Further Observations on the Inhibitory Effect of Myxoviruses on a Transplantable Murine Leukemia[1] (34138)

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In a study on oncolysis by myxoviruses of a syngeneic transplantable murine leukemia induced by the Gross virus it was observed that C_3H mice preimmunized with Newcastle disease virus (NDV) had greater resistance than normal animals to leukemic cells infected with NDV before transplant (1). This phenomenon, which may be characteristic of viruses that are assembled at the cell membrane with local antigenic alterations, is in contrast to results of similar experiments with other viruses such as vaccinia where active or passive antiviral immunity interfered with oncolysis (2). The possible role of immune leukocytes is suggested by the observations of Speel *et al.* (4) who found that splenocytes from animals immune to mumps virus partially destroyed cultures of human conjunctival epithelial cells chronically infected with that virus. Others have demonstrated cytolysis of cells infected with rabies virus by antiviral antibody and complement *in vitro* (7).

The present paper presents additional evidence for antigenic conversion and antiviral immune oncolysis with NDV and Sendai virus and observations on the reaction of infected leukemia cells with antiviral antibodies and complement *in vitro*. The results suggest differences between influenza and parainfluenza viruses in relation to oncolytic activity and the effect of antiviral immunity on oncolysis.

Materials and Methods. Mice and tumors. The source of the inbred C_3H/Bi and C_3H/He strains of mice and the induction by Gross virus of lymphatic leukemia and an ascites lymphoma has been described previously (1). Also detailed in earlier publications are the methods of treating the tumor cells with virus before injection into mice, serial 10-fold dilution of cell suspensions, and estimation of cell numbers from packed cell volumes (5).

Warburg measurements. Cells were incubated, 48–72 hr as 1% (v/v) suspensions in modified Eagle's medium (additional arginine, 0.05% pyruvate, 0.05% glucose) with 10% calf serum, and infected allantoic fluid at 1:20 or 1:100 final dilution. Preparation for the Warburg consisted in washing the cells twice, measuring the volume of cells, and resuspending the yield from 20 ml of the original suspension in 1 ml of BSS[2] with additional 0.05% sodium phosphate, pH 7.6; 0.1% glutamate; and 0.1% lactate as substrates. The packed volume of cells recovered and used in the Warburg was 0.15 to 0.05 ml or 75–25% of initial amounts, depending on viral cytopathic effect. In the *in vitro* studies with antiserum, cells were incubated with virus for 18 hr or less and the experiments were done before marked viral cytopathic effect was evident.

Viruses. The NDV strain Cal had originally been brought to this laboratory by Dr. A. M. Prince and has been passed in chick embryos for over 10 years; GB1, F, L, B_2 Isl, RO1, W, and M2 were furnished by Dr. Michael Bratt of this laboratory; H and Ban were obtained from Dr. van Roekel of the University of Massachusetts. Strain Cal, M2, GB_1, F, Is1, RO_1 have high to moderate virulence for chicks; B_2, W, and H are of low virulence or avirulent. Parainfluenza I Sendai virus (obtained from Dr. J. F. Enders) and influenza WSN from Dr. M. Pons of the Public Health Research Institute

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[2] Hanks' balanced salt solution without bicarbonate.

TABLE I. Effect of Antiviral Immunity to NDV or Sendai on the Oncolytic Effect against Ascites Lymphoma in C_3H/He Mice.

Virus[a]	Antiviral immunity	Extra virus in inoculum[b]	5×10^3 d/t[c]	5×10^3 %	5×10^2 d/t	5×10^2 %	5×10^1 d/t	5×10^1 %
NDV (Cal)	+	+	12/24	50	6/33	18	1/17	6
	0	+	20/22	91	30/60	50	9/47	19
	+	0	—	—	3/57	5	0/8	0
	0	0	—	—	29/38	76	17/27	63
Sendai	+	+	9/49	18	1/18	5	—	—
	0	+	20/21	95	3/16	19	—	—
	+	0	7/22	32	0/9	0	—	—
	0	0	21/27	78	—	—	—	—
None	0	0	—	—	18/26	70	5/6	83

[a] Cells infected in 1:20 final dilution of virus allantoic fluid; HA titer 2500–5000.

[b] Infected cells diluted in 1:20 allantoic fluid before injection into mice.

[c] Number of mice dead of leukemia over number tested.

of the City of New York were nonlethal for C_3H mice by the intraperitoneal route in doses of 100–250 HA units.[3] All stock viruses were carried by the allantoic route in chick embryos. Hemagglutination titrations were done with 0.25% chicken erythrocytes. Antiviral antiserum was obtained by immunizing rabbits with infected allantoic fluid of NDV (Cal), Sendai, or influenza strains.

Results. Effect of preimmunization with virus on rejection of virus-treated lymphoma cells. The C_3H/He mice were immunized by 2–6 injections at intervals of 10–14 days of allantoic fluid infected either with Sendai or NDV (Cal) diluted 1:20. About 1 month later these mice and nonimmune controls were challenged with ascites lymphoma cells incubated 4 hr with virus and diluted either in 1:20 infected allantoic fluid or in medium without virus. As shown in Table I groups of mice immunized with virus were more resistant to various doses of cells treated with NDV or Sendai than controls receiving no preimmunization. In immune mice Sendai showed a significant inhibitory effect against 5000 cells as compared with 500 for NDV and in nonimmune mice, 500 for Sendai versus only 50 for NDV. When NDV-treated cells were inoculated into nonimmune mice dilution of the cell suspensions in excess virus improved oncolysis possibly by greatly increasing virus/cell multiplicity. Other experiments established the specificity of antiviral immunity in augmenting viral oncolysis. Mice immunized with Sendai were no more resistant than normal mice to challenge with NDV-treated cells and immunization with influenza strain PR_8 did not increase resistance to cells treated with Sendai or NDV.

Experiments on systemic leukemia transplanted as leukemic spleen in C_3H/Bi mice gave somewhat different results as shown in Table II. The NDV caused destruction of 10 times as many cells as it did with the ascites lymphoma and oncolysis was not increased by preimmunization provided cells were diluted in excess virus. But when the infected leukemic spleen cells were injected without excess virus more inhibition was observed in immune mice than in nonimmune mice. Sendai inhibited development of leukemia after inoculation of 5000 cells as compared with oncolysis of 50,000 by NDV. In the case of Sendai PR_8 and WSN antiviral immunity did not increase oncolysis of leukemic spleen cells and these results actually suggested an opposite effect, that oncolysis was slightly reduced by preexisting anti-influenzal immunity. This would agree with observations by others (6) with the WS strain. However the influenza strains PR_8 and WSN had only a

[3] In chick embryos infectivity/HA ratios were of the order 105.

TABLE II. Inhibitory Effects of Myxoviruses against Spleen-Transplant Leukemia in C_3H/Bi Mice (strain of origin).[a]

Virus	Antiviral immunity	Extra virus inoculum	No. of cells in challenge	Result d/t	Result %
NDV Cal	+	+	5×10^4	14/59	24
	0	+	5×10^4	7/44	16
	+	0	5×10^4	5/23	22
	0	0	5×10^4	13/25	52
Sendai	+	+	5×10^4	23/24	95
	0	+	5×10^4	14/23	60
	+	+, 0[b]	5×10^3	3/24	12
	0	+, 0[b]	5×10^3	1/21	5
PR_8	+	+	5×10^3	17/18	95
	0	+	5×10^3	8/16	50
WSN	+	+	5×10^2	3/12	25
	0	+	5×10^2	0/10	0
None	0	0	5×10^3	13/14	93
			5×10^2	13/23	56
			5×10^1	6/9	66

[a] Although larger or smaller numbers of cells have been tested only the results of most significance are included in Table II.

[b] Results were similar with or without extra virus.

weak oncolytic effect *in vivo* against leukemic cells.

Relation of viral oncolysis in mice to in vitro *cytopathic effect.* Several strains of NDV and influenza virus were compared for cytopathogenicity *in vitro* when incubated for 48–72 hr in roller tube suspensions of the ascites lymphoma cells. As determined by production of cell associated hemagglutinins NDV (Cal) (BAN) (F_1) influenza (PR_8) (WSN) and Sendai were shown to be infectious for ascites lymphoma cells at dilutions of 1:1000 or higher. Data on growth and infectivity of NDV (Cal) in these cells have been presented previously (1). Survival of cells was estimated by measuring rate of O_2 consumption in a Warburg apparatus in comparison with control suspensions preincubated without virus (the latter showed a reproducible O_2 uptake of 1000–1200 μl/g of wet wt/hr). With equal initial amounts of cells, lysis was indicated by recovery of lower cell volumes from tubes with virus than from the controls. This is reflected in the index of survival (Table III) which represents the ratio of the rates of O_2 uptake (virus/control) not corrected for wet weight. The O_2 measurements correlated well with dye exclusion as previously reported (9).

With various strains of NDV, good correlation was found between reduced *in vitro* survival and oncolysis of the ascites lymphoma or leukemic spleen. Three strains Ban, H, and Cal with the greatest *in vitro* effect were most oncolytic in mice. On the other hand strains L, F_1, and GB, showed little *in vitro* killing or oncolysis in mice. There was no correlation between virulence for chickens and oncolysis. None of the NDV strains was toxic or lethal for mice when injected intraperitoneally as 1:20 infected allantoic fluid.

The relation of *in vitro* cytopathogenicity to oncolysis established for the NDV strains did not hold for the two strains of influenza or for Sendai. The WSN, although as active as the most oncolytic NDV strains *in vitro,* showed 100-fold less inhibitory effect when mixed with leukemic cells injected into mice. Sendai, although less cytopathic in roller tubes, compared favorably with NDV strains for inhibitory effect in mice. Influenza strain PR_8 showed minor *in vitro* or *in vivo* effects

TABLE III. Comparison of *in Vitro* Killing, as Measured by Cell Respiration, to Viral Oncolysis *in Vivo* by Several Strains of NDV, Sendai and Influenza Virus.[a]

Test virus	HA titer allant. fl.	Index of cell survival[b]		Oncolysis; no. of cells[c]	
		1:20	1:100	Ascites lymph.	Leuk. spleen
Infl. WSN	4000	0	38	50	500
NDV-Ban	10,000	0	8	—	50,000
NDV-H	2500	0	28	2000	20,000
NDV-Cal	5000	30	45	500	50,000
NDV-Is_1	2500	0	42	5000	5000
NDV-L	5000	60	80	—	500
NDV-B_2	640	65	85	200	5000
NDV-F_1	5000	65	80	—	500
NDV-GB_1	2500	70	—	—	500
Sendai	2500	75	90	5000	5000
Infl. PR_8	5000	90	—	50	500

[a] LD_{50} of the ascites lymphoma or leukemic spleen not treated with virus was 5–20 cells (1); mice received 0.2 ml of virus–cell mixture ip.

[b] Ascites cells incubated 72 hr with virus in roller tubes before Warburg measurements; index = O_2 (μl/hr) with virus divided by O_2 (μl/hr) without virus; to correct for variations in virus content of the allantoic fluids two dilutions, 1:20 and 1:100, were tested.

[c] Maximum tolerated cell numbers for survival of more than 75% of mice previously immunized with homologous virus. Results for influenza WSN and PR_8 are in nonimmune mice. NDV strains not tested in mice showed the following *in vitro* survival indices at 1:100 dilution; BO_1, 66; W, 75; M_2, 63.

although this virus had been observed by similar methods to be actively oncolytic against Krebs 2 ascites tumor cells (5).

Effect of an interferon inducer on viral oncolysis. It was found that reducing the interval between immunizing injections of NDV or Sendai virus and inoculation of virus-treated cells increased the mortality of mice from leukemia. This could be attributed to stimulation of interferon production by the immunizing virus and such interferon might persist for as long as 2 weeks, inhibiting replication of virus in the injected cells.

Statolon[4] was used as a nonreplicative stimulator of interferon in experiments with ascites lymphoma cells and NDV in mice. As shown in Table IV statolon given 24 hr before and again at the same time as NDV-treated cells completely prevented oncolysis. When administration of statolon was delayed until 24 and 48 hr after the virus-treated cells, no interference with oncolysis was observed. The results suggest that viral oncolysis *in vivo* occurs soon after injection of cells and is dependent at least in part on viral replication. In other experiments it was found that treating NDV with formaldehyde, or heating, under conditions that greatly reduced the infectivity/HA ratio abolished its oncolytic effect.

Reaction of infected cells with antibody in vitro. Ascites lymphoma cells were infected with NDV Cal or Sendai in Eagle's medium containing 2% bovine albumin and 50–250 HA units/ml of virus. After an adsorption

TABLE IV. Effect of Statolon on Oncolysis of Ascites Lymphoma by NDV.

Time (hr) statolon given[a]	No. of mice dead/no. tested with		
	Statolon	No statolon	Control[b]
24 pre and 0	26/34	8/36	27/28
24 and 48 after	6/29	4/25	—

[a] Each dose of statolon was 6 mg, including stabilizers; challenge dose, 500 cells + NDV (Cal) at 0 hour; all mice preimmunized with NDV.

[b] Control mice received 500 cells only; no NDV no statolon.

[4] Obtained through the courtesy of Dr. W. J. Kleinschmidt, Lilly Research Laboratories (10).

period of 1 hr the cells were washed, resuspended in Eagle's medium with 10% calf serum and incubated in roller tubes for 12–20 hr. This period and input multiplicity were adjusted so that most cells would become infected but not yet killed as determined by dye exclusion. Cells were then washed and prepared for Warburg measurements as described in "Material and Methods." To one of the Warburg flasks was added antiviral serum at a concentration of 25–100 HAI units/ml and 1:20 fresh frozen guinea pig serum, to a second flask anti-PR_8 rabbit serum and complement as a control. A third flask received no serum or complement. In some experiments additional controls were uninfected cells with antiserum and complement and infected cells with antiserum only. The rate of O_2 consumption (μl/g of wet wt/ hr) was measured at hourly intervals.

The results are presented in Fig. 1 where it will be seen that the rate of oxygen consumption of cells infected with Sendai declined more rapidly in the presence of the related antiserum than with anti-PR_8 serum which gave results similar to cells incubated without serum. The rate of respiration of ascites lymphoma cells that had been incubated for 24 hr without virus declined slowly during the additional 5 hr in the Warburg. Antiserum and complement accelerated cytolysis of infected cells as shown by the steeper slope of the curves for O_2 and the proportion staining with toluidine blue. With Sendai antiserum at 100 HI units/ml over 80% of the infected cells were killed within 2 hr but a heterophile reaction of the Sendai antiserum with antigens of uninfected cells caused a minor amount of cytolysis. Absorption of the Sendai antiserum with uninfected ascites lymphoma reduced the cytolytic effect so that absorbed serum at 1:10 (50 HI units) was now equivalent in action to unabsorbed serum at 1:40 which did not lyse uninfected cells.

The participation of a heat labile factor in cytolysis was indicated by the observation that heating the rabbit serum to 56° for 20 min greatly reduced the cytolytic reaction [redacted] with addition of guinea pig complement. [redacted] hand complement, although

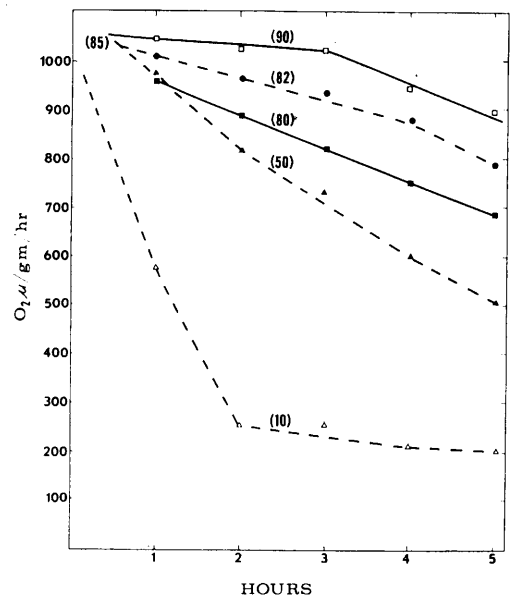

FIG. 1. Measurement by O_2 consumption of the effect of antiviral antibody and complement (fresh guinea pig serum 1:20 on cytolysis of murine ascites lymphoma cells infected with Sendai virus: Uninfected cells, (□) no serum or complement only, (■) with Sendai antiserum 1:10 and complement. (- - -), cells infected with 250 HA units/ml of Sendai virus: (●) PR_8 antiserum 1:10 and complement; (△) anti-Sendai serum 1:10 (100 HI units/ml) and complement; (▲) anti-Sendai serum 1:10 (50 HI units/ml) after absorption to no reaction with uninfected ascites lymphoma cells. The HI unit was defined as the highest dilution of antiserum which would inhibit agglutination by 8 HA units of virus. The PR_8 and Sendai antisera were not inactivated. Numbers in parentheses indicate percentage of cells living as judged by exclusion of toluidine blue at time indicated.

not cytolytic by itself, augmented the destruction of lymphoma cells by unheated rubbit serum. The line for unheated Sendai antiserum 1:10 without complement would occupy a position approximating that of the solid triangles in Fig. 1, as compared with the open triangles where complement was added.

In other experiments results similar to those for Sendai were obtained with NDV (Cal) and the corresponding antiserum. A parainfluenza type 1 horse antiserum obtained commercially showed an HI titer of 1:320 with Sendai virus. This serum at a dilution of 1:5 produced a cytolytic effect,

with complement, against Sendai-infected cells but no reaction with normal lymphoma.

Discussion. Immune cytolytic reactions between virus-infected cells and antibody (plus complement) or immune leukocytes have now been demonstrated with Rauscher leukemia virus (3), mumps (4), rabies (7), and in the present paper Newcastle disease and Sendai viruses for the first time. Active antiviral immunity increases resistance of mice to transplant of tumors infected with NDV or Sendai, and to polyoma and herpes (8); with the latter also by passive immunization with immune lymphocytes. Either reduced viral oncolysis or no effect in virus-immune mice have been reported for vaccinia, lymphogranuloma venerium (2) encephalomyocarditis (1) and influenza viruses (6). Where infection *in vitro* is done at a virus/cell multiplicity less than one some of the cells remain uninfected at the time of injection into mice and the presence of circulating antiviral antibody would prevent a second cycle of viral replication thus permitting such cells to multiply but in our experiments multiplicity was very high because cells were diluted in over 100 HA units/ml of virus. It remains to be determined whether cytolytic effects are due solely to antibody and complement in some cell–virus systems, in others to immune leukocytes, or to both. In cases where destruction of infected cells depended only on immune leukocytes antibody might inhibit lysis by coating the infected cells and preventing adherance of lymphocytes (4).

Rate of oxygen consumption as a measure of cytolysis (9) has certain advantages over the dye exclusion procedure; it is more quantitative and objective, more sensitive, and can be done repeatedly at various times on the same cell suspension.

In the present report some differences between two leukemic tumors induced by the same agent (Gross virus) as to augmented oncolysis in virus immune mice have been noted. For example immunity to Sendai did not increase oncolysis of the leukemic spleen cells but did so with the ascites lymphoma. Also Sendai was more oncolytic for the lymphoma than for leukemic spleen while the reverse was true for NDV (Cal). It is possible that differences of surface characterics and ability to spread systemically in the two kinds of leukemic cells may be a factor in their response to oncolytic viruses.

It is quite likely that the process of immunization against NDV or Sendai and the inclusion of excess virus in the inoculum of virus-treated cells stimulated production of interferon. The diminished viral oncolysis caused by statolon given before the virus-treated cells strongly suggests that interferon brought this about by preventing viral replication. In immune mice giving excess virus with the infected cells does not further improve oncolysis and might under certain conditions be detrimental because of interferon stimulation.

Maturation at the cell membrane by budding through is thought to be the final step in development for viruses both of the influenza and parainfluenza group. Of the several strains in these two groups so far tested only Sendai and NDV show increased oncolysis in virus immune mice while several influenza strains were weakly oncolytic and inhibition of tumor growth was either reduced or unchanged in virus-immune mice. It is also of interest that Sendai or NDV, usually inactivated, are apparently more effective than influenza virus in bringing about fusion of cells. Although aggregations of infected ascites lymphoma cells were observed after 24-hr incubation in the present studies these were dead as judged by staining with toluidine blue. Possible fundamental differences between influenza and parainfluenza viruses in modification of the cell membrane and the role of cell fusion in oncolysis by NDV and Sendai viruses remain subjects for further study.

Summary. Immunization of mice with parainfluenza viruses NDV or Sendai increases the oncolytic effect of these viruses when preinfected leukemic cells are injected into C_3H mice. Variations in oncolytic activity between different strains of influenza and parainfluenza viruses were noted, and also between two leukemic tumors induced by the Gross virus. Statolon given before virus-infected cells prevents oncolysis but has no effect when given later. Antiserum to NDV or Sendai (plus

complement) shows a cytolytic effect *in vitro* against leukemic cells infected with these viruses.

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