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ISOLATION AND CHARACTERIZATION OF ANTIBODIES TO *CLOSTRIDIUM PERFRINGENS* EPSILON TOXIN FROM HYPERIMMUNE HORSE SERUM

R. W. WORTHINGTON and MARIA S. G. MÜLDERS(1)

ABSTRACT

WORTHINGTON, R. W. & MÜLDERS Maria, S. G., 1979. The isolation and characterization of antibodies to *Clostridium perfringens* epsilon toxin from hyperimmune horse serum. *Onderstepoort Journal of Veterinary Research*, 46, 121–124 (1979).

Antibodies against epsilon toxin were isolated from hyperimmune horse serum by affinity chromatography. Purified epsilon prototoxin covalently bound to Affigel 202 was used as immunosorbent, and antibodies were eluted with 6,0 M guanidine chloride. In a single run 80 mg of antibody could be recovered from a 20 mℓ column of immunosorbent. The antibody was shown to belong to the IgG(T) class of immunoglobulins.

Résumé

ISOLEMENT ET CARACTÉRISATION D'ANTICORPS DE LA TOXINE EPSILON DE CLOSTRIDIUM PERFRINGENS *À PARTIR DE SÉRUM DE CHEVAL HYPERIMMUNISÉ*

On a isolé à la chromatographie par affinité des anticorps contre la toxine epsilon à partir de sérum de cheval hyperimmunisé. Comme immunoadsorbant, on a utilisé de la prototoxine epsilon purifiée, en liaison de covalence avec l'Affigel 202; *les anticorps ont été élués par le chlorure de guanidine* 6,0 *M. En un seul passage on a pu récupérer* 80 *mg d'anticorps à partir d'une colonne d'immunoadsorbant de* 20 *mℓ. Ces anticorps se sont avéré appartenir à la classe IgG(T) d'immunoglobulines.*

INTRODUCTION

During the course of a research project on *Clostridium perfringens* epsilon toxin the possibility of concentrating and purifying antibodies against epsilon toxin arose. Since the usual procedures for the purification of gamma globulins result in a great loss of antibody activity, we decided to investigate this problem more fully and to produce purified antibody by affinity chromatography (immunosorption).

MATERIALS AND METHODS

Epsilon toxin antiserum. A horse which had previously been hyperimmunized by the injection of trypsin-digested, formalized epsilon toxin was used*. Its antitoxin titre was boosted by 4 injections given at weekly intervals of 1 400, 2 800, 5 600 and 11 200 Lf units of alum-precipitated, trypsinized toxoid, partially purified by ammonium sulphate precipitation. This schedule raised the antibody titre 400-fold to a level at which the serum contained the equivalent of 625 Lf units of antibody per mℓ as measured by radial diffusion. Four litres of blood was collected from this horse, and the serum freeze-dried. It was reconstituted at 100 mg per mℓ of water for experimental work.

Preparation of gamma globulin

1. Gamma globulins were precipitated from the above serum at 33% ammonium sulphate saturation. After the precipitate had been dissolved in water, the precipitation step was repeated twice more and the precipitate dialyzed against 15 mMNaCl solution until free of sulphate. After clarification by centrifugation the preparation was freeze-dried.

2. Four hundred mg of dried serum was dissolved in 4 ml of water and applied to a 1,5 × 30 cm column of DEAE Biogel** equilibrated with 0,005 M phosphate buffer, pH 7,2. The column was eluted with the same buffer. The first peak eluted was collected and freeze-dried. This method is a slight modification of the procedure described by Nowatny (1969). The purity of gamma globulin preparations was checked by immuno-electrophoresis.

Preparation of Immunosorbent. Epsilon prototoxin was purified by DEAE cellulose chromatography, as described previously (Worthington, Mülders & Van Rensburg, 1973). Two hundred mg of purified prototoxin dissolved in 20 mℓ of water was added to 24 mℓ of Affigel 202* (packed volume 22 ml) and the pH adjusted to 6,0 with 1,0 M NaH_2PO_4. Six hundred mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl* (EDAC) dissolved in 4 mℓ of water was added to the slurry. The suspension was stirred gently for 3 hours during which time the pH was maintained at 6,0. It was then stored overnight in the dark at room temperature.

The gel was packed in a column and washed with phosphate-buffered saline (PBS) until the eluate showed no absorbance at 280 nm. It was further washed with 6,0 M guanidine chloride until it showed no absorbance at 280 nm and then washed again with PBS to remove all traces of guanidine chloride.

Isolation of antibody by immunosorption. Up to 40 ml of epsilon antitoxin serum was applied to the column and eluted with PBS. All fractions eluted were monitored by measuring absorbance at 280 nm and by assaying the antibody content by radial diffusion. When the absorbance of the eluate at 280 nm had returned to zero, the antibody was eluted with 6,0 M guanidine chloride. The eluted material was dialyzed repeatedly against 15 mM NaCl, centrifuged at high speed and the clear supernatant freeze-dried. As much as 167 mg of dried material containing about 50% of protein was recovered in a single run.

Production of antisera to horse serum proteins

1. A rabbit was injected intramuscularly with 2,5 ml of an emulsion containing 3 parts of fresh horse serum to 5 parts of complete Freund's adjuvant**. The injection was repeated after 17 days. Fourteen days later a series of 3 intramuscular injections (½, 1 and 2

(1) Faculty of Veterinary Science, P.O. Box 12580, Onderstepoort 0110, Republic of South Africa

* Supplied by Prof. B. C. Jansen, Veterinary Research Institute, Onderstepoort, 0110

** Bio-Rad Laboratories, Richmond, California, U.S.A.

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* Bio-Rad Laboratories, Richmond, California, U.S.A.

** Difco Laboratories, Detroit, Michigan, U.S.A.

mℓ) and 2 intravenous injections (1 mℓ) of fresh horse serum were given. The rabbit was bled after a further 5 days.

2. Epsilon toxin antibody purified by immunosorption was used to immunize a rabbit. Two injections of 5 mg of antibody emulsified in Freund's adjuvant were given at 14-day intervals. The rabbit was bled 21 days after the last injection.

3. Monospecific antisera to horse IgG, IgG(T) and IgM were purchased from Miles Laboratories*. The anti IgG serum made 2 distinct lines when used in immuno-electrophoresis against whole horse serum. The lines corresponded to IgG and IgG(T) lines. The serum was made monospecific by the addition of 90 μg of epsilon antitoxin, isolated by immunosorption, to each 0,1 mℓ of serum used.

Radial diffusion. One hundred and fifty μg of purified protoxin was added to 30 ml of molten ($\pm$60 °C) 1% agarose solution in PBS containing 0,01% merthiolate. The solution was poured onto a 13 × 13 cm level glass plate and allowed to gel. Small wells were cut into the gel and suitable amounts of serum or antibody preparations added to them (see text). The plates were allowed to stand in a closed container over water for 48 h. The precipitin rings which developed were measured with a vernier calliper. A standard serum containing 625 Lf units/mℓ antibody** was used as reference in all tests.

Immuno-electrophoresis. Immuno-electrophoresis was done in 1% agarose in barbital-acetate buffer pH 8,2 (μ=0,05). Serum or antibody specimens were added to the wells and subjected to electrophoresis at 7 mA/gel for 80 min. Following electrophoresis, 0,1 mℓ of antiserum was added to the central trough. Precipitin lines developed for 24–48 h in a moist atmosphere. Further details are given in the text.

Antibody quantitation. Sober (1970) gives an extinction coefficient $E^{1\%}_{1\,cm}$ at 280 of 13,8 for horse IgG. This extinction coefficient was therefore used for estimating the protein content of all gamma globulin and antibody preparations.

Results

When hyperimmune horse serum was applied to the immunosorbent column, large amounts of antibody were absorbed. An analysis of fractions eluted from the column by radial diffusion showed that it absorbed virtually all the antibody from the first 20 mℓ of serum passed through it. Thereafter, the immunosorbent was saturated and no antibody was absorbed from later fractions. The removal of attached antibody presented some difficulties. Previous experience had shown that flocculated antibody antigen complexes were not soluble in 0,1 M glycine HCl buffer pH 2,7; 1,0 M NaCl; 0,1 M glycine buffer pH 9,4; 1,0 M KI or 1,0 M KSCN. Floccules were partially soluble in 0,3 M KCl in 1,0 M NH_3 after standing for 16 hours at 4 °C and soluble in 8,0 M urea at the same time. Floccules, however, were readily soluble in 6,0 M guanidine chloride. Small amounts of antibody were eluted from the column by 0,3 M KCl with 1,0 M NH_3 and by 0,1 M CH_3COOH with 0,5 M KCl, but only 6,0 M guanidine chloride eluted all antibody satisfactorily from the column. Fig. 1 shows a typical run. After elution from the column and dialysis, in which there is some loss owing to precipation, about 80 mg of antibody was recovered from a single run.

Solutions of gamma globulin (11 mg/mℓ), prepared by DEAE biogel chromatography and ammonium sulphate precipitation, contained only small quantities of epsilon toxin antibody that could not be accurately measured. A solution containing 4,5 mg/mℓ of antibody isolated by immunosorption contained $\pm$500 Lf/mℓ. The antibody content was measured by radial diffusion (Fig. 2).

The antibody and gamma globulin preparations were characterized by immuno-electrophoresis against anti-horse antiserum, anti-horse IgG, anti-horse IgM and anti-horse IgG(T). The epsilon toxin antibody preparation contained IgG(T) only. This class of immunoglobulin has a faster mobility than IgG (Fig. 3). The gamma globulin prepared by ammonium sulphate precipitation contained IgG, IgM and IgG(T) (Fig. 4). Gamma globulin prepared on DEAE biogel contained IgG, IgG(T) and some other unidentified contaminants (Fig. 5).

The rabbit anti-serum prepared against horse epsilon toxin antibodies formed both IgG and IgG(T) lines when used against whole horse serum (Fig. 6).

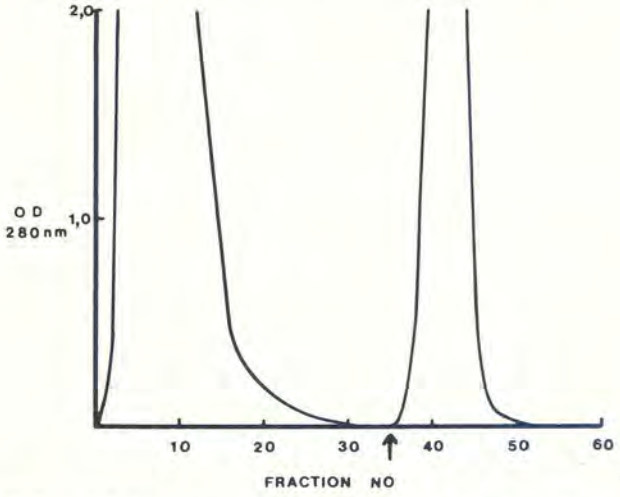

FIG. 1 Affinity chromatography of anti-epsilon toxin antibody on a column containing 20 mℓ of Affigel 202 to which epsilon prototoxin was covalently bound. Twenty mℓ of hyperimmune serum was applied to the column and eluted with PBS. Elution with 6,0 M guanidine chloride was begun at the point marked ↑

FIG. 2 Radial diffusion of epsilon antitoxin preparations. From left to right the wells contained: 4,5 mg/mℓ of anti-epsilon toxin antibody prepared by affinity chromatography; 11 mg/mℓ of gamma globulin prepared by DEAE biogel chromatography; 11 mg/mℓ of gamma globulin prepared by ammonium sulphate precipitation; standard epsilon toxin antiserum containing 625 Lf/mℓ. Each well contained 10 μℓ of solution under test

* Goodwood, Cape Town

** Supplied by Prof. B. C. Jansen, Veterinary Research Institute, Onderstepoort 0110

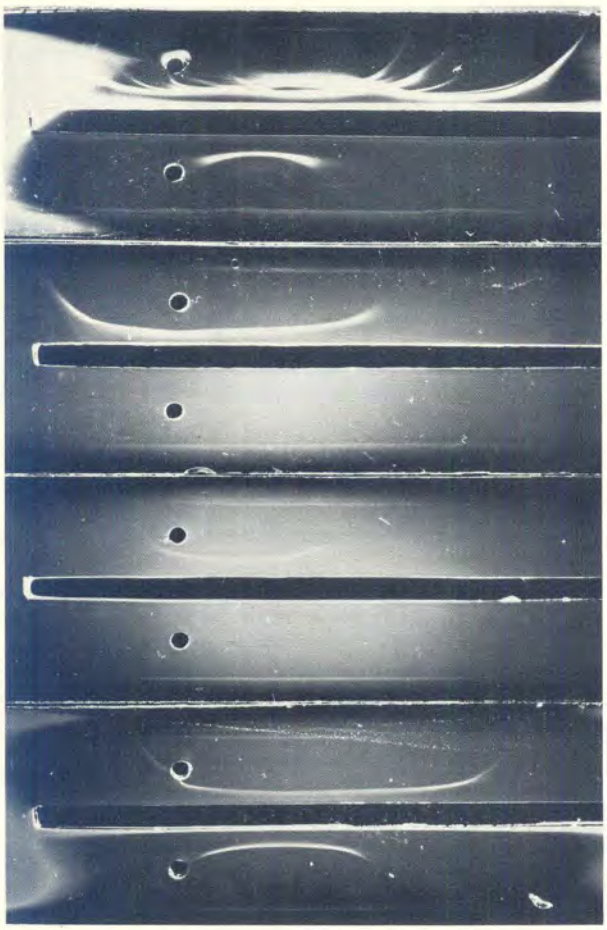

FIG. 3 Characterization of epsilon toxin antibody prepared by affinity chromatography. In each of the 4 preparations the upper well contained whole hyperimmune horse serum and the bottom well epsilon toxin antibody prepared by affinity chromatography. From top to bottom the antiserum troughs contained: anti-whole horse serum; anti-horse IgG; anti-horse IgM and anti-horse IgG(T)

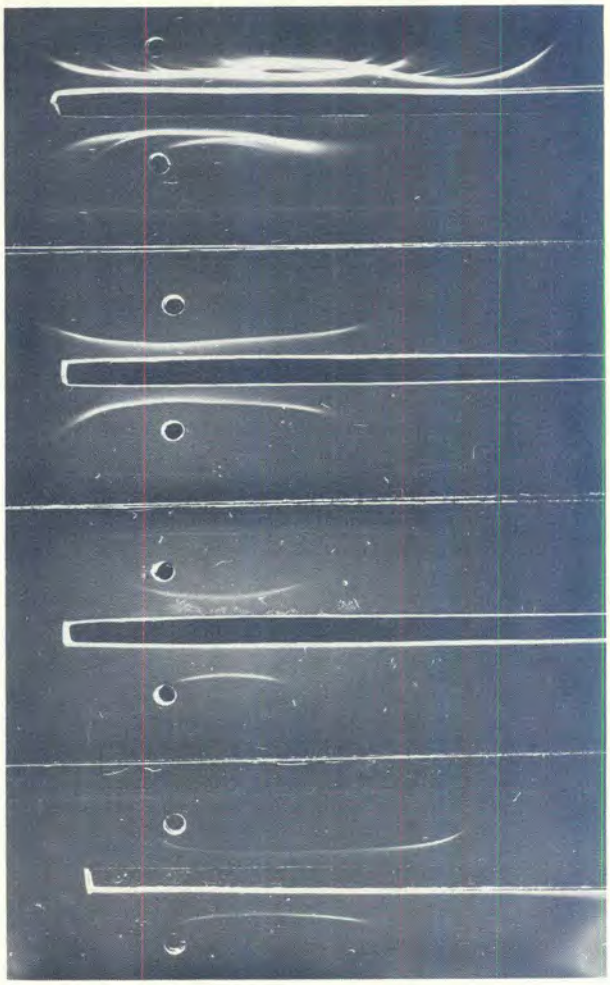

FIG. 4 Characterization of gamma globulin prepared by ammonium sulphate precipitation. In each of the 4 preparations the upper well contained whole hyperimmune horse serum and the bottom well the gamma globulin preparation. From top to bottom, the antiserum troughs contained: anti-whole horse serum; anti-horse IgG; anti-horse IgM; anti-horse IgG(T)

Discussion

Horses hyperimmunized for the production of anti-toxin sera produce a distinct class of antibodies not found in other species. These antibodies have a faster electrophoretic mobility than normal IgG antibodies (Kekwick & Record, 1941; Van der Scheer, Wyckoff & Clarke, 1940; 1941). This class of antibody was originally assumed to belong to the IgA class because of its high carbohydrate content (Hill & Cebra, 1965; Klinman, Rockey, Frauenberger & Karush, 1966; Rockey, 1967). Because of its similarity to IgG in amino acid sequence and antigenic composition, it was suggested, however, that it be termed IgG(T) (Weir & Porter 1966; Weir, Porter & Givol, 1966). It is now generally termed IgG(T) and older synonyms such as $7SB_2A$, T component and IgA(T) have fallen into disuse. IgG(T) antibodies, which become the dominant type of antibody in hyperimmunized horse serum, are responsible for the typical flocculating, as opposed to the precipitating, characteristics of horse antitoxin sera (Relyveld & Raynaud, 1957).

It was not surprising, therefore, to find that the epsilon toxin antibodies in hyperimmunized horse serum belonged to the IgG(T) class. This fact also explains the great loss of antibody activity which occurred when gamma globulin was prepared as described above. By using modified methods of ion exchange chromatography (Weir & Porter, 1966) or taking the 30–50% ammonium sulphate precipitate from serum (Relyveld & Raynaud, 1957), it should be possible to prepare more concentrated preparations of antibodies. It seemed preferable, however, to isolate a pure preparation of antibody by affinity chromatography. This method proved to be most suitable for the preparation of epsilon toxin antibody which appeared to be almost exclusively of the IgG(T) class (Fig. 3). When a rabbit antiserum was made against

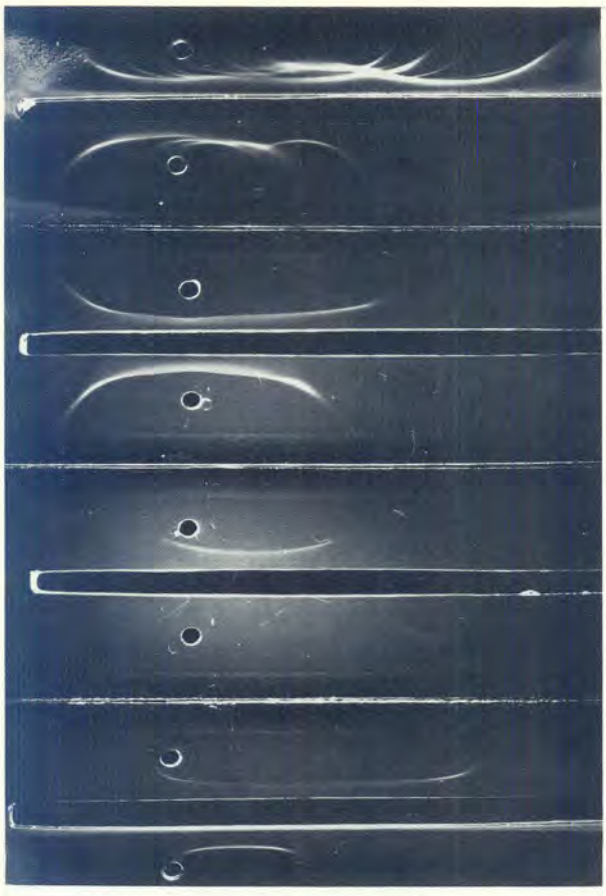

FIG. 5 Characterization of gamma globulin prepared by DEAE biogel chromatography. In each of the 4 preparations the upper well contained whole hyperimmune horse serum and the bottom well the gamma globulin preparation. From top to bottom the antiserum troughs contained: anti-whole horse serum, anti-horse IgG, anti-horse IgM, anti-horse IgG(T)

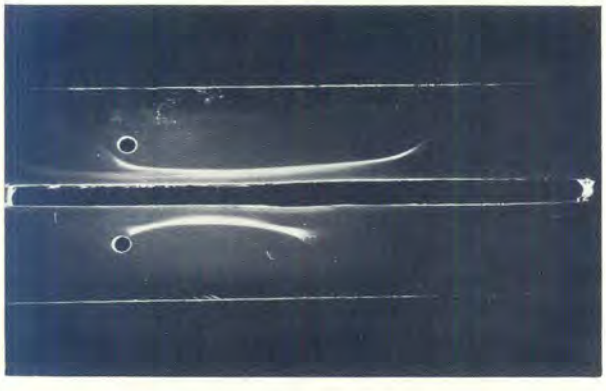

FIG. 6 Immuno-electrophoresis of whole serum (above) and epsilon toxin antibody prepared by affinity chromatography. The antiserum trough contained serum that was raised in a rabbit by injection of anti-epsilon toxin antibody prepared by affinity chromatography

horse epsilon toxin antibodies, the rabbit serum formed precipitin lines against both IgG and IgG(T) (Fig. 6). This was to be expected, however, as IgG(T) antibody is closely related to IgG (Weir & Porter, 1966) and absorption with IgG would be necessary to produce a monospecific antiserum.

Isolated epsilon toxin antibody appears to be biologically active and has been used by us in radial diffusion, rocket electrophoresis and toxin neutralization tests. Affinity chromatography therefore appears to be the method of choice for isolating anti-epsilon toxin antibodies from horse serum.

Acknowledgements

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