Summary. A syndrome closely resembling diabetes insipidus was produced in 4 mongrel dogs by daily injections of cortisone in doses of 50 to 300 mg a day. The period of hormone injection varied from 4 days to 14 days in different dogs. The polydipsia reached values as high as 6200 cc per day and the polyuria reached values as high as 5790 cc per day. Frequent fasting blood sugar determinations and glucose tolerance tests did not reveal any changes in carbohydrate metabolism.

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Agglutination of a Pure Strain of Mammalian Cells (L Strain, Earle) by Suspensions of Vaccinia Virus.* (19704)

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It is the purpose of this paper to record experimental results which demonstrate the ability of suspensions of vaccinia virus to agglutinate altered fibroblastic cells (L strain, Earle)(1). Heretofore, the reported evidence for agglutination of mammalian cells by viruses was limited to hemagglutination.

A variety of viruses have in common the ability to agglutinate erythrocytes(2-4). These viruses fall into two groups(5).† The first group is made up of the viruses of vaccinia, variola and ectromelia. These viruses possess hemagglutinins which are separable from the infectious viral particle. The second group includes the viruses of mumps, Newcastle disease and influenza. These viruses have hemagglutinins which are inseparably associated with the viral particle. It has been shown that viruses from the second group adsorb to cells other than erythrocytes. For example, influenza virus adsorbs to cells from ferret lung(6), and the virus of Newcastle disease has been shown to react with tumor cells to inhibit tumor growth(7). However, in those studies it was not possible to demonstrate that cells were agglutinated by the viruses.

During a study of the host cell-virus relationships between each of a variety of viruses and a strain of mouse cells cultivated *in vitro* (L strain, Earle), it was noted that a hemagglutinating virus which has a soluble hemagglutinin, vaccinia virus, caused cellular agglutination. This strain of cells has been maintained *in vitro* by Earle and coworkers since 1940(1). While being cultivated *in vitro,* the cells developed the capacity to produce sarcomas upon inoculation into mice(8). Since 1948, this strain of cells has had as its parent cell, a single cell(9). Cells from this strain may be considered as altered fibroblastic cells (10).

Materials and methods. Viruses. Three viruses were employed. Vaccinia virus was

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† For a review of the subject of hemagglutinins and hemagglutination, the reader is referred to reference 5.

TABLE I. Results of Viral Agglutination Experiments with Erythrocytes and L Strain Cells.

Virus	Exp. No.	Passage of virus*	Viral titer in rabbit skin	Viral hemagglutinin titer (reciprocal of final dilution of virus)	Agglutination of L strain cells at 36°C
Vaccinia	1	CA-3	10^{-5}	N.T.†	3/3‡
	2	CA-4	$10^{-6.5}$	"	6/6
	3	"	"	"	2/2
	4	CA-6	N.T.	128§	2/2
	5	CA-5	$10^{-8.3}$	16	3/3
	6	CA-6	N.T.	256	4/4
	7	"	"	"	2/2
	8	"	"	"	4/4
Mumps	1	A-1		256	0/2
	2	"		"	
	3	"		"	
Influenza type A	1	A-1		64	0/2
	2	"		"	
	3	A-?		2048	
	4	A-2		16384	

* "CA-3" signifies the third passage of vaccinia virus on chorioallantoic membranes. "A-1" indicates the first passage of mumps or influenza viruses in allantoic sacs of embryonated chicken eggs.

† N.T. signifies not tested.

‡ Numerator indicates number of cultural flasks which showed cellular agglutination; denominator signifies number of flasks inoculated with virus.

§ Discrepancies between titers of vaccinial hemagglutinin shown for passage CA-6 are within the limits of accuracy for the method employed since they represent only a 1 tube difference in results.

utilized as the supernatant fluid following centrifugation for 10 minutes at 1500 rpm of a 5% or 10% suspension of infected chicken chorioallantoic membranes. These membranes had been triturated by mortar and pestle in 5% dextrose in water. Vaccinia virus was quantitated for infectivity by the injection intracutaneously in rabbits of 0.2 ml aliquots of successive decimal dilutions of infected chorioallantoic membranes. The least amount of virus which produced lesions with induration, 5 mm or more in diameter in 4-7 days after inoculation was accepted as the titration or infectivity end point. Mumps virus was used as allantoic fluid from embryonated chicken eggs which had been infected 5 days previously. Infected allantoic fluid harvested after 48-72 hours of incubation at 36°C was the source of influenza virus, type A PR8 strain. Stock supplies of each virus (1 ml aliquots) were stored on dry ice for use during these experiments.

Cells. L strain. The L strain of mouse cells was kindly supplied by Dr. W. R. Earle. Cells were cultivated at 36°C on the glass wall of Porter flasks by the employment of a mixture of horse serum, 40%, chicken embryonic extract (1:1), 20%, and Hanks' solution, 40%. Cells were used for agglutination purposes after periods of from 1 to 8 days of cellular cultivation. The ability of the viruses of vaccinia, mumps and influenza to agglutinate L strain cells was determined by the addition of virus, 0.1 ml, to Porter flask cultures which contained 1.0 ml of liquid medium and sufficient cells to cover 80-100% of the wall of the flask. Cells were observed at 50x and 100x magnification for agglutination at 24 and 48 hours after the inoculation of virus. The results from agglutination tests which were attempted in test tubes with suspensions of L strain cells, were equivocal because spontaneous agglutination of the cells in the absence of virus frequently occurred.

Erythrocytes. Chicken erythrocytes from a single rooster were employed for hemagglutination tests, which were performed by the pattern method described by Salk(11). Dilutions of virus and a suspension of erythrocytes, 0.25%, were used in volumes of 0.4 ml each. The hemagglutination tests were read after 2 hours of incubation at 36°C for vaccinia virus and at room temperature for mumps and influenza viruses.

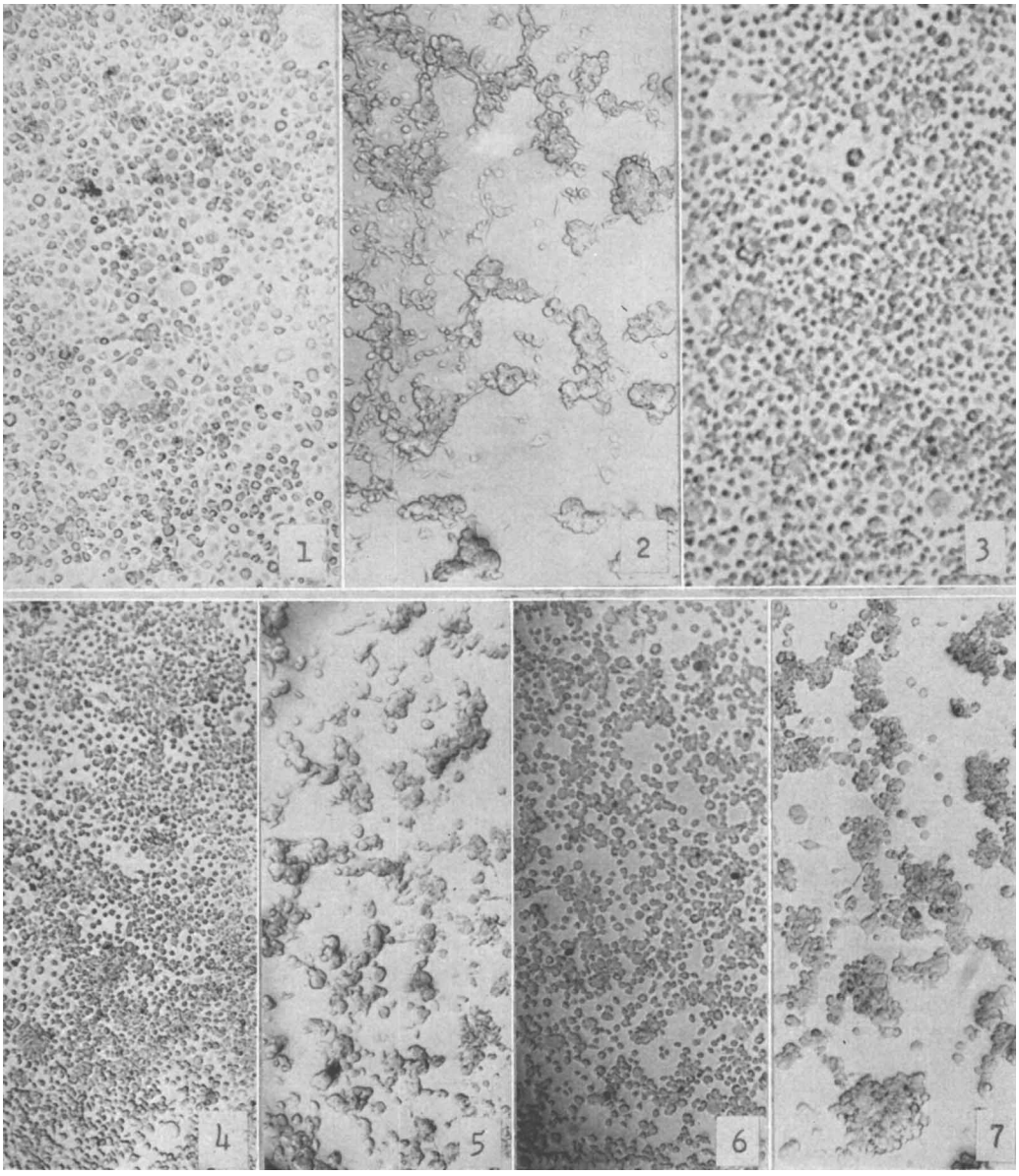

FIG. 1. Normal L strain cells; note the configuration and the even distribution of the cells. × 60.

FIG. 2. L strain cells showing agglutination 48 hr after the addition of a suspension of vaccinia virus. × 60.

FIG. 3. L strain cells from a culture 48 hr after the inoculation of a mixture of vaccinia virus and immune serum. Note the absence of cellular agglutination. × 60.

FIG. 4. L strain cells at 48 hr after the inoculation of mumps virus. × 45.

FIG. 5. Agglutination of the cells shown in Fig. 4 by vaccinia virus, added 5 days after mumps virus. This photograph was made 24 hr after the inoculation of vaccinia virus. × 45.

FIG. 6. L strain cells at 48 hr after the addition of influenza virus, type A, PR8 strain. × 60.

FIG. 7. Agglutination of the cells shown in Fig. 6 by vaccinia virus, added 5 days after influenza virus; at 24 hr after the inoculation of vaccinia virus. × 60.

Vaccinial antiserum. Pre-immunization serum was obtained from a normal rabbit. This animal was subsequently inoculated intracutaneously, for titration purposes, with 0.2 ml aliquots of decimal dilutions ($10^{3.3-9.3}$) of chorioallantoic membranes infected with vaccinia virus. Cutaneous lesions typical of vaccinia resulted from the lower 6 dilutions by 5 days after inoculation. Fifteen days after inoculation, serum was obtained from the rabbit. Both the pre- and post-immunization sera in small aliquots were stored at −20°C. Heat inactivation of complement was carried out prior to use of the sera for agglutination inhibition tests.

Results. Agglutination of L strain cells by suspensions of vaccinia virus. Table I presents the data from 8 experiments performed on separate occasions. It may be seen that L strain cells were agglutinated by vaccinia virus contained in each of 4 suspensions from different passages, CA^3-CA^6, on the chorioallantoic membranes of embryonated chicken eggs. Thus, each passage material readily agglutinated L strain cells even though in different passages, the infectious titers in rabbits ranged from $10^{5.0}$ to $10^{8.3}$, and the hemagglutination titers ranged from 1:16 to 1:256. Fig. 1 and 2 present the appearance of normal L strain cells and of L strain cells agglutinated by a suspension of vaccinia virus. For control purposes, it was determined that a 10% suspension of normal chorioallantoic membrane did not agglutinate L strain cells. It was observed that the extent of agglutination was not affected by the age of the cells in culture provided the cells had appeared normal microscopically at the time virus was added. For Exp. 7, L strain cells were observed for agglutination at 2, 4, and 7 hours, as well as at 24 and 48 hours. No agglutination was present at 2 and 4 hours. Slight agglutination was seen at 7 hours, and by 24 hours, marked agglutination was evident. Usually maximal agglutination existed by 24 hours after the inoculation of virus, but occasionally further clumping of the cells occured by 48 hours.

The presence in the liquid medium of horse serum and embryonic extract was not essential for agglutination of L strain cells to occur, since for Exp. 1 a synthetic medium(12) had been employed for maintenance of the cells during the agglutination experiment.

Titers of agglutinins for L strain cells and for chicken erythrocytes were determined simultaneously in Exp. 6. The final dilution of vaccinia viral suspension which agglutinated L strain cells was 1:32 and that which agglutinated erythrocytes was 1:256. An explanation of the difference in agglutinin titers is unknown. However, the cells obviously are dissimilar in origin, morphology and physiology, and the suspending medium for L strain cells, a mixture of horse serum, embryonic extract, and Hanks' solution, differed markedly from the saline employed for the hemagglutination tests.

For each agglutination experiment either with L strain cells or with erythrocytes, control preparations showed that no spontaneous cellular agglutination occurred in the absence of vaccinia virus.

No evidence was obtained to show that vaccinia virus multiplied in cultures of L strain cells. Vaccinia virus persisted in the liquid phase of L strain cultures for as long as 8 days after viral inoculation, but the titers were found to decrease gradually during that period of time.

Neutralization by immune serum of vaccinia viral agglutination of L strain cells. To establish that the presence of vaccinia virus, its soluble hemagglutinin or similar factor in suspensions of chorioallantoic membranes was causally related to the agglutination of L strain cells, vaccinial antibodies were used to inhibit agglutination. Serum obtained from a rabbit prior to immunization with vaccinia virus, and serum obtained following immunization were employed for viral inhibition tests which were done with both L strain cells and chicken erythrocytes. Equal quantities of serum and viral suspension were mixed and within 2-5 minutes, were added to L strain cells and erythrocytes. Table II presents the results from these agglutination inhibition tests. It is apparent for the dilutions of pre-immunization serum employed, that no inhibition of agglutination occurred for either L strain cells or erythrocytes. In contrast to the failure of pre-immunization serum to inhibit agglutination, the post immunization

TABLE II. Neutralization by Anti-vaccinial Virus Rabbit Serum of Vaccinia Viral Agglutination of Erythrocytes and Altered Mouse Fibroblasts (L Strain, Earle).

Cells	Final dilution of vaccinia virus employed	Reciprocal of final serum dilution — Pre-immunization						Post-immunization						
		32	64	128	256		C*	32	64	128	256	512	1024	C
Chicken erythrocytes	1:32 (4 hemagglutinating units)	+†	+	+	+		0	0	0	0	+	+	+	0
		24	48	96	192	384	C	24	48	96	192	384		C
L strain cells	1:24	+	+	+	+	+	0	0	0	±	+	+		0

* C signifies a control preparation of either the 1:32 dilution of serum for the hemagglutination tests or the 1:48 dilution of serum for L strain cells in admixture with test cells to rule out spontaneous agglutination.

† + denotes cellular agglutination; ±, slight agglutination; 0, no agglutination.

serum effectively prevented hemagglutination and L strain cellular agglutination. For example, L strain cellular agglutination by vaccinia virus was completely inhibited by a 1:48 dilution of the immune serum, and hemagglutination by a 1:128 dilution. Thus, evidence for a rise in the titer of vaccinial antibodies following immunization was demonstrable by the use of cellular agglutination of either L strain cells or of chicken erythrocytes. Fig. 3 shows the absence of cellular agglutination in a culture inoculated with vaccinia virus and immune serum.

Failure of the viruses of mumps, and influenza A to agglutinate L strain cells. For comparison with the results obtained with vaccinia virus, 2 viruses, mumps and influenza A, which agglutinate erythrocytes but do not form soluble hemagglutinins, were employed for experiments with L strain cells. The data that relate to 3 experiments with mumps virus and 4 experiments with influenza virus were included in Table I. It can be seen that although these viruses were potent hemagglutinating agents, they failed to agglutinate L strain cells in a total of 14 cultural flasks (Fig. 4 and 6) even though cultures were observed for as long as 7 days after viral inoculation. The possibility that the viruses of mumps and influenza were immediately inactivated by the horse serum-embryonic extract mixture employed as a nutritive medium for L strain cells, was excluded by the demonstration that the hemagglutinating properties of mumps and influenza A viruses, as well as vaccinia virus, persisted in cell free, horse serum-embryonic extract mixture for at least 24 hours. Attempts to agglutinate L strain cells at 22-25°C with mumps, influenza and vaccinia viruses have failed. However, L strain cells were readily agglutinated when a suspension of vaccinia virus was added at 36°C, to cultures which 1 to 5 days previously, had been inoculated with either mumps or influenza virus (Fig. 5 and 7).

Summary. Mouse "fibroblastic" cells from a pure strain of cells *in vitro* (L strain, Earle) were found to be agglutinated by suspensions of vaccinia virus, but not by the viruses of mumps and influenza A under the experimental conditions employed. The agglutination of L strain cells by suspensions of vaccinia virus was inhibited by vaccinial antibodies. The results of these experiments show that cellular agglutination by suspensions of vaccinia virus is not limited to erythrocytes.

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