Preferential Growth of Mammary Tumors in Intact Mammary Fatpads

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ABSTRACT

Four transplantable mammary tumors, three (66, 410, and 168cl) isolated from a spontaneously occurring strain BALB/cfC3H mammary tumor and one (D2) arising from a BALB/c hyperplastic alveolar nodule were found to grow better in mammary fatpads than at s.c. sites. Furthermore, tumor growth was better (p < 0.05) in intact mammary glands than in cleared mammary fatpads for the D2, 410, and 66 tumors (168cl was not tested). The role of immunity in these differences was investigated using the highly immunogenic 410 tumor. Tumor 410 induced equally effective immunity to subsequent challenge whether it was implanted s.c. or in intact fatpads. Furthermore, in immunized animals, Tumor 410 was rejected equally well when the challenge site was intact fatpad as when s.c. Similarly, Tumor 410 induced immunity after implantation into cleared fatpads and, in immunized animals, was rejected when the challenge site was the cleared fatpad. We thus found no evidence that the mammary fatpad is immunologically privileged, as compared to the s.c. site, with respect to tumor transplantation antigens.

INTRODUCTION

Preneoplastic mammary lesions in mice are optimally transplanted into mammary fatpads which have been cleared of their glandular tissue (5, 8). The presence of normal mammary glandular elements inhibits the growth and progression of preneoplastic HANs (8, 11). The environment of the mammary fatpad is conducive to growth and progression of preneoplasia since such lesions are merely maintained at s.c. sites (5). Tumor cells grow in both cleared and intact fatpads as well as other sites and thus seem neither to require the environment of the fatpad nor to be affected by the presence of normal mammary gland elements. These site dependency characteristics have become operational definitions of preneoplastic versus neoplastic lesions (5, 8, 11). It is important, however, to know whether the differences between the growth of the lesions are absolute or only a matter of degree. In this report, we have reexamined, using low numbers of cells, the growth of mouse mammary tumors in intact and cleared fatpads and at s.c. sites. We have also reexamined the question of the immunologically privileged nature of mammary fatpads.

MATERIALS AND METHODS

Animals. BALB/cCrg1 mice were either purchased from the Cancer Research Laboratory, University of California, or obtained from breeding pairs purchased from Cancer Research Laboratory and maintained at our animal care facilities.

Tumors. The isolation and characterization of Tumors 66 and 168 from a mammary tumor which arose spontaneously in a female BALB/cfC3H mouse has been published previously (7). The 168cl tumor used in these experiments was cloned by the soft-agar technique (7) from Tumor 168. Tumor 410 was derived from a metastatic nodule in the lung of a BALB/cfC3H mouse carrying the tenth s.c. in vivo passage of the same tumor from which lines 66 and 168 were derived (10). Tumor 410 is immunogenic in both BALB/cfC3H mice and in the syngeneic BALB/c strain (13, 14). The designation 410.4 indicates the fourth transplant generation of the line 410 tumor. The D2 tumor arose from a transplanted D2 HAN line (11) in a BALB/c mouse. Tumors were grown in monolayer cultures by treatment with 0.25% trypsin-EDTA for 2 to 5 min and suspended in 0.9% NaCl solution to final concentrations for the proper delivery of tumor cell numbers in 10 µl volumes for i.fp. injections or in 100 µl for s.c. injections. Preliminary experiments indicated no difference in growth of tumor cells after s.c. injection in 100-µl versus 10-µl volumes. At 3 weeks of age, the mammary parenchyma were "cleared" from the inguinal fatpads as described previously (5, 8). Number 4 fatpads were used for injections of both cleared and intact fatpads, and the s.c. injections were done on the ventral surface of the flank at the level of the No. 4 fatpad. Both normal, intact inguinal mammary gland fatpads and gland-free (cleared) inguinal fatpads received injections of cells. Fatpad injections were with modified Hamilton syringes as described previously (6). Mice were examined twice weekly for 180 days to determine the incidence of tumor outgrowths and the mean latency period for growth. The results were expressed as "mean tumor-free days" which combines latency and incidence and is calculated as follows:

Mean tumor-free days = (No. of mice in which tumor grows) (mean latency)
+ (No. of mice tumor free at 180 days postinjection)

Total number of mice given injections

Transplantation of HANs. Samples (0.5 cu mm) of the nodule outgrowth line D2 were transplanted using fine forceps to force the transplant into the substance of the thickest part of the fatpad as described previously (5, 8) or placed s.c. The D2 HAN's were implanted into the center of fatpads of mice which had been cleared at 3 weeks of age and into intact fatpads of 3-week-old mice (at which time normal mammary ducts occupy 20% of the fatpad proximal to the nipple) and into intact fatpads of 8-week-old mice (in which mammary ducts occupy about 80% of the mammary fatpad). The mice were examined for tumors weekly for 14 months. At the end of 14 months, the mammary fatpads of mice without tumors were stained and examined for the amount of fatpad occupied by the nodule outgrowth as described (11).

Immunization and Challenge. Tumor 410 cells were implanted i.f.p. (5 x 10^3 cells) or s.c. (5 x 10^4 cells) into groups of animals, allowed to grow for a period of time, and then surgically removed. The higher dose of cells used for the s.c. injections reflects the preferential growth of the tumor in the fatpad. The cell numbers used for immunization were selected so that tumors in the 2 groups would have the same latency period and tumors would be of the same size at time of surgery.
Along with the tumor, the entire no. 4 fatpad, including the inguinal lymph node, was removed from each animal at surgery. A third group of normal animals received sham surgery consisting of the removal of the no. 4 fatpad and a piece of skin. Challenge was on the contralateral side, either s.c. (at doses of $5 \times 10^5$ or $5 \times 10^3$ cells) or i.fp. (at doses of $5 \times 10^3$ or $5 \times 10^2$ cells), 2 weeks after surgery. The different doses again reflect the site preference of the tumor; the higher dose at each site was selected to produce a 100% tumor incidence, and the lower doses ($5 \times 10^3$ s.c. and $5 \times 10^2$ i.fp.) was selected to produce a 50% tumor incidence. Mice were examined twice a week for palpable tumors.

**RESULTS**

**Growth of Tumors in Intact Fatpads versus s.c.**

Cells from all 4 four tumors grew better after injections into intact fatpads than after s.c. injections (Table 1). Tumor 66 grew at 100% incidence in intact fatpads with all doses tested (including 5 of 5 animals given injections of 500 cells) in 3 separate experiments. Tumor 66 cells injected simultaneously at s.c. sites produced tumors less frequently and with increased latency periods for those tumors that eventually resulted. This effect was most dramatic at the lowest tumor doses injected at which only 3 of 15 tumors grew. Similar results were obtained with the D2 tumor (Table 1, Experiment 4), the 168cl tumor (Table 1, Experiment 5), the no. 410 tumor (Table 1, Experiments 6 and 7), and the 410.4 tumor (Table 1, Experiment 8).

**Growth of Tumors in Intact versus Cleared Fatpads**

Tumors 66, D2, and 410 grew better in intact than in cleared fatpads (Tumors 168cl and 410.4 were not tested). This effect was seen consistently in 5 separate experiments (3 with Tumor 66 and one each with Tumors D2 and 410) but only at very low tumor cell doses. The tumors grew at a decreased incidence in cleared fatpads and displayed an increased latency period for those tumors which did arise in cleared fatpads as compared to the outgrowths from normal fatpads. The data from 3 experiments with Tumor 66 are summarized in Chart 1. In a single experiment, Tumor D2 also grew better in intact glands (4 of 5 tumors after injection of $10^3$ cells) than in cleared fatpads (0 of 5 tumors after injection of $10^3$ cells). In one experiment with Tumor 410, the mean size of 39 tumors, 15 days postinjection into intact fatpads, was 21.2 sq mm and the mean size of 18 tumors, 15 days postinjection into cleared fatpads, was 8.6 sq mm ($p < 0.001$ by Student’s $t$ test).

**Growth of D2 HAN**

It is commonly recognized that HAN implants generally fail to grow and undergo neoplastic transformation either s.c. or in intact fatpads of adult mice (5, 8). The data in Table 2, describing the growth and progression of D2 HAN implants in cleared fatpads, intact glands of 3-week-old and 8-week-old mice, and s.c., are presented to emphasize some less widely appreciated facts. A certain number of HAN implants do form tumors after transplantation either s.c. or into intact fatpads (the exceptional cases may represent tumors which had progressed before transplantation). If HAN’s are implanted into immature (3 week) intact glands, subsequent development of HAN and normal glandular elements are competitive, resulting in a partial filling of the fatpad with each tissue. The latter is the most compelling

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**Table 1**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Tumor 66</th>
<th>Tumor 168cl</th>
<th>Tumor 410</th>
<th>Tumor 410.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor cell dose</td>
<td>1 x 10^2</td>
<td>1 x 10^2</td>
<td>1 x 10^2</td>
<td>1 x 10^2</td>
</tr>
<tr>
<td>Intact fatpad s.c.</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>Statistical test</td>
<td>18 ± 4</td>
<td>13 ± 4</td>
<td>11 ± 3</td>
<td>11 ± 3</td>
</tr>
</tbody>
</table>

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- **a**: Number of animals in which the tumor grew/number of animals given injections of tumor cells at each site injected.
- **b**: Mean Latency ± S.E. is given for all groups in which 3 or more animals developed tumors. Otherwise, the individual latency periods are given or no calculation could be made if no tumors resulted in a group.
- **c**: Mean ± S.E.
- **d**: $p < 0.01$, Mann Whitney $U$ test.
- **e**: NC, no calculation.
- **f**: Not significant, $p > 0.05$.
- **g**: $p < 0.05$, Mann Whitney $U$ test.
- **h**: $p < 0.001$, $\chi^2$ test.
Tumor-associated Immunity in Intact Fatpads versus s.c. Sites

Afferent Response to Tumor in the Mammary Fatpad. Temporary tumor growth in the mammary fatpad renders the mice resistant to a subsequent s.c. injection of tumor cells on the contralateral side of the animal (Table 3). For example, in Experiment 2, a s.c. challenge dose of $5 \times 10^4$ cells produced a tumor in 12 of 12 sham-operated mice, in 0 of 10 mice immunized i.f.p., and in 1 of 8 mice immunized s.c. Thus, i.f.p. immunization is at least as effective as s.c. immunization. This fact is further supported by data in Table 4; temporary tumor growth in the mammary fatpad renders the mice resistant to a subsequent i.f.p. injection of tumor cells on the contralateral side of the animal.

Efferent Response to Tumor in the Mammary Fatpad. The data of Table 4 demonstrate that immunized animals reject tumor cells injected i.f.p. in Experiment 1, an i.f.p. challenge dose of $1 \times 10^4$ tumor cells grew in all sham-operated mice but in only 2 of 5 immunized mice. In a second experiment, an i.f.p. dose of $5 \times 10^3$ cells grew in 7 of 8 sham-operated and 2 of 15 immunized mice. An i.f.p. dose of $5 \times 10^2$ cells grew in 5 of 9 sham-operated mice but did not produce any tumors in immunized mice.

The ability of no. 410 tumor cells to induce an immune response (afferent response) in the mammary fatpad and the ability of systemic immunity to be expressed (efferent response), by suppression of Tumor 410 growth, in the mammary fatpad was found to be tumor specific by comparison with a syngeneic BALB/c rhabdomyosarcoma (3-methylcholan-threne-induced) in a ciss-cross fashion.

Tumor-associated Immunity in Intact Fatpads versus Cleared Fatpads

Both afferent and efferent responses to Tumor 410 are essentially identical in cleared and intact fatpads. Chart 2 illustrates a representative experiment demonstrating that immunization by temporary tumor growth in either intact or cleared fatpads renders mice equally resistant to a subsequent s.c. injection of tumor cells on the contralateral side of the animal. Immunized animals are able to resist tumor cells injected into either intact or cleared fatpads (Chart 3).

DISCUSSION

The ability of normal mammary gland elements to control the growth of normal hyperplastic outgrowths and preneoplastic characteristics of D2 HAN: site dependency

<table>
<thead>
<tr>
<th>Group</th>
<th>Site</th>
<th>Incidence of tumors</th>
<th>Mean % of fatpad filled by HAN</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cleared fatpad (3 wk)</td>
<td>12/20</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>Intact fatpad (3 wk)</td>
<td>8/20</td>
<td>70</td>
</tr>
<tr>
<td>3</td>
<td>Intact fatpad (6 wk)</td>
<td>1/20</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>s.c. (3 wk)</td>
<td></td>
<td>NA</td>
</tr>
</tbody>
</table>

* Samples (0.5 cu mm) of nodule outgrowth line D2 were transplanted into mammary fatpads or s.c.
* Number of animals developing tumors/number of animals implanted with D2 HAN.
* Mean percentage of fatpads occupied by nodule outgrowths in those mice which did not develop mammary tumors. The rest of the space was occupied by host ductal tissue.

By $x^2$ analysis, the probabilities that differences in incidence are due to chance are: Group 1 versus Group 2, $p > 0.05$; Group 1 versus Group 3, $p < 0.001$; and Group 1 versus Group 4, $p < 0.001$.

By $x^2$ analysis: Group 2 versus Group 3, $p < 0.001$; and Group 2 versus Group 4, $p < 0.001$.

NA, not applicable.
lesions was described by Faulklin and DeOme (8) over 20 years ago. These investigators found a growth-regulating system between normal glands, between normal glands and HAN’s, between HAN’s, and between normal glands and tumors, although the ability of the tumor to overcome the control mechanism appeared to them “to be the principal characteristic of neoplastic tissue.” Although Mintz and Slemmer (15, 20) described a synergistic effect between normal gland and HAN tissue on the growth of HAN, inhibition of the growth and neoplastic transformation of HAN’s by normal mammary gland has been described more frequently (5, 6, 8, 11, 12). The outgrowth of tumor from HAN’s has been regarded as representing a qualitative change from a cell type (HAN) which is regulated by normal mammary gland to a cell type (tumor) which is not. The present experiments suggest 2 levels of control of tumor growth in the mammary fatpad. Whereas HAN cells generally grow only in fatpads, not s.c., we show that tumors grow at both sites but prefer the fatpad site. These results suggest that normal mammary cells can enhance the growth of tumor cells rather than inhibit it and argue for a qualitative, rather than quantitative, difference between preneoplastic and neoplastic cell populations.

The mechanisms underlying the effect of transplantation site on the behavior of normal mammary gland, HAN cells, and tumor cells are not known. One obvious possibility for at least some of the effects would be the immunologically privileged nature of the fatpad site. That this is an unlikely mechanism for the present findings is suggested by the fact that tumors that are relatively poorly immunogenic (66 and 168) show the same site specificities as more immunogenic tumors (D2 and 410). In order to test the role of immunogenicity in site preference, we selected our most immunogenic tumor line, line 410 (13, 14), to determine if our observations could be due to the immunologically privileged nature of the mammary fatpad. The mammary fatpad was first reported to be a privileged transplantation site by Blair and Moretti (3) who found that male mammary tissue survived in cleared mammary fatpads of syngeneic female mice even though male skin grafts were rejected by the females. Attempts to immunize with either skin or mammary tissue implanted into fatpads were unsuccessful; however, male mammary tissue did not survive in fatpads of females preimmunized by s.c. implantation of either male skin or male mammary tissue. Similarly, Slemmer (19) found that i.d. inoculation of preneoplastic mammary lesions rendered mice resistant to the i.f.p. growth of the same preneoplastic lesion, but the i.f.p. implantation of the lesions did not induce transplantation resistance. Other investigators have reported relative transplantation privilege for sites other than mammary fatpads (reviewed in Ref. 2). Such privileges seem to be graft-tissue specific; thus, the i.p. site is privileged compared to s.c. for a syngeneic mouse lymphoma (4), but s.c. is privileged compared to i.p. for skin allografts in mice (9). The question of whether mammary tumor antigens are extended privilege in mammary fatpads is important for its implications to surveillance and mammary tumor development. Our results show that the differential growth of tumors in intact fatpads versus s.c. is probably not due to immunological privilege at the former site. Both immunization and challenge with no. 410 tumors were as effective in the intact fatpad as they were s.c. Our data also suggest that the preferential growth of tumors in intact mammary fatpads over cleared fatpads is not based on immunological differences.

Other types of mechanisms that may be responsible for our observations require additional consideration. Auerbach et al. (1) have suggested the “existence of developmental gradients that may influence growth of differentiating cells” to explain caudocephalic differences in s.c. transplantation sites. The existence of similar developmental gradients, possibly involved in tissue specific differentiation, might explain the preferential growth of mammary tumors described. Another mechanism by which mammary tissue might affect the growth of HAN’s and tumors is the production of soluble regulatory factors. Nicoll (17) attempted, unsuccessfully, to demonstrate a soluble factor.
which could control normal mammary ductal growth in vivo, although a stimulatory factor produced by mammary tumors has been demonstrated in vitro (16).

Tumor growth is dependent upon neovascularization and angiogenic factors are produced by tumor cells. Thus, one would expect tumors to grow best in sites well supplied with a vascular network which could rapidly respond with angiogenesis. The vascularization of a cleared and intact fatpad may be somewhat different, but Soemarwoto and Bern (21) found that the clearing procedure did not disrupt the circulation to the fatpad. It is well known that HAN’s grow in cleared fatpads, not in intact fatpads, but the surgical procedure of clearing does more than remove glandular tissue. No such surgical alterations distinguish intact fatpads of 3-week-old mice from 8-week-old mice. Thus, the differences in growth of HAN’s in intact versus 8-week fatpads argues that this site preference is dependent upon the presence of normal mammary gland tissue.

Recent evidence suggests that there are at least 2 levels of regulation of the growth of mammary lesions, one requiring intact glandular architecture (6) and one operating at a cellular level (12). DeOme et al. (6) found that dispersion and transplantation of apparently normal mammary glands of virgin mice resulted in an increased rate of HAN and tumor formation. Medina et al. (12) found that enzymatic dissociation of HAN tissue before transplantation increased tumorigenicity and that adding normal mammary gland cells countered the effect of dissociation.

Although the basis for the described site effects on tumor growth remains to be determined, their existence should encourage the use of sites more natural than s.c. for studies involving transplantation of mammary tumors. Investigation of growth and control of neoplasia must take into account the natural control mechanisms existent for tumors. For example, a much more vigorous therapeutic approach might be necessary to control a mammary tumor growing in a mammary fatpad than to control a mammary tumor growing at a s.c. site. Appreciation of site dependencies of tumors may be necessary for their optimal utilization in the experimental investigation of cancer.

REFERENCES


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