IMMUNOLOGICAL AND CHEMICAL INVESTIGATIONS OF VACCINE VIRUS

VI. Isolation of a Heat-Stable, Serologically Active Substance from Tissues Infected with Vaccine Virus

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A specific flocculation occurs when the serum of a rabbit recently recovered from a vaccinal infection is mixed under proper conditions with an emulsion of tissues infected with vaccine virus (1, 2). Craigie (3) has shown that this flocculation is due to the agglutination of suspended particles, notably elementary bodies, and to the precipitation of soluble antigens. The present paper deals with experiments performed in order to obtain information regarding the nature of one of the soluble antigens.

Craigie has demonstrated that there are apparently two soluble antigens which participate in the vaccinal precipitation reaction (4, 5); one of them, designated as L, is destroyed by heat and formaldehyde, while the other, designated as S, is not affected by these agents. The nature of the heat-stable antigen has already been the subject of investigation, and methods have been described by which it can be freed to some extent from other substances present in extracts of tissues infected with vaccine virus. Smith (6) boiled a saline extract of testicular tissue infected with virus at pH 5.5, 8.0, and 7.0 successively, and removed the coagulated protein at each step. He found that by this means the quantity of protein in an extract could be reduced greatly without materially affecting the precipitating titer. The resulting solution gave negative or weak reactions for protein, but a strong Molisch reaction. In view of these results the suggestion was made that the precipitating antigen might be either a carbohydrate or protein, but that it resembled the bacterial haptenes in its action. Ch'en (7) employed the technic of Smith as a preliminary step, and then purified the active substance further by repeated precipitation with alcohol-ether and alcohol, and by dialysis against running tap water. He obtained a white powder which gave weak reactions for protein and a strong Molisch reaction, and which yielded a precipitate when mixed in a dilution of 1:640 with immune serum. He concluded that the precipitinogen was a poly-

saccharide. Craigie (8) has observed that both L and S antigens are precipitated at a pH of 4.6 from aqueous solutions having a low concentration of electrolyte.

In none of the work already described has the purification of the heat-stable antigen or antigens been carried very far, and the exact nature of the active substance or substances still remains in doubt. We have, therefore, attempted to isolate a stable antigen[1] from tissues infected with vaccine virus in order to study its chemical properties and immunological reactions.

Methods and Materials

Virus.—The C. L. strain of vaccine virus was used.

Extracts of Dermal Vaccine Virus.—Rabbits were inoculated dermally with vaccine virus in the form of a suspension of washed elementary bodies. 3 days later the animals were sacrificed, and the virus was harvested into 30–35 cc. of buffer solution (0.004 M, citric acid-sodium phosphate, pH 7.2). From this emulsion the virus and other particulate material were removed by centrifugation and by filtration through a Seitz disc. The clear, slightly yellow filtrate contained the soluble antigens.

Extracts of Testicular Vaccine Virus.—Rabbits were inoculated intratesticularly with an emulsion of bacteria-free virus. 3 days later the testicles were removed aseptically; each pair was ground with alundum and emulsified in 100 cc. of Locke's solution. The emulsion was placed in a sterile flask and ether was added to prevent bacterial growth. After incubation for 5–7 days at 37°C., the ether was removed, and then the emulsion was centrifuged. The supernatant fluid containing the soluble antigens was saved. By this means an extract of virus-infected tissues was obtained free from the products of bacterial infection.

Hyperimmune Flocculating Serum.—The manner in which hyperimmune antivaccinal rabbit serum is prepared has already been described (3, 9). Serum against the S antigen was prepared by repeated inoculation of rabbits with a suspension of elementary bodies that had been boiled for 30 minutes; three successive intraperitoneal injections of 1.0, 2.0, and 3.0 cc., respectively, were given at intervals of 1 week; the animals were bled 7 days after the last injection. Rigid precautions were observed in order to prevent the accidental infection of the animals with active vaccine virus (10). Serum prepared in this manner and diluted 1:32 or 1:64 gave precipitates with solutions of S antigen in crude extracts; all of its precipitating activity was removed by absorption with S antigen. L serum was

[1] The word antigen used in connection with our purified material signifies a serologically active substance; as yet we have not had the opportunity of ascertaining whether our material is an antigen in the sense that it excites the production of antibodies when injected into animals.

obtained by absorption with S antigen of LS serum from animals hyperimmunized with active vaccine virus; by this means the S antibodies were removed from the serum which then reacted only with L antigen.

Precipitin Reactions.—Each step in the process of purification of the stable, serologically active substance was controlled and guided by the precipitin reaction previously described (3, 9).

EXPERIMENTAL

Many studies of the chemical and immunological properties of an antigen can be carried out only when the substance has been obtained in a pure state. In this communication a method is described by which a stable substance, probably a protein, has been prepared in a relatively pure form from extracts of tissues infected with vaccine virus, and certain observations regarding the chemical and serological properties of the purified material are recorded.

Preparation of a Stable, Serologically Active Substance from Extracts of Dermal and Testicular Vaccine Virus.—Approximately 500 cc. of an extract of dermal vaccine virus prepared as described above were boiled at pH 7.0 for 5 minutes. A slight precipitate formed which was thrown down by centrifugation and discarded. The supernatant fluid was brought to 50 per cent saturation with ammonium sulfate by the gradual addition of an equal volume of the saturated solution of the salt. A heavy precipitate formed; after standing for an hour this was thrown down by centrifugation and the supernatant fluid was discarded. The precipitate was dissolved in approximately 75 cc. of 0.02 M buffer solution at a pH of 7.2; it was then placed in a cellophane bag and dialyzed against running water for 2 days. By the end of that time a slight precipitate of a water-insoluble material had formed which was removed by centrifugation. The clear supernatant fluid was then brought to 25 per cent saturation with ammonium sulfate by the addition of an appropriate quantity of the saturated solution. A moderately heavy precipitate formed which was separated in the centrifuge and discarded. The supernatant fluid was brought to 50 per cent saturation with ammonium sulfate; the precipitate that formed was collected and dissolved in 30–40 cc. of buffer solution, and the solution again was freed of salts by dialysis. To the solution was added 9 volumes of cold neutral alcohol. A flocculent precipitate formed immediately. After standing overnight the mixture was centrifuged; the supernatant fluid was discarded; the precipitate was dissolved in 50 cc. of water, and the alcohol was removed. To the solution 5 cc. of 0.05 M buffer, pH 4.6, were added. A precipitate formed at once; this was thrown down by centrifugation and after removal of the supernatant fluid was dissolved in a buffer solution at pH 7.2. The supernatant fluid was kept overnight in the cold room; by the following morning a slight precipitate had appeared in it which was collected, dissolved in a buffer solution of pH 7.2, and added to the solution of the original precipitate. About 10 to 25 per

cent of the antigen remained in solution at pH 4.6; this amount was lost. The reaction of the solution was adjusted to pH 7.8 and the solution was boiled. The slight precipitate which formed was removed. The reaction was then adjusted to pH 6.0, and the solution was again boiled. A heavier precipitate formed which was likewise discarded. The water-clear supernatant fluid was dialyzed against distilled water until free from electrolytes. It was then frozen and desiccated *in vacuo* over calcium chloride while in the frozen state. This procedure yielded about 15 mg. of a voluminous white substance.

The manner of preparation of a stable antigen from extracts of testicular vaccine virus was similar to that described above. The serological activity of the product obtained, however, tended to be somewhat lower than that of the active material secured from dermal virus.

Serological Characteristics of the Purified Substance

The purified material in a dilution of 1:640,000 gives rise to a precipitate in the presence of immune serum. This occurs with an S serum, but not with a LS serum from which the S antibodies have been absorbed. Experiments to determine whether the substance is capable of stimulating the formation of antibodies against itself have not yet been carried out. It may be noted, however, that boiled elementary bodies containing no L antigen, give rise to antibodies against our stable substance.

Chemical Characteristics of the Purified Substance

The purified substance obtained by the method described above is white and voluminous, tends to cohere, and consequently resists disintegration by grinding. It dissolves readily in distilled water to make a clear colorless solution which forms a persistent foam when shaken. It is almost completely precipitated from an aqueous solution at a pH of 4.6. It is soluble in 80 per cent alcohol to which a small amount of hydrochloric acid has been added, but is insoluble in neutral 80 per cent alcohol. It is salted out by ammonium sulfate when the concentration of the salt is between 25 and 50 per cent saturation. A solution of the antigen gives an intense Molisch reaction and yields a heavy precipitate with trichloracetic acid. It is not affected by boiling for brief periods between pH 6.0 and 8.0. The purified antigen contains 16.5 per cent nitrogen.[2]

[2] Analysis carried out by Dr. W. F. Goebel.

Effect of Enzymes on the Purified Substance

In order to obtain further information regarding the nature of the active substance, the action of certain enzymes upon it was observed.

Trypsin and Chymotrypsin.—Crystalline trypsin and chymotrypsin[3] were dissolved in 0.02 N HCl in the proportions of 5 mg. of enzyme to each cubic centimeter of solution. A solution of commercial (Fairchild) trypsin was prepared by dissolving 50 mg. of dry substance in each cubic centimeter of 0.02 N HCl. In the tests, 0.5 cc. of each of the enzyme solutions was mixed with 0.5 cc. of a solution containing 0.5 mg. of the serologically active substance. The reaction was adjusted to pH 8.4 and the volume made up to 2.0 cc. Ether was added to prevent bacterial growth, and the mixtures were incubated overnight at 37°C. Controls of antigen alone, to determine the effect of incubation at pH 8.4, and of enzyme plus casein, in order to demonstrate the activity of the enzyme, were included in the tests. In all cases the casein was digested, and the precipitating titer of the substance incubated with crystalline trypsin and with chymotrypsin was undiminished, but there was a four- to eightfold reduction in titer of the antigen incubated with commercial trypsin.

In order to show whether the loss in precipitating activity of the substance was due to action of the enzyme on it or to inhibition of the precipitin reaction, the following experiment was performed.

A mixture of the purified substance and enzyme was prepared in the manner described above, and the reaction was adjusted to pH 8.4. It was immediately divided into 2 equal parts, one of which was incubated for 6 hours at 37°C. and then neutralized; the other was neutralized immediately and boiled in order to destroy the activity of the enzyme. The titer of the antigen in the latter mixture was the same as that of the antigen to which no enzyme had been added, while the titer of the antigen in the former mixture which had been incubated at a pH suitable for enzyme activity was reduced eightfold.

Pepsin.—Only commercial enzyme was used to determine the action of pepsin on the purified active substance. A mixture of 0.5 mg. of commercial pepsin and 0.5 mg. of antigen was made, the reaction of the solution was adjusted to pH 2.0, and the volume made to 2.0 cc. The solution was incubated overnight at 37°C. Appropriate controls were included, which indicated that the enzyme was active, and that the antigen was not affected by incubation with buffer. Under the conditions of the experiment there was an eightfold reduction in the activity of the antigen incubated with pepsin.

From the results of the experiment just described it appears that the serological activity of our purified material is greatly reduced by

[3] Obtained from Dr. J. H. Northrop.

digestion with commercial trypsin and with commercial pepsin, but that it is resistant to the action of crystalline trypsin and crystalline chymotrypsin.

DISCUSSION

In the present communication a method has been described by which a heat-stable, serologically active substance has been isolated from tissues infected with vaccine virus. If the substance is not absolutely pure, it approaches purity more closely and is decidedly more active—producing a precipitate in a dilution of 1:640,000—than any soluble antigen that has hitherto been isolated from tissues infected with the virus of vaccinia.

The purified active material contains 16.5 per cent nitrogen, and, if the usual factor of 6.25 is used to express the relation of nitrogen to protein, all of the material is accounted for as protein. Indeed, all of the studies so far conducted are consistent with the idea that our substance is an alcohol-soluble protein. It should be noted, however, that it is soluble only in the form of the acid salt and is insoluble in neutral alcohol. It is not precipitated by boiling in a neutral aqueous solution and is soluble in distilled water. That carbohydrate is present is indicated by the fact that the material yields a strong Molisch reaction. It is possible that the carbohydrate may be present as an impurity, yet it is known that certain conjugated proteins contain carbohydrates of constitution and that these give Molisch reactions (11). The fact that digestion of our material with pepsin and commercial trypsin greatly reduces its precipitating activity indicates that the integrity of the protein molecule is essential for its full serological activity. While proteins possessing characteristics similar to those described are not common, they have been isolated from bacteria, *e.g.*, the type specific protein (M) obtained from streptococci by Lancefield (12).

With the present data it is impossible for us to state definitely that our purified material represents only one antigen. Furthermore, the exact relation of our serologically active substance to those described by Smith (6), Ch'en (7), and Craigie (5) is not known, yet all of them possess certain similarities, and, in view of previous work on crude vaccinal extracts by several investigators (2, 5, 9, 13), one is warranted in concluding that they are specifically associated with vaccinia.

SUMMARY

A method has been described by which a stable, serologically active substance has been isolated in a relatively pure state from tissues infected with vaccine virus. It has the characteristics of an alcohol-soluble protein which is not precipitated by boiling in a neutral aqueous solution. In a dilution of 1:640,000 it gives a precipitate when mixed with a serum containing antibodies against Craigie's S antigen of vaccine virus, but no visible reaction occurs when it is mixed with serum depleted of S antibodies by means of absorption.

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