Immunology of Spontaneous Mammary Carcinomas in Mice
V. Acquired Tumor Resistance and Enhancement in Strain A Mice Infected with Mammary Tumor Virus

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Summary

Immunization of Strain A/Crgl female mice with living tissue and crude cell membrane preparations of recently arisen isogenic spontaneous mammary carcinomas conferred a marked degree of heightened reactivity against a subsequent challenge with the tumors. The heightened reactivity was manifested, under different experimental conditions, by either increased resistance or increased susceptibility to the tumor isografts. Increased tumor susceptibility could be transferred to normal isogenic hosts by means of serum from actively immunized animals and thus resembles the condition of immunologic enhancement. Experience with 1 tumor could evoke enhancement of another, indicating the existence of at least some cross-reacting antigens among spontaneous mammary carcinomas of this strain. Previous experience with living normal tissue or with normal cell membrane preparations of isogenic origin did not confer heightened tumor reactivity.

Introduction

Recent studies in our laboratory have shown that mice can acquire a state of specifically heightened resistance or susceptibility towards spontaneous mammary carcinomas, thus confirming the preliminary observations of other workers (18, 29, 30). Pretreatment with a methanol-insoluble fraction of tubercle bacilli which activates broadly the immunologic reactivity of laboratory animals (41) increased or decreased, depending on several experimental parameters, resistance to isografts of recently arisen spontaneous tumors, including mammary carcinomas (40). Injections of this fraction early in life reduced the incidence, and delayed the onset, of spontaneously arising mammary carcinomas (Weiss and Bonhag, to be published). A 1st experience with a spontaneous mammary carcinoma was found to heighten resistance or susceptibility of the original host to a subsequent challenge with that tumor (42, 43). Isografts of such tumors arising in C3H mice infected with the mammary tumor virus (MTV) were rejected by a significant proportion of MTV-free hosts of the same genotype previously immunized with living and killed tissue preparations of the corresponding tumors. This specifically acquired resistance could be transferred to normal isogenic animals by means of lymph node and peritoneal washing cells (4, 5, 39).

Further studies (23-25, 44) showed that at least some of the antigens associated with the specific immunogenicity of spontaneous mammary carcinomas of mice are already expressed in normal and preneoplastic mammary tissue, and that the immunogenicity of MTV-infected preneoplastic and neoplastic mammary tissues was considerably greater in hosts free of MTV but infected with the morphologically very similar, though biologically distinct, nodule-inducing virus (NIV) (31) than in mice infected with MTV at birth.

These findings suggested the hypotheses that at least some of the antigens associated with the immunogenicity of spontaneous mammary tumors of mice, and of the preneoplastic hyperplastic alveolar nodules (HAN), are possessed by the MTV or are included as a result of MTV-host-cell interaction. It remains entirely possible, however, that there exists in addition another category of antigens of spontaneous mammary tumors of mice, not associated with the presence or activities of the MTV, but developing in the process of progressive neoplasia per se. Some evidence for the existence of such antigen(s) comes from the observation of a definite, albeit limited, immunogenicity of tumors arising from MTV-free C3Hf HAN outgrowths in MTV-free C3H/2 hosts (42, 43).

In the present communication, experiments will be described in which the immunogenicity of MTV-infected mammary tumors was studied in isogenic, MTV-infected A/Crgl hosts. The results, which showed the tumors to be immunogenic, again demonstrate that immunization with neoplastic mammary tissue can evoke some degree of heightened immunologic reactivity even in a system where tumor donor and tumor host are of identical viral status.

Special attention was focused in the present study on the immunogenicity of a membrane-rich concentrate of the carcinoma cells. This emphasis arose from several considerations. Only antigenic changes occurring on the surfaces of cells are likely to be of importance in the rejection of such cells. That events of major importance in the transformation of normal to neoplastic cells do, in fact, occur at, or involve, the cell surface has recently been indicated by a number of investigators (1, 2, 36, 38), and the immunizing efficacy of tumor cell ghosts has
been demonstrated (17). Moreover, the MTV matures at the
cell surface (28, 34). This increases the likelihood of the occur-
rence of cell-surface alterations manifested by changes in anti-
genicitiy, and it has, indeed, been observed that the species-spe-
cific antigenicity of normal mammary parenchymal cells is al-
tered by infection with mammary tumor virus (Heppner and
Weiss, unpublished observations). In addition, the concentra-
tion of MTV on the surfaces of infected cells might make cell mem-
brane preparations strongly immunogenic with regard to anti-
gens associated with the MTV, provided, in this system, that
the presumed immunologic tolerance to such antigens in neo-
natally infected animals (29, 30) is broken as a result of immu-
nization in adult life (11).

Materials and Methods

MICE. All animals were of the A/Crgl strain (8). They were
maintained on a pellet diet (Diablo laboratory or Ralston
purina chow) and water ad libitum. The isogenicity of the strain
throughout the course of these experiments was confirmed by
the acceptance of 2nd set skin grafts exchanged between ran-
domly selected pairs of young adult females, as described pre-
viously (5, 43). In addition, in several experiments animals
which had been immunized with tumor tissue and proven re-
sistant to tumor challenge were found to accept skin grafts from
the autochthonous tumor host as readily as skin autografts.

TUMORS. Five mammary tumors, arising spontaneously in
multiparous breeding females, were employed; they were desig-
nated tumors A1 to A5. The adenocarcinomatous nature of the
tumors was confirmed by histologic examination. All tumors
were maintained in isologous female animals and were employed
no later than the 5th transplant generation; in most instances,
they were used in the 2nd or 3rd transplant generation.

TUMOR IMPLANTATION, BLEEDING, AND SERUM PREPARATION.
The same technic as described previously (5) were employed.

MEASUREMENT OF TUMOR VOLUME. The growth of tumors in
animals is often expressed in terms of increases in the measurable
tumor diameters. This is likely to give an inaccurate impression
of tumor development, however. The growth of a tumor must
be viewed as an increase in its mass. Assuming a fairly constant
relationship of mass to volume, tumor development can be ex-
pressed in terms of volume increment. A given increase in the
dimensions of a body results in a larger proportional increase in
its volume, and tumor development cannot be accurately de-
scribed, therefore, in terms of dimension increments.

In our experience, s.c. and fat-pad implants of spontaneous
mammary carcinomas grow roughly in the shape of spheroids. A

![Chart 1. Relation between the 2 measurable dimensions (a and b) and the volumes of spontaneous mammary carcinomas. The points represent the experimentally determined liquid displacement volumes of 121 newly arisen spontaneous mammary carcinomas. The line represents the slope of tumor volumes calculated from the equation, V = (0.4)(ab^2).](chart.png)
similar observation was recently reported by Perri et al., working with Jensen sarcoma in rats (33). These workers found, however, that neither the formula for oblate nor that for prolate membranes. 

The liquid (saline) displacement volumes of 121 different spontaneous mammary carcinomas of different sizes were ascertained. The \( ab^2 \) values, derived from measurements of the tumors \( \text{in situ} \) immediately before removal, formed a straight line passing through the point of interception on the \( y \) axis when plotted against the measured volumes, \( V_\text{a} \). The coefficient of this linear regression, \( m \), was calculated from the formula:

\[
m = \frac{\sum (V_\text{a})(ab^2)}{\sum (ab^2)^2}
\]

The value for \( m \) thus derived was 0.3986, and was converted for practical application to 0.4. The observed tumor volumes, \( V_\text{a} \), were very close to the calculated volumes, \( V \), derived from the equation (Chart 1):

\[
V = (0.4)(ab^2)
\]

It is thus apparent that the depth dimension of these tumors stands in constant relationship to the value \( b \). The formula \( V = (0.4)(ab^2) \) was accordingly used throughout these studies to obtain an approximation of tumor volumes.

**Preparation of Tumor Cell Concentrates Rich in Cell Membranes.** The method was similar to that employed by O’Neill and Wolpert (32) and Rajam and Jackson (35) and is outlined in Chart 2.

All procedures were carried out at 4°C. Tumor tissue was trimmed free of necrotic areas and sinuses, minced with fine scissors, and suspended 1 part in 10 of barbiturate-buffered saline (pH 7.2). The suspension was then homogenized for 2–3 min in a Waring Blender at low speed; the cup was surrounded by an ice-filled jacket. The homogenate was filtered through nylon, and the filtrate mixed with an equal quantity of a 1.2 M sucrose solution. Fifteen-ml aliquots of the resulting suspension were layered carefully over 30-ml quantities of a 0.7 M sucrose solution in 50-ml conical centrifuge tubes. The layered suspensions were then spun in a refrigerated centrifuge, employing a swinging bucket head at a maximum gravitational field of 1100–1200 \( \times \) \( g \) (\( R_{\text{max}} = 19.7 \text{ cm}; R_{\text{min}} = 8.7 \text{ cm}; \) cf. 9; the gravitational field was thus circa 600 \( \times \) \( g \) at the interface between the 2 sucrose solutions, and the radius from the interface, \( R_t = 10.5 \text{ cm} \). The top 15 ml of the supernatant were removed, pooled, and recentrifuged at 1600 \( \times \) \( g \) for 30 min. The fluffy sediment thus obtained was resuspended in buffered saline, centrifuged at 800 \( \times \) \( g \) for 10 min, washed twice with buffered saline, and recentrifuged at 600 \( \times \) \( g \). As will be described under “Results,” the pellet thus obtained was not a pure membrane preparation, but contained a variety of recognizable organelles and debris as well as structures taken to be membranes. It is therefore only for the sake of convenience that this material will be designated as “tumor cell membrane concentrate” (TCMC). The yield of TCMC was 1–2% by volume of the starting tumor tissue.

A “cytoplasmic” concentrate of tumor cells was prepared by centrifuging the tissue homogenate at 1800 \( \times \) \( g \) for 30 min. The sediment was discarded and the supernate, presumably containing much of the cytoplasmic contents of the cells, is designated “tumor cell cytoplasmic concentrate” (TCCC).  

Analogous normal tissue preparations were made from pooled mammary glands and pooled livers and kidneys of other A/Crgl virgin females. The fatty layers sometimes appearing on the surfaces of the centrifuge tubes after the 1st centrifugation of the sucrose homogenate were discarded. Preparations from the normal tissues are designated “normal cell membrane concentrate” (NCMC) and “normal cell cytoplasmic concentrate” (NCCC), respectively. The concentrates were always prepared immediately before use.

Both the membrane and the cytoplasmic concentrates were diluted in buffered saline to the desired concentrations, expressed as mg dry weight/ml. When the concentrates were administered in Freund’s adjuvant (10), equal volumes of the material and complete adjuvant were emulsified immediately before use.

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The method was similar to that employed by O’Neill and Wolpert (32) and Rajam and Jackson (35) and is outlined in Chart 2.
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STATISTICAL METHODS. The animals in all experiments were distributed randomly into the various treatment groups. The effect of treatment was ascertained in terms of differences in the numbers of animals developing progressive tumors from challenge tumor implants, and differences in the approximated tumor volumes at intervals after challenge. One-way analysis of variance (37) was applied to reveal significance of differences in calculated tumor volumes. Bartlett's test (37) indicated that the variance was heterogeneous in many of the experiments. A square-root transformation was applied to such data before variance analysis, thus reducing the heterogeneity; a higher degree of significance could sometimes be obtained by such transformation. The $\chi^2$ test was employed to determine significance of differences in tumor incidence.

Results are spoken of as "significant" only when the $P$ value of a comparison was 0.05 or less.

Results

THE NATURE OF THE CELL MEMBRANE CONCENTRATE. Examination of the crude tumor homogenate by phase contrast microscopy revealed a heterogeneous mixture, prominent in which were stranded structures which resembled folded membranes, and spherical bodies suggesting cell nuclei (Fig. 1). Examination of the final TCMC preparation revealed a predominantly membranous material, with few if any bodies which resembled nuclei (Fig. 2). Electron microphotographs of the TCMC are shown in Fig. 3. There was an abundance of membranous structures strongly suggestive of cell membranes and endoplasmic reticulum. Numerous type B particles, of identical appearance as those commonly identified as the MTV, could also be seen. Although considerable quantities of other cellular constituents were also present, as well as unidentifiable debris, nuclear elements were not apparent. The absence of nuclear material was also indicated by the failure to detect DNA by means of the diphenylamine reaction (12).

Phase contrast and electron microscopy of NCMC showed a very similar appearance. Examination of the cytoplasmic concentrates derived from homogenates of tumor and normal tissues showed a variety of granular structures resembling mitochondrial and microsomal elements, but did not reveal the presence of membranous or nucleoid bodies.

ACTIVELY INDUCED RESISTANCE. EXPERIMENT 1. In an exploratory experiment, the tumor-protective effect of a previous experience with living tumor alone was ascertained. Ten- to 16-week-old virgin females were given an s.c. implant of carcinoma A1 in the ventral surface of the right hind foot. All the tumors grew. Eighteen days later, the tumors had reached a mean calculated volume of circa 200 cu mm. The tumor-bearing foot was amputated, and 6 weeks later the animals were challenged with 5 x 10^3 living tumor cells placed s.c. in each inguinal mammary area. A group of 10 normal control animals of the same age, whose right hind feet were removed 6 weeks previously, were similarly challenged at the same time.

Tumors appeared at 1 or both sites of challenge implantation in every animal within 6 weeks. As shown in Chart 3, the tumors developed much more slowly in the tumor-pretreated group, however.

EXPERIMENTS 2, 3, AND 4. A study was now undertaken to ascertain the resistance-inducing properties of TCMC when administered in several sequential injections, the 1st administration being in complete Freund's adjuvant. The tumor employed for the TCMC preparation and for the challenge implantation in these experiments was the same mammary carcinoma, designated A5. In Experiments 2 and 3, groups of 16-week-old virgin females were given an i.p. injection of 0.4 ml containing 0.4 mg of TCMC in complete Freund's adjuvant. The animals received a 2nd s.c. injection of 0.2 mg TCMC in 0.4 ml saline in the axillary area 14 days later. Seven days thereafter, they were given a 3rd s.c. injection of 0.1 mg of TCMC in 0.4 ml saline in the axillary area. The animals in Experiment 3 received 2 additional s.c. administrations of 0.1 mg TCMC in the axillary area 7 and 14 days later. In experiment 4, virgin females of the same age were given an initial i.p. injection of 0.2 mg TCMC in 0.4 ml complete Freund's adjuvant, followed by 4 s.c. injections in the axillary area of 0.1 mg TCMC each in saline, administered on Days 20, 34, 56, and 87 after the 1st treatment.

Groups of similar animals were given identically spaced injections of NCMC in the same excipients. Ten days after the last treatment, all animals were challenged with 5 x 10^3 viable tumor cells injected s.c. in each inguinal mammary-gland area. This number of cells constitutes a small implant inoculum and does not usually produce tumors in 100%
of the recipients. The animals were observed for a period of 10 months, in view of the finding that tumors sometimes arise very late following inoculation with small numbers of cells (Weiss and Faulkin, unpublished data). The incidence of tumors developing from the challenge implants in the different groups of animals is shown in Table 1 (A).

As seen from Table 1(A), similar results were obtained in all 3 experiments: Repeated injection of TCMC lowered markedly the proportions of animals developing tumors following a subsequent challenge. When the 3 experiments are considered together, the comparison between TCMC- and NCMC-treated animals reveals a striking difference: only 2 of 35 TCMC-treated animals developed tumors (5.7%) as against 15 of 23 NCMC-pretreated ones (65.2%). The P value for this difference is <0.01.

EXPERIMENT 5. The effect of Freund’s adjuvant as the excipient of the TCMC in the 1st injection was investigated in a parallel experiment. Two groups of 9 16-week-old virgin females each were given a series of injections of either TCMC prepared from tumor A5, or NCMC in the quantities and schedule described for Experiment 2, except that the 1st injections were also in saline rather than in adjuvant. The animals were then challenged with living tumor cells at the same time, and with the same tumor cell preparation as were the animals in Experiments 2-4. The results are presented in Table 1 (B).

It is seen from Table 1 (B) that there was no difference between the TCMC- and NCMC-pretreated animals in tumor incidence. This observation points to the importance of employing Freund’s adjuvant with at least the 1st injection of TCMC in order to elicit a marked degree of heightened tumor resistance.

ACTIVELY INDUCED ENHANCEMENT. The results of Experiments 1-5 show that Strain A mice acquire a considerable resistance to tumor isografts as a result of a previous experience with either that tumor or with a membrane-rich concentrate of the tumor, administered once in Freund’s adjuvant and several times further without adjuvant. In a series of simultaneous experiments, the effect of experience with a living tumor plus additional treatment with the membrane concentrate was investigated.

The animals were 15- to 23-week-old virgin females. The scheme of the experiments was the following: A piece of tumor, or of normal mammary gland and fat pad taken from another virgin female, was placed s.c. into the ventral area of the right hind foot. When the tumors reached a size of circa 200 cu mm—usually 3-4 weeks after implantation—the tumor-bearing limb was amputated at the shank, and the limbs of the control animals carrying the normal mammary implant were removed similarly. Three to 4 weeks later, the animals received an i.p. injection of TCMC, NCMC, TCCC, or NCCC in 0.4 ml complete Freund’s adjuvant. Ten to 13 days thereafter, the animals were challenged with suspensions of living tumor cells or with tumor fragments.

EXPERIMENT 6. Animals, in groups of 6-10 each, received 0.1-mg quantities of TCMC or TCCC, or of NCMC or NCCC, after a previous experience with a living tumor or with a normal mammary isograft, respectively. Animals in a 5th group were given adjuvant alone after an initial tumor experience. The animals of all 5 groups, plus those of an additional group of normal, untreated controls, were then challenged with fragments of tumor tissue implanted into each inguinal mammary fat pad. The tumor employed for immunization as well as for challenge was tumor A1.

The animals pretreated with living tumor and TCMC behaved differently towards the challenge tumor implants than did those of all other groups. At the 1st time of observation, 16 days after challenge, 15 of 20 (75%) implants in the 10 animals vaccinated with TCMC after a living tumor experience had grown to frankly palpable tumors, as compared with only 19 of a total of 41 (46%) animals comprising the other groups (P = 0.05). All tumor implants developed subsequently in all the animals, but as is seen from Chart 4, their development was facilitated in the animals pretreated with living tumor and TMC. The tumors in the other groups of pretreated animals developed at very similar rates, somewhat, but not significantly, less rapidly than those in the untreated controls. This similar degree of slight retardation, regardless of whether the tissue concentrate experience was with tumor or with normal cells, may have accrued from the protective effects of the living tumor experience and/or the killed
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Chart 4. Development of a spontaneous Strain A mammary carcinoma in isogenic animals pretreated with both living and killed tumor or normal tissue preparations. Each point on the curves represents the mean of the calculated tumor volumes in each group of animals. O, animals immunized with living tumor + TCMC in adjuvant; •, animals immunized with living tumor + TCCC in adjuvant; ■, animals immunized with living tumor + adjuvant only; Δ, animals immunized with normal mammary tissue + NCMC in adjuvant; ▲, animals immunized with normal mammary tissue + NCCC in adjuvant; □, untreated controls.

The calculated tumor volumes 50 days after challenge were also significantly greater in the animals immunized with tumor plus TCMC, and their survival time was shorter (Table 2).

Experiment 7. The effect of dosage of TCMC, administered after a previous experience with living tumor, was next investigated. Groups of 10 animals each received an implant of tumor A1 into the foot. Following growth and amputation of the tumor graft, the animals received injections of TCMC of that tumor ranging from 0.005 to 1.215 mg, in complete Freund's adjuvant. One group of animals was given adjuvant only following the living tumor immunization. The animals were challenged with \(5 \times 10^4\) viable tumor cells injected into each inguinal mammary fat pad.

Tumors arose in all implantation sites. As seen from Chart 5 and Table 3, significant enhancement of tumor development occurred at the dose level of 0.135 mg. Some enhancing effect was also observed when the dose was 0.045 mg, but not with smaller quantities. Quantities larger than 0.135 mg had no obvious effect, although there was a slight suggestion of retardation of tumor growth in the animals given the largest amount, 1.215 mg.

Experiment 8. This experiment was designed to determine whether immunization with 1 tumor could enhance the growth of another, i.e., whether different spontaneous mammary carcinomas share common tumor antigens, assuming that the phenomenon of enhancement here described is indeed of an immunologic nature.

Groups of 8 animals each were immunized with living tumor grafts or with normal mammary gland. After removal of the limb carrying the implant, the animals received respective i.p. injections of 0.1-mg quantities of TCMC made from the corresponding tumor, or of NCMC, in Freund's adjuvant. Three tumors—A2, A3, and A4—were used in this experiment. Tumor challenge was by s.c. injection of suspensions of tumor cells into each inguinal mammary fat pad.

Table 2

Effect of Immunization with Living Tumor Plus Tumor Cell Membrane Concentrate on Resistance of Strain A Mice to Subsequent Tumor Challenge

<table>
<thead>
<tr>
<th>Immunization*</th>
<th>No. of animals/ group</th>
<th>Mean of calculated tumor volumes 50 days after challenge implantation (ml)</th>
<th>Mean survival time after challenge (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor tissue</td>
<td>TCMC + adjuvant</td>
<td>10</td>
<td>7.1 ± 0.8(d)</td>
</tr>
<tr>
<td>Tumor tissue</td>
<td>TCCC + adjuvant</td>
<td>10</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>Tumor tissue</td>
<td>Adjuvant only</td>
<td>9</td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td>Normal mammary</td>
<td>NCMC + adjuvant</td>
<td>6</td>
<td>3.2 ± 0.5</td>
</tr>
<tr>
<td>Normal mammary</td>
<td>NCCC + adjuvant</td>
<td>6</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>10</td>
<td>4.1 ± 0.8</td>
</tr>
</tbody>
</table>

* See text for details.

\(d\) Day 50 after challenge was the day of termination of the experiment.

TCMC, tumor cell membrane concentrate; TCCC, tumor cell cytoplasmic concentrate; NCMC, normal cell membrane concentrate; NCCC, normal cell cytoplasmic concentrate.

S.E.
Immunology of Mammary Carcinomas.

Each inguinal area. Groups of animals immunized with the tumors or with the normal tissue were challenged with the corresponding tumors or with one of the other 2 tumors. In addition, groups of control animals that had been injected only with saline into the hind foot, and, subsequent to amputation, with Freund's adjuvant alone, were challenged with the 3 tumors. Because tumor A3 was slow growing, a large challenge inoculum, \(5 \times 10^6\) living cells at each site, was employed; the animals challenged with the other 2 neoplasms received only \(5 \times 10^3\) cells in each inguinal area.

All 3 mammary carcinomas were capable of causing enhanced growth of 1 or more of the tumors. Tumor A4 was significantly enhanced by pretreatment of the mice with either the corresponding tumor or with 1 of the others (Chart 6 and Table 4). In contrast, growth of tumor A3 was not affected by pretreatment of the animals with tumors A3 and A4, and only slightly by pretreatment with tumor A2 (Chart 7 and Table 4). It is thus

**TABLE 3**

**EFFECT OF DOSE OF TUMOR CELL MEMBRANE CONCENTRATE ON TUMOR ENHANCEMENT IN STRAIN A MICE**

<table>
<thead>
<tr>
<th>Immunization(^a) (in Freund's adjuvant)</th>
<th>Means or calculated tumor volumes 45 days after challenge implantation (ml) (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor cell membrane concentrate, 0.005 mg</td>
<td>2.9 ± 0.5 (^c)</td>
</tr>
<tr>
<td>Tumor cell membrane concentrate, 0.015 mg</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>Tumor cell membrane concentrate, 0.045 mg</td>
<td>4.0 ± 0.5</td>
</tr>
<tr>
<td>Tumor cell membrane concentrate, 0.135 mg</td>
<td>5.5 ± 0.7</td>
</tr>
<tr>
<td>Tumor cell membrane concentrate, 0.405 mg</td>
<td>2.9 ± 0.7</td>
</tr>
<tr>
<td>Tumor cell membrane concentrate, 1.215 mg Adjuvant only</td>
<td>2.3 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>3.0 ± 0.5</td>
</tr>
</tbody>
</table>

\(^a\) All animals received an initial immunization with living tumor tissue; see text for details.

\(^b\) Day 45 after challenge was the day of termination of the experiment.

\(^c\) S.E.

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**Chart 5.** Development of a spontaneous Strain A mammary carcinoma in isogenic animals pretreated with a living tumor graft and with different quantities of the tumor cell membrane preparation (TCMC). Each point on the curves represents the mean of the calculated tumor volumes in each group of animals. ▲, animals immunized with living tumor + 0.005 mg TCMC in adjuvant; Δ, animals immunized with living tumor + 0.015 mg TCMC in adjuvant; ●, animals immunized with living tumor + 0.045 mg TCMC in adjuvant; ○, animals immunized with living tumor + 0.135 mg TCMC in adjuvant; ▼, animals immunized with living tumor + 0.405 mg TCMC in adjuvant; ▼, animals immunized with living tumor + 1.215 mg TCMC in adjuvant; ■, animals immunized with living tumor + adjuvant only.

**Chart 6.** Cross enhancement. Growth of tumor A4 in isogenic animals pretreated with living tissue and cell membrane concentrate derived from either tumor A4, tumors A2 or A3, or normal tissue, or pretreated with saline and adjuvant only. Each point on the curves represents the mean of the calculated tumor volumes in each group of animals. ○, animals immunized with tumor A2 preparations; △, animals immunized with tumor A3 preparations; □, animals immunized with tumor A4 preparations; ●, animals immunized with normal tissue preparations; ■, animals immunized with adjuvant only.
seen that a tumor capable of enhancing another one is not neces-
sarily one against which enhancement is readily manifested. It
was also found that a tumor capable of enhancing the growth
of another one could, in turn, only be enhanced by pretreatment
with itself. Thus, tumor A2 enhanced the growth of tumor A4
(Chart 6) but was itself enhanced significantly only by pre-
exposure to its own tissue (Chart 8 and Table 4).

PASSIVELY INDUCED ENHANCEMENT. Experiments were under-
taken to ascertain whether immunization with TCMC results in
the production of humoral factors possessing immunologic reac-
tivity. It was found that the sera of animals immunized both
with a living tumor graft and with TCMC (and showing en-
hanced tumor susceptibility upon subsequent challenge with
tumor isografts) conferred such heightened susceptibility upon
normal hosts, indicating thereby the immunologic nature of the
phenomenon (20, 22). These results have already been com-
municated briefly (3) and will be reported in detail elsewhere.
The results with sera of animals in whom a state of heightened
resistance was produced by tumor immunization are described
here.

Groups of 14 or 15 males each, 3-4 months old, were given
i.p. injections of 0.1 ml of pooled serum obtained from the ani-
mals which were immunized by repeated injections of TCMC
from tumor A5 (Experiments 2-5). Control groups received sera
from the animals which had been treated with NCMC. Six hr
after receiving the serum, the recipients were challenged by s.c.
injections, in each inguinal area, of 5 × 10^4 living cells of tumor
A5. The results are shown in Chart 9 and in Tables 5 and 6.

It is seen from Chart 9 and from Table 5 that 0.1-ml quanti-
ties of pooled serum from animals immunized initially with
TCMC in adjuvant and subsequently with TCMC alone pas-
sively transferred a significant degree (P = >0.01<0.05) of
tumor enhancement. In contrast, the serum of animals whose
initial TCMC vaccination was not given in adjuvant failed to
induce enhancement (Table 6). As was shown above (Table 2),
only the animals whose 1st TCMC injection was with adjuvant
proved to be tumor resistant upon challenge. The present find-
ings thus indicate that the sera of mice which had acquired a
state of heightened tumor resistance nonetheless contain factors,
presumably antibodies directed at tumor-associated antigens,
which are capable of conferring a degree of tumor enhancement.

It was found in further experiments that sera from animals
with acquired tumor resistance could confer either tumor en-
hancement or resistance on normal recipients, depending on the
quantity of serum employed. Thus, pooled serum which bestowed
enhancement when 0.1 ml was given prior to challenge with
5 × 10^4 tumor cells conferred some resistance when 0.4 ml was
given before challenge with 5 × 10^3 tumor cells (4). This ob-
servation is also in keeping with the known properties of enhanc-
ing sera (20, 22).

Discussion

The present findings provide further evidence for the specific
immunogenicity of newly arisen spontaneous mammary carci-
nomas of mice: The specific acquisition of heightened tumor re-

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**Table 4**

CROSSENHANCEMENT: EFFECT OF PRETREATMENT OF STRAIN A MICE WITH
1 TUMOR ON GROWTH OF ANOTHER

| TISSUE EMPLOYED FOR IMMUNIZATION | CHALLENGE | NO. OF ANIMALS/ GROUP | NO. OF TUMORS/ IMPLANTS | MEANS OF CALCULATED TUMOR VOLUMES ON TERMINATION DAY
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mammary tissue</td>
<td>Tumor A2</td>
<td>2 × 10^4</td>
<td>8</td>
<td>13/16</td>
</tr>
<tr>
<td>Normal mammary tissue</td>
<td>Tumor A3</td>
<td>2 × 10^4</td>
<td>8</td>
<td>11/16</td>
</tr>
<tr>
<td>Normal mammary tissue</td>
<td>Tumor A4</td>
<td>2 × 10^4</td>
<td>7</td>
<td>11/14</td>
</tr>
<tr>
<td>Normal mammary tissue</td>
<td>Normal</td>
<td>2 × 10^4</td>
<td>5</td>
<td>10/10</td>
</tr>
<tr>
<td>Normal mammary tissue</td>
<td>Adjuvant</td>
<td>2 × 10^4</td>
<td>8</td>
<td>9/16</td>
</tr>
<tr>
<td>Normal mammary tissue</td>
<td>Tumor A2</td>
<td>2 × 10^4</td>
<td>7</td>
<td>14/14</td>
</tr>
<tr>
<td>Normal mammary tissue</td>
<td>Tumor A3</td>
<td>2 × 10^4</td>
<td>8</td>
<td>16/16</td>
</tr>
<tr>
<td>Normal mammary tissue</td>
<td>Tumor A4</td>
<td>2 × 10^4</td>
<td>8</td>
<td>16/16</td>
</tr>
<tr>
<td>Normal mammary tissue</td>
<td>Adjuvant</td>
<td>2 × 10^4</td>
<td>5</td>
<td>10/10</td>
</tr>
<tr>
<td>Normal mammary tissue</td>
<td>Tumor A4</td>
<td>2 × 10^4</td>
<td>8</td>
<td>16/16</td>
</tr>
<tr>
<td>Normal mammary tissue</td>
<td>Adjuvant</td>
<td>2 × 10^4</td>
<td>6</td>
<td>12/12</td>
</tr>
</tbody>
</table>

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* See text for details.

**Discussion**

The present findings provide further evidence for the specific
immunogenicity of newly arisen spontaneous mammary carci-
nomas of mice: The specific acquisition of heightened tumor re-

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Mohammed A. M. Attia and David W. Weiss
Resistance or heightened tumor susceptibility as a result of a previous tumor experience is now seen to occur in still another strain of inbred mice, A/Crgl. In addition, the present study reveals the specific immunogenicity of a tumor cell concentrate rich in cell membranes.

The isogenicity controls included in this, as well as in the preceding studies in this series of communications, preclude the possibility that the acquired resistance or enhancement accrues from a residual heterozygosis of normal isoantigens. The immunizing capacity of spontaneous mammary carcinomas must arise, therefore, from their content of antigens specific to the neoplastic state and/or from antigens associated with the presence of the etiologic viral agent.

Strong evidence for the existence of antigen(s) of the latter category has already accrued (23–25, 44). The findings reported in the present communication show that some degree of immunogenicity is manifested by mammary tumors even though the hosts as well as the immunizing and challenging tumor tissues are identically infected with MTV. It is not yet clear, however, whether the immunogenicity of the tumors under such conditions indeed indicates the presence of tumor-specific antigens independent of the MTV, or whether it is accounted for by the development of some degree of immunologic reactivity towards antigens associated with the MTV despite infection of the animals from birth. A breaking of immunologic tolerance has been demonstrated for other antigens and has already been invoked as a possible means of developing experimentally antitumor reactivity in autochthonous hosts (11). (It has also been shown that the sera of some mice infected at birth with MTV nonetheless contain antibody specifically reactive with the MTV (9).)

The finding of some cross reactivity among several of the tumors studied could be taken as support for this explanation. It is also possible, however, to view such cross reactivity as reflecting a related chemical structure of MTV-independent antigens characteristic of this type of neoplasm. Moreover, the failure of normal, though MTV-infected, tissue preparations to induce heightened tumor reactivity might seem to argue against the MTV-associated nature of all the antigens of spontaneous mammary carcinomas. However, this argument, too, fails to convince: It is quite possible that neoplastic tissue contains larger quantities of MTV-associated antigen(s), or that these are present in an immunogenically more reactive state in neoplastic tissue.

Further experiments have been initiated to answer the question of the nature of the antigen(s) involved in the development of immunologic reactions of MTV-infected hosts against MTV-
infected mammary tumors. Whatever this answer will be, it is clear that tumors initiated by the action of oncogenic viruses, even where transmission of the virus is vertical, need not be considered a priori as immunologically unreactive in isogenic, and presumably in autochthonous, hosts. Preliminary results obtained in other experiments currently in progress have indeed indicated that C3H mice can develop some resistance against autochthonous mammary tumors in situ as a result of hyperimmunization with tissue preparations derived from each tumor (Vaage and Weiss, to be published).

Immunologic activity could be demonstrated for the tumor cell membrane concentrate only when the 1st of several immunizing injections was administered in complete Freund's adjuvant. The requirement for the adjuvant does not necessarily indicate a weak immunogenicity of the tumor-associated antigen(s) in this system, because mice quite often fail to develop pronounced immunologic reactions unless antigens are administered in adjuvants or are given repeatedly. It is also possible that the adjuvant has a function here other than prolonged retention of antigen or the incitement of a marked cellular response at the site of antigen deposit (14): Mice of strains which carry the mammary tumor virus are probably immunologically unresponsive, or partially so, to antigens possessed by the virus or controlled by the viral genome (23, 24, 29, 30, 44). If all or most of the antigens responsible for acquired resistance or enhancement to spontaneous mammary carcinomas are of this category (23-25, 44), it may be very difficult to evoke an immunologic response against them by the usual technics. As has been suggested by Cinader (11), such a response might be achieved, however, by breaking the immunologic unresponsiveness through immunization with antigens chemically related to the ones towards which the unresponsiveness is manifested. Such related antigens can be produced by a partial denaturation or other mild chemical change of the original substance. Freund's adjuvant, which contains a powerful surface active agent, could conceivably bring about such a change in antigenicity.

Tumor challenge in the present experiments was either with small numbers of living tumor cells in suspension or with small tumor fragments. It is not unlikely that more massive challenge would have obscured or reduced the considerable degree of tumor resistance and enhancement obtained by tumor tissue vaccination. One reason for the past failure of some other workers to detect a significant degree of acquired immunologic reactivity to spontaneous mammary carcinomas may, indeed, lie with their use of challenge inocula so large as to overwhelm acquired immune defenses (other possible reasons for this failure are discussed elsewhere (5, 23, 24, 44)).

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**Table 5**

<table>
<thead>
<tr>
<th>Immunization of donor animals (1st injection in adjuvant)</th>
<th>No. of recipient animals</th>
<th>No. of tumors/implants</th>
<th>Means or calculated tumor volume 2 mo. after challenge (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor cell membrane concentrate</td>
<td>15</td>
<td>29/30</td>
<td>1.0 ± 0.2*</td>
</tr>
<tr>
<td>Normal cell membrane concentrate</td>
<td>15</td>
<td>24/30</td>
<td>0.4 ± 0.2</td>
</tr>
</tbody>
</table>

* See text for details.

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**Table 6**

<table>
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<tr>
<th>Immunization of donor animals (1st injection in saline)</th>
<th>No. of recipient animals</th>
<th>No. of tumors/implants</th>
<th>Means or calculated tumor volume 2 mo. after challenge (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor cell membrane concentrate</td>
<td>15</td>
<td>26/30</td>
<td>0.6 ± 0.1*</td>
</tr>
<tr>
<td>Normal cell membrane concentrate</td>
<td>14</td>
<td>24/28</td>
<td>0.9 ± 0.1</td>
</tr>
</tbody>
</table>

* See text for details.

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**Chart 9.** Passive transfer of enhancement. Growth of tumor A5 in isogenic animals pretreated with serum from donors immunized with tumor or normal cell membrane concentrate administered in Freund's complete adjuvant. Each point on the curves represents the mean of the calculated tumor volumes in each group of animals. O, animals treated with serum from donors immunized with TCMC; •, animals treated with serum from donors immunized with NCMC.
Experience of Strain A mice with either a living tumor graft or with the tumor cell membrane concentrate resulted in heightened tumor resistance, whereas an experience with both the living tumor tissue and the membrane concentrate enhanced the growth of the challenge tumor implants in the present experiments. It appears from this, and from the preceding experiments (5, 23, 24, 43, 44) that both the resistance and the enhancement produced by immunization with spontaneous mammary carcinomas are of immunologic nature. Available evidence suggests that immunologic rejection of solid grafts and of cell suspensions of certain tissues is brought about primarily by the activity of sensitized cells (6, 7) and can also be effected, under certain circumstances, by large amounts of free antibody (15, 27). Immunologic enhancement, in contrast, is caused by the action of moderate quantities of antibody (15, 27). Whether antigenic stimulation with a given foreign tissue will result, in a particular animal, in the development of resistance or enhancement depends on a number of variables—dose and route of administration of antigen; nature, quantity, and site of implantation of the test tissue; length of vaccination-challenge interval (16, 20, 26, 27)—and the simultaneous presence in an immunized animal of resistance and enhancing factors to antigenically related foreign tissues has been clearly shown by other workers (13, 16, 21) and is again demonstrated here. Immunologic resistance and immunologic enhancement are thus seen to stand in a very labile relationship to each other, and it is of considerable experimental, and clinical, importance to define precisely the conditions which will favor the manifestation of one or the other. It would be tempting, therefore, to conclude that dual immunization with the living cells and the membrane concentrate of the tumors here studied constitutes a condition favorable for the manifestation of enhancement, whereas vaccination with either preparation alone results in an equilibrium of immunologic factors making for resistance. This conclusion is not yet justified, however, because other variables existed in the design of the experiments in which the phenomena of resistance and enhancement were respectively found. Work is in progress to clarify the experimental parameters which govern the nature of the outcome of immunization with spontaneous mammary carcinomas, and also to determine whether some such tumors favor the one, while others tend to favor the other, of these manifestations of the immune response.

The narrow dose range over which the tumor cell membrane concentrate (administered after a previous living tumor experience) could induce enhancement also indicates the necessity of an empirical approach to the characterization of the circumstances requisite for the development of this aspect of the immune response. The failure of quantities of the concentrate much greater than 0.1 mg to evoke either marked enhancement or marked resistance when given subsequent to a living tumor experience could well have resulted from a neutralization of resistance-promoting (cellular) and enhancement-promoting (humoral) factors, or from the induction of a state of immunologic paralysis.

In 1 experiment, animals immunized with a living tumor implant and subsequently treated with tumor cell cytoplasmic or normal cell preparations in Freund's complete adjuvant, or with the adjuvant alone, exhibited a lesser degree of tumor resistance than did animals in other experiments vaccinated with living tumor only. The difference in results could perhaps be accounted for by unrecognized differences in the experimental conditions existing at the times of the several experiments, but it may also have arisen from an ill-timed employment of the adjuvant. It has recently been demonstrated that the time of administration of endotoxin adjuvant in relation to the time of antigenic stimulus determines what effect is exerted by the adjuvant (A. Johnson, personal communication, 1964), and the possibility that Freund's adjuvant may have an adverse effect on immunologic responsiveness has also been indicated (19).

Acknowledgments

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Fig. 1. Phase contrast microphotograph of tumor tissue homogenate before separation of the cell membranes. Prominent are starded structures resembling cell membranes and spheric bodies which are probably nuclei. × 250.

Fig. 2. Phase contrast microphotograph of a tumor cell membrane concentrate preparation. The nuclear bodies seen in Fig. 1 are no longer visible in this preparation. × 250.
Fig. 3. Electron microphotographs of a tumor cell concentrate preparation. The preparation was rich in membranous structures, but still contained quantities of other cell constituents and debris. Discrete particles usually identified as the mammary tumor agent are seen clearly in Fig. 3b, X 30,000.
Immunology of Spontaneous Mammary Carcinomas in Mice: V. Acquired Tumor Resistance and Enhancement in Strain A Mice Infected with Mammary Tumor Virus

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