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Augmented Immunogenicity of Tumor Cell Membranes Produced by Infection with Influenza Virus as Compared to Moloney Sarcoma Virus

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ABSTRACT

The tumor-associated transplantation antigens (TATA) of crude membrane extracts from SV40-transformed BALB/3T3 tumor cells lytically infected with influenza virus were markedly more immunogenic than were extracts from uninfected cells measured either by the ability to induce heightened resistance to tumorgraft challenge or by heightened lymphocyte-mediated cytotoxicity against tumor cells *in vitro*. When intact tumor cells (as opposed to membrane extracts) were productively infected with Moloney sarcoma virus, they were made so immunogenic that they would only grow in X-irradiated syngeneic animals. Yet crude membrane extracts from the Moloney sarcoma virus-infected tumor cells showed no increase in TATA activity analogous to that seen after infection with influenza virus. Thus, influenza virus augmentation of tumor membrane TATA may operate by a different mechanism than does the oncornavirus augmentation of intact tumor cell TATA reported by others. It appears that Moloney sarcoma virus and possibly other oncornaviruses cannot be used to augment the TATA activity of tumor cell membranes in the same way that other surface-budding viruses can.

INTRODUCTION

The phenomenon of augmented TATA[2] activity associated with antigenic modification of tumor cells by means of virus infection has been reported by a number of investigators (1–12, 14–16, 19–20, 23–25). The subject naturally falls into 2 categories: (*a*) the augmented antigenicity of tumor cell homogenates or membrane extracts after infection with cytopathic viruses, such as influenza virus (4, 5, 9, 15, 16), VSV (14, 24), Newcastle disease virus (3, 6), and vaccinia virus (23); and (*b*) the augmented TATA activity of intact tumor cells capable of proliferation *in vivo* after persistent infection with partially cytopathic or noncytopathic viruses such as Sendai virus (6), measles virus (8), lymphocytic choriomeningitis virus (7), various oncornaviruses (Gross, Friend, Moloney, and Rauscher), and others (10–12, 18–20). Our previous work has been concerned with (*a*), where we have shown that immunization of mice with tumor cell membrane extracts prepared from cultured tumor cells infected with influenza or VSV produces heightened immunity to tumor graft challenge (4, 5, 9). Information regarding (*b*) has principally been supplied by Kobayashi *et al.* (10, 11) and recently by Kuzumaki *et al.* (12).

Kobayashi *et al.* infected chemically induced rat ascites fibrosarcoma cells with Friend virus by passaging the cells for 2 to 3 days through mice previously infected with Friend virus. When the virus-infected cells were transplanted back into syngeneic rats, they no longer grew continuously but instead grew for 1 to 2 weeks and then regressed over the next week. After regression of the virally "xenogenized" tumor, the host became highly immune to challenge with the original ascites tumor cells that were not infected with Friend virus, even though these tumor cells possessed only a very weak TATA. Sendo *et al.* (18) showed that virally xenogenized tumor cells were effective in tumor immunotherapy. In rats bearing tumors inoculation at another site of tumor cells that had been xenogenized with Friend virus produced suppression of tumor growth.

In this paper we confirm that tumor cell membrane extracts with augmented TATA activity are produced if they are obtained after infection of the tumor cells with influenza virus. In a parallel series of experiments, when the intact tumor cells were productively infected with MSV, they became so immunogenic that they would only grow in X-irradiated animals. In a critical test of mechanisms, the membranes from the MSV-infected tumor cells were tested for augmented immunogenicity, and none was found. The details of these findings are presented in this paper.

MATERIALS AND METHODS

Mice. Male and female BALB/cAnN mice, 3 to 5 months of age, were obtained from the Animal Production Branch, Division of Research Services, NIH, Bethesda, Md.

Tumors. E4 cells and a methylcholanthrene-induced fibrosarcoma (MCA 6) were used in this study. All cultured tumor lines were maintained in Roswell Park Memorial Institute Medium 1640 or DV-MEM containing 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml).

Infection of Tumor Cells with MSV. A stock of regressor MSV was kindly provided by Dr. R. B. Herberman (Laboratory of Immunodiagnosis, National Cancer Institute, Bethesda, Md.). This virus has been described in detail in previous papers (13, 20). The MSV stock had a titer of 10^4 to 10^5 focus-forming units/ml on SC-1 cells and a Moloney leukemia virus titer of 10^6 to 10^7 infectious units on XC cells (17). For infection of E4 cells with MSV, 10^6 cells were planted in a 75-sq cm plastic tissue-culture flask (Falcon Plastics, Oxnard, Calif.). When the cells were 70 to 80% confluent (about 3 to 4 days later), the tissue culture medium was decanted, and the tumor cells were washed twice with Hanks' balanced salt solution. Ten ml of a 1:10

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[2] The abbreviations used are: TATA, tumor-associated transplantation antigens; VSV, vesicular stomatitis virus; MSV, Moloney murine sarcoma virus; E4 cells, an SV40-transformed BALB/3T3 fibrosarcoma; DV-MEM, Dulbecco-Vogt modified Eagle's minimal essential medium; FBS, fetal bovine serum; CM, crude membranes.

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dilution of stock MSV was added, and the monolayers were incubated at 37° for 1 hr, after which an adequate volume of DV-MEM plus 10% FBS was added. Before use in experiments MSV-infected tumor cells were cultured for at least 3 generations and were checked by the indirect membrane immunofluorescence test (22) for the presence of MSV-induced cell surface antigens. For this test we used anti-MSV serum obtained from rats bearing a syngeneic transplantable tumor initiated over 50 passages previously in newborn rats by inoculating them with MSV (Huntingdon Research Center, Baltimore, Md.). At a dilution of 1:256, this antiserum produced membrane fluorescence in over 90% of MSV-infected E4 cells examined, whereas no fluorescence was produced by the same antiserum in noninfected E4 cells.

Infection of Tumor Cells with Influenza. WSA influenza virus, a mouse-adapted strain of WSN/A_0 influenza, was the kind gift of Dr. J. Lindenmann, Institute for Medical Microbiology, Zurich, Switzerland. This virus strain remains cell associated after propagation in cell culture and gives a recognizable cytopathic effect in 9 to 12 hr when the multiplicity of infection is between 5 and 50. The virus was propagated in the allantoic cavity of 10-day embryonated chicken eggs. For infection of a tumor cell monolayer, it was first washed twice with DV-MEM without serum. The egg-grown virus was then added and allowed to adsorb for 1 hr at 37°. The monolayer was then refed with DV-MEM with 10% FBS. A more complete infection was obtained if the cells were subconfluent at the time of infection.

Production of CM Extracts of Virus-infected Tumor Cells. Infected cells were harvested (usually 20 to 24 hr after infection with influenza virus) by scraping and centrifuged at 2000 rpm for 15 min. The pelleted cells were resuspended in a small volume of DV-MEM and centrifuged again. The packed cells were then resuspended in 4 volumes of 10^{-3} M $MgCl_2$ and allowed to swell for 3 to 5 min. They were then disrupted in a Dounce apparatus until no intact cells could be seen by phase microscopy (50 to 100 down-and-up cycles of the pestle). The homogenate was sonically disrupted in a Sonifier-Cell Disruptor W200P (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) with a cup-horn filled with ice-water at Setting 8 for 2 min. Nuclei and any remaining intact cells were removed by centrifuging at 1500 rpm for 4 min. The membrane protein concentration was determined by pelleting the membranes from a measured 0.1-ml aliquot (at 100,000 × g; 1 hr), resuspending the pellet in phosphate-buffered saline (0.85% NaCl solution containing 0.01 M NaH_2PO_4), titered to pH 7.4 with NaOH and determining the protein concentration of the membranes by the Lowry method. Influenza virus-infection of the cells was monitored by hemagglutination assay of the CM extracts. Homogenates of uninfected or persistently infected cells were harvested in the same manner.

Preparation of Tumor-immune Mice. E4 immune mice were produced by excision of a growing tumor in the front footpad followed by a s.c. inoculation of 10^6 viable tumor cells 7 days after excision of the primary tumor. These mice were considered tumor immune 7 to 10 days after the s.c. inoculation of viable cells. MSV-immune mice were produced by i.m. inoculation in the right front thigh with 0.05 ml of a 1:20 dilution of MSV stock. Primary tumors were detected at about 7 days, reached maximum size at 12 to 16 days, and spontaneously regressed between 21 and 28 days. At 28 days after injection of virus, mice with tumors larger than 10 mm in diameter were eliminated from the group; the rest were considered MSV immune and were used in the experiments.

Microcytotoxicity Assay. The procedure for this assay has been described in detail by Takasugi and Klein (21). Trypsinized tumor cells were seeded into the wells of microtest plates (Falcon 3034; Falcon Plastics, Oxnard, Calif.). Each well (6 wells/group) received approximately 500 tumor cells. The cells were allowed to attach during a 24-hr incubation period after which the medium was removed, and spleen cells at different effector/target cell ratios were added to each well in a 10-μl volume of Roswell Park Memorial Institute Medium 1640 plus 10% FBS. Normal and immune spleen cells were generally obtained from groups of 2 to 3 mice. At 24 to 48 hr after incubation in a CO_2 incubator, the plates were carefully washed twice with warm Hanks' balanced salt solution, dried, fixed for 30 min with methanol, and stained with Giemsa stain. The number of tumor cells in each well was counted at ×40, and the mean and S.E. were determined. Spleen cells from immune mice were considered active when the number of remaining target cells in the group was significantly ($p < 0.05$) lower than the number of target cells remaining in the wells exposed to control spleen cells. The p value was determined by the 2-tailed Student t test.

RESULTS

Markedly Increased Immunogenicity of Tumor Cells Productively Infected with MSV. Table 1 illustrates the phenomenon of "xenogenization," which is that tumor cells that ordinarily would grow to kill the host will instead regress if they have been productively infected with an oncornavirus, in this case MSV. In Group 2, noninfected E4 tumor cells injected s.c. into groups of 10 normal mice at doses of 1×10^6, 2.5×10^6, and 5×10^6 cells produced lethal tumors in a total of 29 of 30 mice. By marked contrast, in Group 1 the same E4 tumor cells infected with MSV and injected into normal mice at the same dosages produced

Table 1
Transplantability of MSV-infected E4 cells and uninfected E4 cells in syngeneic BALB/c mice

Group	Tumor cells	No. of cells injected s.c.	No. of mice with lethal tumors/no. of mice given injections: Normal mice	X-irradiated mice
1	MSV E4 cells	1×10^6	2/10	10/10
		2.5×10^6	1/10	10/10
		5×10^6	0/10	10/10
			3/30	30/30
2	E4 cells	1×10^6	9/10	10/10
		2.5×10^6	10/10	10/10
		5×10^6	10/10	10/10
			29/30	30/30

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lethal tumors in only 3 of 30 mice. The fact that MSV-E4 tumor cells grew readily in X-irradiated mice shows that their inability to grow in normal mice was not due to attenuation of their growth potential by infection with MSV but more likely to a pronounced increase in antigenicity associated with the MSV infection. MSV antigens on the surface of the infected E4 cells were demonstrated by immunofluorescence (see "Materials and Methods"). Additional proof that MSV antigens are present in the surface membranes of the E4 tumor cells is given in Table 2. Spleen lymphocytes from mice immunized with MSV alone were cytotoxic to MSV-infected E4 cells.

Lack of Augmented TATA Activity of CM from Tumor Cells Infected with MSV. Table 3, Groups 1, 2, and 3, demonstrates the phenomenon previously studied by us and others, that CM from tumor cells infected with a surface-budding virus such as influenza virus retain a degree of immunogenicity approaching that of whole cells, whereas CM from uninfected tumor cells are only poorly immunogenic (3–6, 9, 14–16, 24). This virus augmentation effect is specific for the tumor antigen of the infected cell; immunization with CM from influenza virus-infected but antigenically distinct MCA-6 tumor cells or with egg-grown influenza virus had no protective effect against challenge with E4 tumor cells (Groups 5 and 7).

In contrast to the result obtained with influenza virus, CM from E4 tumor cells persistently infected with MSV did not show augmented immunogenicity over that shown by CM from E4 cells not infected with MSV. The findings in Table 3 are confirmed in Table 4. The immunogenicity of CM from MSV-infected E4 cells was not increased over that of membranes from uninfected E4 cells, as measured by a microcytotoxicity assay. This significant result establishes that the augmentation of TATA activity produced by productive infection of intact E4 cells with MSV must occur by some mechanism other than that which operates to produce augmented TATA activity of CM from influenza virus-infected E4 cells.

Table 3

Comparison of the immunogenicity of CM from influenza virus- and MSV-infected E4 cells

Tumor graft rejection assay.

Group	Mice immunized with[a]	Result of s.c. challenge with 2×10^6 E4 cells: Tumor incidence (no. of mice with tumors/no. of mice given s.c. injections)	Tumor wt (g)[b]
1	X-irradiated E4 cells	0/20 (0)[c]	0 ± 0[d]
2	Flu-E4 CM[e]	2/20 (10)	0.10 ± 0
3	E4 CM	14/20 (70)	0.47 ± 0.16
4	MSV-E4 CM	14/20 (70)	0.40 ± 0.13
5	Flu-MCA-6 CM	18/20 (90)	0.74 ± 0.22
6	MCA-6 CM	20/20 (100)	0.81 ± 0.18
7	Egg-grown influenza alone	10/10 (100)	0.80 ± 0.07
8	Phosphate-buffered saline	10/10 (100)	0.83 ± 0.14

[a] Recipient mice were immunized twice at weekly intervals with 10^6 E4 cells or 0.5 mg CM.
[b] Tumors weighed 4 weeks after challenge with E4 cells.
[c] Numbers in parentheses, percentage.
[d] Mean ± S.E.
[e] Crude membranes from influenza virus-infected E4 cells.

DISCUSSION

Two mechanisms have been proposed to explain the augmented TATA activity of membranes from tumor cells infected with surface-budding viruses such as influenza virus and VSV. One is the helper antigen effect; a strong host immune reaction against virus antigen in the tumor cell plasma membranes generates soluble and cellular mediators that augment the immune reaction against the nearby TATA in the membrane. The second is chemical stabilization of the TATA; insertion of the virus antigen in the plasma membrane in some way stabilizes the labile tumor antigen against denaturation or enzymatic degradation by lysosomal hydrolases. Three pieces of evidence supporting the helper antigen mechanism are: (*a*) priming mice with egg-grown influenza virus either increases (14, 16) or abrogates (5) the augmented response to subsequent immunization with CM from influenza virus-infected cells; (*b*) mixing antibody to the virus with a homogenate of influenza virus-infected tumor cells abrogates the immunogenicity of the homogenates (16); and (*c*) mice made tolerant to influenza virus can no longer be immunized with virus-augmented CM (5).

The experiments reported here were undertaken to determine whether the augmented TATA activity associated with oncornavirus infection of intact tumor cells reported by Kobayashi *et al.* (10, 11) occurred through mechanisms similar to those described previously. In spite of the proven presence of highly active MSV antigens in the membranes of the MSV-infected E4 tumor cells both by membrane

Table 2

Antitumor cell-mediated immune reaction to MSV-E4 cells assayed by lymphocyte-mediated microcytotoxicity

Group	Spleen cells from mice immunized with	Target cells	No. of remaining target cells/well: 200:1	100:1	50:1	25:1
1	X-inactivated 10^6 E4 cells[a]	MSV-E4 cells	37 ± 5[b,c]	132 ± 5[b]	272 ± 27[b]	352 ± 46[b]
2	MSV alone	MSV-E4 cells	123 ± 15[b]	178 ± 11[b]	304 ± 29[d]	486 ± 38
3	None	MSV-E4 cells	251 ± 21	322 ± 22	500 ± 39	578 ± 29

[a] Donor mice were immunized twice at weekly intervals with 10^6 X-irradiated E4 cells (5000 rads).
[b] $0.01 < p < 0.001$, compared with spleen cells from normal mice.
[c] Mean ± S.E.
[d] $0.05 < p < 0.001$, compared with spleen cells from normal mice.

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Table 4

Comparison of the immunogenicity of CM from influenza virus- and MSV-infected tumor cells by microcytotoxicity assay

Group	Spleen cells from mice immunized with[a]	No. of remaining target E4 cells/well			
		200:1	100:1	50:1	25:1
1	X-irradiated E4 cells	268 ± 27[b,c]	353 ± 12[b]	434 ± 35[b]	412 ± 26[b]
2	Flu-E4 CM	211 ± 41[b]	414 ± 17[b]	479 ± 24[b]	705 ± 74
3	E4 CM	332 ± 30[b]	457 ± 37	574 ± 36	689 ± 43
4	MSV-E4 CM	339 ± 31[b]	458 ± 36	571 ± 39	585 ± 39
5	UV-inactivated influenza	454 ± 30	474 ± 23	515 ± 39	615 ± 51
6	None	459 ± 36	524 ± 46	613 ± 47	706 ± 66

[a] Donor mice were immunized twice at weekly intervals with 10^6 X-irradiated E4 cells (5,000 rads), 0.5 mg CM, or 0.2 ml influenza virus.
[b] $p < 0.05$ by Student's t test, compared with Group 7.
[c] Mean ± S.E.

immunofluorescence and by specific cell-mediated immune interactions (Tables 1 and 2), the crude membrane fraction prepared from these cells was no more immunogenic than were CM from non-MSV-infected E4 cells. Thus it appears that MSV and possibly other oncornaviruses cannot be used to augment the TATA activity of tumor cell membranes in the same way that other surface-budding viruses can. Why this is so is not readily apparent. It may be that the membrane antigen produced by each virus as it buds from the cell surface forms structurally different complexes with the tumor antigen and that only the influenza virus antigen-tumor antigen complex exhibits augmented immunogenicity, or the MSV-induced membrane antigen may form no complex with the tumor antigen at all.

What is the mechanism for the augmented TATA activity of intact MSV-infected E4 cells as opposed to uninfected E4 cells (Table 1)? It would seem reasonable that the augmented TATA activity of intact oncornavirus-infected tumor cells might be due to the prolonged antigenic stimulation by the TATA during the 2- to 3-week period of initial tumor growth and subsequent regression produced by the immune response to the oncornavirus antigen on the surface of the cells adjacent to the TATA. Prolonged antigenic stimulation may not be the entire mechanism, however, because recently Kuzumaki *et al.* (12) reported that some but not all (see also Ref. 1) tumors infected with endogenous mouse virus retained augmented immunogenicity even if they were X-irradiated before being used to immunize mice against subsequent challenge with uninfected cells. In this case the initial growth by the tumor was prevented by X-ray treatment, so that the period of antigenic stimulation was shortened. On the other hand, even after X-ray inactivation of growth potential, the period of continuous antigenic stimulation by the cells probably lasted many days, so that a state of relatively prolonged antigenic stimulation was in effect as compared to the probably much shorter survival time *in vivo* of TATA on CM extracts. Indeed, the explanation of why X-ray-inactivated tumor cells themselves are so much more powerfully immunogenic than are membrane extracts of tumor cells may lie in the fact that their survival time *in vivo*, and therefore the period of antigenic stimulation, is much longer than that of the membrane extracts.

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