An Hypothesis for the Mechanism by Which an Hemagglutinin Inhibitor Affects Vaccinia Virus[1] (35178)

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The conversion *in vivo* and *in vitro* of vaccinia virus to hemagglutinin-negative has been associated with the presence of an hemagglutinin inhibitor found in the plasma of the Ehrlich ascites tumor of mice (1, 2). The rapidity of this conversion is impressive, and it is of interest to determine whether it is a genetic or nongenetic process. Evidence from the present study suggests the change is nongenetic and results from the inhibitor complexing with the hemagglutinin, thus preventing its function as a template for the formation of more hemagglutinin.

Materials and Methods. Viruses. The vaccinia viruses employed in this study were strains IHD-E, HS-33, and TF-33 (2). The last two viruses were derived from a common inoculum carried divergently for 33 passages in L cells with or without inhibitor (ascites tumor plasma) present. The virus with inhibitor (TF-33) lost its ability to produce hemagglutinin.

Virus assay and cell culture. Unless otherwise stated, the virus was assayed on primary monkey kidney (MK) cells on lactalbumin hydrolysate (LAH) medium in 60-mm plastic dishes (3). In 4 days, either the plaques were counted, after staining the cell layer with 0.02% crystal violet, or they were tested for hemadsorbing capacity. Counted plaques were recorded as plaque-forming units (pfu) per 0.25 ml. In one case the virus was assayed on L cells that had been grown in medium 199 with 15% calf serum. At each virus dilution, 4 tubes of L cells received 0.25 ml/tube. Each tube contained 0.75 ml of medium 199 with 2% calf serum. After 50 hr at 37° the tubes were stained with 0.02% crystal violet and the plaques were counted and recorded as pfu per 0.25 ml. In L cell studies with viruses HS-33 and TF-33, a special medium was employed consisting of 10% of a 10X stock of medium 199, 5% calf serum, and 85% cell-free Ehrlich ascites tumor plasma.

Hemadsorption (HAd) by viral plaques. After removal of the agar overlay the MK cells were washed twice with 0.85% NaCl. Three ml of a 1% suspension of chicken erythrocytes (RBC) were then added to each plate. After 20 min at 37°, the unadsorbed RBC were washed free.

Virus cloning. Part of the agar above a plaque was removed with a capillary pipette and expelled into a tube of MK cells, which was then incubated until the cells came off the glass. Each specimen was recloned 3 more times. Before the third and fourth clonings the virus was filtered through a 650-μ Millipore disk to remove any virus clumps (4). Immediately after the plaque sections had been picked from a given plate, the agar overlay was removed and RBC were added to determine whether the plaques were HAd-positive or HAd-negative. Cloning was performed on virus IHD-E after the specified number of tumor passages.

Virus neutralization. Virus was added to LAH growth medium (control) or to Ehrlich ascites tumor plasma (containing inhibitor) to give approximately 1.6×10^4 pfu/ml. At appropriate intervals 0.1-ml samples were removed, diluted 1:100 in cold LAH medium, and 0.25 ml was inoculated/plate of MK cells. After 1 hr, the cell layers were washed twice with phosphate-buffered saline (5) and 4 ml of LAH overlay medium was added/plate. Four days later plaques were counted

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and their hemadsorbing capacities were determined.

Viral hemagglutinin (HA). A 20% suspension of ground, chorioallantoic membranes infected with virus IHD-E served as the HA (1). The HA was assayed by first making 2-fold serial dilutions in 0.3-ml volumes of a buffered diluent (6). To each tube was added 0.3 ml of a 0.5% suspension of RBC. After 35 min, the dilution in the last tube showing hemagglutination was designated as the titer and is referred to as 1 hemagglutinating dose (1 AD).

Tumor. The Lettré, hyperdiploid line of the Ehrlich ascites tumor was maintained by weekly passage of 0.2 ml (4×10^7 cells) intraperitoneally in female, NIH mice.

Virus passage in the tumor. Six-day-old tumors were inoculated with 1×10^4 pfu of virus. The tumor was aspirated in 7 days, and after sedimentation of the cellular debris the ascitic fluid was stored at −60°.

HA inhibitor. Ascites was aspirated from 13-day tumors, the cells were sedimented, and the cell-free fluid was stored at −30°. The inhibitor-containing plasma is referred to as "tumor fluid." For assay, 2-fold serial dilutions were made in 0.3-ml volumes of buffered diluent (6), and 2 AD of virus, in 0.3 ml, were added to each tube. After 40 min, 0.3 ml of a 0.5% suspension of RBC was added to each tube. The resulting titer was read in 1 hr and 15 min. The dilution in the last tube showing no agglutination was the inhibitor titer.

Results. Reversibility of the inhibitor–HA interaction. HA and inhibitor were reacted so that no excess HA and very little excess inhibitor remained. This was accomplished by determining the highest inhibitor dilution that would cause HAI when mixed with undiluted HA. After adding an equal volume of 0.5% trypsin 300 (Nutritional Biochemicals) to this mixture, and holding it at 37° for 1 hr, all the initial HA activity was recovered, thus showing that inhibitor acts by complexing with HA without destroying it (Table I).

The released HA was concentrated, by centrifugation at 105,000*g* for 2 hr at 4°, and resuspended in buffered diluent. Two AD of this and 2 AD of stock HA, which had been processed in parallel, were tested against inhibitor. An HAI titer of 256 was obtained in each case, indicating the full capacity of trypsin-released HA to react with fresh inhibitor.

TABLE I. Effect of Trypsin on Optimally-Reacted HA-Inhibitor Mixture.[a]

Preparation	HA titer	HAI titer
HA (stock virus)	128	
+ trypsin[b]	128	
Tumor fluid (inhibitor)	<2	256
+ trypsin[b]	<2	<2
HA-Inhibitor mixture	<2	
+ trypsin[b]	128	

[a] The titers are corrected for dilution factors involved in testing.

[b] An equal volume of 0.5% trypsin was added to the corresponding experimental material and reacted at 37° for 1 hr.

Examination of the inhibitor for virus-neutralizing activity. IHD-E virus, a population composed entirely of HAd-positive infectious units, was added to LAH medium (control) or to tumor fluid (inhibitor). Samples were removed after 0-, 0.5-, 1-, 3-, 24-, and 72-hr exposure at 37° and assayed on MK cells. The pfu counts of the control were, respectively, 33, 33, 37, 37, 28, and 10 per 0.25 ml. The inhibitor preparation showed 34, 30, 37, 34, 21, and 13 pfu. This, and repeated tests, failed to reveal any neutralization by the inhibitor. All plaques were HAd-positive.

Stability of viral clones. An HAd-positive clone was selected from IHD-E virus having had 1 tumor passage. HAd-negative clones were selected from IHD-E virus having had 5 and 26 tumor passages. The fifth passage virus population had just become HA-negative. The clones were designated respectively as 1T-CL, 5T-CL, and 26T-CL. A plating of 5000 plaques of each clone showed that 1T-CL produced only HAd-positive plaques, and 26T-CL produced only HAd-negative plaques. Clone 5T-CL was unstable and produced 0.1% HAd-positive plaques.

Ten serial passages of each clone were made in MK cells, by inoculating 5000 pfu on to 4.5×10^6 cells and harvesting in 3 days at each passage. The HAd status of

TABLE II. Stability of Viruses 5T, 5T-CL, and 26T-CL During Passage in Eggs.[a]

Egg passage	Virus 5T		Virus 5T-CL		Virus 26T-CL	
	HA titer	% of HAd-positive plaques	HA titer	% of HAd-positive plaques	HA titer	% of HAd-positive plaques
0	<2	8	<2	<1	<2	0
1	2	16	<2	<1		
2	2	20	<2	1		
3	16	24	<2	1		
4	16	32	<2	3		
5	32	38	<2	4		
6	32	38	<2	5		
7	32	43	<2	7		
8	32	40	<2	7		
9	64	47	<2	8		
10	64	56	2	9		
11	64	55	2	15		
12	64	64	4	22		
13			16	30		
14			16	33		
15			32	33		
16			32	37	<2	0

[a] Each passage in embryonated chicken eggs was made with approximately 5×10^4 pfu of virus.

clones 1T-CL and 26T-CL remained unchanged. At passages 1, 5, and 10 clone 5T-CL showed an increase in HAd-positive plaques, respectively, to 0.4, 1.7, and 3.5%. To determine if this resulted from a growth advantage of HAd-positive virus, growth curves were performed by inoculating triplicate cultures, each containing 4.5×10^6 MK cells, with 2000 pfu of each type of virus. Samples were removed daily for 5 days. Since stable clones could not be derived from 5T-CL, and because two viruses with a similar background were needed for comparison, HAd-positive HS-33 and HAd-negative TF-33 were employed in this study. The daily assay values of the two viruses did not differ from each other by more than 0.3 log, thereby indicating no growth advantage relating to HAd capacity.

A more pronounced increase in HAd-positivity, in populations containing some HAd-positive virus, was noted during passage in embryonated chicken eggs. A comparison of virus 5T, its clone 5T-CL, and virus 26T-CL is shown in Table II. Viruses 5T and 5T-CL produced, respectively, 64 and 37% HAd-positive plaques in 12 and 16 passages. Virus 26T-CL remained HAd-negative after 16 passages.

If clone 1T-CL, which contained no detectable HAd-negative virus, should rapidly evidence HAd-negative virus upon tumor passage it would indicate strongly that genetic selection is not involved in the change. Upon examination it was found that clone 1T-CL produced 3% HAd-positive plaques after 1 tumor passage and 90% after 2 passages. The respective virus concentrations from passages 1 and 2 were 1.0×10^5 and 3.5×10^6 pfu/0.25 ml of tumor fluid.

The influence of inhibitor on a mixture of HAd-positive (HS-33) and HAd-negative (TF-33) viruses was studied. The mixture, containing 38% HAd-positive virus, was inoculated on to L cells in medium 199 with either 15% calf serum or 85% tumor fluid added. The tumor fluid gave the medium an HAI titer of 512. Five passages, each of 5-days duration, were made in each medium, using 0.25 ml of the preceding passage as the inoculum. The changes in HAd status are shown in Table III. In the presence of inhibi-

TABLE III. Population Changes During Passage in L Cells of a Mixture of HAd-Positive (HS-33) and HAd-Negative (TF-33) Viruses.[a]

L cell passage[c]	In cell culture medium containing 15% calf serum[b]		In cell culture medium containing 85% tumor fluid[b]	
	Virus titer (pfu/0.25 ml)	% of HAd-positive plaques	Virus titer (pfu/0.25 ml)	% of HAd-positive plaques
1	6.5×10^5	36	6.0×10^5	4.9
2	3.5×10^5	47	5.0×10^5	2.4
3	1.7×10^6	63	7.0×10^5	0.9
4	2.0×10^5	64	2.5×10^5	1.1
5	1.3×10^6	86	4.0×10^5	1.0

[a] The initial mixture contained 38% HAd-positive plaque-forming units.
[b] Given values are from the fifth day of each passage.
[c] Virus passage was made with 0.25 ml from the preceding passage.

tor there was a rapid decrease in HAd-positive virus. In the absence of inhibitor the population became more HAd-positive. Growth curves were determined by inoculating 1500 pfu of virus HS-33 or virus TF-33 on to 1×10^7 L cells and removing samples daily for 5 days. The daily assay values of the two viruses did not differ from each other by more than 0.3 log, indicating that the emergence of HAd-negative virus in the presence of inhibitor did not result from a growth advantage in L cells.

Discussion. It is evident that the inhibitor complexes with HA without destroying it. HA released from the complex is not altered radically and can react fully with fresh inhibitor.

The mechanism by which the inhibitor can cause an HAd-positive virus to change to a stable, HAd-negative population does not appear to involve the selection of an HAd-negative mutant. The reasons which lead to this conclusion are as follows: (i) No HAd-negative virus could be detected in the clone-derived HAd-positive stock, which was readily convertible to HAd-negative. (ii) The inhibitor exhibited no neutralizing (selective) action against the HAd-positive virus. (iii) The HAd-negative clones (5T-CL) derived from early tumor passage were unstable. This is consistent with the hypothesis of nongenetic change proposed below. (iv) No growth advantage of HAd-negative virus in the presence of inhibitor could be demonstrated under *in vitro* conditions in the present study. In a previous study (7) it was shown that under *in vivo* conditions the HA status of the virus did not influence viral replication. (v) It is improbable that the inhibitor is mutagenic. Even so, one would have to assume a phenomenal rate of mutation to account for the rapid change.

The data appear most consistent with a nongenetic alteration. It is suggested that this might involve the requirement of HA as a template for the production of more HA. This is analogous to cell wall being necessary for further wall synthesis in L forms of bacteria (8). In the case of vaccinia virus the inhibitor might complex with HA and prevent it from acting as a template. A few passages of HAd-positive virus in the presence of inhibitor does not result in a stable change to HAd-negative, and reversion occurs on additional passage in the absence of inhibitor. This could result from the occurrence in the virus population of a small amount of uncomplexed HA which acts as a template. After many passages in the presence of inhibitor the HAd-negative virus produced is completely stable on passage in the absence of inhibitor, suggesting that all the HA template has been inactivated and removed by dilution with passage. The same mechanism could explain the emergence of HAd-positive virus when a mixture of it and HAd-negative virus is allowed to proliferate in L cells in the absence of inhibitor. The fact that there is no obvious selective growth advantage of HAd-positive virus in the

absence of inhibitor (7) or of HAd-negative virus in the presence of inhibitor lends support to this hypothesis.

Summary. A study was made of the loss of hemagglutinin by vaccinia virus under the influence of an hemagglutinin inhibitor originating from a mouse ascites tumor. It was found that the inhibitor reacted with the hemagglutinin by forming a dissociable complex. In examining the possible mechanisms by which stable, hemagglutinin-negative virus is formed, it was noted that the inhibitor exhibited no neutralizing action against hemagglutinin-positive virus, that hemagglutinin negative virus could not be found in cloned populations of hemagglutinin-positive virus, and that there was no obvious growth advantage shown by the hemagglutinin-negative virus in the presence of inhibitor. For these and other reasons the hypothesis is presented that the inhibitor facilitates the emergence of stable, hemagglutinin-negative virus by complexing with and gradually eliminating the hemagglutinin, which might be necessary as a template for the formation of more hemagglutinin.

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