Generation of Immunoprotection Against Squamous Cell Carcinomas by *In Vitro* Cultivation and a Possible Mechanism of Action[1]

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ABSTRACT. **The immunogenicity of individual diethylnitrosamine (DEN)-induced forestomach carcinomas in female BALB/c mice was investigated following *in vitro* and *in vivo* cultivation. Of the five transplantable tumor lines studied, (DEN_1, DEN_3, DEN_6, DEN_8, and DEN_9) only two (DEN_6 and DEN_8) showed some degree of immunogenicity. DEN_1, DEN_3, and DEN_9 were highly tumorigenic with very little immunogenic potency as judged by tumor transplantation-excision assay, Winn neutralization, and antibody binding tests. These three tumors grew rapidly and showed a high degree of malignancy. DEN_1 and DEN_3 also metastasized readily. Cell lines from DEN_6 and DEN_9 lost their tumorigenicity at the 5th and 50th passage of culture, respectively. Although DEN_1 and DEN_3 did not lose their tumorigenicity, the number of tumor cells required to produce tumors increased substantially and their ability to metastasize was lost. Tumor transplantation studies, with these cultured cell lines in normal and x-irradiated recipients, suggested that the decrease in tumorigenicity may be immunologically mediated. Mice immunized with the *in vitro* lines demonstrated transplantation resistance against the respective *in vitro* and *in vivo* lines. The treatment of *in vivo* or *in vitro* propagated cells with periodic acid or neuraminidase enhanced antigen-antibody binding significantly. The effect of these chemicals became less pronounced as *in vitro* culture continued. It appears that during *in vivo* cultivation the antigenic determinants are masked or modulated by some glycoprotein or glycolipid molecules which render them non-, or very weakly, immunogenic.**

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INTRODUCTION

Multiple factors appear to be involved in the control and development of oncogenic diseases. Among these factors, elements of immune response undoubtedly play an important role in some tumor systems. However, for the immune mechanisms to be effective in controlling oncogenic diseases, tumors must express appropriate tumor-associated antigen(s). The presence of tumor-associated antigen(s) is common on cell surfaces of tumors induced by chemical carcinogens or oncogenic viruses (North 1984, Schreiber et al. 1988). Such antigen(s), however, has not been demonstrated as a common feature of many spontaneously arising tumors (Hewitt et. al. 1976, Klein and Klein 1977). For this reason, many attempts have been made using different chemicals (Bonmassar et al. 1970, Contessa et. al. 1981, Frost et. al. 1984, Ishikawa et. al. 1987), viruses (Kobayashi et. al. 1969, Boon 1983, Altevogt 1986, Fearon et. al. 1988), mutagens (Boon 1983), and factors such as cholesterol derivatives (Ludes et al. 1990) and interleukin 6 (Mullen et al. 1992), to enhance immunogenicity and to reduce tumorigenicity of some malignant tumor cells.

In some of the previous investigations, we reported that with increased *in vitro* passage, the immunogenicity of several respiratory squamous cell carcinomas (that is, the ability of tumor cells to induce cellular and humoral immunity in syngeneic hosts) was increased while their tumorigenicity decreased (Jamasbi and Nettesheim 1977, 1979). Similar observations have also been reported by other investigators using different tumor models (Ossowski and Reich 1980, Correll et. al. 1983, Yamashina et. al. 1986, Chiba et. al. 1987). Although some speculations concerning the mechanism(s) of increased immunogenicity of cultured cell lines have been made, the exact nature of this phenomenon remains unknown.

The main objectives of the present investigation were to study the biological and immunological characteristics of five different mouse forestomach carcinoma lines and to determine whether the tumorigenicity and immunogenicity of some of the highly malignant tumor lines could be altered by *in vitro* cultivation. If so, efforts would be made to determine whether the underlying mechanism(s) can be elucidated.

MATERIALS AND METHODS

Animals

Syngeneic BALB/c female mice, 8-12 weeks of age were used throughout these experiments. They were bred and maintained in a conventional animal facility and had free access to food and water.

Tumors

Five forestomach carcinoma lines were used. These tumors developed in the forestomach of female BALB/c mice which had received DEN in their drinking water for eleven weeks (40 μg/l, cumulative dose 350 μg/kg of body weight) as reported previously (Jamasbi and Perkins 1990). These tumors were designated DEN_1, DEN_3, DEN_6, DEN_8, and DEN_9. Histologic examination of transplanted tumors showed that all of the tumors were invasive squamous cell carcinomas. The syngeneic sarcoma cell line, designated MSC (Kennel et al. 1985), was used as a control.

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In all experiments tumor cells were inoculated intramuscularly (i.m.) in the thigh of syngeneic mice. The transplantability of each tumor line was determined by serial passage *in vivo*. At the fifth *in vivo* passage, tumors were removed, single cell suspensions were prepared by trypsinization, and a large cell pool was established and stored in liquid nitrogen. Cells from the fifth *in vivo* passage were used for the majority of *in vivo* experiments and for the establishment of tissue culture cell lines.

The relative tumorigenicity of each carcinoma line was determined by injecting graded doses of tumor cells. Tumor development, growth rates, and time and incidence of mortality were determined.

Metastatic characteristics of these tumors were studied as described previously (Jamasbi and Perkins 1990). Briefly, mice were inoculated with 10^5 tumor cells and the tumor was removed when it reached 1-1.5 cm in diameter. Thirty days later the animals were killed and examined for the incidence of metastasis.

In Vitro Culture

Culture media, sera, and antibiotics were obtained from Grand Island Biological Co. (Grand Island, NY). Tissue culture procedures and the establishment of epithelial cell lines from these tumors were as previously described (Jamasbi and Perkins 1990). Briefly, cell lines were derived from the fifth *in vivo* tumor passage by explant culture of tumor fragments in McCoy's medium supplemented with 10% fetal bovine serum (FBS). All cultures were incubated at 37° C in a humidified atmosphere of 5% CO_2 in air. When sufficient epithelial outgrowth was observed, cells were subcultured. All of the cell lines were screened periodically for mycoplasma contamination (Hayflick 1965). Tissue culture cell lines were developed from all tumors except DEN_8.

In Vitro Growth Properties

For determination of seeding efficiency, growth rate, and cell doubling times, cell lines were plated at a density of 10^5 cells per 100 mm plastic dish. Cells from two dishes were independently harvested by trypsinization each day and counted using a hemacytometer. Results were plotted on semilogarithmic paper, and doubling times calculated.

Transplantation Tests

To test the tumorigenicity of *in vivo* or *in vitro* passaged tumor cells, different doses of cells were inoculated into syngeneic hosts. Cultured cells were tested for their tumorigenicity after every fifth passage for 25-50 passages. *In vivo* propagated cells were serially transplanted into syngeneic hosts for an additional five times. Thighs of inoculated mice were checked at weekly intervals for tumor development. Rates of tumor growth and incidence of mortality were recorded.

Fluorescent Antibody Assay

In order to analyze cells for viral antigens, tumor cells were grown on coverslips. When the cells reached near confluency, the coverslips were washed in PBS and adherent cells were fixed in cold acetone. The cells were then incubated with broadly reactive antibodies from a rat immunized against mouse Moloney leukemia and Moloney sarcoma viruses. The cells were washed in PBS, incubated with goat anti-rat serum (fluorescein conjugated), and counter-stained with rhodamine. Positive cells were observed using an ultraviolet microscope.

Immunization and Challenge (*In Vivo* Tumor Lines)

To determine tumor immunogenicity, previously described immunization procedures were used (Jamasbi et al. 1978). For this purpose, mice were injected i.m. with 10^3-10^5 live tumor cells. Tumors were removed 3-4 weeks later when tumor size reached 1-1.5 cm in diameter. One week later the animals were challenged with graded doses of *in vivo* parental tumor cell lines. Because of the apparent absence of immunogenicity of some of these tumor lines, transplantation-excision procedures were repeated 2-3 times to determine whether immune responses (resistance) could be generated. Immunization by x-irradiated cells (Jamasbi and Nettesheim 1979) was also attempted. Mice immunized with MSC or receiving sham surgery served as controls.

Immunization and Challenge (*In Vitro* Tumor Lines)

For immunization with *in vitro* grown cell lines, 10^6 cells in 0.1 ml PBS from different *in vitro* passages were inoculated into syngeneic recipients. Tumors which developed in some of the hosts were surgically removed when they grew to about 1-1.5 cm in diameter (3-5 weeks later), and the animals were challenged with appropriate *in vivo* or *in vitro* cell lines 7-10 days later. In all of the preceding experiments normal mice which had undergone sham surgery or mice immunized with MSC served as controls.

Winn Neutralization Test

To determine the cytotoxic effect of the immune spleen cells on the tumor development, the Winn neutralization test was conducted. For this purpose, spleens from either normal or immunized mice were aseptically removed and pooled (3-5 spleens/group). Single-cell suspensions were prepared and mixed at a ratio of 200:1 with tumor cells using the smallest tumor cell dose that produced 100% tumor takes for each respective tumor line. The mixture was incubated at 37° C for 1 hr, and inoculated i.m. into the thighs of normal or x-irradiated mice (450r x-rays). Control groups received tumor cells only or tumor cells mixed with spleen cells from either normal mice or mice which had been immunized with MSC tumor cells.

Antibody-binding Test

To demonstrate an antibody in the sera of mice immunized against each tumor, an enzyme-linked immunosorbent assay (ELISA) was used. To perform ELISA, *in vitro* propagated cells were cultured into 96-well plates (Corning) and fixed with 0.1% glutaraldehyde (Sigma) for 10 min at room temperature. The fixed cells were treated with 0.1 M glycine for 5 min and were then

exposed to 1% bovine serum albumin in phosphate-buffered saline, pH 7.2 (BSA-PBS) for 1 h. Properly diluted (1:500) immune or non-immune sera were added at 100 µl/well and allowed to react for 1 h at 37° C. The plates were washed three times with PBS and incubated at 37° C for 1 h with 100 µl/well of a 1:250 dilution (in 2% BSA-PBS) of ß-galactosidase-conjugated goat anti-mouse immunoglobulin (Southern Biotechnology Associates, Inc.). After the incubation, the plates were washed three times with PBS and incubated with 100 µl/well of p-nitrophenyl-ß-D-galactopyranoside (Sigma) solution (1 mg/ml in phosphate buffer, pH 8.5) at 37° C for 1 h. The optical density (at 410 nm) was measured with an MR-600 microwell plate reader (Dynatech Laboratories, Inc.). In some ELISA experiments, when *in vivo* propagated cells were to be tested, a 10^5 cells/well of either *in vivo* or *in vitro* (for comparison) were attached to poly-L-lysine treated wells, fixed and examined as above. Cultured MSC cells served as controls.

Periodic Acid Treatment

To determine whether the binding of immune sera to the tumor cells can be altered by periodic acid treatment, glutaraldehyde-fixed cells in 96-well plates were treated with 100 mM periodic acid solution (Sigma) at 37° C for 30 min. The wells were washed with PBS three times and the reactivity of the antibody with the treated and untreated cells was determined by ELISA.

Enzyme Treatment

The effect of neuraminidase (Sigma) treatment on the binding of immune sera to *in vivo* and *in vitro* propagated cells was also assessed. Glutaraldehyde-fixed cells in 96-well plates were exposed to a 0.5 mU/well of neuraminidase (in 50 mM sodium acetate buffer, pH 5.5), for 1 h at 37° C. Control wells were exposed to 100 µl of sodium acetate buffer. Following enzyme treatment, the wells were washed 3 times with PBS; then antigen-antibody binding was assessed by ELISA.

RESULTS

Growth Characteristics of *In Vivo* Propagated Tumor Lines

The degree of tumorigenicity varied considerably among the forestomach carcinomas of BALB/c mice (Table 1). Whereas the 10^3 viable cells of DEN_1, DEN_3, or DEN_9 produced tumors in 100% of the inoculated hosts, a much higher (approximately 100-fold) tumor cell dose (10^5) of DEN_6 or DEN_8 was needed to obtain similar results. This experiment was repeated several times using a different range of tumor cell concentrations. Only those which gave approximately 10-100% tumor takes are presented (Table 1). Growth rates of *in vivo* propagated DEN_1, DEN_3, and DEN_9 tumor lines (data not shown) were also much greater than those observed for DEN_6 and DEN_8.

The rate of mortality (Table 2) also differed considerably among mice that received cells from different tumor lines, i.e., 10^5 cells of DEN_1 killed all mice between 6-8 weeks whereas the same dose of DEN_3, DEN_6, DEN_8, or DEN_9 tumor cells killed the injected mice between 9-11, 20-24, 18-23, and 7-11 weeks, respectively. Highly malignant, fast-growing DEN_1 and DEN_3 metastasized readily (Table 2). DEN_1 metastasized faster and produced micrometastases and secondary tumors in many organs, including the lungs, kidneys, and renal lymph nodes, while DEN_3 primarily metastasized to the lungs and occasionally to the renal lymph nodes. Surprisingly, aggressively growing, highly malignant DEN_9 did not metastasize. Relatively slow growing DEN_6 and DEN_8 did not metastasize, either.

Growth Characteristics of *In Vitro* Propagated Tumor Cell Lines

Epithelial outgrowths from the tumor explants in the culture grew slowly and subcultures were generally not possible until 5-6 weeks after the primary culture (Jamasbi and Perkins 1990). Once fibroblast-free epithelial cultures were established, the cells grew more rapidly and subcultures were routinely made at weekly intervals.

TABLE 1

Demonstration of tumorigenic potency of in vivo *propagated carcinoma lines.*[a]

Time after tumor cell injection (weeks)	DEN_1			DEN_3			DEN_6			DEN_8			DEN_9		
	10^3	$5X10^2$	10^2	10^3	$5X10^2$	10^2	10^5	$5X10^4$	10^4	10^5	10^4	$5X10^3$	10^3	$5X10^2$	10^2
2	2/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
4	5/5	0/5	0/5	3/5	0/5	0/5	2/5	1/5	0/5	5/5	4/5	1/5	3/5	0/5	0/5
6	5/5	2/5	0/5	4/5	1/5	0/5	5/5	2/5	1/5	5/5	4/5	1/5	5/5	2/5	0/5
8	5/5	3/5	2/5	5/5	3/5	0/5	5/5	3/5	1/5	5/5	4/5	1/5	5/5	2/5	1/5
10	5/5	4/5	2/5	5/5	3/5	0/5	5/5	3/5	1/5	5/5	4/5	1/5	5/5	2/5	1/5
12	5/5	4/5	2/5	5/5	3/5	1/5	5/5	3/5	1/5	5/5	4/5	1/5	5/5	2/5	1/5

[a]Graded doses of single cell suspensions of each tumor line (fifth *in vivo* passage) were injected i.m. into the thighs of five syngeneic mice. Only the tumor cell doses that gave ~10-100% tumor takes are reported here. Number of mice with tumors/number of mice injected.

TABLE 2

Time to mortality of mice receiving 10^5 cells from different carcinoma lines[a] and the incidence of metastasis.

Tumor Line	Time to mortality (weeks)[b]	Incidence of metastasis[c]
DEN_1	6-8	100%
DEN_3	9-11	80%
DEN_6	20-24	0
DEN_8	18-23	0
DEN_9	7-11	0

[a]Single cell suspensions (fifth *in vivo* passage) of each cell line were injected i.m. into thighs of 10 syngeneic mice and incidence of mortality was recorded.

[b]First number indicates the week when the first mouse died and the second number when the last mouse died.

[c]Mice were injected i.m. (thigh) with 10^5 tumor cells. The tumors were removed when they reached 1-1.5 cm. Animals were killed 30 days later and organs examined for the presence of metastasis.

Each cell line exhibited its own distinct morphological characteristics (Fig. 1) and, when injected into syngeneic hosts at early passage *in vitro* (P_{10}); these cell lines produced squamous cell carcinomas similar to those produced by the parental tumor line propagated *in vivo* (Fig. 2).

The *in vitro* growth characteristics of the various cell lines were determined at the 10th-15th passage. Seeding efficiency (as determined by the number of cells attached at 24 hr) was 63, 54, and 71% for DEN_1, DEN_3, and DEN_9, respectively. The population doubling times for these tumors were 14, 20, and 16 h, respectively.

At every fifth *in vitro* passage, graded cell doses of each carcinoma line were inoculated into syngeneic recipients to determine if the tumor line retained its tumorigenicity. Results (Table 3) show that the highly malignant, metastasizing DEN_1 and DEN_3 cell lines retained a relatively high degree of tumorigenicity up to the 50th *in vitro* passage (more recent examination of these cell lines showed that they retained tumorigenicity to the 100th passage or greater). However, both lines showed considerable reduction in their tumorigenicity as judged by the increasing number of cells required to induce a tumor take at late passages. DEN_9, also highly malignant but non-metastasizing, lost its tumorigenicity at about the 50th in vitro passage.

Although DEN_6, was not tumorigenic at the highest dose tested (10^5) at the fifth *in vitro* passage, tumors readily developed in x-irradiated hosts (100%). The tumorigenicity of the other cell lines was also enhanced when inoculated into x-irradiated mice (data not shown). Thus, the results reveal diverse biological characteristics of these tumor lines.

Determination of Transplantation Immunity of the *In Vivo* Line

Transplantation-excision-challenge experiments were conducted to determine immunogenic potency of the *in vivo* tumor lines. "Immunized" mice were challenged with graded doses of tumor cells. Untreated, sham-operated mice and mice immunized with MSC cells were also tested. For the sake of brevity, results of these experiments were not tabulated. Briefly, no transplantation resistance was demonstrated in mice immunized (receiving 2-3 transplantation excisions) against DEN_1 or DEN_3. Mice immunized with DEN_9 showed marginal resistance to a subsequent challenge (7 of 10 mice developed tumors). However, the slow growing, non-metastasizing DEN_6 and DEN_8 tumor cell lines showed a significant degree of protection (2 of 10 mice developed tumors). Other methods of immunization (e.g., use of irradiated cells) failed to confer protection against any of these tumors. Control mice, immunized against MSC or receiving sham surgery, showed no protection against these tumors.

DEN_1, DEN_3, and DEN_9 were found negative for cell-associated viral antigens by the indirect fluorescent antibody test using antibody to mouse-leukemia and sarcoma viruses (Jamasbi and Perkins 1990). DEN_8 was not tested and DEN_6 was positive. Whether the higher degree of

TABLE 3

Demonstration of tumorigenic potency of tumor cell lines at different in vitro *passages.*[a]

Tumor cell line	P_5			P_{10}			P_{20}			P_{30}			P_{40}			P_{50}		
	10^5	10^4	$5X10^3$	10^5	10^4	$5X10^3$	10^5	10^4	$5X10^3$	10^5	10^4	$5X10^3$	10^5	10^4	$5X10^3$	10^5	10^4	$5X10^3$
DEN_1	100	100	100	100	100	100	100	100	100	100	80	60	100	80	40	80	60	20
DEN_3	100	100	100	100	100	ND	100	80	40	80	50	20	60	30	10	60	20	10
DEN_6	0	0	0	0	0	0	0	0	0	ND	ND	ND	ND	ND	ND	ND	ND	ND
DEN_9	100	100	100	100	100	100	100	80	40	60	20	0	20	0	0	0	0	0

[a]Graded doses of single cell-suspensions were injected into the thighs of 10 syngeneic mice. Tumor takes (percentage) were recorded for 24 weeks. DEN_6 were tumorigenic at passage 3 *in vitro*. DEN_6 (P_{10}) and DEN_9 (P_{50}) produced tumors in x-irradiated (450 r) mice.

ND = not done.

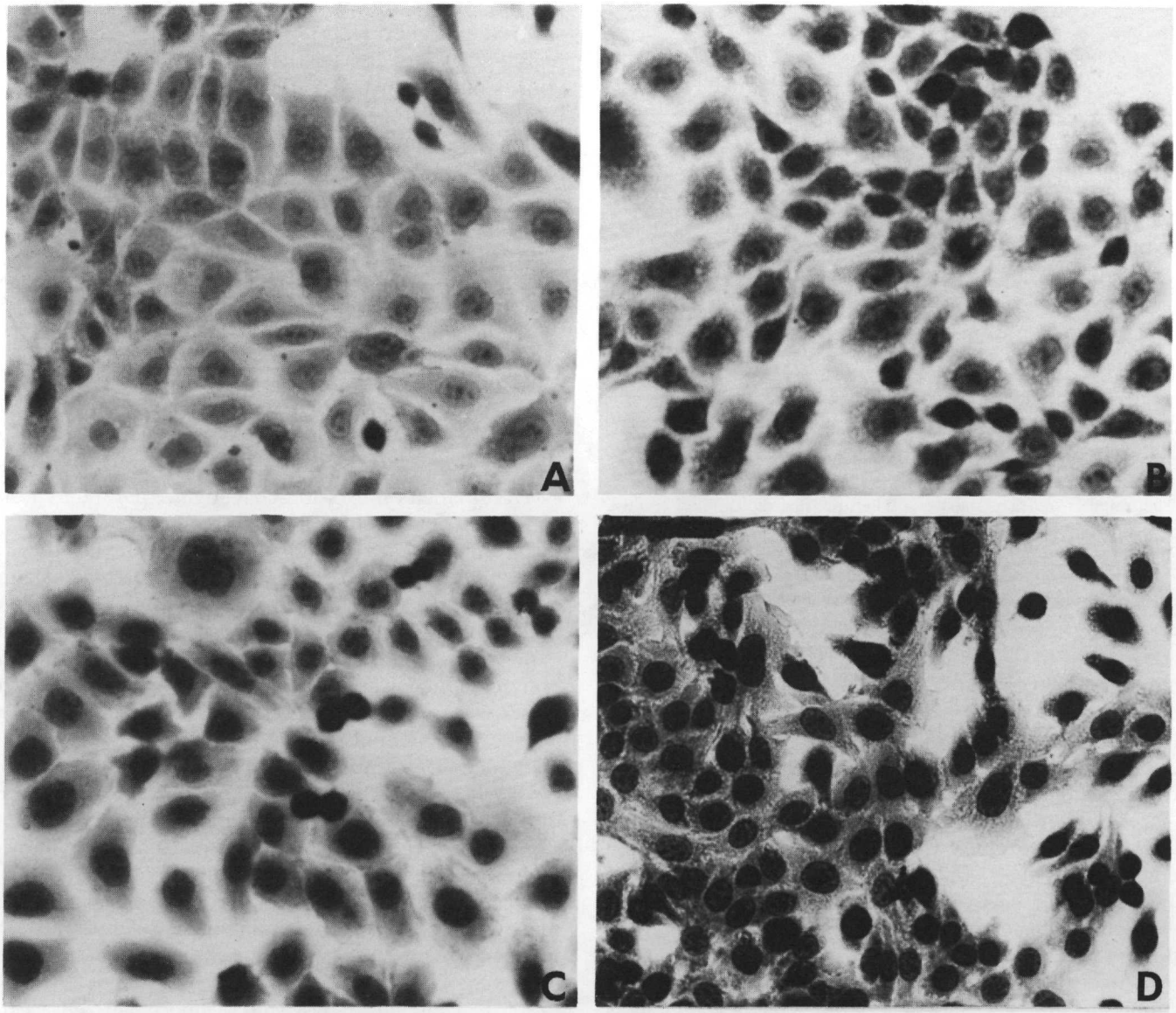

FIGURE 1. Cytological appearance of epithelial cell lines derived from different forestomach carcinomas propagated *in vitro* for 15 passages (Phase Contrast X 250). A, DEN_1; B, DEN_3; C, DEN_6; D, DEN_9.

immunogenicity of DEN_6 tumor cell line was related to viral antigen has yet to be determined.

The cytotoxic effect of spleen cells from "immunized" mice (receiving 3 transplantation excisions) on the development of the homologous tumor was examined by the Winn neutralization test. Spleen cells from mice immunized against DEN_6 or DEN_8 caused 60% and 50% inhibition, respectively. This type of inhibition was not observed with the other tumors, indicating that only DEN_6 and DEN_8 could induce detectable immunity. Normal spleen cells or spleen cells from mice immunized with the MSC line had no inhibitory effect on the incidence of tumor development (data not shown). The results were very similar whether normal or x-irradiated recipients were used.

For the detection of binding antibody in the sera of mice immunized against their respective *in vivo* or *in vitro* tumor cells, indirect ELISA was used. No detectable binding could be demonstrated in the sera of mice immunized against the *in vivo* propagated DEN_1, DEN_3 or DEN_9 (Table 4), indicating that the *in vivo* tumor lines are incapable of inducing detectable binding antibody. Sera of mice immunized against DEN_6 (*in vivo*) showed some reactivity against the respective tumor cells and sera of mice immunized with *in vitro* propagated tumor cells demonstrated significant binding capacity (Table 4). The binding of immune sera to control cells (MCS) was insignificant (data not shown).

Determination of Transplantation Immunity of the *In Vitro* Line

To determine the immunogenic potency of the cell lines grown *in vitro* and to compare these results with those obtained using their *in vivo* counterpart, cultured cells of the highly malignant DEN_1, DEN_3, and DEN_9 tumor cell lines were selected for detailed investigation. Syngeneic mice were injected with the cultured cells (P_{30}); and, if tumors developed, they were excised and the procedure was repeated one or two times. These mice

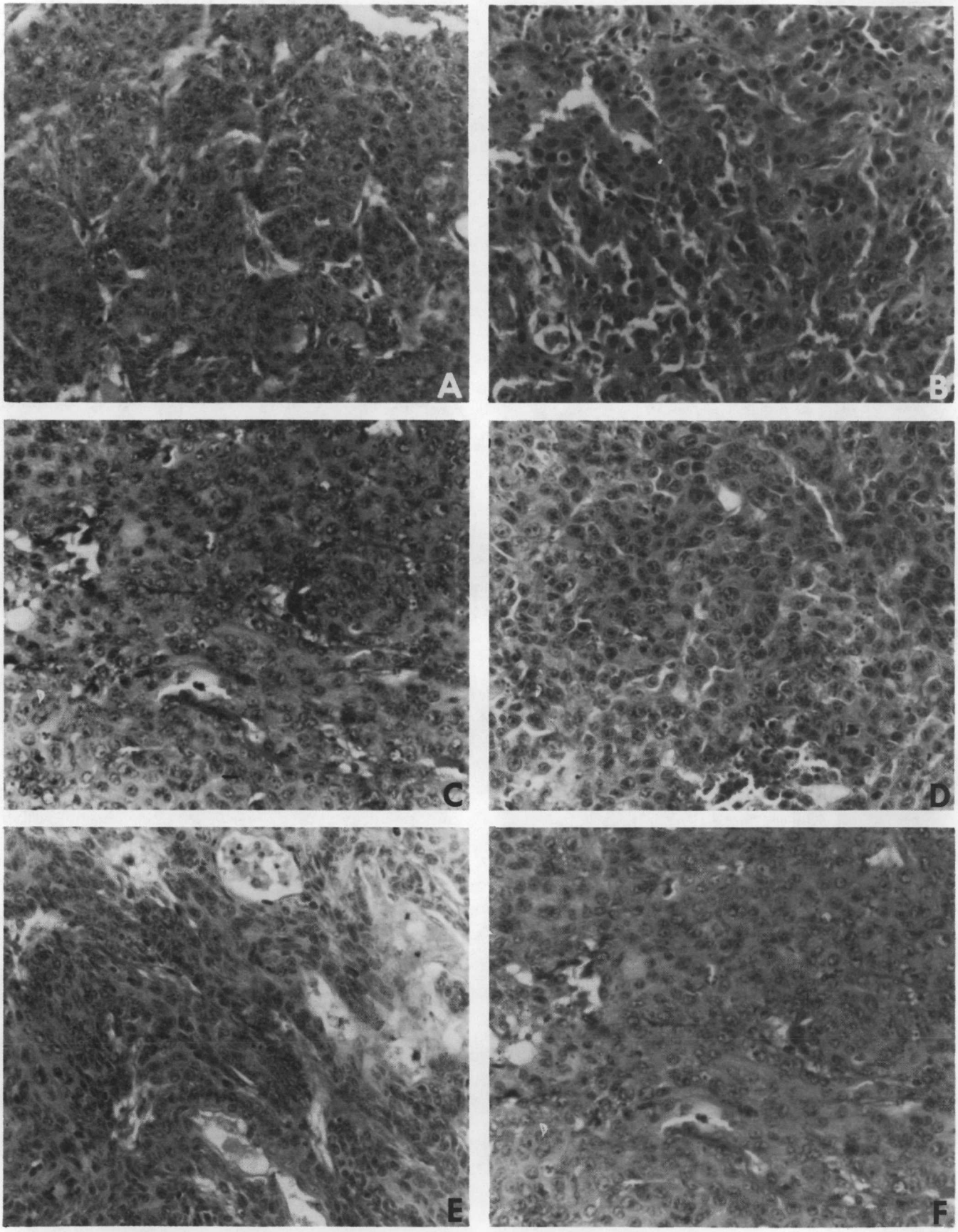

FIGURE 2. Histological appearance of different forestomach carcinoma produced by injection of tumor cell lines grown *in vitro* (X 250). DEN_1 (A), after 10 serial *in vivo* passages; (B), after 10 serial *in vitro* passages and 1 *in vivo* passage; DEN_3 (C), after 10 serial *in vivo* passages; (D), after 10 serial *in vitro* passages and 1 *in vivo* passage; DEN_9 (E), after 10 serial *in vivo* passages; (F), after 10 serial *in vitro* passages and 1 *in vivo* passage.

TABLE 4

Detection of binding antibody in the sera of mice "immunized" against in vivo *or* in vitro *propagated tumor cells by ELISA.*[a]

Cell Line	Absorbance at 410 nm[b]: Sera from mice immunized with *in vivo* cells	Sera from mice immunized with *in vitro* cells	Sera from normal mice
DEN_1	0.15 ± 0.02	1.00 ± 0.05	0.12 ± 0.03
DEN_3	0.20 ± 0.03	1.30 ± 0.10	0.10 ± 0.02
DEN_6	0.85 ± 0.10	1.70 ± 0.20	0.20 ± 0.06
DEN_9	0.20 ± 0.05	1.50 ± 0.10	0.10 ± 0.04

[a]Sera of mice "immunized" with *in vivo* and *in vitro* propagated tumor cells (1:500 dilution) were tested against each respective cultured tumor cells by ELISA. No significant bindings were detected with MSC cells (data not shown).

[b]The values are the means of triplicate samples ± S.D.

were then challenged with *in vivo* (passage 5) or *in vitro* (passage 30) parental cell lines. Results of these experiments (Table 5) showed that with this procedure, not only could we demonstrate a significant degree of protection against challenge with the *in vitro* line, but also against the fast-growing-metastasizing *in vivo* parental cell line. Such protection suggested enhanced immunogenicity of these tumor lines during *in vitro* cultivation.

TABLE 5

Effect of immunization with in vitro *propagated* DEN_1, DEN_3, *or* DEN_9. *Cells on the transplantability of their respective* in vitro *and* in vivo *tumor cells.*[a]

	Challenge dose	Recipients: Immunized	Recipients: Sham operated
In vitro grown tumor cells used for challenge			
DEN_1	10^5	3/10	10/10
DEN_3	10^5	2/10	10/10
DEN_9	10^5	0/10	10/10
In vivo grown tumor cells used for challenge			
DEN_1	10^3	6/10	10/10
DEN_3	10^3	5/10	10/10
DEN_9	10^4	4/10	10/10

[a]Mice were immunized with DEN_1, DEN_3, or DEN_9 *in vitro* lines (3x) and were then challenged with their corresponding *in vitro* and *in vivo* propagated lines. Number of mice with tumors/number of mice challenged. Mice immunized with the control syngeneic sarcoma line (MSC) showed no protection against the challenges (data not shown).

Effect of Periodic Acid and Neuraminidase Treatment on Antigen-antibody Binding

It is well known that periodic acid can cleave carbohydrate moieties from glycoprotein or glycolipid molecules (Ravindranath et. al. 1989, Naito et. al. 1990) and neuraminidase can cause sialic acid release (Bekesi et. al. 1972, Ogasawara et al. 1988). To determine whether the exposure of *in vivo* or *in vitro* propagated tumor cells to the above chemicals influences the antigen-antibody binding test, experiments were conducted in which the cells were exposed to periodic acid or neuraminidase. The antigen-antibody binding was determined by ELISA. The results (Table 6) showed that exposure of the cells to these chemicals enhanced antigen-antibody binding significantly. It should be noted that periodic acid had a greater effect than the neuraminidase treatment. It appears that during the *in vivo* or early *in vitro* cultivation, the antigenic determinants of these tumor cells are masked by some glycoprotein or glycolipid molecules that interfere with antigen-antibody binding and render them very weak or non-antigenic. The removal of the carbohydrate side chains or sialic acid residue from the interfering glycolipids and/or glycoproteins appears to increase epitope accessibility, thus enhancing their binding capacity to the antibody.

When the effect of periodic acid on the antigen-antibody binding was studied using different *in vitro* passages (Table 7), it was found that the influence of these chemicals on the antigen-antibody binding decreased as the passage in culture continued. It appears that during *in vitro* cultivation, concentrations of masking or interfering molecules gradually decreased, the antigenic determinants become exposed, and consequently the tumor cells demonstrated greater antibody binding capability. However, concentrations of the masking or interfering substances appeared to be different on different tumor cells, since some of the cell lines lost their tumorigenicity at an early *in vitro* passage, while others retained their tumorigenicity for a much longer period of time. It is also possible that quality and/or quantity of the antigenic determinants are not the same on the different cell lines.

DISCUSSION

Existing evidence suggests that most chemically-induced tumors possess unique tumor-associated antigens capable of generating immune responses in syngeneic hosts (Baldwin 1974, Schreiber et al. 1988). In contrast, in our *in vivo* experiments, three (DEN_1, DEN_3, and DEN_9) of the five DEN-induced tumor lines used were fast growing, highly malignant, and showed very little immunogenicity despite repeated attempts using different methods of immunization and/or protocols (e.g., use of irradiated tumor cells, repeated transplantation-excision procedures). None, or only marginal, protection was demonstrated even against the smallest tumor cell challenge (10^3). Furthermore, demonstrable cellular or humoral immunity could not be detected using the Winn neutralization test or the antibody-binding assay.

As we increased the number of *in vitro* passages, a marked decrease in tumorigenicity with a loss of metastatic

TABLE 6

Effect of periodic acid and neuraminidase treatment on the antigen-antibody binding by ELISA.[a]

	% Increase in antigen-antibody binding[b]	
	Periodic Acid	Neuraminidase
Tumor cells propagated *in vitro* (P_5)		
DEN_1	109.5 ± 5.0	42.60 ± 1.9
DEN_3	114.3 ± 6.2	38.00 ± 1.4
DEN_9	140.6 ± 5.6	20.60 ± 1.3
Tumor cells propagated *in vivo* (P_5)		
DEN_1	81.50 ± 5.5	ND
DEN_3	93.20 ± 6.0	ND
DEN_9	105.6 ± 8.5	ND

[a]Tumor cells (10^5/well) were fixed into poly-L-lysine treated, microtiter plates, fixed with 0.1% glutaraldehyde and treated with 100 mM periodic acid in PBS (100 µl/well) and 0.5 mU/well of neuraminidase (in 50 mM sodium acetate buffer, pH 5.5) at 37° C for 30 minutes. The untreated cells, incubated with appropriate buffers, served as controls.

The binding of antibody (1:500 dilution of sera of mice immunized with different cell lines) to the treated and untreated cells was measured by ELISA.

% increase in the binding of antibody was calculated by the following formula:

$$\% \text{ increase} = \frac{\text{O.D. (untreated) - O.D. (treated)}}{\text{O.D. (untreated)}} \times 100$$

[b]The values are the means of triplicate samples ± S.D.

ND = not done.

Normal mouse serum did not show any significant binding to the treated or untreated cells (data not shown).

capability was observed. This occurred concomitantly with an increase in immunogenicity, as judged by the increasing number of cells that were required to produce tumors, as passage in culture continued, and by the presence of binding-antibody in the immune sera. Loss of metastatic capability as a result of *in vitro* cultivation has been observed in other tumor systems, indicating that this phenomenon is not uncommon (Ossowski and Reich 1980, Belardelli et. al. 1984). The increasing experimental evidence indicates that the integrin family of adhesion molecules plays a major role in tumor invasion and metastasis (Natali et al. 1993, Dedhar 1990). Whether the loss of metastatic potential observed in the present study is caused by alteration in the expression of adhesion molecules is not clear and requires further study.

It has been shown that concentrations of FBS in growth media can influence the immunogenicity of tumor cell lines (Chiba et. al. 1987). This was not the case in our experiments since the tumor cells cultured in medium containing 0.5% or 10% FBS showed no significant differences in antigen-antibody binding. In addition, the growth of tumor cells in media containing syngeneic mouse sera did not influence the results (data not shown), indicating that not all tumor systems react similarly to the environment of *in vitro* cultivation.

The demonstrated increase in immunogenicity appears to be significant. This became evident with our protection studies wherein animals that were immunized with the *in vitro* line were protected not only against the immunizing *in vitro* line but also, at least to some degree, against the highly malignant, metastasizing parental *in vivo* line. In contrast, immunization with these *in vivo* lines repeatedly failed to confer any significant degree of protection. Furthermore, the tumorigenic potency of *in vitro* lines was enhanced and/or recovered when the cells were inoculated into x-irradiated (immunosuppressed) mice. Similar phenomena have been observed by other investigators (Correll et. al. 1983, Altevogt 1986). Thus, the present data, as well as observations made by other investigators (Mullen et al. 1992), indicate that concomitant with the development of decreased tumorigenicity, tumor cells become more immunogenic and less metastasizing.

It has been shown that periodic acid can cleave carbohydrate moieties from glycolipid or glycoprotein molecules (Ravindranath et. al. 1989, Naito et. al. 1990). In the present study, it was found that exposure of the tumor cells to this oxidizing agent enhances antigen-antibody binding considerably, suggesting that some glycolipid and/or glycoprotein molecules on surfaces of the cells interfere with antigen-antibody bindings. The

TABLE 7

Effect of periodic acid treatment on antigen-antibody binding test using different in vitro passages of DEN_1 or DEN_9.[a]

	% increase in antigen-antibody binding[b] *in vitro* passage			
Tumor Line	P_{10}	P_{30}	P_{50}	P_{100}
Periodic Acid Treated				
DEN_1	91.5 ± 2.30	74.6 ± 4.20	50.0 ± 3.50	45.3± 4.60
DEN_9	102.3 ± 3.50	ND	30.3 ± 3.50	10.2 ± 1.30

[a]*In vitro* cultured, glutaraldehyde-fixed tumor cells were treated with 100 mM periodic acid (in PBS) and incubated at 37° C for 30 minutes. Control wells received PBS. The binding of antibody (1:500 dilution of sera of mice immunized with tissue culture cell lines) were measured by ELISA.

% increase in the antibody binding was calculated by the following formula:

$$\% \text{ increase} = \frac{\text{O.D. (untreated) - O. D. (treated)}}{\text{O.D. (untreated)}} \times 100$$

[b]The values are the means of triplicate samples ± S.D.

ND = not done.

Periodic acid treatment did not change the binding of antibody to control (MCS) cells.

removal of the carbohydrate side chains from these molecules appears to make the antigenic determinants more accessible to the antibody and thus enhances antigen-antibody binding capacity. The increased expression of mucin-associated carbohydrate molecules on the tumor cell surface was also shown to correlate with aggressive tumor growth and poor prognosis of several human cancers (Kobayashi et al. 1992, O'Boyle et al. 1992), suggesting that certain carbohydrate moieties on the cell surface can protect the tumor cells from the host immune destruction. The exposure of tumor cells to neuraminidase, an enzyme that releases sialic acid from cell surfaces, also enhanced antigen-antibody binding (Table 6), further indicating that sialic acid-containing carbohydrate chains can also mask the antigenic determinants in this system. Similar observations have been reported by other investigators (Bekesi et. al. 1972, Ogasawara et al. 1988).

Although the treatment of the *in vivo* grown cells with periodic acid or neuraminidase enhanced antigen-antibody binding considerably, the highest percentage of increase was obtained when early *in vitro* propagated cells were used (Tables 6 and 7). It is possible that concentrations of the interfering substances are much greater on the surface of *in vivo* grown cells and longer exposure time and higher concentrations of the enzymes or periodic acid are required for their removal or inactivation. However, the influence of other host factors on the antigen-antibody binding cannot be excluded at this time. It is also very likely that normal host cells, that are inevitably mixed with *in vivo* tumor cell suspensions, may have influenced the results and caused the apparent reduction.

Interestingly, as *in vitro* cultivation continued, the percentage of increase in antigen-antibody binding between the treated and untreated cells became smaller, suggesting that during *in vitro* cultivation the masking or interfering substances are gradually released from the cell surface, and consequently the tumor cells become more antigenic and bind the antibody more efficiently. In contrast to *in vitro* experiments, the passages of *in vivo* propagated cells serially into syngeneic mice caused no detectable changes in antigen-antibody binding tests (data not shown), further confirming that during *in vivo* cultivation antigenic determinants are not exposed and are possibly masked by the interfering glycolipid or glycoprotein molecules.

Although the mechanism(s) underlying the increase in immunogenicity has not been well described, from the data presented some credence might be given to each of the following scenarios: 1) The majority of tumor cells within a single tumor carry a tumor-associated antigen. The antigen, however, may not be expressed on the cell surface, or perhaps is modulated or masked by some host factors (glycoproteins, glycolipids) which render them non- or very weakly immunogenic. However, when these cells are cultured *in vitro,* antigenic determinants are gradually exposed and thus the cells become more immunogenic. Immunization with immunogenic cultured cells would then induce significant degrees of immunity capable of acting on the *in vivo* propagated parental cell line which, although unable to induce transplantation immunity, is obviously immunosensitive. 2) All or the majority of tumor cells within a single tumor possess tumor-associated antigen, but the rapid rate of growth and metastasizing capability of many malignant cells kill the host or at least overcome any natural resistance before the host can mount a specific immune response against tumor-associated antigen(s) (Table 2). As a result of *in vitro* cultivation, modification could occur in both the rate of growth and ability of tumor cells to spread rapidly *in vivo* (Table 3). Thus, the modified growth kinetics of *in vitro* propagated tumor cells could allow the induction of an effective immune response which would subsequently inhibit the growth of more tumorigenic, metastasizing *in vivo* tumor cells. 3) The tumor system consists of heterogeneous cell subpopulations (Jamasbi and Perkins 1990). As a result of *in vitro* selection, subpopulations of cells with higher degrees of immunogenicity become major components of the cell culture. Thus the *in vitro* line would be more immunogenic than the *in vivo* counterpart. If the majority of tumor cells carry different quantities or qualities of the same antigen(s), then immunization with higher passaged cultured cells could inhibit the growth of the less immunogenic subpopulation which appears to have *in vivo* growth advantages. None of these possibilities can be excluded at this time, and it is reasonable to believe that each one may play a role. The first possibility, however, appears to correspond more closely with the data presented here.

Periodic acid or neuraminidase treatment enhanced antigen-antibody binding *in vitro,* indicating inhibitory effects of glycoprotein and/or glycolipid molecules on the antigen-antibody binding. The presence of tumor-stimulating factors (Vaage 1978), suppressogenic determinants (Klein et. al. 1981), antibody-mediated epitope masking (Manson 1991) and shedding of the antigen into circulation (Vinuela et al. 1991) which can interfere with immune responses, has been described by other investigators.

It has also been shown that the immunogenic potency of poorly immunogenic tumor cells can be enhanced by viral (Kobayashi et. al. 1969, Fearon et. al. 1988), chemical (Bonmassar et. al. 1970, Ishikawa et. al 1987), and mutagenic agents (Boon 1983). More recently, however, procedures such as gene transductions have also been applied for the same purpose (Mullen et al. 1992, Fearon et al. 1988). The antigenic properties of these cell variants may be explained by the concept of association-recognition (Fearon et. al. 1988), which states that a single cell surface antigenic determinant is insufficient to induce effective host immune responses (Lake and Mitchison 1976, Boon 1983) and that the development of effective immune responses require additional surface antigen or a helper determinant to enable the host to respond to the newly acquired antigen as well as to the tumor-associated antigens (Kenne and Foreman 1982). Whether more than one antigenic determinant would become exposed during *in vitro* cultivation or whether association-recognition plays any role in our study is not known and warrants additional investigation.

In summary, our observations demonstrated that: 1) different squamous cell carcinoma lines induced by diethylnitrosamine (DEN) in the forestomach of BALB/c mice show different degrees of tumorigenicity, metastatic capability, and immunogenicity; 2) although some degree of transplantation protection could be generated against some of these tumor lines, this was not achieved for others (e.g., the fast growing, highly malignant tumors); 3) it was possible to induce a significant degree of transplantation protection and circulating antibody against these tumors when the animals were injected with *in vitro* propagated cell line counterpart; and, 4) more importantly, the antigen-antibody binding could be enhanced substantially when the tumor cells were exposed to periodic acid or neuraminidase prior to antigen-antibody binding tests, suggesting that inaccessibility of antigenic determinants on the surface of tumor cells *in vivo* may be responsible for the difficulties associated with the immunotherapy of certain cancers.

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