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Forssman Antigen and Phase Specifiic Fetal Antigens: an Evaluation of Their Role in SV40 Tumor Immunity[1] (38691)

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Transformation of cells by oncogenic viruses has been shown to result in modifications of surface membrane components (1, 2). Among these changes, the appearance of new antigenic determinants on the cell membrane or alterations in the antigenic expression of existing determinants (i.e., histocompatibility antigens) have been of particular interest to tumor immunologists. Two membrane associated antigenic sites which have been demonstrated to undergo such transitions during or following cellular transformation are the Forssman heterophile antigen and fetal or phase-specific antigens (3–5).

Forssman antigen was demonstrable on the surface membrane of virus transformed cells, but was not detectable on the nontransformed control cells (5, 6). The "cryptic" or masked nature of this heterophile antigen within the membrane of normal hamster cells has been suggested by experiments involving the exposure of intramembrane antigens following treatment with protease (7). The expression of immunogenic fetal antigens is transient in nature, being detectable on hamster cells through the tenth day of gestation but not thereafter in either fetal tissue, neonate, or adult hamster cells. Proteolytic treatment of hamster fetal cells from 14-day embryos suggested that reexpression of cryptic embryonic or fetal antigens can also be determined (8).

These reports suggested that the Forssman antigen which occurs on hamster fetal cells early in gestation (9) might be related to fetal antigens which evoke immunity to SV40 and adenovirus induced tumors in hamsters (10, 13). The role of the Forssman heterophile antigen in tumor immunity against SV40 induced tumor has been examined and resolved in the present study.

Methods. Tumor cells. An SV40 transformed cell line derived *in vivo* in syngeneic hamsters and subsequently cultured *in vitro* in medium 199 containing 10% heat-inactivated calf serum (11) was free of infectious or inducible SV40 and mycoplasma. The tumor cell line possessed tumor or T antigen, surface or S antigen, cytostatic antigen, SV40 tumor specific transplantation antigen, cross reactive with other SV40 tumor cells and fetal or phase specific antigens. Culture passages 27–44 were used for these studies.

Tumor immunity assay. The procedure employed to assay for SV40 tumor immunity was essentially that described by Goldner *et al.* (14) and Coggin *et al.* (15). Syngeneic hamsters bred in the University colony received three weekly inoculations of either 5×10^6 irradiated (5000 R) trypan blue excluding SV40 tumor cells or 5×10^6 X-irradiated (5000 R) trypan blue excluding guinea pig kidney cells, or 1×10^7 X-irradiated (5000 R) sheep erythrocytes (GIBCO, Madison, WI) followed, 10 days after the last inoculation, by a subcutaneous challenge with 10^4 live SV40 tumor cells. Tumor appearance was determined by weekly palpation of the animals.

Antisera preparation. For routine immunization, hamsters were given three weekly injections of 5×10^6 irradiated (5000 R), viable cells suspended in Hank's balanced salt solution at weekly intervals. Some animals were hyperimmunized with more than five such inoculations. Hamsters were bled by cardiac puncture or from the retro-orbital sinuses and the blood allowed to clot overnight at 4°. The serum was harvested by centrifugation, heat inactivated at 56° for 30 min, sterilized by filtration, and stored in small aliquots at −70°.

Cell suspensions. Pregnant hamsters were obtained from matings conducted under observation. Fetuses were harvested just prior to the 10th, 11th, 12th, and 14th days of gestation, pooled within each age group and rinsed several times with phosphate

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buffered saline, pH 7.2 (PBS). Suspensions were prepared from 10- and 11-day fetuses forcing them through a 22 gauge needle and washing the brei by centrifugation in PBS. Older fetuses, guinea pig kidneys, and suckling hamsters were minced finely, suspended with a loose fitting Teflon pestle homogenizer, and filtered through sterile cheese cloth. These crude suspensions were then forced through an 18 gauge needle followed by a 22 gauge needle, and the cell suspensions washed in PBS by centrifugation. All preparations of fetal cells were subjected to 5000 R X-irradiation prior to use.

Hemolysis and inhibition of hemolysis assays. Hemolytic titers of antisera were measured in the microtiter system (Cooke Engineering Co). Test wells of the microtiter plates contained 0.025 ml of the appropriate serum dilution (in PBS), 0.025 ml of guinea pig complement (GIBCO, titer 1:320) diluted 1:10 in PBS and 0.25 ml of a freshly washed one percent suspension of SRBC in PBS. Plates were agitated for several minutes on a rotary shaker and incubated for 90 min at 37° with occasional mixing. The positive control for each test was commercial rabbit hemolysin (Grand Island Biological Co.) that consistently produced a titer of 1:5120 in this assay. Fifty percent hemolysis was taken as the endpoint for the titration.

The assay for inhibition of hemolysis of standard rabbit and hamster anti SRBC was a modification of the procedure used by Burger (7). The degree of inhibition of their hemolytic reactivity was monitored using cells or purified Forssman antigen (gift of Dr. S. Hakomori) suspended in PBS as possible inhibitors. An aliquot of 0.5 ml of the test inhibitor was added to 0.5 ml aliquots of serial twofold dilutions of antisera using several dilutions near the endpoint. Controls consisted of either the serum or inhibitor diluted with PBS. After incubation at 37° for 90 min with frequent agitation, the cell-antibody complexes were removed by centrifugation at 2000 rpm for 10 min. The supernatant fluid was then assayed in duplicate for hemolytic reactivity. Aliquots of 0.2 ml of the supernatant fluid were mixed with 0.3 ml of guinea pig complement diluted 1:10 in PBS and 0.2 ml of a fresh one percent suspension of washed SRBC in PBS. The test included controls for autolysis of SRBC and guinea pig complement induced hemolysis. After incubation of the mixtures at 37° for 1 hr, the intact cells were removed by centrifugation at 2000 rpm for 10 min. Hemolysis was measured in the supernatant fluid by monitoring the absorption of free hemoglobin at 550 nm. The percent inhibition of hemolysis was determined by relating the extent of hemolysis in the inhibitor-treated antiserum to that of the untreated antiserum at the same dilution. Possible errors due to a hemolytic reactivity of the inhibitor were eliminated by analysis of the supernatant fluid from the inhibitor controls alone which indicated no release of free hemoglobin in these assays.

Results. The serological response of hamsters to the Forssman heterophile antigen is presented in Table I. The immunization of hamsters with either guinea pig kidney cells or SRBC induced high hemolytic titers indicative of a response to the Forssman antigen. In animals challenged with live tumor cells, the sera taken prior to and throughout tumor development were negative for hemolytic reactivity. Sera from animals receiving three inoculations of cultured SV40 tumor cells were also negative for hemolytic activity; however, when hamsters were subjected to six inoculations a positive, though weak response was detected. The sera from animals hyperimmunized with 10 day hamster fetal cells also indicated a weak hemolytic reactivity while sera taken from primiparous and multiparous hamsters at the 10th day of gestation revealed no hemolytic reactivity. It is most interesting to note that SV40 transformed tumor cells and the 10-day hamster fetal cells that reportedly possess the Forssman heterophile antigen (7, 9) failed to produce demonstrable hemolytic reactivity with three inoculations; only after a course of six inoculations could any hemolytic activity be detected and then only at low test dilutions of the sera. One to three immunizations with these immunogens is sufficient to confer tumor

TABLE I. THE HEMOLYTIC ACTIVITY OF SERUM FROM HAMSTERS INOCULATED WITH CELLS POSSESSING FORSSMAN ANTIGENS.[a]

Immunogens tested	Sex of hamsters	Hemolytic activity (Titer)
Untreated controls	Male	Negative (undiluted)
	Female	Negative (undiluted)
Sheep erythrocytes (three immunizations)	Male	Positive (1:2560)
	Female	Positive (1:2560)
Guinea pig kidney cells (three immunizations)	Male	Positive (1:160)
	Female	Positive (1:320)
SV40 tumor bearer[b]	Male	Negative (undiluted)
	Female	Negative (undiluted)
SV40 tumor cells (three immunizations)	Male	Negative (undiluted)
	Female	Negative (undiluted)
SV40 tumor cells (hyperimmune antiserum—6 immunizations)	Male	Positive (1:4)
	Female	Positive (1:4)
10-day hamster fetal cells (hyperimmune antiserum—6 immunizations)	Male	Positive (1:4)

[a] The titers represent the median value obtained from at least four independent assays. All sera were tested at varying dilutions. Results are reported above as negative if found to be nonreactive when undiluted.

[b] The results obtained for the antisera from SV40 tumor-bearers presented were representative of sera taken weekly throughout tumor development.

transplantation resistance to SV40 tumors. Fetal or embryonic antigens present in hamster fetus are expressed at the cell surface of the unfertilized egg and in the developing fetus until the eleventh day of gestation (2, 3). An evaluation of the expression of Forssman antigen was initiated to determine whether its expression was transient in the developing hamster fetus. Results for the absorption of rabbit anti-SRBC serum containing Forssman antibody with hamster fetal cells obtained at the different days of gestation are given in Fig. 1A. These data indicate that the Forssman antigen is continuously expressed on fetal cells from the 10th day through the 14th day of gestation, and on cells from 2-day old suckling hamsters. It appears that the cells from 2-day old suckling hamsters certainly possess as much Forssman antigen as cells from fetuses of the 10th day of gestation. Results following adsorption of hamster anti-SRBC serum are shown in Fig. 1B, and are very different from those found with rabbit anti-SRBC serum (Fig. 1A). None of the cells from fetus or 2-day suckling hamsters demonstrate any capacity to remove the hemolytic reactivity from hamster anti-SRBC serum.

The absorption of rabbit anti-SRBC serum shown in part A of Fig. 2 with cultured SV40 tumor cells indicated that these cells and the fetal cells (Figure 1A) display a common antigenic determinant. This determinant appeared, in comparison to that seen for fetal cells (Fig. 1A), to be expressed to a lesser extent on the SV40 tumor cells. When SRBC, guinea pig kidney cells and purified Forssman antigen were used for absorption (Fig. 2A) all reactivity was removed. These results suggest that the hemolytic reactivity was induced by the Forssman heterophile antigen. Similar absorptions were performed with hamster anti-SRBC serum in place of rabbit serum (Fig. 2B). The hemolytic reactivity of this serum was not reduced by treatment with guinea pig kidney cells, with cultured SV40 tumor cells, or with purified Forssman antigen. A reduction in the activity was only obtained when the serum was treated with SRBC. These results suggested that the antigen inducing the hemolytic reactivity in hamster serum was unique to SRBC and was not the Forssman heterophile determinant.

Male and female hamsters immunized with SRBC or guinea pig kidney cells were

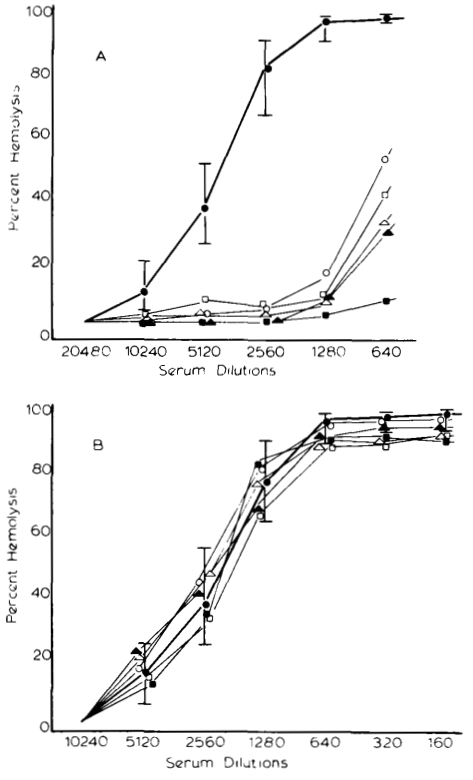

FIG. 1. The cross-reactivity of hemolytic immunogens on sheep erythrocytes (SRBC) and hamster fetal cells. (●——●) indicates the mean hemolytic activity and the range of activities at each two-fold dilution for ten independent assays of the untreated anti-SRBC serum. Serum dilutions are expressed as the reciprocal of the twofold dilution. The hemolytic activity remaining after absorption with viable, intact hamster cells is indicated as the percentage of activity related to the same untreated serum at that particular dilution. The points illustrated on these curves represent the mean of at least two assays. The absorption of rabbit anti-SRBC serum (A) and of hamster anti-SRBC serum (B) are shown: (○) with 2×10^7 10-day hamster fetal cells, (■) with 4×10^7 11-day hamster fetal cells, (□) with 1×10^7 12-day hamster fetal cells, (△) with 2×10^7 14-day hamster fetal cells, and (▲) with 1.5×10^7 2-day suckling hamster cells.

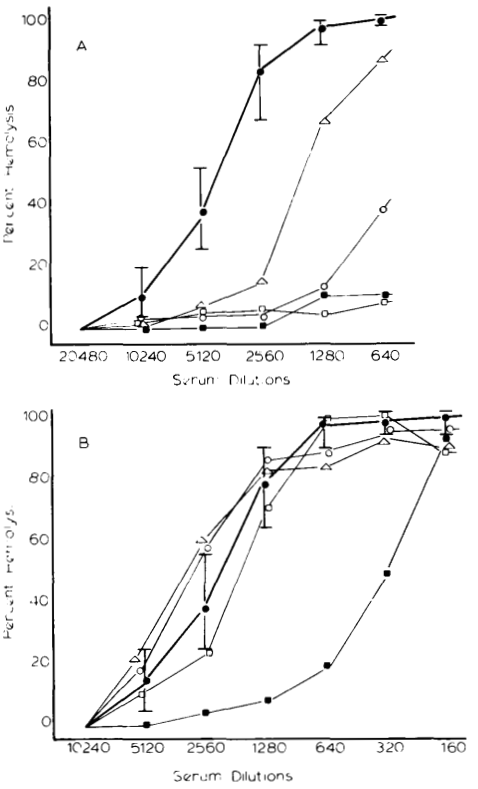

FIG. 2. The characterization of the hemolytic activity of the serum from hamsters inoculated with sheep erythrocytes (SRBC). (●——●) indicates the mean hemolytic activity and range of activities at each twofold serial dilution for ten independent assays of the untreated anti-SRBC serum. Serum dilutions are expressed as the reciprocal of the twofold dilution. The hemolytic activity remaining after absorption is indicated as the percentage of activity relative to the same untreated serum at that particular dilution. The points illustrated on the curves represent the mean of at least two assays. The absorption of rabbit anti-SRBC serum (A) and of hamster anti-SRBC (B) are demonstrated: (△) with 4×10^7 cultured SV40 tumor cells, (□) with 0.070 mg of purified Forssman antigen, (■) with 5×15^7 SRBC, and (○) with 4×10^7 guinea pig kidney cells.

tested for the development of protection against SV40 tumor challenge. Immunizations identical to those described for achieving immunization with hamster fetal cells were employed (10). Results given in Fig. 3 reveal that these immunizations failed to produce any significant protection relative to the untreated control animals. Complete protection was achieved with SV40 tumor cells. Hamster fetal cells from the 10th day of gestation, although not included in this particular experiment, routinely produced 50% or greater protection in similar experiments (10, 13).

Discussion. Guinea pig kidney cells and SRBC, known to possess the Forssman

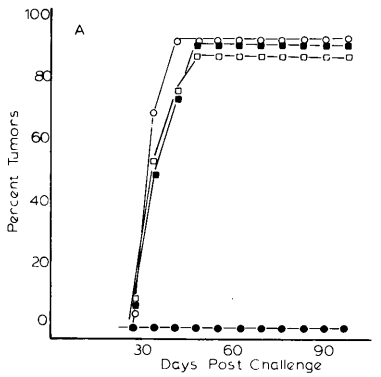

FIG. 3. An examination of the capacity of cells possessing Forssman antigen to immunize hamsters against challenge with live SV40 tumor cells. Hamsters received weekly vaccines of either (■) 5×10^6 X-irradiated guinea pig kidney cells, or (●) 5×10^6 X-irradiated SV40 tumor cells, or (○) 1×10^7 X-irradiated sheep erythrocytes, or (□) no vaccine. All hamsters were challenged with 10^4 live SV40 tumor cells 10 days after the last vaccine. Tumor appearance was monitored by weekly palpation. Negatives were confirmed by autopsy.

determinant on their surface membranes (9, 10, 13) produced high titers of hemolytic reactivity in hamster serum. The failure of animals to respond in a similar manner when exposed to the Forssman determinant on virally transformed cells (4, 5) and fetal cells (9) was a perplexing result. Hyperimmunization with these cells could produce weak hemolytic reactivity. A possible explanation was that the procedures used in the treatment of fetal and transformed cells in these experiments had altered the Forssman antigen; however, the absorption of rabbit anti-SRBC serum with purified Forssman antigen, fetal cells and SV40 tumor cells revealed that the determinant was still present. An examination of the hemolytic reactivity of hamster anti-SRBC serum using absorption with these same cell types and purified Forssman antigen demonstrated that the reactivity in hamster serum was not produced by the Forssman determinant of SRBC. It had been noted previously that absorption with Forssman material failed to remove the hemolytic reactivity from purified immunoglobulins of hamster anti-SRBC serum (16) which had been previously prepared for a protocol for immunization different from that used in our experiment. Evidently guinea pig kidney cells and SRBC possess isoantigenic determinants on their membranes capable of inducing unique hemolytic reactivities when these cells are used for immunogens in hamsters. Furthermore, it appears that the antigenic recognition for the Forssman determinant was particularly poor in hamsters.

The fetal antigen which evokes immunity against SV40 tumors had been demonstrated to be abruptly phased out at the 11th day of fetal development (2) and was only reexpressed after viral transformation (10) or proteolytic treatment (8). A key test in this study was to determine whether the Forssman antigen was still expressed on fetal cells after the disappearance of the fetal antigen. If it was not also phase specific, then Forssman antigen could clearly be distinguished from fetal antigen. A phasing out of the Forssman antigen on hamster fetus was not detected during this defined period of gestation.

Previous studies using purified membranes and only two ages of fetus had suggested that the quantity of Forssman antigen decreased with fetal hamster development (9). If anything, our data suggest that the quantity of Forssman antigen increased with fetal development, and that in agreement with others (9), the SV40 transformed cells express less of this determinant than the fetal cells. Several possibilities exist which may explain this difference in the quantity of Forssman determinants found on fetal cells. The differences could be a reflection of the type or preparation of material used for absorptions. In another view, the test employed may not be sufficiently sensitive for such quantitative comparisons.

The critical parameter for distinguishing the immunogenic differences between Forssman antigen on fetal cells and embryonic antigens would be the demonstration of the induction of tumor immunity following the injection of SRBC's or guinea pig kidney cells. If Forssman antigens present on these cells could confer tumor protection to the same extent that we had previously observed for fetal cells then a firm correlation be-

tween Forssman and fetal antigens would be established. Immunization of hamsters with either guinea pig kidney cells or SRBC failed to prevent or retard SV40 tumor development, although serum from these animals demonstrated a significant hemolytic reactivity. Subsequent studies later showed, however, that this hemolytic reactivity was not induced by the Forssman determinant but rather to another antigen present in sheep erythrocytes. These studies demonstrate that the hamster fetal antigens evoking immunity to the SV40 tumor are not of the Forssman type. Further, our data establish that a particular isoantigen can exist on fetal cells during gestation and on tumor cells after oncogenic transformation, but that such an antigen(s) does not play a significant role in eliciting tumor immunity in the system studied.

Summary. Forssman heterophile antigen was detected on hamster fetal cells which had been previously shown to be capable of eliciting transplantation resistance to syngeneic hamster SV40 tumors. The expression of Forssman antigen continued throughout fetal development and could be detected postpartum in the tissues of neonate hamsters. In contrast, fetal antigen(s) evoking immunity to SV40 tumors was also present on early fetal cells but, unlike Forssman antigen, was not expressed after the tenth day of gestation. Immunization of hamsters with guinea pig kidney cells or sheep erythrocytes which carry Forssman antigen failed to demonstrate significant protection against SV40 tumor development. Again by contrast, immunization with fetal cells was effective in evoking tumor immunity. Evaluation of serological responses to the Forssman antigen in hamsters indicated that the hemolytic reactivity produced by immunization with guinea pig kidney cells or sheep erythrocytes was elicited against isoantigens and not the Forssman antigen. A response to the Forssman determinant could only be detected in the serum from animals receiving exhaustive hyperimmunization with fetal cells or SV40 tumor cells. These data would eliminate a possible role of the Forssman heterophile antigen in the tumor protection evoked by immunization with fetal cells bearing embryonic antigens.

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