VIRAL ONCOLYSIS: INCREASED IMMUNOGENICITY OF HOST CELL ANTIGEN ASSOCIATED WITH INFLUENZA VIRUS*

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Influenza viruses have been known for more than 20 yr to incorporate antigens derived from the cells in which they are grown (1, 2). Components resembling Forssman, blood group, and infectious mononucleosis antigens have been described (3, 4). The most widely studied of these host antigens is a sulfated mucopolysaccharide present in chick liver, bile, and allantoic cells that becomes intimately associated with influenza viruses growing in the chick allantoic cavity (5, 6).

Some strains of influenza A virus, derived either from fowl plague (7) or from neurotropic variants of human influenza virus (8), can be grown in nonspecific mouse tumors where they exert an oncolytic effect. When the experiment is performed in mice that are genetically resistant to the lethal action of the oncolytic virus, viral oncolysis is followed by the development of a solid antitumor immunity mediated by γG-immunoglobulins (9, 10). This in itself is not surprising, since nonspecific tumors represent allografts. More surprising, however, are the following observations: Whereas tumor cell homogenates prepared by mechanical disruption and lyophilization are not immunogenic, or at best are very weakly so, similar homogenates prepared from virus-infected tumors are highly immunogenic (10).

In order to explain the enhancing role of the virus on the immunogenicity of the tumor, it was conjectured that weak antigens or haptens derived from the host tumor cell might become associated with or incorporated into the virus and thus turn into potent immunogens (10, 11). We present evidence here that strongly suggests that this indeed is the case.

Materials and Methods

Mice.—ICR/Vet mice (random-bred) were obtained from the Department of Animal Husbandry, Veterinary School, University of Zurich, Switzerland. A2G/Exm mice (henceforth called A2G) were bred by brother-sister mating from nuclei of A2GfCFW/Lac supplied

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by the Laboratory Animals Centre, Carshalton, Surrey, England. A2G mice are genetically resistant to influenza A viruses (12, 13). Mice were 8–10 wk old at the beginning of experiments. Experimental groups were matched with respect to age and sex.

Tumor.—The strain of Ehrlich ascites (EA) tumor used in the present experiments has been described previously (11). The tumor was maintained in male A2G mice by weekly intraperitoneal (i.p.) injections of 10^6 washed tumor cells. Cells from the 125th to the 160th serial passage in A2G mice were used. Periodic checks on the minimum number of cells required to induce fatal ascites in A2G mice of both sexes confirmed that the LD_{50} was close to 10 cells per mouse (11). Tumor challenge in the experiments to be described was always 1000 washed EA cells administered i.p. (100 LD_{50}). In each experiment, an additional untreated control group of four mice (not shown in the tables) received a challenge of 100 EA cells. All these mice died of tumor ascites.

Viruses.—The WSA strain of neurotropic, tumor-adapted influenza A virus (8) used was the same as that used in previous experiments (9, 10, 11). Virus stock was prepared from Ehrlich ascites tumor grown in ICR/Vet mice and infected on day 6 with 10^4 EID_{50} of WSA virus. After 48 hr, the oncolytic tumor was harvested, homogenized, and lyophilized in 0.2 ml amounts. The ampoules were sealed under vacuum and kept at −10°C.

The TUR strain of fowl plague virus was A/Turkey/England/63 obtained from Dr. H. G. Pereira, World Influenza Centre, Mill Hill, London. This virus was adapted to growth in EA cells by serial passages. From the fourth passage, it produced changes in EA tumor indistinguishable from those induced by WSA. Stock tumor-grown TUR was prepared from the 10th serial passage of TUR in EA tumor cells grown in ICR/Vet mice in the same manner as that described for WSA.

The Lee strain of influenza B virus was an old laboratory strain. Stock capillary tubes containing infected allantoic fluid were kept over dry ice.

Egg-Grown Virus.—

WSA: A lyophilized ampoule of stock WSA was diluted 10^{-6} and inoculated allantoically into 10 day old embryonated eggs. The virus was harvested after 2 days incubation at 35°C. Capillary tubes were prepared from one infected fluid and stored over dry ice. Capillaries from this stock were then used to inoculate batches of 10 day old eggs at a dilution of 10^{-4}. These eggs were incubated for 3 days at 35°C. The allantoic fluids were harvested, pooled, and subjected to two cycles of differential centrifugation in the cold, each consisting of a low speed run at 1000 *g* for 10 min and a high speed run at 105,000 *g* for 30 min. The final sediment was resuspended in about one twentieth of the original volume and subjected to a third low speed centrifugation. The final suspension was adjusted to a hemagglutinin (HA) titer of 1:1600.

TUR: The preparation of egg-grown TUR virus was similar to that described for WSA, with the following exceptions: The inoculum consisted of infected allantoic fluid from an early egg passage of TUR virus which had never been passed in ascites tumor or in mice; eggs were incubated for 48 hr only. The final suspension was adjusted to a HA titer of 1:1600.

Lee: The preparation of egg-grown Lee virus was similar to that described for WSA, except for the inoculum which consisted of Lee allantoic virus stock. The final suspension was adjusted to an HA titer of 1:3200.

Antisera.—Rabbit antisera to egg-grown WSA and TUR viruses were prepared by one intramuscular injection of egg-grown virus emulsified in Freund's complete adjuvant (Difco Laboratories, Inc., Detroit, Mich.), followed 21 days later by one intravenous injection of egg-grown virus. Sera were collected 10 days after the intravenous injection, and inactivated at 56°C for 30 min before use. Hemagglutination-inhibition (HAI) titers of the two sera most extensively used in the present experiments are shown on Table V.

Anti-WSA gamma globulin was prepared from 20 ml of rabbit anti-WSA serum by precipitation with 0.37 saturated ammonium sulfate and passage through a DEAE-cellulose

column equilibrated with 0.0175 M sodium phosphate buffer pH 6.3 (14). At 2 mg/ml this preparation had an HAI titer of 1:1600 against WSA and showed a single sedimentation peak with an $s_{20,w}$ value of 6.5 in the analytical ultracentrifuge.

HA Titrations and HAI Tests.— HA titrations were done in Perspex hemagglutination trays with 0.5% washed fowl erythrocytes. Sedimentation patterns were read when the negative control cups had formed compact buttons. HA titers indicate highest virus dilution giving partial or complete agglutination. HAI titrations were performed by mixing serial serum dilutions with four agglutinating doses of virus. After 30 min at room temperature, fowl red cells were added. The patterns were read as above. HAI titers indicate highest serum dilution at which partial or complete inhibition of agglutination occurred.

Preparation of Viral Oncolysate.—Adult ICR mice of either sex were inoculated i.p. with 10^6 washed EA cells. After the tumor had grown for 7 to 8 days, 10^5 EID_{50} of WSA- or TUR-lyophilized stock virus were inoculated into the ascites. Within 48 to 72 hr oncolysis occurred, as evidenced by a striking reduction of abdominal tension and ruffling of hair (9). Animals showing definite tumor collapse were sacrificed, their peritoneal cavities were opened, and the solidified tumor masses were removed aseptically with forceps. This pooled material was stored at −20°C. To prepare homogenates, the material was thawed, homogenized with a Sorvall omnimixer, frozen and thawed again, and centrifuged in the cold at 5000 *g* for 30 min. The supernatant was distributed in ampoules and lyophilized. The ampoules were sealed under vacuum and stored at −10°C. WSA oncolysates, when reconstituted with distilled water, had HA titers of 1:32 to 1:320 and egg infectivity titers of 10^8 to 10^9 EID_{50} per milliliter. TUR oncolysates had HA titers of 1:800 to 1:1600 and egg infectivity titers of 10^8 to 10^9 EID_{50} per milliliter.

Egg Infectivity Titrations.—Infectivity for eggs was determined by allantoic inoculation of 10 day old eggs as described previously (12). Eggs inoculated with WSA virus were opened after 3 days and their allantoic fluids were tested for HA. Eggs inoculated with TUR were observed for 3 days and the deaths were recorded. Titers are expressed as 50% egg-infecting doses (EID_{50}) per milliliter.

Absorption of WSA Oncolysate with Fowl Erythrocytes and Elution with Receptor-Destroying Enzyme.—The following procedures refer to the experiment presented in Table III. 2 ml of packed, washed, fowl erythrocytes were added to 100 ml of WSA oncolysate (HA titer 1:32). The mixture was gently agitated every 10 min for 1 hr at +4°C. After centrifugation for 10 min at 300 *g*, the supernatant (which had no measurable HA activity) was set apart. The sedimented erythrocytes were washed four times with saline and once with calcium saline (saline with 0.1% $CaCl_2$ added). A portion of these cells was set apart for testing of immunogenicity. The remaining erythrocytes were resuspended in 5 ml of calcium saline containing 1000 μg units of purified neuraminidase (General Biochemicals, Chagrin Falls, Ohio) and incubated at 37°C for 30 min in a roller drum. The neuraminidase-erythrocyte mixture was then centrifuged at 300 *g* for 10 min. The sedimented erythrocytes were washed once with saline and the wash supernatant added to the first supernatant containing most of the virus. These combined supernatants were centrifuged at 105,000 *g* for 30 min to sediment the virus. The pellet was resuspended in 3 ml of saline (HA titer 1:320). Recovery of the virus in terms of hemagglutinin was about 30%. Erythrocytes were tested for their immunogenic properties at a concentration of 2.5% (v/v).

Parallel absorption-elution procedures were carried out on a batch of allantoic fluid infected with WSA and adjusted to the same HA titer. Recovery of egg-grown virus was close to 50%. Aliquots of the material obtained at various stages of absorption and elution of egg-grown virus were tested in parallel with the corresponding materials from tumor-grown virus. None of the egg-derived samples showed any immunizing activity against EA tumor. For the sake of simplicity, only one of these tests is shown on Table III (group 3).

Saline.—Saline refers to a solution containing 8.0 g NaCl, 2.7 g $Na_2HPO_4 \cdot 7H_2O$ and 0.4 g KH_2PO_4 per liter, pH 7.2.

RESULTS

1. Immunizing Power of Viral Oncolysates.—When 0.02—0.5 ml of reconstituted lyophilized viral oncolysates (see Materials and Methods) were inoculated once intraperitoneally into A2G mice, significant protection against challenge with 1000 to 100,000 EA cells (100 to 10,000 LD_{50}) could be observed. The challenge was given 11 days after the immunizing injection, but no study was made of the optimal time interval for challenge. WSA oncolysates and TUR oncolysates behaved similarly. Table I shows a representative experiment with a WSA oncolysate. The oncolysates induced a serologic antitumor response, detectable on the 9th day, in vitro by tumor cell agglutination and in vivo by

TABLE I

Induction of Immunity to Ehrlich Ascites Tumor in A2G Mice by WSA Oncolysate

Treatment*	Result of challenge*
Saline	0/8
WSA oncolysate 1:5‡	8/8
WSA oncolysate 1:15	8/8
WSA oncolysate 1:45	2/8

* 0.5 ml i.p. on day 0. Challenge, 1000 EA cells. 0.5 ml i.p. on day 11. Final reading on day 39. Number of survivors per total number of mice in group.

‡ For preparation of oncolysates as mentioned in this and the next tables, see Materials and Methods.

passive protection of syngeneic mice against EA cells (10, 11). Mice also responded to the virus contained in the oncolysate with a specific antiviral antibody response. HAI titers against the homologous virus ranged from 1:160 to 1:1240, 10–20 days after the injection of WSA oncolysate, and from 1:40 to 1:160 after TUR oncolysate.

To assess the possibility that a continuing viral infection was a cause of resistance to reimplantation of tumor cells, the following data were obtained:

(*a*) The peritoneal cavities of six mice were rinsed with saline at the time of challenge, and of six other mice 2 days after challenge when virus capable of multiplying in the newly introduced tumor cells should have been at a peak. Each peritoneal washing was inoculated undiluted and diluted 10^{-1}, 10^{-2}, and 10^{-3} into 12 ten day old eggs. No virus could be recovered from any of the washings.

(*b*) Antiviral antibody was determined at the time of challenge in the sera of 10 mice immunized with WSA oncolysate. The average HAI titer against WSA was 1:640.

(*c*) A WSA oncolysate with an HA titer of 1:64 and an egg infectivity titer of 10^8 EID_{50}/ml was treated with 0.08% (v/v) of formaldehyde overnight at 37°C. No residual infectivity could be detected in this material when dilutions 10^0 through 10^{-2} were inoculated into eggs. Each of eight A2G mice received 0.5 ml of the inactivated oncolysate intraperitoneally. All these mice survived challenge with 1000 EA cells 11 days later. Their average HAI titer against WSA was 1:160.

TABLE II

Failure of Mechanical Lysates of Tumor Cells to Immunize A2G Mice Against Ehrlich Ascites Tumor

Group	Treatment*	Result of challenge*
1	Saline	0/8
2	WSA oncolysate 1:5	8/8
3	Mechanical lysate of Ehrlich ascites tumor cells‡	0/8
4	Egg-grown WSA virus§	0/8
5	Mechanical lysate of Ehrlich ascites tumor cells mixed with egg-grown WSA virus‖	0/8

* 0.5 ml i.p. on day 0. Challenge, 1000 EA cells. 0.5 ml i.p. on day 11. Final reading on day 39. Number of survivors per total number of mice in group.

‡ 10^8 EA cell equivalents per milliliter obtained by freezing-thawing and homogenization in a Sorvall omnimixer.

§ Influenza A virus, strain WSA, concentrated and partially purified from infected allantoic fluid by differential centrifugation (see Materials and Methods). HA titer 1:1600.

‖ Ultracentrifugal pellet of virus prepared as above (group 4) resuspended in EA cell lysate (as used in group 3).

(*d*) Finally, as shown below (section 4), mice actively immunized against the oncolytic virus could be protected by oncolysate and even by oncolysate to which antiviral antiserum had been added. It had previously been shown that active antiviral immunization of mice prevented oncolysis by WSA virus (9).

In contrast to the ability of lysates from virus-infected tumor cells to induce antitumor immunity, both lysates from noninfected tumor cells and egg-grown virus were inactive. Thus a mechanical lysate of noninfected tumor cells prepared in their own ascitic fluid and containing an equivalent of 10^8 EA cells per milliliter was not immunogenic. Egg-grown WSA virus, either as crude infected allantoic fluid (HA titer 1:160) or concentrated by differential centrifugation (HA titer 1:1600, see Materials and Methods) induced high levels of HAI antibodies but failed to immunize against the tumor, even

after repeated injections. Mechanically lysed noninfected tumor cells mixed with egg-grown WSA virus also failed to immunize against the tumor. A typical experiment is presented in Table II.

2. Attempts at Separating the Immunogen from the Virus Particles in Viral Oncolysates.—Since lysates from virus-infected tumor cells were immunogenic but lysates from noninfected tumor cells were not, we tried to separate the virus from the bulk of the oncolysate. The immunogenic activity could not

TABLE III

Absorption and Elution of Immunizing Principle from Viral Oncolysate

Group	Treatment*	Result of challenge*
1	Saline	0/6
2	Chick red blood cells‡	0/6
3	Egg-grown WSA virus adsorbed onto chick red blood cells	0/6
4	WSA oncolysate	11/12
5	WSA oncolysate after absorption with chick red blood cells	1/6
6	Washed chick red blood cells used to absorb WSA oncolysate	10/12
7	Same as above, but after RDE treatment	1/6
8	RDE eluate from above, concentrated by ultracentrifugation	6/6
9	Same as above diluted 1:10	3/6

* 0.5 ml i.p. on day 0. Challenge, 1000 EA cells. 0.5 ml i.p. on day 11. Final reading on day 39. Number of survivors per total number of mice in group.

‡ 2.5% suspension in saline. For details on the other preparations, see Materials and Methods and text.

be separated from the virus particles by differential centrifugation. An experiment on absorption and elution is shown in Table III.

Absorption of viral oncolysate with chick red cells diminished its immunogenicity (group 5). The chick red cells that had absorbed the virus proved immunogenic (group 6). The immunogen could be eluted from such cells with neuraminidase, leaving inactive red cells behind (groups 7, 8, 9). Parallel tests on virus grown in eggs showed lack of immunogenicity (group 3, and Materials and Methods).

More refined attempts at purifying the virus also failed to separate the hemagglutinating and infectious activities of viral oncolysates from their

capacity to immunize against the tumor. Among the methods used were fractionation on DEAE cellulose (10) and sedimentation in sucrose and potassium tartrate gradients. We shall not insist on details of these experiments here since they are open to the criticism that perfect purity of a virus suspension is difficult to prove.

3. Inhibition of Immunogenic Activity of Viral Oncolysates by Rabbit Antiserum to Egg-Grown Virus.—When viral oncolysates were mixed with potent rabbit antiserum prepared against egg-grown WSA virus and then injected into A2G mice, both the antiviral and antitumor responses were suppressed. The amount of antibody added was sufficient to neutralize the infectivity of the oncolysate for eggs. The same effects could also be produced with the gamma globulin fraction of the antiserum. Normal rabbit serum, including the preimmunization serum of the rabbit from which the anti-WSA had been obtained, was without effect. Table IV shows a representative experiment. Similar inhibition of immunogenicity was observed when the rabbit antiserum was first injected intravenously 5 hr before intraperitoneal injection of viral oncolysate.

TABLE IV

Inhibition of Immunizing Activity of Viral Oncolysate by Rabbit Antibody to Egg-Grown Virus

Group	Treatment*	Antiviral response‡	Result of challenge§
1	Saline	<10	0/6
2	WSA oncolysate diluted 1:5 in saline	640	6/6
3	WSA oncolysate diluted 1:5 in normal rabbit serum	640	6/6
4	WSA oncolysate diluted 1:5 in rabbit anti-WSA	<10	0/6
5	WSA oncolysate diluted 1:5 in rabbit anti-WSA γ-globulin (2 mg/ml)	<10	3/12

* 0.5 ml i.p. on day 0. For details of the various preparations used, see Materials and Methods.

‡ As measured by hemagglutination-inhibition against four agglutinating doses of egg-grown WSA. Figures show reciprocal of HAI titer of pooled orbital bleedings on day 14.

§ Challenge, 1000 EA cells. 0.5 ml i.p. on day 11. Final reading on day 39. Number of survivors per total number of mice in group.

Rabbit antisera prepared against egg-grown influenza B virus, strain Lee, did not inhibit the immunogenicity of WSA oncolysates when used at the same HAI concentrations as anti-WSA in experiments of the type shown in Table IV.

However, when larger amounts of anti-Lee were used, a slight degree of inhibition became apparent. Since adjustment of antiserum concentration by HAI titer seemed an arbitrary procedure, it was felt that a satisfactory demonstration of serologic specificity required a different approach.

Experiments of the following type were therefore contemplated: Two oncolytic viruses, X and Y, were to provide oncolysates OX and OY, and the same viruses grown in eggs would be used to prepare rabbit antisera anti-EX and anti-EY. Proof of serologic specificity would be obtained if it could be shown that anti-EX inhibited the immunogenicity of OX more than that of OY, while at the same time anti-EY inhibited OY more than OX.

We chose the WSA virus strain for X and the TUR virus strain for Y. Adaptation of the TUR strain to Ehrlich ascites tumor was easily achieved. A rabbit antiserum was prepared from the unadapted strain passed exclusively

TABLE V

Hemagglutination-Inhibition Studies with Rabbit Antisera to the WSA and TUR Strains of Influenza A Virus

Test virus*	Anti-WSA‡	Anti-TUR§
WSA	2560	<10
TUR	<10	5120

* Four agglutinating doses of tumor-grown virus. Figures show reciprocal of HAI titer.
‡ Serum of a rabbit immunized with egg-grown WSA (see Materials and Methods).
§ Serum of a rabbit immunized with egg-grown TUR (see Materials and Methods).

in eggs. Thus the virus used for immunizing the rabbit had never encountered tumor cell antigens. TUR behaved very similarly to WSA during oncolysis. Both strains were influenza A viruses and hence shared the complement-fixing nucleoprotein antigen. Their surface antigens showed little cross-reactivity as judged from HAI tests (Table V).

TUR oncolysates had essentially the same characteristics as have already been described for WSA oncolysates. The results of a cross-inhibition experiment with rabbit antisera are shown in Table VI. All control mice died (group 1), as did mice pretreated with rabbit antiviral antiserum alone (not shown). Oncolysates mixed with homologous antiserum (WSA oncolysate + anti-WSA serum or TUR oncolysate + anti-TUR serum) had lost their immunizing power (groups 3 and 6). However, the same concentration of antisera either did not inhibit the immunogenicity of heterologous oncolysates (group 7) or inhibited it very weakly (group 4). The antiviral response (not shown) followed the same pattern.

4. Enhancement of Immunogenic Activity of Viral Oncolysates by Prior Active Immunization with Egg-Grown Virus. When A2G mice were first actively

immunized against the virus by intraperitoneal injections of egg-grown WSA and then immunized with WSA oncolysate, the dose of oncolysate needed to achieve a given level of immunity against the tumor was reduced. Table VII shows a typical experiment. High levels of HAI antibodies (titers 1:640 to 1:12,560) were induced by this preimmunization schedule. In several experiments, primed mice required 4–16 times less oncolysate as unprimed mice to attain comparable levels of antitumor immunity. The relatively large numbers

TABLE VI

Specificity of Inhibition of Immunizing Activity of Viral Oncolysates by Antiviral Antiserum

Group	Treatment*	Result of challenge*
1	Saline	0/6
2	WSA oncolysate diluted 1:5 in saline	6/6
3	WSA oncolysate diluted 1:5 in rabbit anti-WSA‡	0/6
4	WSA oncolysate diluted 1:5 in rabbit anti-TUR§	5/6
5	TUR oncolysate diluted 1:5 in saline	6/6
6	TUR oncolysate diluted 1:5 in rabbit anti-TUR§	0/6
7	TUR oncolysate diluted 1:5 in rabbit anti-WSA‡	6/6

* 0.5 ml i.p. on day 0. Challenge, 1000 EA cells. 0.5 ml i.p. on day 11. Final reading on day 39. Number of survivors per total number of mice in group.

‡ Serum of a rabbit immunized with egg-grown WSA (see Materials and Methods). HAI titer 1:2560.

§ Serum of a rabbit immunized with egg-grown TUR (see Materials and Methods). HAI titer 1:5120.

of animals required was a drawback of these experiments. Thus one would hesitate to conclude from Table VII that priming with influenza B virus (group 9) was without effect. A useful amplification of the difference between primed and unprimed mice was obtained in the following experiment.

Mice were either primed by an injection of egg-grown WSA, by an injection of egg-grown B/Lee, or an injection of saline. 2 wk later, one-third of each group received injections of WSA oncolysate, one-third injections of WSA oncolysate mixed with rabbit anti-WSA, and one-third injections of saline. 11 days later, all mice were challenged with 1000 EA cells. The results are shown in Table VIII.

All control mice died (groups 1, 4 and 7). All mice which had received on-

colysate alone survived (groups 2, 5 and 8). The unprimed mice that had received the mixture of oncolysate and antiserum died (group 3) as did the mice primed with heterologous virus (group 6). In mice primed with WSA, however, immunogenicity of the oncolysate was not abolished by antiserum (group 9). The results of these experiments are in line with the observation that a primary

TABLE VII

Effect of Priming with Egg-Grown WSA on Immunogenicity of WSA Oncolysate

Group	First treatment*	Second treatment*	Result of challenge*
1	Saline	Saline	0/6
2	Saline	WSA oncolysate diluted 1:20	6/6
3	Saline	WSA oncolysate diluted 1:40	3/6
4	Saline	WSA oncolysate diluted 1:80	0/6
5	Egg-grown WSA‡	Saline	0/6
6	Egg-grown WSA	WSA oncolysate diluted 1:40	6/6
7	Egg-grown WSA	WSA oncolysate diluted 1:80	6/6
8	Egg-grown WSA	WSA oncolysate diluted 1:160	5/6
9	Egg-grown B/Lee§	WSA oncolysate diluted 1:40	4/6

* First treatment, 0.1 ml i.p. on day 0. Second treatment, 0.5 ml i.p. on day 14. Challenge, 1000 EA cells. 0.5 ml i.p. on day 25. Final reading on day 53. Number of survivors per total number of mice in group.

‡ Influenza A virus, strain WSA, concentrated and partially purified from infected allantoic fluid (see Materials and Methods). HA titer 1:1600.

§ Influenza B virus, strain Lee, concentrated and partially purified from infected allantoic fluid (see Materials and Methods). HA titer 1:3200.

antigenic stimulus can easily be inhibited by antibody excess, whereas a secondary stimulus cannot (15, 16, 17).

DISCUSSION

A single intraperitoneal injection of a viral oncolysate induced a significant level of resistance to challenge with tumor cells. This resistance could not be attributed to a recapitulation of oncolysis for the following reasons: No virus could be recovered at the time of challenge; high levels of antiviral antibodies were present; inactivation of the virus by formalin did not abolish immunogenicity; mice actively preimmunized against the virus could be protected by

viral oncolysate and by viral oncolysate to which neutralizing amounts of antiviral antibody had been added.

When we first observed antitumor immunity following viral oncolysis, we considered the possibility that the immunogenicity of certain structures of the

TABLE VIII

Effect of Priming with Egg-Grown WSA on Immunogenicity of WSA Oncolysate Mixed with Rabbit Anti-WSA

Group	First treatment*	Second treatment*	Result of challenge*
1	Saline	Saline	0/4
2	Saline	WSA oncolysate diluted 1:5 in saline	4/4
3	Saline	WSA oncolysate diluted 1:5 in rabbit anti-WSA‡	0/4
4	Egg-grown B/Lee§	Saline	0/4
5	Egg-grown B/Lee	WSA oncolysate diluted 1:5 in saline	4/4
6	Egg-grown B/Lee	WSA oncolysate diluted 1:5 in rabbit anti-WSA‡	0/4
7	Egg-grown WSA‖	Saline	0/4
8	Egg-grown WSA	WSA oncolysate diluted 1:5 in saline	4/4
9	Egg-grown WSA	WSA oncolysate diluted 1:5 in rabbit anti-WSA‡	4/4

* First treatment, 0.1 ml i.p. on day 0. Second treatment, 0.5 ml i.p. on day 14. Challenge, 1000 EA cells. 0.5 ml i.p. on day 25. Final reading on day 53. Number of survivors per total number of mice in group.

‡ Serum of a rabbit immunized with egg-grown WSA (see Materials and Methods). HAI titer 1:2560.

§ Influenza B virus, strain Lee, concentrated and partially purified from infected allantoic fluid (see Materials and Methods). HA titer 1:3200.

‖ Influenza A virus, strain WSA, concentrated and partially purified from infected allantoic fluid (see Materials and Methods). HA titer 1:1600.

tumor cell might be increased when they became associated with or incorporated into the virion (11). We can now offer some evidence in favor of this view.

(*a*) Egg-grown WSA virus alone or mixed with mechanically lysed, uninfected tumor cells was incapable of inducing antitumor immunity.

(*b*) When the virions were isolated from the bulk of the viral oncolysate by differential centrifugation, adsorption to and elution from red cells, and other

procedures, the capacity to induce antitumor immunity could not be separated from the virus. This is a comparatively weak argument at the present stage of our attempts to purify the virus.

(*c*) The immunizing activities of viral oncolysates both with respect to antiviral (HAI) antibody and antitumor immunity, could be abolished by rabbit antibody to egg-grown virus. This effect was mediated by the gamma globulin fraction of the rabbit antiserum and showed serologic specificity.

(*d*) The immunizing activities of viral oncolysates were enhanced by prior vaccination of mice with homologous egg-grown virus. This effect again showed serologic specificity.

Since the rabbit antisera were prepared against egg-grown virus, they should have recognized virus-specific and chick-specific antigens only. The host component of tumor-grown virus may have cross-reacted with antibody to the chick-specific component of egg-grown virus. If this were so, one would have expected that mice could be immunized against the tumor with egg-grown virus. Several attempts to demonstrate this have failed. Also, antisera induced by different strains of egg-grown influenza virus should have shown extensive cross-inhibition of immunogenicity. But the serologic specificity observed points instead to the virus-specific component of tumor-grown virus as the target of the rabbit antibody.

One difficulty in this concept is that antibody directed against only part of the antigenic determinants of a complex antigen seems to inhibit the immunogenicity of the whole antigen. This probably means that the host antigen is an integral part of the virus and not merely an adventitious contaminant. Similar intimate integration of host antigen has indeed been shown to occur in influenza virus: Antibody directed against one particular host component inhibits hemagglutination by the virus (5, 6). A possible analogy is presented by the decreased risk of Rh sensitization of mothers who are incompatible with their foetus with respect to the ABO group (18). Whatever explanation is contemplated, it is difficult to escape the conclusion that the immunogenicity of viral oncolysates must be in some way associated with the virus, since it was the virus which provided the only link between oncolysate and rabbit antibody in our experiments.

The same conclusions follow experiments on mice primed with egg-grown virus. Here again it could be argued that the chick-specific component of egg-grown virus, although by itself unable to induce antitumor immunity, primed the mice with respect to cellular antigens in viral oncolysates. Additional assumptions are necessary to account for the serologic specificity observed. We prefer the alternative explanation that the mice were primed by the virus-specific antigens. It may seem difficult at first to understand why priming with one antigen should be followed by an anamnestic response to a novel antigen. This, however, is a well-recognized phenomenon and represents the less obvious facet of so-called "original antigenic sin" (19). In the words of Fazekas de St.

Groth and Webster, "the Original Antigenic Sin is manifested by a secondary response to a foreign substance the organism is experiencing for the first time" (20). Another useful analogy is provided by examples of enhanced antibody response to a hapten-carrier complex after priming with carrier alone (21). This is said to occur only when the priming injection elicits delayed hypersensitivity (22). We have not studied this aspect in our mice, but it is known that influenza viruses induce a state of delayed allergy in man (23).

All mice passively immunized with rabbit antiviral antiserum and mice actively immunized with egg-grown virus had high titers of HAI antibody directed against the oncolytic virus. Whereas the first procedure (passive immunization) abolished immunogenicity, the second procedure (active immunization) enhanced the immunogenicity of the viral oncolysate. This paradox is easily resolved. Primary immunization can be inhibited by antibody excess; a secondary response, however, is little affected by either actively acquired or passively administered antibody (15, 16, 17). This was strikingly demonstrated in experiments in which the same oncolysate-antibody mixture was not immunogenic in unprimed mice or in mice primed with heterologous virus, but was immunogenic in mice primed with homologous egg-grown virus (Table VIII).

Quantitation in our experiments was relatively crude, and we cannot state the precise degree of serologic specificity involved. No exact parallelism need be expected with any of the serologic groupings currently recognized among influenza viruses, particularly if delayed hypersensitivity should prove essential for priming. A number of experiments suggest themselves. For instance, rabbit antisera to a whole spectrum of influenza viruses could be used to pinpoint the antigenic structure most closely associated with the immunogen. Another approach to the same problem would be the preparation of highly purified virus from virus-infected tumor and its fractionation into subunits. Also, the time at which tumor homogenates first become immunogenic after viral infection might give important clues.

Several reports indicate that viral oncolysis leads to tumor immunity. Thus, oncolysis by arboviruses was followed by solid immunity to several transplantable tumors (24, 25). The same was true of oncolysis by tumor-adapted influenza virus (9, 10, 11, 26). Tumor cells infected in vitro with influenza virus induced immunity to the tumor (27). A tumor-adapted strain of Newcastle disease virus led to oncolysis and postoncolytic tumor immunity (28), as did a strain of reovirus type 3 (29).

The question arises whether incorporation of host materials into the virus is a common feature of all of these experiments. Influenza and Newcastle disease viruses are known to incorporate host materials into their envelopes (1, 2, 3, 4, 5, 6, 30, 31, 32). Sindbis, an arbovirus, contains host-derived antigens (4). The situation is less clear with reovirus, possibly because of lack of work on this aspect. Besides incorporation of host antigens into virions, other means of

enhancing immunogenicity by viral infection are conceivable (33). The increase of cellular immunogenicity just described might be related to some forms of induction of "neoantigens" by oncogenic viruses. Although it is usually assumed that these antigens are coded for in the genome of the virus, alternative explanations may exist. For instance, it has been suggested that the common complement-fixing antigen of the avian leucosis group of viruses could be a normal chick component (34).

The possibility of vaccinating animals with the viral agent prior to injecting them with virus-infected cells opens new perspectives for the study of virus-enhanced immunogenicity of tumors and other tissues. It would no longer be necessary to use mice genetically resistant to certain oncolytic viruses, such as PRI (25) or A2G (9), or to use viruses of intrinsically low virulence, such as NDV (28) or reo 3 (29). Neither would the choice of viruses have to be limited to those having dramatic oncolytic effects. Infection of the cells could take place either in vivo, as in the experiments presented above, or in vitro, as performed by others (27). In vivo infection would offer the advantage of much higher cell density.

There is no evidence that tumor-specific target antigens rather than alloantigens are involved in the system we have studied. It remains for future experiments to elucidate the behavior of tumor-specific antigens during viral infections.

Much speculation but relatively few facts support the idea that viruses play an important role in initiating autoimmune disorders (33). It has recently been possible to induce an autoantibody to an antigen present in chicken bile by immunization of chickens with egg-grown influenza virus (35). Our experiments demonstrate that, in the mouse, cellular structures of low immunogenicity, perhaps haptens, become potent immunogens when integrated into the makeup of viral particles. Similar phenomena may be involved in the immunopathology of certain diseases. Experiments modeled on the oncolytic system outlined above might shed some light on this area.

SUMMARY

A2G mice could be solidly immunized against the Ehrlich ascites tumor by single intraperitoneal injections of homogenized and lyophilized tumor cells which had been infected with oncolytic strains of influenza A virus. Similar homogenates from noninfected tumor cells were not immunogenic, even when mixed with egg-grown virus.

The immunizing principle in viral oncolysates could not be separated from the oncolytic virus by differential centrifugation or adsorption to and elution from red cells. It could be inhibited by antibody raised in rabbits against the egg-grown oncolytic virus. This reaction showed serologic specificity. Thus, the immunogenicity of an oncolysate produced with the WSA strain of neurotropic influenza virus could be inhibited by rabbit anti-WSA, but not by rabbit anti-

body to the TUR strain of fowl plague virus. Conversely, the immunogenicity of an oncolysate prepared with the TUR strain could be inhibited by rabbit anti-TUR, but not by anti-WSA.

When mice were preimmunized (primed) with egg-grown WSA virus, their antitumor response to a later injection of WSA oncolysate was of the anamnestic type. Priming with egg-grown influenza B virus had no such effect.

It was concluded that the immunogenicity of certain host cell components was greatly increased by incorporation into the makeup of the oncolytic virus.

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