also efficiently eliminated from the foot pad. It seems reasonable to propose, therefore, that an understanding of the reason why anti-microbial resistance is not fully expressed in an established progressive tumor, will also allow an understanding of the reason why concomitant anti-tumor immunity is not expressed in the same site.

## Summary

Mice carrying any one of three murine tumors in their right hind foot pad were incapable of eliminating an inoculum of the bacterial parasite *Listeria monocytogenes* from the progressive tumor. In contrast, they were as capable as control mice in efficiently eliminating the organism from their contralateral tumor-free foot pad, and from their lymph nodes and livers. The results serve to show, therefore, that conditions within an established tumor are not only antagonistic to the expression of concomitant anti-tumor immunity, but that they are also antagonistic to the expression of T-cell-mediated anti-bacterial immunity. The possibility was discussed that the tumor contains factors that act pharmacologically to locally suppress the function of sensitized T cells and activated macrophages.

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