Removal of Cell-Associated Myxoviruses by Antibody[1] (35858)

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It is generally assumed that viruses after crossing the cell membrane become inaccessible to antibody. This concept has formed the basis for measurements of viral penetration with myxoviruses (1–3) and other viral species (4). Antibody treatment (during the first hour at 37°) of cells that have adsorbed influenza virus shows that the proportion of noneutralizable virus increases with time. In earlier experiments (1), we had observed that the concentration of antibody and period of exposure were important factors affecting the neutralization of membrane associated virus.

Further studies have revealed that certain paramyxovirus–host-cell systems show extreme sensitivity to antibody and that neutralization of virus, probably membrane associated, can occur under some conditions 24 hr after infection. Other strains under the same conditions show up significant sensitivity to antibody after penetration is complete. A strain highly sensitive in one kind of host cell may be much less so in another. Reversibility of the surface neutralization phenomenon has also been investigated.

Materials and Methods. Krebs 2 ascites tumor cells were propagated by intraperitoneal passage in Swiss mice. The ascites lymphoma, originally induced by Gross virus, was similarly grown in C3H/Bi mice. Chick embryos 10–11 days old were minced and trypsinized to produce minute fragments and free cells. Chorioallantoic membrane, also minced but not trypsinized, was used in some experiments.

Viruses were propagated in the allantoic sac of chick embryos. Two strains of influenza type A, PR_8, and WSN, three strains of Newcastle disease virus (NDV) Cal, BA, and IS, and the Sendai strain of parainfluenza I were studied. Allantoic fluids contained approximately 5000 hemagglutinin (HA) units/ml. After 1-hr incubation of cell suspension with virus, this was removed by centrifugation and washing. Input multiplicity for the ascites tumor cells was estimated at 1–5 EID_{50}/cell and for the chick embryo tissue 5×10^5 EID/mg which, at 10^5 cells/mg, would give a similar multiplicity. All strains were identified as influenza, parainfluenza, or NDV by HA inhibition (HAI) with known antiserum. In ascites tumor cells much more noninfectious HA was produced than in chick embryo tissues. After 48 hr in ascites cells EID_{50}/HA ratios of cell extracts varied from 1 for Sendai and WSN to 10^2 or 10^3 for the NDV strains. Generally less HA was found in the culture fluids than in cell extracts (5).

Antiserum. Rabbits and guinea pigs were immunized by repeated injections of egg passage virus strains PR_8, WSN, NDV, and Sendai. HAI titers were measured vs 8 HA units of virus. Antiserum (inactivated at 56°) added to the infected cell suspensions at 4 hr or later was either left in for the 48-hr duration of the experiment, or removed at various times by washing. Continued presence of antibody in the medium limited growth of cell-associated virus to one cycle at most. Cells were carefully washed to remove antibody before preparation of extracts for HA titration (1). Antisera used with chick embryo tissue were absorbed with 10% chicken erythrocytes to remove tissue antibodies. With ascites tumor cells, removal of tissue antibodies was not necessary.

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TABLE I. Comparison of Antibody Sensitivity of Influenza, Parainfluenza (Sendai), and Newcastle Disease Viruses in Mouse Ascites Tumor and Chick Embryo Cells.[a]

Virus	Strain	Cell	Antibody HAI/ml	Cell-associated hemagglutinin titer (48 hr)		
				(A) Control	(B) Experimental	A/B
NDV	IS	AL	0.8	160	20	8
	IS	AL	6.4	320	5	64
	BA	AL	128	640	80	8
INF	WSN	AL[b]	250	160	160	1
Sendai		AL	25	40	5	8
NDV	CAL	K_2	2	32	4	8
INF	PR_8	K_2	250	1024	32	32
NDV	Cal	CAM	25	128	3	16
INF	PR_8	CAM	25	2048	128	16
NDV	IS	CE	25	1280	1280	1
	IS	CE	250	2560	10	256
Sendai		CE	25	2560	16	16
		CE	100	640	10	64
INF	WSN	CE[b]	100	20,000	1280	16

[a] Antibody added 4 hr after infection. AL = mouse ascites lymphoma; K_2 = Krebs mouse ascites tumor; CAM = minced chorioallantoic membrane; CE = minced whole chick embryo, trypsinized.

[b] 10% calf serum in medium, in all other experiments 1% bovine albumin.

Results. Comparison of sensitivity to antibody at 4 hr after infection. Previous studies (1, 6) have indicated that penetration of influenza virus in chick embryo and mouse ascites cells is completed within 4 hr after adsorption. This period was therefore selected in experiments comparing the minimal inhibitory antibody concentrations for the various strains. Representative results of a number of experiments, which were reproducible, are presented in Table I. The most dramatic effect of antibody (present 4–48 hr) was seen with NDV-IS in ascites lymphoma where 0.8 HAI/ml produced significant inhibition. In contrast 128 HAI/ml or 160 times as much antibody were required for comparable inhibition of NDV-BA. With influenza WSN 250 HAI/ml of influenza antibody were ineffective. A few experiments with Sendai, which grew poorly in ascites cells, indicated an antibody sensitivity intermediate between that of NDV-IS and NDV-BA. In Krebs 2 cells the minimal concentration of homologous antibody producing inhibition was 2 HAI/ml for NDV-Cal as compared with 250 for influenza PR_8.

Viral adsorption by AL at 4 or 36° did not clearly relate to antibody resistance or sensitivity. NDV-IS was unique in being the only strain not strongly adsorbed at 4°. Two sensitive strains, IS and Sendai, and one resistant, PR_8, showed relatively weak adsorption at 36°. Others adsorbed and did not elute within 3 hr.

In chick embryo tissues all strains grew well; and differences between paramyxoviruses and the influenza group were less remarkable. NDV-Cal, influenza PR_8, and Sendai were about equally inhibited by 25 HAI/ml. In contrast IS was definitely less sensitive to antibody in CE than in AL. Influenza WSN was inhibited by 100 HAI/ml in CE even though growth in the controls was much greater in CE than in AL.

Late addition of antibody. By 24 hr after infection, cell-associated HA had almost reached maximum titer. It was of interest to determine whether or not antibody at this time would actually remove HA. This was found to be the case (Table II) with NDV-IS in AL and Sendai in CE. However BA, PR_8, and WSN in AL; and BA and IS in CE

TABLE II. Effect of Antibody Added 24 hr After Virus.

Virus	Strain	Cell	Antibody HAI/ml	HA titer control/expt.	Cells excluding dye at 24 hr (%)
NDV	IS	AL	6.4	16	70
	BA	AL	250	4	18
INF	PR_8	AL	50	1	44
	WSN	AL	250	2	17
NDV	IS	CE	25	1	50
	IS	CE	250	256	
Sendai		CE	25	16	35
NDV	BA	CE	250	1	50
INF	WSN	CE	25	1	75[a]
	WSN	CE	100	16	

[a] At 18 hr postinfection.

were found to be resistant at 24 hr; WSN in CE was inhibited by 100 HAI/ml as at 4 hr.

If there were extensive cell disintegration at 24 hr after infection, the disappearance of cell-associated HA might be explained merely as neutralization of release or exposed virus. The ratios of cell-associated HA/supernatant HA constituted evidence against this. In AL at 24 hr, this ratio was 32 for NDV-IS and 20 for BA; in CE it was 8 for Sendai and WSN, 16 for IS, and 32 for BA. The patterns of sensitivity or resistance at 24 hr resembled those after the 4-hr additions but resistance to antibody was somewhat increased at 24 hr.

Dye exclusion. The last column in Table II is included to compare the destructive effect of the viruses with sensitivity to antibody at 24 hr. Toluidine blue was used in dye exclusion tests as previously described (7). AL infected with NDV-IS, the most antibody-sensitive strain, showed strong dye exclusion at 24 hr; whereas cells with BA, a resistant strain, showed considerable membrane disruption. The influenza strains, both resistant in AL showed poor dye exclusion for WSN and moderate for PR_8. Other comparisons for infected CE are shown in Table II. At 24 hr, stained cell debris was not evident in any case. Antibody at 4 hr did not prevent cytopathology. There was no difference in dye exclusion between infected cells incubated with, and without, antibody from 4 to 24 hr.

Reversibility. Cells infected with viruses sensitive to antibody at low or high HAI/ml were treated at 4, 12, or 24 hr and the antibody removed at various times by centrifugation and washing (Table III). The next to last column of Table III shows the reduction in cell-associated HA titer when the antibody was removed at the times indicated by resuspension of the cells. When antibody was added at the times indicated and left in there was in some cases a much greater reduction in HA (last column) indicating reversal of inhibition. With IS there was evidence of partial reversal especially after late addition of antibody. Sendai in CE showed complete reversal when antibody was added at 24 and removed at 30 hr. WSN, inhibited by antibody added at 4 hr, did not grow out when antibody was removed. One-hour exposure to 25 HAI/ml of antibody 4–5 hr after infection resulted in normal growth of Sendai in CE and PR_8 in CAM. However, in the latter case, irreversible inhibition occurred with 100 HAI/ml.

Discussion and Summary. The fact that the two strains IS and BA in AL showed such a large difference in sensitivity to the same NDV antibody makes it seem likely that the disappearance of cell-associated viral hemagglutinins is due to a reaction at the cell membrane, not inside the cell. Since the HAI titer was approximately the same for IS as for BA, entry of antibody into the cell should

TABLE III. Reversal of Inhibition by Removal of Antibody at Various Periods After Injection.

Virus	Strain	Cell	Antibody			HA control/HA expt.[a]	
				Time (hr)		Serum	Left
			HAI/ml	Added	Removed	removed	in[b]
NDV	IS	AL	6	4	24	16	64
	IS	AL	25	12	24	8	32
	IS	AL	25	24	30	4	16
Sendai		CE	50	4	24	4	16
		CE	25	4	5	1	16
		CE	25	24	30	1	16
INF	WSN	CE	100	4	24	16	16
	PR_8	CAM	25	4	5	2	16
	PR_8	CAM	100	4	5	64	64

[a] Cell-associated HA at 48 hr; ratio of titers as for A/B in last column of Table I.
[b] Data partly from Tables I and II.

cause equal inhibition. In the antibody-sensitive cell–virus systems, virus budding through the membrane probably is removed as fast as it is formed and it follows that maturation is exclusively at a site accessible to antibody. With virus strains where large amounts of cell associated hemagglutinins are formed, even in the presence of extracellular antibody, intracellular HA production seems likely. Evidence for association of influenza virus with internal membranes of the cell has been presented by Holland and Kiehn (8). Also it is of interest that ascites lymphoma cells infected with influenza are more resistant to antiviral immune cytolysis, early or late in infection, than are those infected with Sendai or NDV (9). Part of the increased resistance in CE might be attributed to small clumps of cells which were difficult to eliminate from the suspensions. For example, NDV-IS, highly sensitive in AL, was resistant in CE. However, Sendai was inhibited by about the same antibody concentration in either cell species and WSN was apparently more resistant in AL than in CE.

Resistance was overcome by increasing the antibody concentration. In CE, the strains NDV-IS, NDV-BA, and influenza, became sensitive to high antibody levels. An explanation of this phenomenon must await further investigation but concentration dependence suggests forced diffusion across a barrier. The alternative is entrance of small amounts of antibody into the cell through pinocytosis.

The action of antibody at 24 hr in causing disappearance of cell-associated HA does not seem related to cell disruption by the virus, at least those cases where dye exclusion remained effective or the inhibition was reversible.

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