ELEMENTARY BODIES OF VACCINIA FROM INFECTED CHORIO-ALLANTOIC MEMBRANES OF DEVELOPING CHICK EMBRYOS

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PLATE 8

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Paschen bodies (1) or elementary bodies, have been observed in many types of cells in various animal species infected with vaccine virus (2). Suspensions of these minute structures in a relatively pure state have been prepared by means of differential centrifugation from infected skins of rabbits (3–5) and guinea pigs (6). The presence of large amounts of protein in emulsified organs has made it difficult in the past to obtain from such sources clean suspensions of virus elementary bodies. Infected skin, however, is peculiarly well adapted to the methods previously employed since the highly infectious dermal pulp, which results from scraping the skin (4, 5), contains less tissue proteins than do ground infected organs.

The preparation of suspensions of vaccine virus elementary bodies in a relatively pure state from infected tissues of a generic class different from that previously used would add to the existing evidence for the intimate association of the elementary body with the causal agent. Moreover, such an experiment would offer an opportunity for further observation on the soluble precipitable substances (6) that accompany the virus infection. Studies (7, 8) on the chick chorio-allantoic membrane inoculated with vaccine virus indicated that this tissue, which has a high infective titer and contains numerous elementary bodies, might be suitable material from which to obtain suspensions of relatively pure Paschen bodies. The purpose of this paper is to describe a method for preparing suspensions of elementary bodies from vaccine virus-infected chorio-allantoic membranes of developing chick embryos, and to present the results of serological studies on the elementary bodies and virus-free filtrates of infected membranes.

Materials and Methods

Chorio-Allantoic Membranes.—The chorio-allantoic membranes of 12 to 14 day chick embryos were exposed by Burnet's modification (9) of the technic of Woodruff and Goodpasture (10). Usually 0.1 cc. of a 10^{-3} dilution of virus suspension was dropped on the membrane surface; then the egg was gently rotated to insure spread of the inoculum. After incubation for 36–48 hours at 37°C., the infected portion of the chorio-allantoic membrane was removed. Occasional membranes that were contaminated with bacteria were discarded.

Virus.—Vaccine virus of the C. L. strain,[1] which had been carried for 4 years in this laboratory by passage of elementary bodies on rabbit skins, was rendered bacteriologically sterile by storage with ether. This virus served initially to infect chick membranes. Experiments were performed with the 2nd to the 27th membrane subculture of virus.

Antisera.—Antivaccinal sera were obtained from rabbits and guinea pigs after hyperimmunization with washed elementary bodies of rabbit origin. Monkeys recovered from intravenous inoculation of similar preparations of virus also yielded immune sera. Antisera against the heat-labile and heat-stable precipitable substances, respectively, of vaccinia which had been prepared in rabbits (11) were supplied by Dr. Robert F. Parker.

Infective Titers.—Infective titers were determined by intradermal injection into rabbits of serial tenfold dilutions of virus. Duplicate titrations were always performed.

Preparation of Elementary Body Suspensions

Suspensions of elementary bodies of vaccinia in a relatively pure state were prepared from infected chorio-allantoic membranes of chicks in the following manner.

The infected portions of 10–25 membranes were placed in Locke's solution immediately following removal from the eggs. After the last membrane was harvested the entire lot was rinsed in fresh Locke's solution in order to remove additional mucilaginous material adherent to the surface as well as blood which had oozed from the cut vessels of the membranes. The tissues were next individually dipped into ethyl ether to remove part of the lipid substances and were again rinsed in Locke's solution. Excess fluid having been drained from the membranes, they were placed in a mortar, minced with scissors, and ground vigorously. 10–20 cc. of dilute buffer solution, pH 7.2, (standard citric acid-disodium phosphate buffer, pH 7.2, diluted 50 times with distilled water) were added to the triturated material and the suspension was thoroughly shaken in a test tube. Sediment thrown down at 1000 R.P.M. in the International horizontal centrifuge was resus-

[1] Virus was obtained from Dr. J. Craigie in 1933.

pended in a similar volume of buffer solution and recentrifuged. Supernatant fluids from these two centrifugations were pooled and again spun at 1000 R.P.M. for 10 minutes. The resultant supernatant suspension was distributed in flat pyrex tubes, that have an inside diameter of 4 mm. and a capacity of 5 cc., and run in a Swedish angle centrifuge for 1 hour at 3500 R.P.M. Fluid was poured off and saved for titration of precipitinogens. The angle sediment, which contained practically all of the elementary bodies as well as considerable amounts of tissue materials, was taken up in 5–10 cc. of dilute buffer solution, pH 8.0. 0.5 cc. of a filtered 1 per cent solution of commercial trypsin that had been freed from lipids by successive extraction with ethyl ether and petrol ether was added to the suspension; the mixture was incubated at 37°C. for a half to 1 hour. Fat-extracted trypsin was used because Pirie (12) found that the lipids in commercial trypsin—not the enzymes—inactivated vaccine virus. The digested material was then washed 3 to 7 times by sedimentation in the angle centrifuge and resuspension in dilute pH 7.2 buffer solution. After the final washing, the suspension was spun in the horizontal centrifuge for half an hour at a speed of 2500 R.P.M. in order to remove particles larger than elementary bodies.

In the manner described, suspensions of relatively pure elementary bodies were prepared by a method of differential centrifugation similar to that used by other workers (4, 5). However, one important additional procedure, namely, tryptic digestion, was employed. Suspensions of virus still contained some soluble tissue proteins after 3 washings. This was indicated by the presence of amorphous, brown, precipitate interspersed among the round, black, elementary bodies in smears stained by Morosow's technic (13). When such preparations were subjected to further washing the amorphous precipitate was reduced to a minimum. Fig. 1 is a photograph of a stained preparation made from a suspension of elementary bodies treated with trypsin and washed 7 times in all.

Protein digestion was an important step in purification since repeated washing of the sediment from the angle centrifugation with dilute buffer solution alone failed to remove adequately tissue proteins. Physiological salt solution was not employed because Craigie (14) found that vaccine virus elementary bodies in the presence of 0.85 per cent saline readily underwent spontaneous agglutination. Treatment with trypsin usually reduced the amount of the angle sediment to one-half to two-thirds of its original volume.

Only a portion of the elementary bodies present in infected membranes was secured by the method described as routine. In 3 in-

stances, cells and debris, sedimented during preliminary horizontal centrifugations and discarded in the regular procedure, were first subjected to tryptic digestion and then to differential centrifugation. The resultant material, after repeated washing and additional enzymatic treatment, was found to be rich in elementary bodies. The infective titer, moreover, was in each case the same as that of the elementary body suspension prepared according to routine. These suspensions were not so clean as the regularly prepared ones, as evidenced by the precipitated amorphous material in stained smears. Such suspensions were not satisfactory for use in immunological tests because marked spontaneous agglutination occurred.

Infective Titer of Elementary Body Preparations

The most important single fact that has led to an acceptance of the close relationship between elementary bodies of vaccinia and the agent responsible for the disease is the correlation of the infective titer of suspensions with their elementary body content. The virus accompanies the elementary bodies from rabbit dermal pulp (3–5) even when filtered (15); the bodies increase in tissue culture with multiplication of the virus (16); and a definite numerical relationship exists between the bodies and a single infectious dose (17); moreover, materials freed from elementary bodies are not infectious (4, 5).

Additional evidence for the idea that elementary bodies represent the virus itself or are closely associated with the virus was brought out in the present set of experiments. The infectivity of membrane material was not appreciably altered by tryptic digestion and repeated washing; the final preparation of elementary bodies was as infectious as the original membrane emulsion. Experiment 5, summarized in Table I, is illustrative of the data supporting this statement. After the removal of coarse particles by preliminary centrifugation, the membrane emulsion had an infective titer of 10^{-6}. The material sedimented in the angle centrifuge was digested with trypsin and washed 5 times; this represents a dilution of the original angle sediment of the order of 1×10^{-4}. At this stage the infective titer was 10^{-7}. Further washing diluted the first angle sediment to approximately 1×10^{-6} times its original volume; nevertheless, the titer remained 10^{-7}. Two factors operated to increase the titer of the original emul-

TABLE I

Infectivity of Suspension of Elementary Bodies of Vaccinia

Experiment 5	Day	Treatment	Volume of suspension	Dilution of 1st angle sediment	Infectivity of suspension						
					10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
			cc.								
21 membranes infected with 12th passage virus	1	Membrane emulsion after horizontal centrifugation	22	0	++++	++++	++++	+++	++	±	
	2	Washed total of 5 times. Treated with trypsin after 2nd sedimentation	5	1×10^{-4}							
	4	Stored at 3°C.	"	" " "	++++	++++	++++	+++	++	+	±
		Washed 2 additional times (total 7)	"	1×10^{-6}							
	11	Stored at 3°C.	"	" " "	++++	++++	++++	+++	++	+	

TABLE II

Agglutination of Elementary Bodies of Vaccinia

Suspension	Antivaccinal serum	Dilution of serum										Type of agglutination
		1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	
Chick E.B. No. 9	Rabbit 6022			+++	+++	+++	++	++	++	+		Granular
Rabbit E.B. No. 7660	" "			+++	+++	+++	+++	+++	++	+	−	Large flocks
Chick E.B. No. 8	Guinea pig 5972	−	±	+	++	+	±					Granular
Chick E.B. No. 9	" " "	+	++	++	++	−	−	−	−			"
Rabbit E.B. No. 7078	" " "	+++	+++	+++	+	+	+	−	−			Large flocks

E. B. = elementary bodies of vaccinia.

sion. Tryptic digestion probably liberated virus from cell particles and from cells killed by immersion of the membranes in ether, for, as observed in another experiment, tryptic digestion alone raised the titer of a crude membrane emulsion from 10^{-5} to 10^{-6}. The second factor was the decrease in volume of suspending fluid; the particulate matter originally present in 22 cc. was later taken up in only 5 cc. of fluid.

Decrease in the number of elementary bodies and in the infective titer occurred to a certain extent during the manipulation incident to purification of elementary body suspensions. This was apparent when relatively few infected membranes constituted the source of virus, *e.g.*, in Experiment 3, in which 8 membranes were employed. The titer following tryptic digestion, and before washing, was 10^{-6}; after 4 washings, which diluted the original angle sediment approximately 1×10^{-7} times, the titer had fallen to 10^{-5}. Another source of loss was the thin layer of elementary bodies that always adhered to the walls of the glass tubes. Moreover, the small fraction of elementary bodies which had been sedimented along the outer wall of the centrifuge tube, but which had not yet been thrown to the bottom, was often lost in pouring off the supernatant fluid. Finally, the last horizontal centrifugation also contributed to the losses, because in several instances, the sediment which resulted from this procedure was found to be rich in elementary bodies, when stained by Morosow's technic, and had an infectivity equal or almost equal to that of the suspension prepared according to routine. Nevertheless, the final horizontal centrifugation was useful, notwithstanding the waste that accompanied it, because debris and clumped elementary bodies were removed.

Agglutination of Elementary Bodies

Agglutination of elementary bodies (18, 3) by antivaccinal serum was another significant step in establishing the importance of these structures in the disease. It seemed worth while to determine whether or not elementary bodies prepared from hosts of different generic classes were equally agglutinable with a given immune serum.

Agglutination Reaction.—Agglutination of elementary bodies was carried out in the manner described by Craigie (4) and Parker and Rivers (5). Dilutions of

immune sera were made with freshly prepared physiological salt solution buffered to pH 7.2 by the addition of 1 cc. of standard citric acid-disodium phosphate buffer solution to each 100 cc. of saline solution. Elementary body suspensions were brought to the proper concentration for testing by the addition of dilute buffer solution, pH 7.2. 0.25 cc. of elementary body suspension was added to 0.25 cc. of diluted serum which had been placed in a pyrex tube with outside dimensions of 10 x 75 mm. Tubes were placed in a covered rack and incubated overnight at 50°C.

The data presented in Table II show that elementary bodies prepared from either chick membrane or rabbit skin are agglutinated approximately to the same extent by immune sera from two mammalian species. Hyperimmune rabbit serum, in a dilution of 1:1280, agglutinated elementary body suspension 9 obtained from membranes of chick embryos; the serum had the same titer when tested with an elementary body suspension derived from dermal vaccine pulp of rabbits. The guinea pig and monkey antivaccinal sera, although not so potent as the rabbit immune sera, respectively agglutinated chick membrane and rabbit dermal elementary bodies to an approximately equal degree. A consistent difference between the two virus suspensions was noted in the type of clumping. Elementary bodies of rabbit origin formed large loose flakes when mixed with immune sera from various species; in higher concentrations of serum the floccules settled and left a clear supernatant fluid. Chick membrane elementary bodies, however, agglutinated only in a granular form. Repeated washing tended to reduce the agglutinability of bodies; the highest titers were obtained when suspensions were washed 3 or 4 times.

Soluble Precipitable Substances in Filtrates of Infected Membranes

It has been pointed out by Sabin (19) that soluble antigens obtained from tissues infected with vaccinia might be "specific products of infection which are not derived from the virus substance" rather than products of the virus. Craigie and Wishart (6) subsequently demonstrated the immunological identity of L and S precipitinogens found in dermal pulp filtrates from infected rabbits, guinea pigs, and calves, and Ch'en (20) found a soluble antigen in tissue culture vaccine virus prepared according to the method of Li and Rivers (21) in which minced chick embryo tissue suspended in Tyrode's solution was used.

Further information regarding these soluble antigens was sought by means of precipitin tests on the virus-free filtrates from infected chick membranes.

Precipitin Reactions.—Precipitin tests were carried out on extracts of infected membranes. The supernatant fluid which resulted from the first angle centrifugation was filtered through a Seitz pad; the filter had been previously prepared by the passage of 10 cc. of broth and 2 cc. of normal rabbit serum. These filtrates were not infectious. Serial dilutions of the clear serum-colored filtrates were prepared with buffered saline solution and mixed with equal volumes of diluted immune serum, the optimal dilution of the immune serum having been determined by preliminary titration. Tubes and racks similar to those employed for agglutination tests were used; the mixtures were incubated overnight at 50°C.

Precipitin tests on Seitz filtrates of suspensions of infected membranes demonstrated the presence of heat-labile and heat-stable antigens. Results of such a test are shown in Table III. Hyperimmune rabbit serum, which contained antibodies against L and S fractions, gave a slightly higher titer with pooled rabbit dermal filtrate than with membrane filtrate. Rabbit serum that had L but no S antibody reacted equally well with chick membrane and rabbit skin filtrates. Pure anti-S rabbit serum, like the L-S serum, precipitated membrane filtrate at a slightly lower titer than rabbit filtrate.

The results of precipitin tests, which were done on all filtrates of infected membranes, were found to vary. No attempt was made to remove inhibitive substances (6) from the membrane filtrates; this may account for considerable variation in titer of different preparations. Membrane emulsions were filtered with difficulty through Seitz pads, even after removal of most of the particles by centrifugation. The absence of demonstrable precipitinogens in Experiments 6 and 9 probably depended on technical manipulation, such as extraction with ethyl ether and the use of several pads for filtering a small volume of material. There was no obvious explanation for the inability to demonstrate the presence of precipitinogens in filtrate 10; of interest also was the failure to find agglutinogens in the elementary body suspension prepared from this group of membranes which had been inoculated with the 24th membrane passage virus. This failure to demonstrate serologically active substances was not dependent on a change in the virus, because precipitinogens and agglutinogens were

TABLE III

Soluble Precipitable Substances in Filtrates of Chick Membranes Infected with Vaccine Virus

Filtrate		Type of antivaccinal rabbit serum	Dilution of filtrate								
Source	No.		1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Chick membrane	8	L-S serum 6022, diluted 1:12		++++	++++	++++	++	+	−	−	−
Rabbit skin	Pool I	" " " " "		++++	++++	++++	++++	+++	++	+	−
Chick membrane	8	L serum 5110, diluted 1:2	++	++	++	+	+	−			
Rabbit skin	Pool I	" " " " "	++	++	+++	++	+	−			
Chick membrane	8	S serum 5199, diluted 1:4	++++	++++	+++	++	−	−	−		
Rabbit skin	Pool I	" " " " "	++++	++++	++++	+++	++	+	−		

present in lot 11 which had been infected with the 26th membrane passage.

DISCUSSION

The present observations suggest that by use of appropriate methods the infectivity of tissues from animals with vaccinia may be found, in each case, to be associated with their elementary body content. The experiments described in this paper throw no light on the idea of Craigie and Wishart (22) that there are two forms, resistant and active, of vaccine virus elementary bodies. Nor do the observations bring direct evidence to bear on Sabin's suggestion (19) regarding host production of the soluble precipitable substances. The finding of L and S precipitinogens in the filtrates of infected chick membranes by means of immune sera prepared in rabbits by repeated injection of elementary bodies from rabbits, might be considered additional circumstantial evidence that these antigens are products of the virus rather than the result of a response of the host to infection with the active agent.

SUMMARY

By a method of differential centrifugation and tryptic digestion suspensions of elementary bodies have been prepared from chorioallantoic membranes of chick embryos infected with vaccine virus. The infective titer of the final suspension of elementary bodies was usually the same as that of the original tissue emulsion. Elementary bodies from infected chick membranes were agglutinated as well by antivaccinal serum obtained from different mammalian species as were bodies prepared from inoculated rabbit skin. Seitz filtrates of infected chick material contained soluble precipitable substances of vaccinia; these filtrates and filtrates from infected rabbit skin, respectively, reacted equally well with rabbit serum which contained either L or S antibodies.

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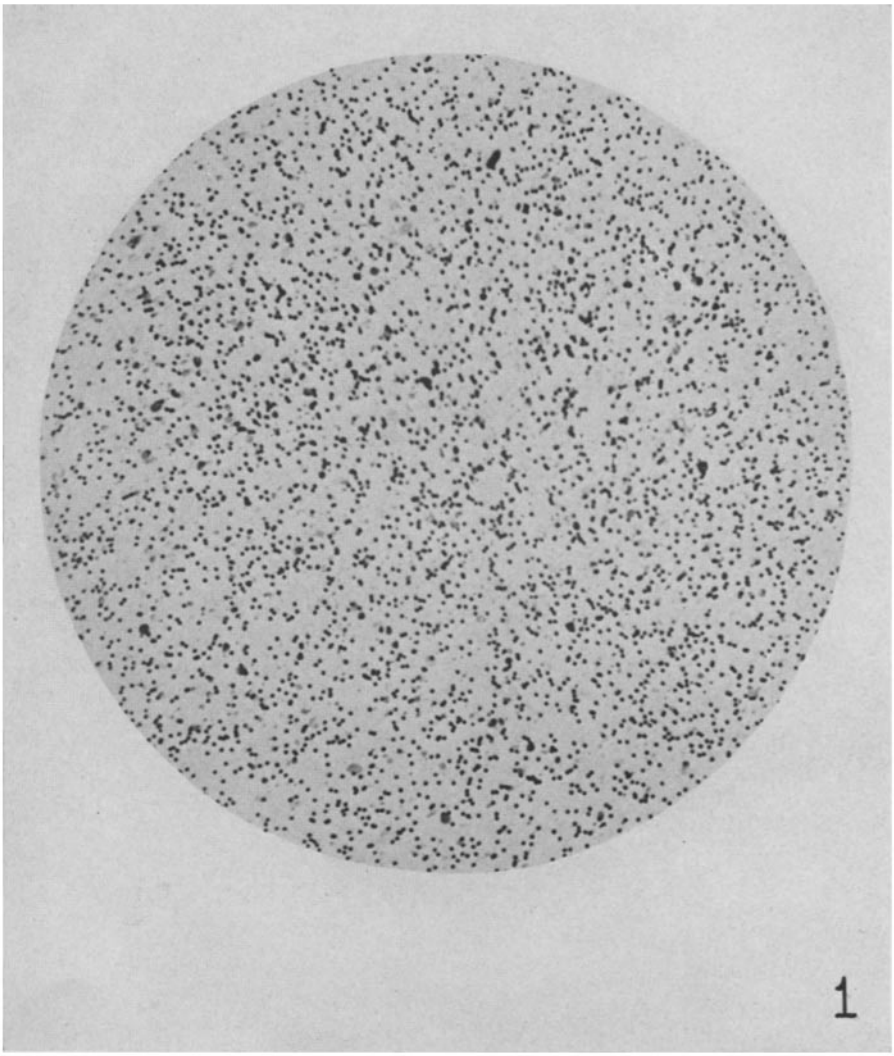
EXPLANATION OF PLATE 8

FIG. 1. Photograph of a preparation of elementary bodies in a relatively pure state obtained from chorio-allantoic membranes of chick embryos infected with vaccine virus. Morosow stain. × 1200.



Photographed by Joseph B. Haulenbeek

(Smadel and Wall: Elementary bodies of vaccinia)