SUBVERSION OF HOST DEFENSE MECHANISMS BY MURINE TUMORS

II. Counter-Influence of Concomitant Antitumor Immunity*

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It was shown in the preceding paper (1) that subcutaneous injection of cells of any one of five unselected murine tumors resulted within 24 h in the presence of a factor in circulation that severely suppressed the ability of mice to resist experimental infection with *Listeria monocytogenes* and *Yersinia enterocolitica*. It was suggested, on the basis of the knowledge (2) that both native and acquired resistance to *Listeria* infection are expressed by macrophages, that the tumor-suppressor factor exerted its effect by either directly or indirectly interfering with the function of these phagocytic cells. It was suggested, in turn, that the evidence is consistent with the proposition that at least some malignant cells are naturally selected to avoid destruction by a macrophage-mediated mechanism of native antitumor resistance. Obviously, this view would have more credence if it were shown that mice with suppressed antibacterial resistance displayed at the same time a reduced capacity to resist the growth of a challenge of tumor cells.

Again, the preceding paper only dealt with the events that immediately followed injection of transplantable tumor cells. It remained a possibility, therefore, that suppression of antibacterial resistance is only a short-term effect of tumor cell implantation. Indeed, this could be suggested on the grounds that it is by no means a commonly reported finding that death from acute natural infection is a consequence of injecting experimental animals with tumor cells. On the contrary, there is a recent demonstration (3) that mice bearing the Lewis lung carcinoma display increased resistance to *Candida albicans* infection.

The purpose of this paper is to show that the state of severely impaired antibacterial resistance which immediately follows subcutaneous injection of murine tumor cells is short lived, in that it is soon replaced by a contrasting state of greatly increased antibacterial resistance; in spite of the continuous presence of the suppressor factor in circulation. Evidence will be presented to show that the tumor-induced states of decreased and increased antibacterial resistance correspond to states of decreased and increased resistance to the tumor itself. Consequently, the results support the proposition that antibacterial and antitumor resistance are expressed by a common defense mechanism.

Materials and Methods

The materials and methods employed were the same as those employed in the preceding paper (1) except for additional procedures that were used to measure resistance to a tumor cell challenge.

Antitumor Resistance. Mice bearing a progressive subcutaneous tumor in the right-hind foot pad were compared with normal control mice in terms of their capacity to resist the growth of

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either a subcutaneous or intraperitoneal challenge of tumor cells. Resistance to subcutaneous tumor cell challenge at progressive times during growth of the primary tumor was determined by measuring the growth of a standard number of tumor cells injected into the contralateral foot pad. The cells were injected in a vol of 0.05 ml of phosphate-buffered saline (PBS),[1] and tumor growth was monitored by measuring changes in the dorso-ventral thickness of the foot pad with dial calipers.

Changes in resistance to growth of an intraperitoneal challenge of tumor cells was determined by measuring changes in the quantity of tritiated thymidine ([^{3}H]TdR) incorporated into total peritoneal cell DNA according to a published method (4). This involved challenging mice intraperitoneally with a standard dose of tumor cells in a vol of 0.2 ml of PBS at progressive times during the growth of the primary subcutaneous tumor. At 24-h intervals over the next 3 days the mice were given a single intravenous pulse of 20 μCi [^{3}H]TdR of sp act 3 Ci/mmol (New England Nuclear, Boston, Mass.). 30 min later, the peritoneal cells were harvested in 3 ml of heparinized PBS in a standard fashion, washed two times over a period of 1 h in ice-cold 5% TCA, and then extracted for 1 h in 2 ml of hot (90°C) TCA. ^{3}H-DNA in the extract was counted by liquid scintillation spectrometry.

Results

Conversion from Suppressed to Enhanced Antibacterial Resistance during Tumor Growth. The preceding paper showed (1) that subcutaneous injection of tumor cells resulted in rapid suppression of resistance to intravenous *Listeria* infection. The following experiments were designed to determine whether this state of severely suppressed antibacterial resistance persists during subsequent growth of the primary tumor. The experiment consisted of injecting semisyngeneic mice subcutaneously with 10^6 SA1, P-815 mastocytoma, or Meth A cells, and measuring the capacity of the mice at progressive times thereafter to resist a standard 5×10^3 intravenous *Listeria* challenge infection. Antibacterial resistance was expressed as the $\log_{10}$ difference between the growth of the challenge organism in the livers of tumor-bearing mice and its growth in livers of control mice at 24 h of infection.

The results in Fig. 1 reveal, in agreement with those in the preceding study, that subcutaneous injection of tumor cells quickly resulted in a state of severely suppressed antibacterial resistance. It can be seen in addition, however, that the state of negative resistance rapidly waned after 24 h, and was soon replaced by a contrasting state of greatly increased antibacterial resistance. The speed at which this conversion from suppressed to enhanced antibacterial resistance occurred, moreover, was related to the rate of growth of the primary tumor. The experiments were terminated when the condition of the mice had deteriorated because of the massiveness of the primary tumors.

To exclude the possibility that conversion from negative to positive antibacterial resistance was the unlikely result of a nonimmunological response of the F_1 hybrid against parental tumor cells, the same experiment was performed with syngeneic mice. That syngeneic A/J mice injected with 10^6 SA1 cells gave the same results is shown in Fig. 2.

Comparison between States of Negative and Positive Antibacterial Resistance. The meaning, in terms of overall resistance to infection, of the states of negative and positive resistance as revealed by the 24-h growth assay shown

[1] *Abbreviations used in this paper:* [^{3}H]TdR, tritiated thymidine; PBS, phosphate-buffered saline.

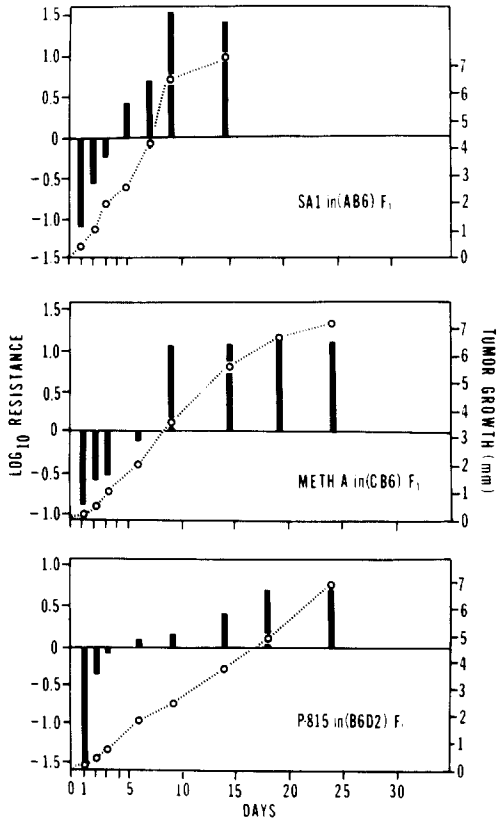

FIG. 1. Changes in resistance to *Listeria* infection (bar graphs) during growth of three murine tumors in semisyngeneic mice. Subcutaneous implantation of tumor cells first resulted in a state of severely suppressed resistance and then in a contrasting state of enhanced resistance. Resistance is expressed as the $\log_{10}$ difference between the 24-h growth of the organism in livers of control and tumor-implanted mice. Means of five mice per time point.

above is more convincingly demonstrated in Fig. 3, which compares the 2-day growth of *Listeria* in the livers of 1-day and 9-day tumor bearers. It can be seen that whereas a 2×10^3 *Listeria* inoculum showed enhanced growth for 2 days in the livers of 1-day tumor bearers, no bacterial growth occurred in the livers of 9-day tumor bearers. Thus while 1-day tumor bearers were highly susceptible to infection, 9-day tumor bearers were highly resistant.

Presence of Suppressor Factor in Circulation in Spite of Acquisition of Enhanced Antibacterial Resistance. It is known (1) that the short-term state of suppressed antibacterial resistance that rapidly follows injection of tumor cells is mediated by a small molecular weight factor in circulation. To determine whether the production of this molecule continues in spite of the change from suppressed to enhanced antibacterial resistance, an experiment was performed to test for its presence in serum during a 9 day period of tumor growth. Thus, serum obtained from (AB6)F_1 donor mice at progressive intervals after initiating a subcutaneous tumor with 10^6 SA1 cells was tested for its capacity to

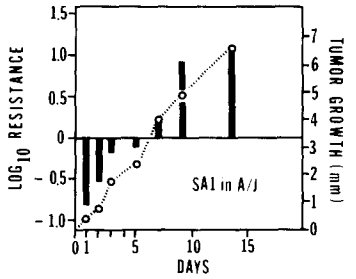

FIG. 2. Same experiment as Fig. 1, but in syngeneic mice.

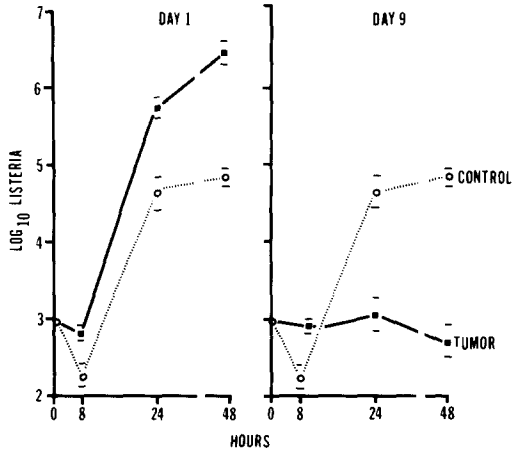

FIG. 3. Additional evidence for conversion from suppressed to enhanced antibacterial resistance during subcutaneous growth of an SA1 tumor. The liver growth curves show that whereas 1-day tumor bearers allowed enhanced growth of a sublethal *Listeria* challenge for a 2 day period, 9-day tumor bearers were highly resistant to the same challenge. Means ± 2 SE of five mice per time point.

suppress anti-*Listeria* resistance when infused into normal recipients. Serum was injected intraperitoneally in a vol of 0.2 ml 1 h before intravenous challenge with 2×10^3 *Listeria*. Bacterial growth was measured by the 24-h growth assay employed in a preceding section.

Fig. 4 shows that an infusion of 0.2 ml of serum collected at any time during growth of the primary tumor was able to significantly suppress the antibacterial resistance of normal recipients. It will be noted, however, that there was a drop in the potency of the serum after about the 5th day of tumor growth, i.e., after the conversion from negative to positive antibacterial resistance (Fig. 1). Indeed, the resuts of a limiting dilution assay employed in the preceding paper (1) indicate that this drop may have represented as much as a 50-fold reduction in concentration of the suppressor factor.

Nevertheless, when sera from 1-day tumor-bearing donors and 9-day tumor-bearing donors were compared in terms of their capacity to increase bacterial growth in the livers of normal recipients over a 3 day period, it was found (Fig. 5)

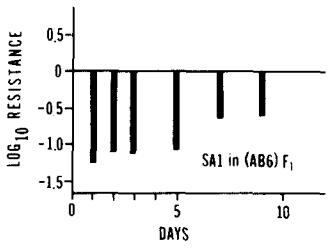

FIG. 4. Evidence that a suppressor factor persisted in circulation for at least 9 days of tumor growth. An intraperitoneal infusion of 0.2 ml of tumor-bearers' serum collected at any of the times indicated after implanting tumor cells, suppressed the capacity of normal recipients to resist sublethal *Listeria* infection. Means of five mice per time point.

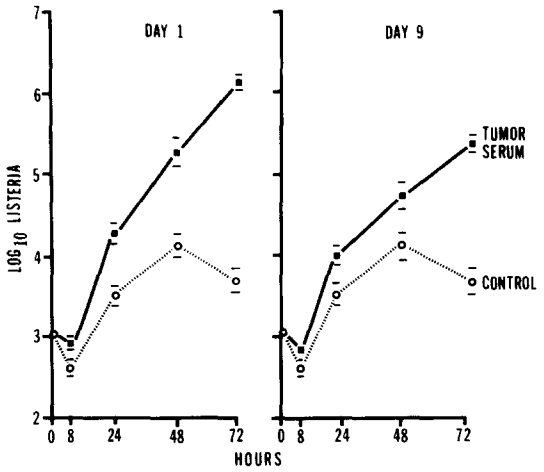

FIG. 5. Additional evidence that 9-day tumor bearers contained a suppressor factor in circulation. A 0.2 ml infusion of 9-day as well as 1-day tumor-bearers' serum caused continuous bacterial growth for 3 days in the livers of normal recipients. Means ± SE of five mice per time point.

that 0.2 ml of 9-day serum as well as 0.2 ml of 1-day serum caused enhanced bacterial growth over this period. It can be concluded, therefore, that in spite of conversion from a state of negative to a state of highly positive antibacterial resistance, a suppressor of antibacterial resistance persisted in circulation.

Failure of Allogeneic and Lethally Irradiated Tumor Cells to Cause Enhanced Antibacterial Resistance. It was important for the design of future experiments to know whether the suppressed antibacterial resistance which results from subcutaneous injection of lethally irradiated tumor cells or of allogeneic tumor cells (1) is also followed by a state of enhanced antibacterial resistance. The possibility that increased bacterial resistance might fail to develop is suggested by the knowledge that lethally irradiated tumor cells cause no tumor growth, and that allogeneic tumor cells result in only a limited period of tumor growth. This question was investigated by an experiment that involved measuring changes against time in resistance to a *Listeria* challenge infection of (AB6)F_1 mice that were injected subcutaneously with either 10^6 lethally irradi-

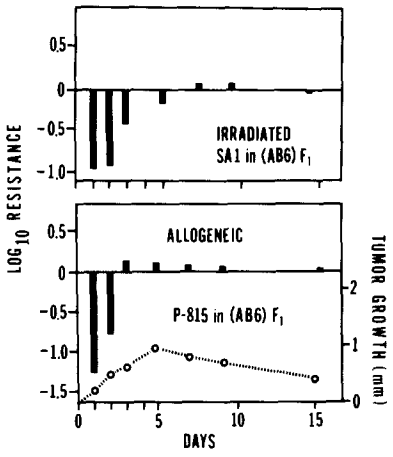

FIG. 6. Evidence that implantation of lethally irradiated or allogeneic tumor cells failed to result in the generation of a state of increased antibacterial resistance. In both cases, the state of severely suppressed resistance to a *Listeria* challenge that occurred after implantation of tumor cells was followed by a return to normal levels of antibacterial resistance. Means of five mice per time point.

ated SA1 cells as a semisyngeneic tumor, or with 10^6 Meth A cells as an allogeneic tumor. Changes in anti-*Listeria* resistance were measured by the 24-h growth assay described above.

It was found (Fig. 6) that although lethally irradiated tumor cells caused a short-lived state of suppressed antibacterial resistance, there was no subsequent conversion to a state of increased antibacterial resistance. Instead, the level of antibacterial resistance returned to normal by day 5. Fig. 6 shows that a similar result was obtained with an allogeneic tumor, the early rejection of which was associated with a return to a normal level of antibacterial resistance. Taken together, these results indicate that the generation of a state of increased antibacterial resistance depends on progressive growth of the primary tumor.

Changes in Antibacterial Resistance Reflect Changes in Resistance to the Tumor Itself. The experiments presented in this section were designed to test the prediction that changes in the level of antibacterial resistance that occur during growth of a primary tumor are the result of changes in the level of host resistance to the tumor itself. This prediction was tested by injecting $(AB6)F_1$ mice subcutaneously in the right-hind foot pad with 10^6 SA1 cells, and measuring their capacity at progressive times thereafter to resist the growth of a challenge of 5×10^5 tumor cells given either subcutaneously in the contralateral foot pad, or intraperitoneally.

The results obtained with the subcutaneous challenge are shown in Fig. 7 where it can be seen that initiation of a subcutaneous primary tumor first resulted in a short-term state of suppressed resistance as evidenced by "enhanced growth" of a challenge given on day 3, and then in a contrasting state of greatly increased resistance as evidenced by a striking suppression of growth of the same challenge given on days 6 or 9. The timing of the change from suppressed to enhanced antitumor resistance thus shows a striking concordance with the change from suppressed to enhanced antibacterial resistance (Fig. 1).

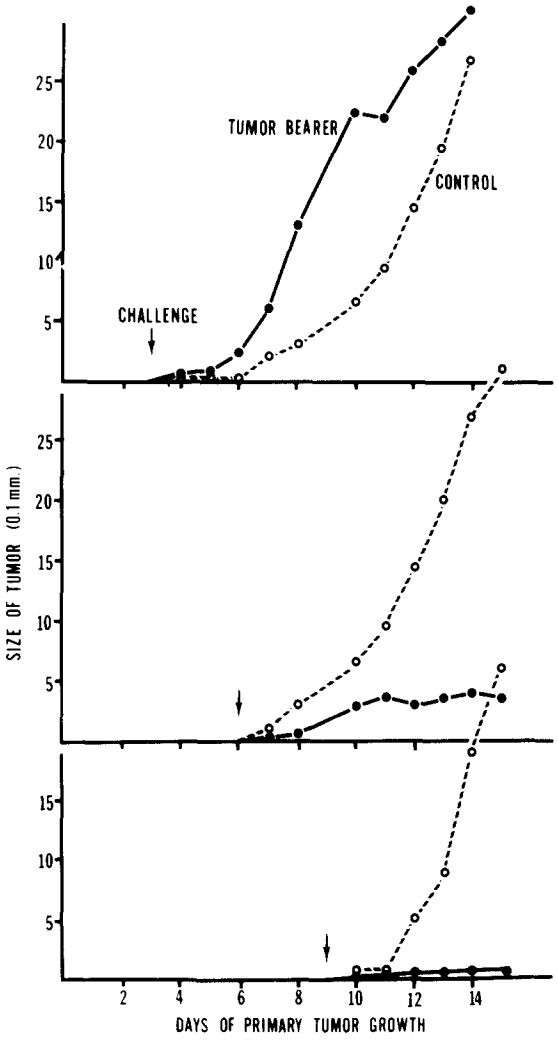

FIG. 7. Evidence that subcutaneous implantation of 10^5 SA1 cells first resulted in a state of suppressed resistance ("enhanced growth") to a subcutaneous 10^5 challenege of tumor cells, and then in a contrasting state of increased resistance to a challenge of the same cells. Means of five mice per time point.

The same time relationship was found when the peritoneal cavity was used to test for antitumor resistance. It can be seen in Fig. 8 that whereas the period that immediately followed initiation of the primary subcutaneous tumor was characterized by a state of greatly reduced resistance to growth of the intraperitoneal tumor cell challenge ("enhanced" [^{3}H]TdR incorporation), this was soon followed by the development of a powerful mechanism of resistance to the same challenge (suppressed [^{3}H]TdR incorporation). Differences between the control and experimental groups on day 3 of the assay are plotted at the bottom of Fig. 8 to show more clearly the time-course of conversion from suppressed to enhanced resistance.

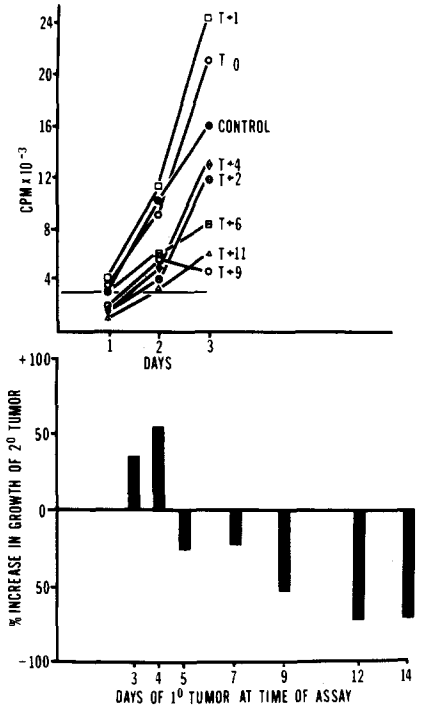

FIG. 8. Conversion from negative to positive resistance to an intraperitoneal tumor cell challenge. The 3-day [^{3}H]TdR incorporation curves (top graph) show that an intraperitoneal challenge of tumor cells underwent "enhanced growth" (increased [^{3}H]TdR incorporation) when injected either at the time of, or 1 day after initiating a subcutaneous primary tumor. In contrast, growth of the same challenge was suppressed (decreased [^{3}H]TdR incorporation) when given at later times. The bar graph plots the 3-day differences in [^{3}H]TdR incorporation between tumor-bearing and control mice in order to better illustrate conversion from suppressed to enhanced antitumor resistance. Means of five mice per time point.

It will be noted that the switch from negative to positive antitumor resistance occurred faster according to the peritoneal assay. It should be pointed out, however, that additional experiments have revealed that the speed at which the switch occurs depends on the number of tumor cells used to initiate the primary tumor and the number used for challenge.

Discussion

The results of this study confirm those of the preceding study (1) which showed that subcutaneous injection of murine tumor cells results in rapid and severe suppression of the capacity of mice to resist experimental infection with the bacterial parasite, *L. monocytogenes*. The present results show, in addition, that the tumor-induced state of suppressed antibacterial resistance was short lived and was soon replaced by a contrasting state of greatly enhanced antibacterial resistance. Again, conversion from suppressed to enhanced resistance was shown to depend upon progressive growth of the primary tumor, and to correspond with conversion from suppressed to enhanced resistance to growth of a tumor cell challenge. The results show, therefore, that changes in the level of

antibacterial resistance reflect changes in the level of resistance to the tumor itself. This implies that antibacterial resistance and antitumor resistance are expressed by a common mechanism of defense.

The generation of the capacity to strikingly resist the growth of a tumor cell challenge during rapid growth of the primary tumor means that the host generated a state of concomitant antitumor immunity (5), the nature of which will be dealt with in a forthcoming publication. It can be suggested here, however, that because it appeared coincidentally with the development of macrophage-mediated anti-*Listeria* resistance, it is almost certain that concomitant immunity itself is expressed by activated macrophages. This seems a reasonable suggestion in view of the large body of evidence which shows (6–8) that macrophages activated in vivo as a result of microbial infection can recognize and destroy neoplastic cells in a nonimmunological way in vitro. The suggestion is also supported by earlier in vivo findings (9) which showed that animals with macrophage systems activated as a result of treatment with infectious and noninfectious agents acquire the capacity to retard the growth of a tumor cell challenge. The results of this paper show that the converse is true in that the response to the tumor itself results in the generation of an activated macrophage system. In fact, the results suggest that the level of nonspecific macrophage-mediated antibacterial resistance eventually generated in response to the SA1 sarcoma is at least equal to, or even higher than the level of nonspecific antibacterial resistance generated in response to an intravenous BCG infection. This is in keeping with publications which show that tumor-bearing humans (10) as well as tumor-bearing animals (9) can show an increased capacity to clear intravenously injected colloids from their circulation.

The present study lends more credence to the view that activated macrophages play an important role in antitumor defense. More direct evidence to suggest that concomitant antitumor immunity is expressed by macrophages, and that it can therefore be expressed nonspecifically against antigenically unrelated tumors, has recently been reported (11). Our findings serve to emphasize the nonspecific nature of the resistance since they clearly show that it is expressed against infectious agents. It is not surprising, therefore, that in spite of an initial state of severely suppressed antibacterial resistance, death from acute natural infection is not a commonly reported consequence of injecting experimental animals with syngeneic tumor cells.

The coincident development of a state of increased antitumor and antibacterial resistance is not the only reason for postulating a common mechanism of defense against microorganisms and neoplasia. The postulate is also supported by the finding that resistance to both agents is simultaneously suppressed after initiating a primary tumor. In this case, however, a native mechanism of resistance is involved. Indeed, it is difficult to escape the conclusion that an initial implant of tumor cells survives and grows because of its capacity to rapidly suppress a native defense mechanism that would otherwise cause its destruction. Since the only cells in the mouse that can destroy *Listeria* are macrophages, it seems likely that the suppressor factor described in the preceding paper (1) serves to protect the primary tumor from destruction by macrophages. Direct evidence that tumor-induced suppression of antitumor resist-

ance, like suppression of antibacterial resistance, is caused by a factor in circulation will appear in a forthcoming publication.

In spite of the acquisition of a mechanism of macrophage-mediated resistance to a tumor cell challenge, the host remained unable to reject its primary tumor. It is highly significant in this connection that the factor which interferes with macrophage function continues to be produced and liberated into the circulation during growth of the primary tumor. This implies that its most likely source is the tumor. If so, it may be present in a large enough concentration in the tumor bed to protect the primary tumor from macrophage-mediated antitumor immunity. It is well to realize that the demonstration of concomitant resistance is an artificial procedure in that the introduction of tumor cells with a needle is in itself sufficient to cause a local inflammatory response. It is considered possible that this would result in an influx of enough circulating activated mononuclear-phagocytes into a nonvascularized deposit of loose tumor cells to cause tumor cell destruction. The conditions in the vascularized bed of a large established tumor, obviously, are quite different from those that exist at the site of a tumor cell challenge.

An additional artificiality in this study was the initiation of growth of transplantable tumors by subcutaneous injection of 10^5–10^6 cells. This bears little resemblance to the way in which autochthonous malignancies naturally emerge. It is therefore right to question the significance to tumor biology of the rapid creation of the short-term state of severely suppressed antitumor and antibacterial resistance that resulted from injection of this comparatively enormous number of tumor cells. It can be stated in defense of the model, however, that it has been shown in this laboratory (unpublished observations) that a similar degree of suppression occurs after injection of as few as 10^3 tumor cells, but not until after a delay of 2 days. In this case, moreover, antibacterial resistance remains suppressed for a much longer period of time, a period that corresponds to the period of "latency" before tumor growth becomes manifest. The employment of small numbers of tumor cells for studying the mechanism that enables a small incipient tumor mass to avoid destruction by the host's defenses over a long period of time will be the subject of a paper now in preparation.

Summary

Subcutaneous injection of murine tumor cells first resulted in a state of severely suppressed macrophage-mediated antibacterial resistance and then in a contrasting state of greatly enhanced antibacterial resistance. Whereas, the state of suppressed antibacterial resistance corresponded to a state of suppressed resistance to a tumor cell challenge, the generation of enhanced antibacterial resistance corresponded to the acquisition of concomitant antitumor immunity. It was suggested on the basis of this evidence that changes in the level of macrophage-mediated antibacterial resistance that occur during growth of the primary tumor reflected changes in the level of the host's resistance to the tumor itself. It was further suggested that the coincidental suppression of antibacterial and antitumor resistance that occurs during the initial stages of growth of the primary tumor represents the operation of a mechanism that enables the tumor

to avoid destruction by macrophages. The results support the view that macrophages play an important role in native and acquired resistance to malignant tumors.

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