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Preparation of the Specific Soluble Substance from Vaccinia Virus.

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The presence of a specific precipitable substance in tissues infected with vaccinia virus has long been known. The nature of this substance remains, however, obscure. Recently Craigie[1] and Smith[2] reported on the preparation of a comparatively pure fluid extract from vaccinia virus. The extract exhibited definite immunological specificity, on the basis of which Smith regarded the substance responsible for this specificity as analogous in its behavior to the bacterial haptenes. If such be the case, it would seem reasonable to attempt its isolation in a form which would permit more or less accurate determination of the main characteristics of the substance.

The present communication deals with the successful experiment along this line. The technic used here was essentially that described by Smith with certain modifications. The precipitable substance was prepared both from vaccinia virus culture in River's medium consisting of a suspension of chick embryo tissue in Tyrode's solution and from testicular vaccinia virus (lapine). Control substances were also prepared from chick embryo tissue medium and from the testes of normal rabbits. To obtain a necessary supply of vaccinia virus culture, large Erlenmeyer flasks were used. Each flask contained from 25 to 50 cc. of culture. To avoid possible bacterial contamination of the medium during the process of inoculation, a horizontal short and narrow glass neck was attached to each flask near its bottom. Inoculation of flasks was done through this narrow glass neck.

In all instances the procedure of the preparation of the substance was the same. Infected tissue was ground in a glass mortar operated by motor until a very fine and homogeneous emulsion was obtained. In case of the testicular vaccinia virus or normal rabbit testes 5% suspension of the tissue in normal saline was used.

To the suspension 5% ether was added, to prevent deterioration of the tissue due to bacterial growth. The whole was then placed in an incubator for 7 days to allow autolysis to take place. Following this the suspension was centrifuged and the supernatant fluid

[1] Craigie, J., *Brit. J. Exp. Path.*, 1932, **13**, 259.

[2] Smith, W., *Brit. J. Exp. Path.*, 1932, **13**, 434.

passed through Seitz filter. The clear filtrate was adjusted to pH 5.5 and boiled for 5 minutes. This resulted in the formation of a heavy precipitate which was removed by centrifugalization and filtration. The solution was then adjusted to pH 8.0 and boiled again. Usually slight turbidity developed and this again necessitated filtration through Seitz filter.

After this the solution was brought up to the point of neutrality and evaporated over a water bath to one-third of its original volume. If any cloudiness appeared it was filtered off. To such a solution 9 volumes of mixture containing equal parts of ether and absolute alcohol were added, and the mixture was left in an ice-box over night. The white precipitate formed in the solution was collected, dried over a water bath, redissolved in distilled water and dialyzed in a cellophane bag for 48 hours in running water. After dialysis the solution was clarified by filtration and the dissolved substance thrown down by addition of 9 volumes of absolute alcohol. The precipitate was again collected and the whole procedure of precipitation with alcohol repeated. After 3 such precipitations a white and easily soluble substance was obtained. The yield was in average from 5 to 10 mg. per 10 gm. of the testicular vaccinia virus or per 200 cc. of the vaccinia virus culture.

Similarly a white and soluble substance was prepared from the control lots of the normal testicular tissue of a rabbit and chick embryo tissue medium. In the latter 2 cases the yield was, however, considerably smaller than that obtained in cases of infected tissues.

Chemical tests performed with each of 4 substances gave the following result: Biuret test negative, Ninhydrin test slightly positive and Molisch test strongly positive. Each substance was hydrolyzed in the presence of 2-N sulphuric acid and showed positive Benedict's test for reducing sugars. Both the substance prepared from the vaccinia virus culture and that derived from the testicular vaccinia virus were tested for antigenic power through the intravenous injections into rabbits. Subsequent examination of the sera of these animals showed no detectable antibodies. Both substances were also tested for their power to produce positive skin reaction in rabbits previously vaccinated or hyperimmunized with vaccinia virus. In all instances this test was negative. Although both experimental and control substances appeared to be similar so far as the above mentioned chemical tests were concerned, their behavior in the serological test was entirely different.

For this test rabbit anti-sera were prepared by vaccination, using

single intradermal injection of vaccinia virus culture, or testicular vaccinia virus or calf lymph. One month after such injection the sera were collected and used in the experiment as convalescent sera. The same rabbits were then immunized with vaccinia virus culture or testicular vaccinia virus using 4 intravenous injections each consisting of 1 cc. of the material given at weekly intervals. The sera obtained from such immunized animals were used here as hyperimmune sera. Control sera prepared through the immunization of rabbits with a suspension of normal testicular tissue of a rabbit or chick embryo tissue medium and serum from a normal rabbit were used in the experiment to rule out possible non-specific reactions.

The serological test consisted of precipitin and complement fixation reactions. Precipitin ring test was performed using various dilutions of each of 4 substances in normal saline against undiluted sera. In complement fixation test Kolmer's technic was followed with the only modification that a constant amount of the serum diluted 1:10 was tested against different concentrations of the substances.

TABLE I.

Results of Precipitin and Complement Fixation Reactions Observed with S.S.S. Prepared from Vaccinia Virus.

		Soluble specific substances			
Anti-sera	Method of preparing anti-sera	Vaccinia virus culture	Testicular vaccinia virus	Chick embryo tissue medium	Normal rabbit testis
Hyperimmune	Injections of vaccinia virus culture	1:640 *1:800*	1:320 *1:800*	1:80 *Not tested*	0 *0*
"	Injections of testicular vaccinia virus	1:320 *1:800*	1:640 *1:1600*	0 *0*	0 *0*
"	Injections of chick embryo tissue medium	1:80 *Not tested*	0 *0*	1:160 *1:200*	0 *0*
"	Injections of normal rabbit testis	0 *0*	0 *0*	0 *0*	0 *0*
Convalescent	Single intradermal injection of vaccinia virus culture	1:160 *1:400*	1:160 *1:400*	0 *0*	0 *0*
"	Single intradermal injection of testicular vaccinia virus	1:160 *1:400*	1:160 *1:400*	0 *0*	0 *0*
"	Single intradermal injection of calf-lymph	1:160 *1:400*	1:160 *1:400*	0 *0*	0 *0*
Normal rabbit serum		0 *0*	0 *0*	0 *0*	0 *0*

Upper figures for precipitin titre; lower figures (in italics) for complement fixation titre; 0 denotes negative result.

The result secured in these 2 serological tests is given in Table I. As it is seen from the table positive precipitin and complement fixation reactions occurred regularly when specific antibody and antigen were brought into contact. In our case the possibility of non-specific reactions is excluded by various controls which were always negative. It was found that somewhat higher specific titre was recorded both in precipitin and complement fixation reactions when the antigen used in the tests was derived from a homologous material. It is only natural that higher titres for both reactions were observed when hyperimmune sera were used. It is to be noted that the reactions occurring in case of the antigen prepared from vaccinia virus culture and homologous anti-serum exhibited considerably higher titre than the same reactions developing in case of the substance derived from chick embryo tissue culture and the same serum. In this particular case one would expect to obtain non-specific result. This fact indicates that the precipitin and complement fixation reactions as reported in this work were mainly due to the activity of the specific substance derived from virus bodies. The specificity of the reaction is furthermore indicated by positive results obtained in case of substances prepared both from vaccinia virus culture and testicular vaccinia virus and anti-serum prepared through vaccination of a rabbit with calf lymph.

In the present work evidence was produced supporting the view that the precipitin reaction observed by several workers with extracts from the tissue infected with vaccinia virus and homologous anti-serum is caused by the presence in such extracts of a specific product of the vaccinia virus which can be isolated in the form of a polysaccharide similar in its behavior to the bacterial carbohydrate haptenes.

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Intermediate Hosts of Microfilaria Malayi in Chekiang, China.

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Of the 2 species of human filaria found in China *Filaria bancrofti* is transmitted by *Anopheles hyrcanus* var. *sinensis, Culex pipiens* and *Culex fatigans* according to the researches of Feng,[1] Hu[2] and

[1] Feng, L. C., *Am. J. Hyg.*, 1931, **14**, 502.

[2] Hu, S. M. K., *Chinese Med. J.*, 1933, **47**, 1359, 1367.