This report extends this observation and demonstrates that concomitant immunity develops so slowly in mice bearing this cancer that the metastases that are shed in the 1st week of growth survive and ultimately kill the host.

MATERIALS AND METHODS

Mice. The mice used throughout these experiments were 3-month-old BALB/c males and females (single sex within an experiment). The mice were housed 8/cage with food and water provided ad libitum. Details of the total-body X-ray exposures have been given (12).

Line 1 Alveolar Cell Carcinoma. The origin of this cancer from a BALB/c mouse and its propagation have been described in detail elsewhere (11–14). In vivo tumor samples, which were used for all studies except the i.v. injections, were obtained from the 25th to 38th transplant generations. Injections were made i.v. with tumor cells harvested from the 22nd to 38th in vitro passages. Both tumors yield identical results in terms of transplantability, immunogenicity, and pathological appearance of resultant tumors.

Tumor Growth Assays. At selected intervals after s.c. transplantation, the mice were killed and the tumors were excised and weighed. The assay of lung tumor colonies from i.v.-injected cells (9) involved killing the mice 21 days after injection and inflating, clearing, and staining their lungs (10). Tumor colonies were counted under a dissecting microscope.

Surgical Tumor Removal. Ten million line 1 carcinoma cells were injected i.m., and 7 to 10 days later the tumor-bearing leg was amputated. Attempts to remove the tumor-bearing leg at later intervals were unsuccessful due to a 60 to 80% incidence of local recurrence.

Bioassay of Early Lung Metastasis. At intervals of 6 to 21 days after s.c. transplant of 5 × 10^5 tumor cells, the entire lung mass of recipient mice was removed, minced, and injected i.p. into normal recipients. These mice were observed for 7 months and autopsied at death.

RESULTS

Rate of Development of Concomitant Immunity. Groups of mice (n = 8) were given s.c. transplants of 0 or 5 × 10^7 tumor cells and 0, 3, 7, or 14 days later were given an i.v. injection of 0 or 2 × 10^4 tumor cells. Twenty-one days after the i.v. injections, the mice were killed and the number of
tumor colonies in the lungs and the weights of the s.c.
tumors determined. Chart 1 is a plot of the number of lung
tumor colonies observed in mice given i.v. tumor cell
injections at each stage of s.c. tumor growth, expressed as
a percentage of the number observed in mice given only the
i.v. injection. As reported elsewhere (11), s.c. tumor growth
does not result in detectable inhibition of i.v.-injected tumor
cells given on Day 0 (Chart 1), but when these artificial
metastases are injected at later stages of tumor growth a
gradually increasing inhibition of their development into
lung tumor colonies is observed (Chart 1). For comparison,
we have included in Chart 1 the results of an identical
experiment in which Milas et al. (7) studied a chemically
induced fibrosarcoma. Relative to this fibrosarcoma, which
is a typical murine tumor, the rate at which line 1
carcinomas induce concomitant immunity is very slow.

Chart 2 is a plot of the s.c. tumor weights observed in the
mice given i.v. tumor cell injections at different stages of s.c.
tumor growth, expressed as a percentage of those observed
in mice that did not receive the artificial metastases. Tumor
cell injection i.v. during the early stages of growth, which

result in the development of many lung tumor colonies
(Chart 1), inhibits the s.c. tumor growth; but similar
injections at later stages have little or no effect (Chart 2).
Again, the data of Milas et al. (7) are included for
comparison (Chart 2), and at none of the stages studied was
the growth of the fibrosarcoma inhibited by the i.v. injection
of identical tumor cells.

**Deceleration of the Development of Concomitant Immunity.** In the hope of slowing the rate of development of
concomitant immunity even further, we performed an
experiment identical to the one described above, except that
all tumor recipients were exposed to 500 R of total-body
X-rays 2 hr before the s.c. transplant. This exposure
accelerates the development of both spontaneous and artifi-
cial metastases, but it slows the growth rate of s.c.
transplants (11). In these irradiated mice, detectable con-
comitant immunity did not develop even when the i.v. cells
were injected 14 days after s.c. transplant (Chart 1).
Correspondingly, the many lung tumor colonies that devel-
oped inhibited the growth of the s.c. tumors (Chart 2) even
further than that observed in mice that were only exposed to
X-rays.

**Acceleration of the Development of Concomitant Immunity.** The relatively slow rate of induction of concomitant
immunity by the line 1 carcinoma is not due to a slower
tumor growth rate. In fact, this cancer grows at the same
rate as the fibrosarcoma (Chart 3) of Milas et al. (7). It
would appear, therefore, that individual line 1 cells possess either fewer or weaker tumor antigens than the fibrosarcoma cells do. After failing to increase the immunogenicity of individual line 1 cells through enzymatic treatment (13), we attempted to increase the rate of development of concomitant immunity in the line 1 system by increasing the host's tumor burden. Through rigorous selection of tumor samples, use of plastic tissue grinders with wide tolerances, and repeated washing of tumor cell suspensions, we obtained tumor preparations that would produce s.c. tumors weighing in excess of 5 g within 21 days after transplant, as opposed to the 2-g tumors that we normally obtain. Preliminary experiments demonstrated that mice bearing these rapidly growing tumors developed sufficient concomitant immunity within the 1st 21 days of growth to suppress almost totally the development of lung tumor colonies from tumor cells injected i.v. on Day 0 of growth. These data were equivocal, however, since we could not discount the possibility that the new procedure for preparing tumors selectively preserved or concentrated a highly immunogenic subpopulation of tumor cells.

In order to provide a more definitive test of the possibility that the antigen burden determined the rate of development of concomitant immunity, a tumor cell suspension was prepared as described above and graded cell doses were given s.c. to groups of 16 mice. Two hr later, one-half of each group was given an i.v. injection of $2 \times 10^4$ tumor cells. As expected, the s.c. tumor weights, observed 21 days later, were directly proportional to the s.c. cell dose (Chart 4). Mice bearing the larger s.c. tumors were able to inhibit, almost totally, the development of lung tumors from the i.v.-injected tumor cells. Over the range of s.c. cell doses studied, the ability to suppress the development of lung tumor colonies was directly proportional to s.c. cell dose (or tumor size). At the 2 lowest s.c. cell doses studied, the i.v.-injected cells produced enough lung tumor colonies to reduce the growth of the s.c. tumor significantly ($p < 0.05$) relative to that observed in mice given only the s.c. injections. We conclude from these data that the rate of development of concomitant immunity in the line 1 carcinoma system is directly proportional to the tumor antigen burden.

Concomitant Immunity and Spontaneous Metastasis. The experiments reported above would suggest that concomitant immunity develops slowly in mice bearing the line 1 carcinoma but that it eventually reaches significant levels (Chart 1). For a number of reasons (see "Discussion"), we suspected that the artificial metastasis system would tend to overestimate the effectiveness of concomitant immunity in controlling spontaneous metastases, and we therefore assayed concomitant immunological control of metastases that were spontaneously shed by growing line 1 carcinomas. Groups of unirradiated and irradiated (500 R) mice were given s.c. transplants of $5 \times 10^7$ line 1 tumor cells in the dorsal-cervical region, and their lungs were bioassayed for the presence of transplanteble tumor cells between 6 and 21 days later. Chart 5 is a plot of the frequency of successful tumor transplantation by the lung cell suspensions as a function of time after receipt of the tumor. By the 18th day after transplant, 100% of the unirradiated tumor-bearing mice possess transplantable tumor cells in their lungs. In irradiated mice, it requires only 11 days of s.c. tumor growth to reach this same concentration of tumor cells in the lungs (Chart 5). Also included in Chart 5 are the survival times of recipients in all groups showing 100% transplantability. The progressively shorter survival time with the later times of assay (Chart 5) suggests that the tumor cell concentration continues to increase throughout the observation period. The curve for recipients of lungs from irradiated mice is displaced 7 days earlier than that of unirradiated mice, again indicating that metastatic spread occurs earlier in this group. A 100% incidence of macroscopically detectable spontaneous metastases is first observed on Day 28 posttransplant in irradiated mice and on Day 35 in unirradiated mice (11). Since it requires 18 to 21 days for i.v.-injected cells to grow into macroscopically detectable lung colonies (11-14) in the line 1 system, the early metastases that we have detected develop into macroscopic colonies as rapidly as do artificial metastases in normal mice. This would suggest that the growing s.c. tumors are not inducing sufficient concomitant immunity to inhibit the growth of the early spontaneous metastases.

A 2nd method was used to determine the rate of early metastasis and their eventual contribution to host lethality. Groups of 32 mice each were given i.m. transplants of $5 \times 10^7$ line 1 cells. One group was allowed to live out their
remaining life intact, while the other had their tumor-bearing legs amputated 7 to 10 days after transplant. Surgical removal of the tumor was effective in all but 2 of the animals, but 40% (12 of 30) of the remainder had already developed spontaneous metastases, which eventually killed them (Table 1). These surgically treated mice possessed fewer metastases at death than did their intact counterparts, but the size of the individual metastases in the lungs was appreciably larger. The fact that tumor removal prolonged the survival time of these mice by only 4 weeks would suggest that the early metastases make a major contribution to lethality in intact mice and that the remaining small difference in survival between intact and surgically treated mice is due to the continued metastatic spread in the former.

DISCUSSION

The data presented above have demonstrated that the rate of development of concomitant immunity in mice bearing line 1 carcinomas growing s.c. is directly related to their tumor burden (Chart 4). Further, at equal tumor burdens (Chart 3) the line 1 carcinoma induces these reactions at a slower rate than do more conventional murine tumors (Chart 1). The fact that the line 1 carcinoma is far less immunogenic than tumors that rapidly induce concomitant immunity (5, 14) would appear to be the most logical explanation for these observations.

Deceleration of the rate of development of concomitant immunity by preirradiation of the host is due presumably to the immunosuppressive effects of the exposure, but an additional factor, reduced tumor burden (12), must be taken into account. Preirradiated mice possess smaller s.c. tumor burdens throughout the posttransplant period (12), and it would be expected therefore that the s.c. tumors would provide less of a stimulus for the development of concomitant immunity. This would appear to be a relatively minor contribution relative to the immunosuppressive effects of the exposure, however. As an example, the i.v.-injected cells given to preirradiated mice on Day 14 of s.c. tumor growth are exposed to a larger average tumor burden than are the i.v. cells given to normal mice on Day 3 of tumor growth, but only the latter group demonstrates significant concomitant immunity.

In a number of experiments, we have found that one cannot directly extrapolate from the artificial metastasis system to the process of spontaneous metastasis, at least not quantitatively. This would also appear to be the case in the present experiments. Artificial metastasis studies suggest a slow but eventually significant level of concomitant immunity in the line 1 carcinoma system (Chart 1), but spontaneous metastasis studies (Table 1; Chart 5) indicate that the concomitant immunity that is induced by the growing tumor has negligible effects on those metastases that are shed soon after transplantation. We suggest that the reason for this discrepancy is that artificial metastases accentuate the underlying immunological reactions induced by the growing s.c. tumor. Spontaneous metastasis is characterized by a progressively increasing number of tumor cells being re-

![Chart 4. Mean tumor weights in BALB/c mice receiving either an s.c. transplant only (O) or both s.c. and i.v. transplants (•), and mean number of lung tumor colonies in mice receiving both s.c. and i.v. transplants (•). No lung tumor colonies were observed in mice that received only an s.c. transplant. Mice receiving only i.v. transplants developed 83 ± 8 lung tumor colonies. Bar, S.E.](chart4)

![Chart 5. Fraction of lungs from unirradiated (O) and 500 R preirradiated (•) s.c. tumor-bearing mice that contained transplantable line 1 carcinoma cells as a function of time after s.c. transplant of 5 × 10⁶ line 1 carcinoma cells and the survival time of recipients of these transplantable cells obtained at varying times from unirradiated (O) and 500-R-preirradiated (•) s.c. tumor-bearing donors.](chart5)
Concomitant Immunity Development

Table 1

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* Mean survival time ± S.E.
^ Individual survival times.

leased into the circulation, while in the artificial metastasis system a massive number of tumor cells are suddenly introduced. Fewer than 1 in 1000 of these cells develop into metastases, leaving the remainder to stimulate or accentuate the effectiveness of the concomitant immunity induced by the s.c. tumor. In our own work, we continue to use both methods but rely for quantitative estimates on the spontaneous metastasis system.

The line 1 carcinoma system is not unique in any single respect, but its combination of characteristics (11-14), including poor control over spontaneous metastases, makes it an excellent system for the study of lung cancer therapy. Irradiation therapy protocols that have been highly successful in nonmetastasizing tumor systems (10) have negligible effects in the line 1 carcinoma system because they fail to address the problem of metastatic spread. None of the studies that we have performed in this system have yielded successful therapy, but it would appear that we are failing because we are addressing the full complexity of the problem.

REFERENCES

Development of Concomitant Immunity in Mice Bearing the Weakly Immunogenic Line 1 Lung Carcinoma

John M. Yuhas, Nelson H. Pazmiño and Elizabeth Wagner


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