A Further Study of Hemagglutinin Loss by Vaccinia Virus. (27433)

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In considering the occurrence of mutant forms of animal viruses, the nature of selective, nonantibody factors in the animal body has received little attention. Studies on this subject have been reported in connection with influenza virus(1,2), poliovirus(3,4) and vaccinia virus(5). The present report deals further with a vaccinia virus inhibitor, which acts against the hemagglutinin of the virus. The change of a hemagglutinin-producing population to one which is hemagglutinin-negative was first noted during serial passage of vaccinia virus in the Ehrlich ascites tumor(6). This change occurred also on serial pasage in L cell cultures, as long as the inhibitor was present in the cell culture medium(7). This report answers a criticism concerning our study with the L cell passage system, *i.e.*, that the loss of hemagglutinin might have occurred because of viral multiplication in the small number of stray tumor cells remaining in the so-called "cell-free" tumor fluid used in the L cell culture medium. This report also demonstrates that the loss of hemagglutinin, in presence of inhibitor, is not limited to the IHD strain of vaccinia virus which was used in our first study(6).

Materials and methods. Tumors. The tumors employed were a hyperdiploid line of the Ehrlich ascites tumor of the mouse, with a modal chromosome count of 46 per cell, and a hypotetraploid line of the tumor with a mode of 72 chromosomes per cell. They were passed weekly, using 0.2 ml of tumor stock from the previous week. For brevity, the hyperdiploid tumor is designated as 2X, and the hypotetraploid tumor as 4X.

Mice. The mice employed were of the N.I.H. general-purpose strain. Females were used exclusively, and weighed 20-25 g.

Viruses. IHD-E refers to the IHD strain of vaccinia virus, which had been passed 4 times on the chorioallantois of the chick embryo(6). The other vaccinia viruses employed were the Nelson(8) and Levaditi(9) strains. The last 2 viruses were adapted to the tumors by extensive manipulations during another study, and need not be elaborated upon here.

Virus assay. This procedure has been reported(6), and involved the counting of viral pocks on the chorioallantois of the chick embryo.

Hemagglutinin titration. This method was the conventional tube type of assay, as described elsewhere(6).

Inhibitor titration. Starting with a 1:10 dilution of cell-free tumor fluid, prepared by centrifugation, a series of 2-fold dilutions was made in 0.3 ml dilution blanks of 0.85% NaCl. Five agglutinating doses of the IHD-E virus, contained in 0.3 ml, were added to each tube. After 15 minutes, 0.3 ml of a 1% suspension of susceptible chicken erythrocytes was added to each tube. The agglutination pattern was read in 40 minutes. The titer refers to the initial dilution, *i.e.*, before addition of 5 agglutinating doses of the virus and the chicken cells.

L cell procedure. These methods have been described(7). The culture medium containing inhibitor was made using medium 199 as a base, and consisted of 90% by volume of cell-free tumor fluid having an inhibitor titer of 1:160. The control medium contained 15% horse serum, and no tumor fluid or inhibitor activity. Approximately 2×10^5 pock-producing units of virus were inoculated at each serial passage.

Cell-free tumor fluid. Tumorous ascitic fluid was removed from mice bearing 13-day 2X tumors, and the major cell mass removed by centrifugation at 5°C and $1200 \times g$ for 10 minutes. The decanted fluid was then filtered by positive pressure through a Pressure Filter Holder (Millipore Filter Corp.; item XX 40 047 00) containing 2 of the absorbent pads which are found in packages of Millipore filter discs. This preparation was centrifuged

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TABLE I. Hemagglutinating Response of IHD-E Virus during Serial Passage in L Cells in Presence or Absence of Inhibitor (Ascitic Fluid).

Inhibitor present	HA titer*	Virus titer‡	Inhibitor absent	HA titer	Virus titer
Passage 25	40	5.0	Passage 25	160	4.9
" 26	20	4.9	" 26	160	5.2
" 27	0†	5.1	" 27	80	4.8
" 29	0	5.1	" 29	80	5.3
" 31	0	5.3	" 31	160	4.9

* Hemagglutinin titer of a 20% suspension prepared from chorioallantoic membranes bearing confluent lesions as a result of inoculation with virus from the respective passage.

† The designation 0 indicates the titer was less than 1:10.

‡ Log of the pock-producing units per 0.05 ml of tissue culture medium on 7th day after virus inoculation.

at 5°C and 25,000 × *g* for 30 minutes, then decanted into the filtration apparatus, and by positive pressure was passed through a filter disc with a pore size of 3 μ. The final fluid had a hemagglutinin-inhibition titer of 1:160, and was stored at −20°C for a minimum of 6 weeks prior to use. Positive pressure was a necessity in the filtration, since the extreme frothing under negative pressure made filtration impossible. The tumor fluid showed a low surface tension of 56.3 to 59.2 dynes per cm at 25°C. This range was maintained regardless of tumor age.

Virus passage in tumors. The virus inoculum in each case consisted of 0.1 ml of infected tumor from the previous passage. Virus was passed in 5-day-old 4X tumors, and 6-day-old 2X tumors. The infected tumor fluids were harvested in 6 days.

Results. On passage of the IHD-E virus in L cells, bathed in a medium composed mainly of cell-free tumor fluid, the virus became hemagglutinin-negative at the 27th passage (Table I). It remained so up to the 32nd passage, which is as far as it was carried. As in our previous study(7), hemagglutinating ability was determined with a 20% suspension prepared from chorioallantoic membranes bearing confluent viral lesions. A parallel series in a medium without tumor fluid (inhibitor) did not show hemagglutinin loss. This has been discussed in a previous report(7).

As a result of serial passage in either the 2X or 4X tumor, the Nelson and Levaditi viruses lost their hemagglutinating activity. The points at which this change developed are shown in Table II. Tumor passage was continued for 8 passages beyond the point of hemagglutinin loss, and the viruses remained hemagglutinin-negative. No effort was made to maintain a constant viral inoculum at each passage, since we were interested only in whether or not hemagglutinin loss would occur. The results, therefore, cannot be accurately compared in relation to the number of passages required for the loss to occur in each case.

Discussion. It has been our opinion that in our previous studies, stray tumor cells, which might have been present in the tumor fluid used as a cell culture medium, played no role in the loss of viral hemagglutinin, because we have found tumor cells to survive at −20°C for only about 6 weeks(10), the minimum storage period for the tumor fluid before it was used in the medium. Also, we have been unsuccessful in direct attempts to obtain vaccinia virus multiplication in Ehrlich ascites tumor cells *in vitro*. In the present study it is inconceivable that any tumor cells could have remained in the tumor fluid after the final passage through the highly uniform Millipore filter disc. Since no pellet was formed on further high-speed centrifugation of the fluid, nothing could be recovered for direct microscopic examination. Filar micrometer measurements of 100 randomly selected tumor cells, mounted in tumor fluid in a Neubauer counting chamber, showed the arithmetic mean diameter to be 13.68 μ, with a standard deviation of 2.86 μ. Minimum and maximum diameters were respectively 7.8 μ and 21.67 μ.

It is of interest that a conversion to the hemagglutinin-negative state by tumor passage is not limited to the IHD virus, but occurs with the Nelson and Levaditi viruses in both

TABLE II. Hemagglutinin Loss by the Nelson and Levaditi Viruses during Serial Passage in the 2X and 4X Tumors.

Virus	Passage in 2X tumor	HA titer*	Virus titer‡	Passage in 4X tumor	HA titer	Virus titer
Nelson	10	160	4.5	8	40	3.9
	11	160	5.0	9	20	4.7
	12	80	4.1	10	10	4.4
	13	0†	3.9	11	0	4.0
Levaditi	7	80	4.7	4	20	4.3
	8	40	5.0	5	20	4.1
	9	10	5.5	6	10	4.6
	10	0	5.7	7	0	4.2

* Hemagglutinin titer of a 20% suspension prepared from chorioallantoic membranes bearing confluent lesions as a result of inoculation with virus from the respective passage.
† The designation 0 indicates the titer was less than 1:10.
‡ Log of the pock-producing units per 0.05 ml of tumor on 6th day after virus inoculation.

2X and 4X tumors. We could not demonstrate this earlier, because only recently have we succeeded in adapting these originally very refractory viruses to the tumors.

These results provide supporting evidence for a role of the hemagglutinin inhibitor in bringing about the hemagglutinin loss in vaccinia virus. In an effort to establish a better control in our experiments, various methods, including heat treatment and exposure to antibody, have been tested for removing inhibitor from the tumor fluid without altering other constituents of the fluid. No method has proved to be satisfactory. We have concluded that the best evidence for a role of the inhibitor in hemagglutinin loss would be through addition of chemically pure inhibitor to a system such as the L cell in medium 199, and examination of this system for its effectiveness in bringing about hemagglutinin loss. Inhibitor purification studies are now being carried out in another laboratory.

Summary. The present study strengthens the evidence for the role of a hemagglutinin inhibitor in the loss of hemagglutinin by vaccinia virus, and answers a criticism of a previous study. It has also been demonstrated that on serial passage in the Ehrlich ascites tumor loss of hemagglutinin is not limited to the IHD strain of vaccinia virus, but occurs also with the Nelson and Levaditi strains.

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Comparison of the Lipids in Maternal and Cord Blood and of Human Amniotic Fluid.* (27434)

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Preliminary studies by Boyd and Wilson (1) indicated that cephalins were disproportionately low in umbilical cord blood. Mod-

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