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Influence of Hemagglutinating Viruses on Tumor Cell Suspensions: I. Growth Inhibition and Reversal of the Effect.* (19479)

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In the course of experiments with mixtures of Newcastle Disease (NDV) virus and suspensions of sarcoma 180 cells it was found that after being kept at 4°C, the tumor cells failed to grow when inoculated subcutaneously into mice. This result was accompanied by a fall in hemagglutination titer of the virus. When similar mixtures were kept at 4°C for 10 minutes and subsequently at room temperature for 1, 2, or 3 hours, subcutaneous inoculation resulted in tumor growth. This was accompanied in most instances by the reappearance of hemagglutinating activity of the virus. The similarity of these findings to those observed when hemagglutinating viruses are brought in contact with red cells (1) has led to further experiments to determine to what extent the parallelism of the phenomenon holds. Most have been conducted with the NDV and the sarcoma 180 suspensions, but other tumor-virus systems have been used with success. The preliminary results are herewith reported.

Materials and methods. Viruses. The Massachusetts strain of NDV† was used in most of the experiments. In a few, however, the PR8† strain of influenza and the WS† strain of neuro-influenza were employed. All were used as pools of allantoic fluids. *Tumor suspensions.* Animals bearing 7-day-old sarcoma 180 were sacrificed, their tumors removed and made into a suspension by pushing them through a fine 40 mesh monel metal screen with a pestle. The suspensions were made to 10% in Locke-Ringer buffered with phosphate to make the pH 7.2. Glucose was added to make 600 mg %. The suspensions consisted of single cells, clumps of 8-10 cells and larger clumps up to the size of the apertures of the sieve. After the larger clumps had been permitted to settle a fairly uniform suspension was obtained. Both settled and unsettled suspensions were used in the experiment. Suspensions of Erhlich carcinoma were made in the same way.

Experimental procedure. In most experiments 10% sarcoma 180 and virus were mixed at the desired temperature. At different time intervals 0.5 cc of tumor-virus mixture was inoculated subcutaneously in each flank of Carworth Farm white mice. A readily palpable and visible bleb formed which gradually disappeared in about 1 hour. When tumor grew it assumed the form of the bleb. The mice were examined at weekly intervals and tracings of their tumors made. They were kept for 1 month after which they were autopsied to ascertain the presence of internal tumors. The results are expressed as per-

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† The NDV was obtained through the courtesy of Dr. G. Sharpless of the Lederle laboratories, the PR8 strain from Dr. F. Horsfall of the Rockefeller Institute and the WS strain from Dr. H. Koprowski of Lederle laboratories.

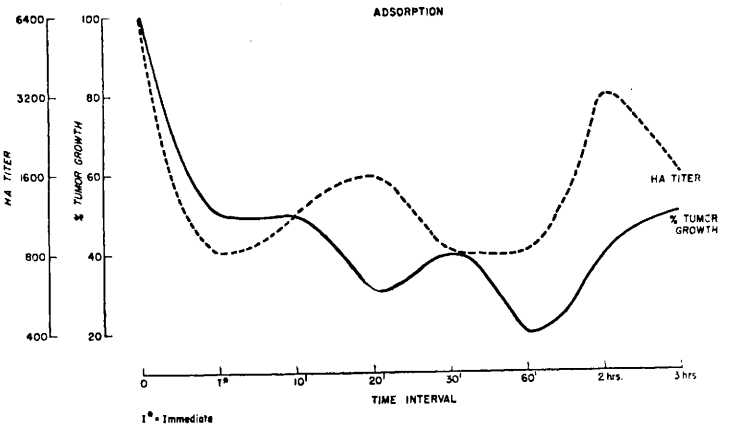

FIG. 1. Inhibition of sarcoma 180 by NDV at 4°.

centage of tumor growth regardless of the size of the tumor. In all experiments a comparable tumor suspension was mixed with normal allantoic fluid and the same procedures carried out. *Titrations.* All titrations for the hemagglutination (HA) of the virus were carried out by making two 1-10 dilutions in saline for the first two tubes and doubling dilutions thereafter. An equal volume (0.5 cc) ¼% of washed adult chicken red blood cells in saline were added and the tests were read at the end of 1 hour at room temperature. Titrations for infectivity of the virus were made by inoculation of 0.2 cc of each dilution into the allantoic sac of three 10- or 11-day eggs. The dilution which killed ½ the embryos was taken as the end point and the result expressed logarithmically.

Experimental. The experiments will be presented under two headings, 1) adsorption of the virus as indicated by the fall in HA titer and as correlated with the failure of the tumor to grow and 2) elution of virus accompanied in most instances by an increase in HA and tumor growth. It is realized that absolute proof that the virus adheres to the tumor cells and results in their inability to grow is lacking and must await further work. For convenience of discussion, however, it is felt justifiable to carry over the terms used in discussing the hemagglutination of red cells by viruses.

Adsorption of NDV on sarcoma 180 tumor cell suspensions. 1) Effect of temperature. Experiments were carried out simultaneously at 4°C, room temperature which varied from 22-24°C, and 37°C. It was found that at 37° the control suspensions of tumor cells in normal allantoic fluid lost their viability so rapidly that no direct comparison could be made with the experiments done at the other temperatures. There was evidence, however, that the virus was adsorbed with inhibition of tumor growth. Undiluted NDV allantoic fluid was brought to the temperature employed in the experiment and then rapidly mixed with a 10% sarcoma 180 cell suspension (settled) at the same temperature. Directly thereafter

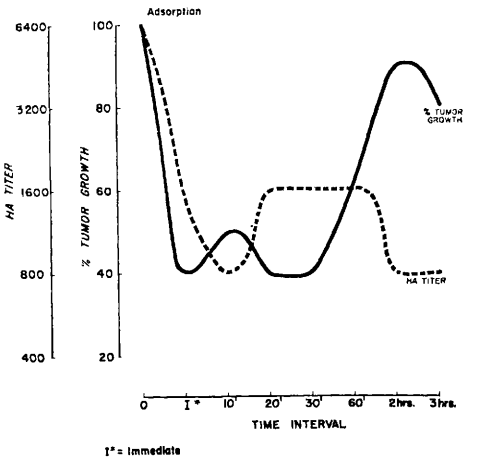

FIG. 2. Inhibition of Sarcoma 180 by NDV at room temperature.

6 cc was removed, 1 cc rapidly centrifuged to sediment the tumor cells and the remaining 5 cc was inoculated in 0.5 cc quantities into both flanks of 5 white mice. A hemagglutination titration was done on the centrifuged supernatant. This procedure was repeated at stated time intervals. As a control, another aliquot of tumor cell suspension was mixed with equal parts of normal allantoic fluid and mice were inoculated at the same time intervals. All of these tumors grew.

Fig. 1 and 2 show the result of a representative experiment. The tumor inhibitory effect was apparent immediately at both temperatures. It was maximum at 4°C in 60 minutes and at room temperature remained at its lowest level for 30 minutes, after which spontaneous elution, as measured by tumor growth, appeared to take place. The HA titer seldom coincided absolutely with the inhibition of tumor growth although the immediate fall in titer after mixing always occurred. No attempt was made to interpolate between the relatively large range of dilutions in the HA test. This might have resulted in a smoother curve. However, in experiments carried on for 8 to 12 hours it was found

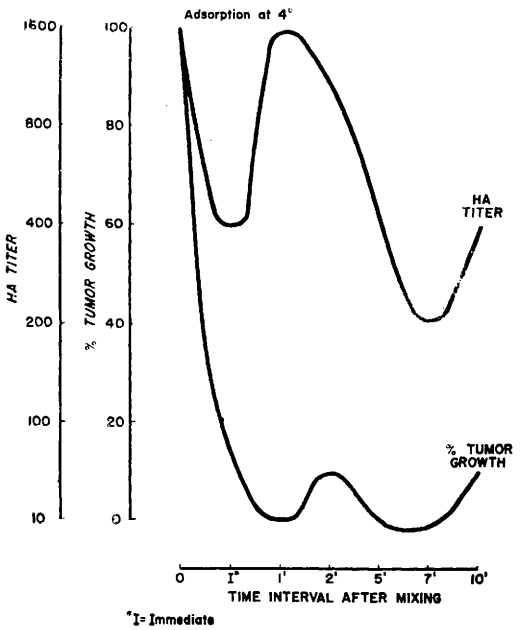

FIG. 3. Inhibition of growth of Sarcoma 180 suspensions by NVD.

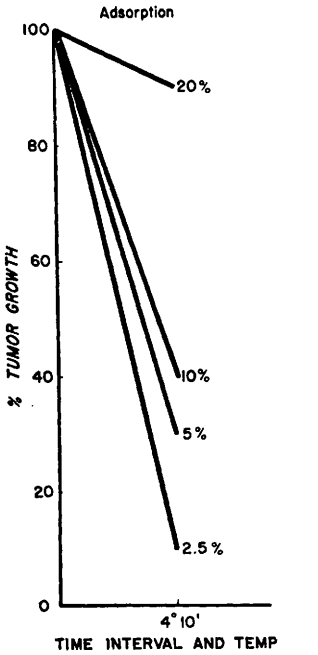

FIG. 4. Inhibition of Sarcoma 180 by NVD with different amounts of tumor suspension.

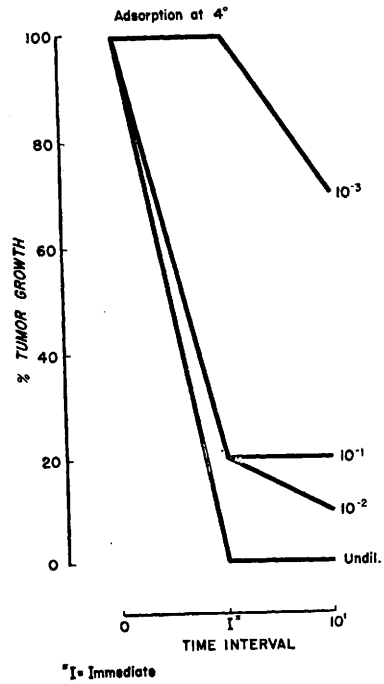

FIG. 5. Inhibition of sarcoma 180 by NDV with different dilutions of virus.

that the virus HA titer varied in an undulating fashion from high to low, whereas the inhibi-

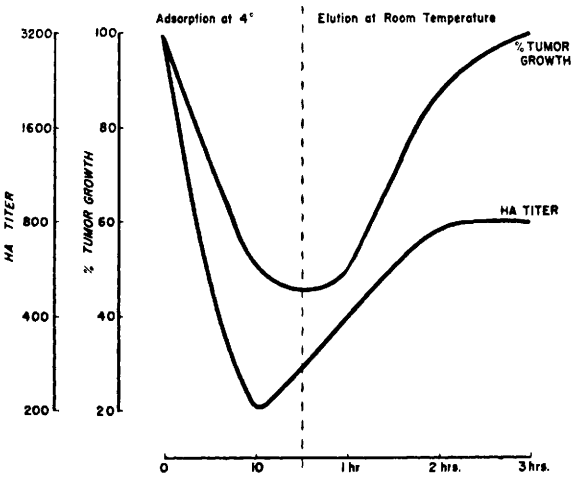

FIG. 6. Adsorption of Sarcoma 180 by NVD at 4° and elution at room temperature.

tion of tumor growth varied only slightly. In most experiments in which the effects of 4°C and of room temperature were compared the extent of tumor inhibition was approximately the same although some elution took place at room temperature.

2) Rapidity of the reaction. A sarcoma 180 suspension was settled for 10 minutes at 4°C to remove all the clumps. It was mixed in equal portions with NDV infected allantoic fluid and samples were removed at intervals of 1 minute for inoculation into mice and for determination of the HA titer. Fig. 3 shows the results of the experiment. It demonstrates that the inhibitory effect of the virus on the growth of the tumor cell takes place very rapidly.

3) Relation between the number of tumor cells and the inhibition of their growth. The sarcoma 180 suspension was made up in buffered Locke-Ringers to concentrations of 20%, 10%, 5% and 2.5%. Each concentration was mixed with an equal part of NDV infected allantoic fluid at 4°C. After 10 minutes mice were inoculated subcutaneously and a HA titer done on a sample of the supernatant. Fig. 4 shows that very little inhibition occurred with the 20% suspension but was demonstrable with all the others. The smaller the concentration of cells the more inhibition occurred. The control tumor suspensions grew in all instances. The HA titer of both the 20% suspension and 10% suspension fell from 1280 to 320, and for both the 5% and 2.5% suspension the fall was from 1280 to 160.

4) Influence of the amount of virus on tumor inhibition. NDV infected allantoic fluid was mixed with normal allantoic fluid to make dilutions of 10^{-1}, 10^{-2} and 10^{-3}. These, along with the undiluted fluid, were then mixed with an equal amount of settled sarcoma 180 suspension and adsorption carried out at 4°C. Mice were inoculated and HA titrations done immediately and after 10 minutes. The results given in Fig. 5 show that maximum tumor inhibition occurred with the undiluted NDV infected allantoic fluid

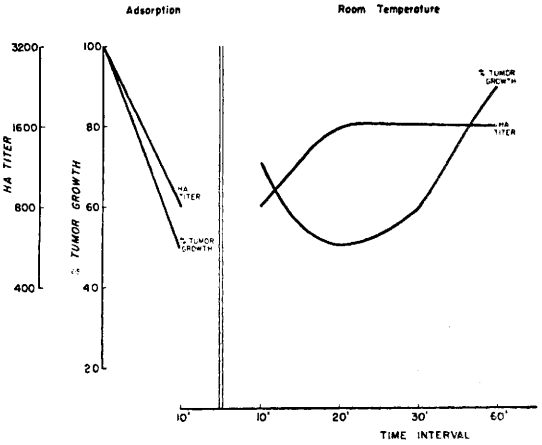

FIG. 7. Elution of NDV from tumor suspension at room temperature for a one hr period.

TABLE I. Persistence of NDV in Sarcoma 180 in the Mouse.

Time after inoc.	Titer
Immed.	7.5*
10 min	5
30 "	5
60 "	5
1 day	>3
2 "	3
3 "	1
4 "	0

* Expressed as the reciprocal of the LD_{50}.

and was least with that diluted 1-1000. HA titers showed a drop concurrent with tumor inhibition except for the 10^{-3} dilution where no HA titer was demonstrable. Repeated experiments have given the same general result and show that there is a critical level beyond which the virus cannot be diluted to obtain the inhibitory effect.

Elution of NDV from sarcoma 180 suspension. After adsorption at 4°C for short periods as described above, it was found that placing the flask at room temperature for varying periods of time resulted in an increase in the hemagglutination titer and concurrent with this the tumor cells "regained" their ability to grow. Fig. 6 demonstrates the result of adsorption at 4°C for 10 minutes and subsequent elution at hourly intervals. In another experiment adsorption was carried out at 4°C for 10 minutes and then the flasks placed at room temperature for 1 hour. During this time aliquots were removed at regular intervals for inoculation of mice and for HA titers. The results are given in Fig. 7. It can be seen that elution does not take place until 1 hour and is not quite complete. Control tumor suspensions with normal allantoic fluid grew in all instances. The elution process is much slower than the adsorption and is not always demonstrable. The failure of elution is probably also related to the amount of virus and the number of cells. Further work is being done to elucidate this point.

Failure of the NDV to grow in the sarcoma 180. When NDV was inoculated in 0.1 cc quantities directly into the tumor of sarcoma 180 bearing animals it persisted but there was no evidence of multiplication, Table I. Animals were killed at regular intervals and the virus content of their tumors titered by egg inoculation. The experiment was carried out for 11 days and only traces of virus could be detected on the 8th and 9th days when 1 chick embryo out of 3 given a 10^{-1} dilution of tissue died and showed a positive HA. In addition, in tissue culture experiments in which virus was inoculated into minced sarcoma 180 in Tyrode's solution it was impossible to keep the virus living for more than 3 consecutive passages. Bioassays showed the tumor to be viable. Parallel cultures made in chick embryo showed that the virus multiplied readily in this medium. In summary, there is no evidence that the NDV multiplies in the sarcoma 180 whether in the intact animal or in tissue culture where the source of living cells is the sarcoma 180.

Effect of immune serum on the reaction. Immune serum was prepared by inoculation of rabbits with 10 cc of NDV infected allantoic fluid intravenously twice at weekly intervals. Normal allantoic fluid (NAF) was inoculated in the same way. Two weeks after the final inoculation the rabbits were bled and serum collected. Sarcoma 180 tumor cell suspensions were prepared in the usual manner and mixed with equal parts NDV infected allantoic fluid and placed at 4°C for 10 minutes for adsorption. HA titers and animal inoculations showed that adsorption had taken place. Table II. The cells were removed, centrifuged and washed in twice the volume of buffered Locke-Ringer solution. They were again sedimented and resuspended in fresh diluent to 5 cc. One cc of anti-NAF serum, buffer, pre-vaccination sera and sera from the vaccinated rabbit at different dilutions was added. The flasks were allowed to stand at room temperature for ½ hour after which mice were inoculated subcutaneously. It is apparent that the anti-NDV serum even when added in high dilution to the cell suspensions resulted in complete tumor growth in contrast to the cells to which the other sera and buffered Locke-Ringers had been added. Since the reaction can be inhibited by specific serum it indicates that the virus particle is responsible for the inhibition of tumor growth.

TABLE II. Effect of NDV Immune Serum on Inhibition of Tumor Growth by NDV.

	4° 10′		Resuspended S-180 at ½ hr RT°		
	HA	T.G., %*	Materials added to cells (1 cc)	HA	T.G., %*
Orig.	3200		Buffered Locke-Ringers	10	40
4° 10′	1600	60	Anti-NAF serum	0	50
			Pre-vaccination serum	0	60
			Anti-NDV serum—undil.	0	100
			" " " -10^{-1}	0	100
			" " " -10^{-2}	0	100

* T.G. = Tumor growth.

Experiments with other tumors and other viruses. Another tumor which lends itself to this type of experiment is the Ehrlich carcinoma which will cause subcutaneous tumors when inoculated subcutaneously or a fatal ascites when inoculated intraperitoneally. When a 10% suspension was made from a solid tumor and mixed with equal parts of NDV, tumor inhibition associated with a fall in hemagglutination titer occurred in the same manner as in the experiment with sarcoma 180 when these mixtures were inoculated either subcutaneously or intraperitoneally. Elution also took place in the same manner.

Other hemagglutinating viruses have been used with the sarcoma 180 suspensions. Preliminary experiments lead us to believe that when sufficient PR8 strain influenza virus is present, it has the same inhibitory effect in tumor growth as the NDV. WS strain of influenza virus appears also to produce a profound inhibitory effect on the sarcoma 180 cells suspensions. With this virus (WS) no elution has been noted under the same conditions in which it takes place with the NDV. All of the details of the PR8 and WS viruses have not been worked out as yet. They will be the subject of another communication.

Discussion. The results here presented seem to be quite analogous to the hemagglutination of red blood cells by the Influenza-NDV-Mumps groups of viruses. It has been impossible to demonstrate agglutination of the tumor cells themselves because of their tendency to agglutinate spontaneously. The differences so far appear to lie mainly in the fact that the cells involved are tumor cells which ordinarily have the power of reproducing themselves when implanted into the proper host. When brought in immediate contact with the virus they lose this ability but later on after a change in temperature from 4°C to room temperature they slowly "regain" their ability to grow. Since this is always associated with a fall in hemagglutination titer during the "adsorbing" phase, and usually followed by an increase in hemagglutination titer during the "elution" phase it is tempting to consider the two systems as due to the same process. Since we have not been able to demonstrate actual multiplication of the virus in the sarcoma 180 in the intact animal or in tissue culture it seems that the inhibition of tumor growth may be associated with the presence of virus on the tumor cell. This would be analogous to that seen with inactivated bacteriophage which also is capable of inhibiting bacterial growth during the "adsorption" stage(2). It is difficult to explain the ability of the tumor cells to grow again after elution if we postulate on actual entrance of the virus particle into the cell.

The points which are under investigation now and which it is hoped will elucidate the mechanism more fully are 1) investigation of the tumor cell itself following adsorption and elution, 2) the effects of virus which has undergone physical treatment such as ultraviolet and heat, 3) attempts at receptor removal by chemical or enzymatic means, 4) the role of non-specific inhibitors in this reaction.

Summary. 1. When mixtures of sarcoma 180 and active NDV allantoic fluid are mixed at 4°C and inoculated subcutaneously into mice an inhibition of tumor growth takes place. This is associated with a fall in hemagglutination titer. After the mixtures have been brought to room temperature for one to 3 hours subcutaneous inoculation re-

sults in tumor growth. This is usually associated with an increase in hemagglutination titer. 2. No evidence has been found for the multiplication of the virus in the sarcoma 180. The results are discussed as an analogy with the hemagglutination of red blood cells by the Influenza-Mumps-NDV group of viruses.

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Prompt Effect of Adrenal Stimulation on the Free Amino Acid Content of Rat Tissues. (19480)

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In studies involving the prolonged administration of the hormone preparation, it was found(1) that various endocrine factors influenced the free amino acid concentration of rat tissues. In connection with certain of our investigations it was important to know if these effects could be elicited upon short term treatment. We are reporting the effects within 2 hours of adrenocorticotropin and of various adrenal steroids on the free amino acid concentration of rat tissues.

Experimental. Male Sprague-Dawley rats, weighing approximately 160 g, were used in groups of 4 to 8 for each experiment. The hormone was administered subcutaneously after a preliminary fast of 16 hours and the animals were sacrificed 2 hours later. Picric acid filtrates of various tissues were immediately prepared and analyzed for amino acid content by the ninhydrin gasometric method (2). Analytical values are presented as mg of amino N/100 g of tissue, wet weight.

Results and discussion. The experimental results are presented in Table I. Deviations from normal are considered significant only if $P < 0.02$(3) and, in the table, are in italics. It can be seen that, of the tissues studied, only liver, kidney, and adrenal showed a short term response to hormone administration. This is in contrast to the report of Friedberg and Greenberg(1) where it was shown that, following several days treatment, kidney showed no response and there was a sharp rise for plasma. The inertness of cortisone acetate probably might be explained on the basis of poor absorption from the site of injection. That the liver, kidney, and adrenal changes represent specific target organ responses is supported by the lack of significant alteration in plasma levels.

An unexpected finding was the marked change in the free amino acid content of the adrenal following adrenocorticotropin ($P < 0.001$). In view of the ease and precision

TABLE I. Influence of Various Adrenal Hormones and of Adrenocorticotropin on Free Amino Acid of Tissue Concentration of Rat Tissues.

Treatment	Dose/ 100 g	Tissue					
		Heart	Liver	Kidney	Spleen	Adrenal	Plasma
Control	—	24.1±1.56	22.7±1.03	26.8±.85	35 ± .59	43.3± .86	4.21±.24
Adrenocortical extract (Upjohn)	.2 ml	25 ± .74	*28.7*± .87	*33.3*±.58	37.4±1.34	44.8±1.73	5.40±.40
Desoxycorticosterone glucoside	.5 mg	24.7± .59	*27* ± .47	27.4±.91	34.5± .45	43.8±1.47	5.10±.48
Cortisone acetate	.5 "	27.5± .76	26.4±1.02	28.9±.37	34.6± .66	46.3±1.98	4.19±.31
Adrenocorticotropin	.01 "	27.7± .69	*28.8*±2.00	*30.2*±.46	36.5±1.10	*53.6*±1.38	4.91±.31

Values are presented as means ± stand. errors.