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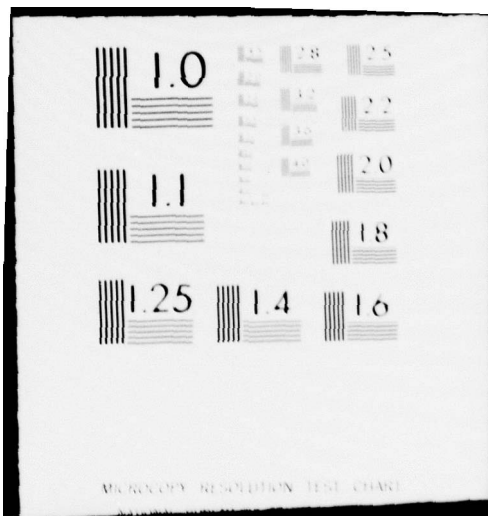
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Use of reversed passive hemagglutination in the
detection of Clostridium botulinum Type A,
B and E toxin

by

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Received: Oct. 10, 1977

With 3 illustrations

Summary:

With the reversed passive hemagglutination technique it is possible to detect minimal amounts of botulinum type A, B and E toxins (s. Tab. 3). The antisera used were prepared by foot-pad injection of rabbits with purified toxoids in Freund's complete adjuvant (s. Tab. 1). Antitoxin globulin were prepared from rabbit antisera with $(\text{NH}_4)_2\text{SO}_4$ to 50%. Formalinized and tanned human erythrocytes were sensitized with these specific antitoxin globulins.

Only slightly cross reactions were encountered between the type A, B and E antiglobulin sensitized cells and culture filtrates of *C. butyricum*, *C. sporogenes* (type A and B anti-globulin only) and *C. perfringens* type C (s. Tab. 4).

MUL 0572

Passive hemagglutination (PHA) for in vitro detection of *Clostridium botulinum* was ... by Rycaj (1956), Sinitsyn (1960), Yafaev and Chepelev (1961), Bulatova (1964), Johnson et al. (1966), Azarenok (1970), Uemura and Saka-

(NOTE: NEXT TWO PAGES MISSING)

7. Preparation of toxoids

After a repeated testing of the type specificity of the purified toxins in mouse protective experiments, they were inactivated by addition of 0.6% formalin. The tolerance of the toxoids was tested by intraperitoneal application of 0.5 ml to 2 mice per week.

8. Preparation of type-specific sera

The toxoids were used by both as AlPO_4 -adsorbate vaccines and mixed with the same quantity of complete Freund's adjuvant (Difco Laboratories, Detroit). The immunization procedures were carried out with toxoids type A, B and E on 4 rabbits each.

8.1. Immunization with AlPO_4 adsorbate vaccine.

8.1.1. Two subcutaneous injections of 1.0, 1.5 or 2.0 ml toxoid at intervals of 14 days. After a 4 and 8 week pause, 2 subcutaneous applications per week over a period of 4 weeks with the same quantities of toxoid (1.0, 1.5 or 2.0 ml).

8.1.2. Seven subcutaneous injections of 1.0 ml toxoid every 2nd day - 4 week pause - 14 subcutaneous injections of 1.0 ml every second day.

Blood was drawn 14 days after the last application.

8.2. Immunization using complete Freund's adjuvant.

8.2.1. First injection: 0.1 ml toxoid/adjuvant into the central foot pad of the two rear legs, 0.5 ml on the right and left in the musculature of the upper arms, 0.1 ml subcutaneously in 8 dorsal sites. Second injection after 2 or 4 weeks:

0.6 ml toxoid/adjuvant right and left in the musculature of the upper arms, 0.1 ml subcutaneously in 8 dorsal sites. Third injection 14 days after the second injection: as for the second injection. Blood was drawn 14 days after the third injection and also directly before each application.

8.2.2. First injection: as described in 8.2.1. Second injection after 2 or 4 weeks: as described in 8.2.1. Third injection 14 days after the second injection: 2 ml toxin diluted to 10 LD₅₀-doses intravenously. Blood was drawn as described in 8.2.1.

8.2.3. First injection: as described in 8.2.1. Second injection after 2 or 4 weeks: as described in 8.2.2. (3rd injection). Third injection 14 days after the second injection: As described in 8.2.2. Blood was drawn as described under 8.2.1.

9. Fractionation of type-specific sera

Fractionation of the type-specific sera took place at 50% ammonium sulfate saturation (Coons and Kaplan, 1950; Coons, 1958).

10. Determination of antitoxin content

Determination of the antitoxin content took place as described by Sonnenschein (1973).

11. Immune electrophoresis

Immune electrophoresis took place with a Boskamp Microphoresis apparatus (Boskamp Co., Hersel) for 90 and 180 min.

The buffer used was a veronal buffer (stock solution: 40.14 g barbital-Na, 26.49 g Na-acetate (free of H₂O) for 3000 ml distilled water, pH 8.2). In addition, a 1% agarose solution (agarose of maximum purity, Serva Co., Heidelberg) prepared with the above buffer was used and the slides were stained with amido black.

12. Agar gel precipitation

Agar gel precipitation took place according to the slide microtechnique of Wadsworth and Crowle, modified according to Untermann (Sonnenschein, 1973).

13. Osmolarity measurements¹

The osmolarity of PBS, phosphate buffered NaCl solution of pH 7.2, pH 6.4 and pH 4.8 and of phosphate buffer solutions I² and II³ (Mai and Rosin, 1969) was determined by establishing the lowering of the freezing point with a half micro-osmometer (Knauer Co., Berlin).

14. Erythrocytes used

In addition to previously formlinized sheep erythrocytes (Difco Laboratories, Detroit, Best-no. 3136-65-3), Rh-positive

¹ The measurements were made by Dr. Hedrich at the Central Institute for Animal Breeding, Hannover.

² Phosphate buffer solution I, pH 7.2:

NaCl	4.80 g
NaHPO ₄ · 2 H ₂ O	7.60 g
KH ₂ PO ₄	1.45 g

³ Phosphate buffer solution II, pH 6.4:

NaCl	4.500 g
Na ₂ HPO ₄ · 2 H ₂ O	4.299 g
KH ₂ PO ₄	6.915 g

human erythrocytes of blood group O were used. Blood was drawn under sterile conditions in quantities of 100 ml by means of blood transfusion instruments. To prevent blood coagulation, either Na citrate or heparin was used. Before further processing, the erythrocytes were washed once with phosphate buffer solution I.

15. Formalin fixation of erythrocytes

Formalin treatment of human erythrocytes took place in accordance with the specifications of Ingraham (1958). A 50% erythrocyte suspension was mixed with 4 volumes formaldehyde solution (37% formaldehyde dissolved in 3 parts PBS) and next stored for 10 days at 4°C and shaken daily. After washing 5 times in phosphate buffer solution I, the erythrocytes were suspended in 0.038 M NaHSO₃ to neutralize free aldehydes and stored for 18 hours at 4°C (Silvermann et al., 1968), followed by 18 hours of dialysis against tap water. After another centrifugation the erythrocytes were stored, with addition of merthiolate (0.01%), as a 10% suspension in 0.15 M NaCl solution at 4°C.

16. Tannin treatment

From the store of fixed erythrocytes the required quantity was removed, washed once in 0.15 M NaCl solution and resuspended as a 2.5% suspension with 0.15 M NaCl solution. A pure, powdered tannin (Merck Co., Darmstadt, no. 773) was mixed with PBS, phosphate buffered NaCl (pH 7.2, 6.4, 4.8), 0.15 M NaCl and

phosphate buffer solutions I and II, up to a final concentration of 1:20,000 and 1:40,000. The same volumes of erythrocyte suspension and tannin solution (1:20,000 or 1:40,000) were incubated for 10, 15, 30 and 45 min. with gentle shaking at 37°C, and for 15 and 30 min. at 56°C, in the water bath. Next the erythrocytes were centrifuged, washed twice in the appropriate buffer or 0.15 M NaCl solution and taken up in 0.15 M NaCl solution as a 2.5% suspension.

17. Sensitization

The globulin fractions used for sensitization of the tannin-treated erythrocytes must be inactivated before use at 56°C for 30 min. in the water bath. In order to achieve optimal loading of the erythrocytes, the globulin quantity used must be tested in preliminary experiments by appropriate dilutions against known quantities of toxin. The procedure is the same as in the main experiment, in which only one certain, optimal globulin concentration is used. The globulin dilutions are prepared with the buffer solutions described under 16. and with 0.15 M NaCl solution. The same quantity of serum dilution and a 4-fold quantity of the buffer solutions or 0.15 M NaCl solution is added to the tannin-treated erythrocytes and they are incubated in flasks for 15, 30 and 45 min. with gentle shaking in the water bath at 30°C. After centrifugation and two washings in the appropriate buffer solutions or 0.15 M NaCl solution, with addition of 0.5 or 1% inactivated rabbit serum, the erythrocytes are taken up in the washing liquid as a 1% suspension.

18. PHA

PHA was carried out with the micro-titer procedure (Cooke Co., Alexandria, USA) on microtiter plates (U-form, Greiner Co., Nürtingen). The preparation of the toxin dilution series took place with a factor of 2^{-1} in quantities of 0.025 ml using the buffer or 0.15 m NaCl solutions referred to under 16., with addition of 0.5 or 1% inactivated rabbit serum as dilution medium. To each dilution stage the same volume of the suspension of sensitized erythrocytes was added. After 2 min. mixing on a mixer (Köttermann Co., Hänigsen), the plates were stored at 30°C. In each experiment mixtures with tannin-treated, non-sensitized erythrocytes - with or without toxin - and with sensitized erythrocytes without a supply of toxin were also carried through as controls.

Evaluation took place after 1 and 3 hours. Agglutination was designated as + (questionable), ++ and +++ , a negative as -. All experimental procedures were carried out a total of 5 times.

Results

1. Properties of C. botulinum toxins and toxin filtrates

The toxicity of C. botulinum toxin filtrates in the mouse lethality test was between 10^4 and 10^7 LD₅₀/ml toxin. By purification of toxins type A, B and E, an increase in toxicity could be achieved by 1 to 2 powers of ten. Upon absorption of the purified toxins with sheep erythrocytes at pH 8.0 there was a loss of toxicity, so that the lethality of the purified toxins for mice after absorption was within

the range of the toxin filtrates.

Absorption of the hemagglutinin components could be detected from the loss of a line of precipitation in the agar gel precipitation reaction.

The nitrogen content of the purified toxins was 0.05-0.07 mg N/ml.

2. Properties of type-specific sera

Immunization of rabbits with highly purified toxoids proved to be very difficult, since with the methods of immunization used there were marked variations in the titer level within a group of 4 rabbits. In some cases no antitoxin titers could be detected in 1 or 2 animals, although at the same time the sera of the two other rabbits of the same group revealed high titers. This circumstance renders comparability of the various processes of immunization considerably more difficult.

2.1. Immunization with $AlPO_4$ -adsorbed toxoids.

With these toxoids only 1-10 IU/ml serum antitoxin titers could be achieved.

2.2. Immunization using complete Freund's adjuvant.

The highest antitoxin titers of each immunization method are compiled in Table 1.

Table 1. Maximum antitoxin titers upon immunization of rabbits using complete Freund's adjuvant

Type	Method	Titer IU ¹ /ml	2nd antigen injection
A	8.2.1. ³	316.23	2 ²
		316.23	4
	8.2.2.	10	2
		212.8	4
	8.2.3.	10	2
	177.83	4	
B	8.2.1.	31.62	2
		316.23	4
	8.2.2.	100	2
		100	4
	8.2.3.	67.61	2
	316.23	4	
E	8.2.1.	3162.3	2
		3162.3	4
	8.2.2.	3162.3	2
		3162.3	4
	8.2.3.	632.46	2
	3162.3	4	

¹IU = International antitoxin units

²Given in weeks after the first toxoid injection

³The numbers give the chapter in which the corresponding method of immunization is described.

The sera with the highest antitoxin titers of each type were pooled and fractionated. The antitoxin content of the globulin fractions for Type A was: 316.23 IU/ml, for Type B: 316.23 IU/ml and for Type E: 3162.3 IU/ml.

The protein content of these fractions for Type A was 19.77 mg/ml; for Type B, 25.65 mg/ml; and for Type E, 32.68 mg/ml.

The immune electropherograms shown in Fig. 1-3 indicate that the globulin fractions consist exclusively of various subfractions that can be classified with the globulin complex.

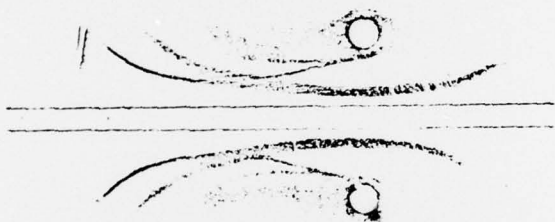


Fig. 1 Immune electropherogram of Type A globulin fraction against goat anti-rabbit full serum. Time of travel: 180 min.

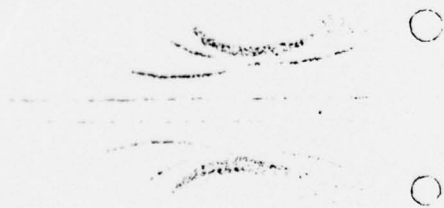


Fig. 2 Immune electropherogram of Type B globulin fraction against goat anti-rabbit full serum. Time of travel: 180 min.

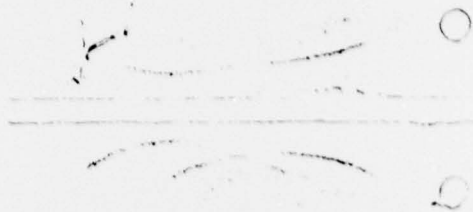


Fig. 3 Immune electropherogram of Type E globulin fraction against goat anti-rabbit full serum. Time of travel: 180 min.

3. Osmolarity measurements

Only buffer solutions were used whose osmolarity was between 270 mosm/1000 g H₂O and 295 mosm/1000 g H₂O.

4. Results of PHA

All experimental runs carried out with formalinized sheep erythrocytes led to spontaneous agglutinations. Therefore, in the following, we shall only give results obtained with formalinized human erythrocytes.

4.1. Tannin treatment.

Of the buffer solutions used, phosphate buffered NaCl solution of pH 7.2 and phosphate buffer solution I were the most suitable. However, in the specific mixture with the phosphate buffered NaCl solution of pH 7.2, spontaneous agglutinations occurred fairly frequently. When carrying out the main experiments, therefore, phosphate buffer solution I was used for tannin treatment. The different times of action

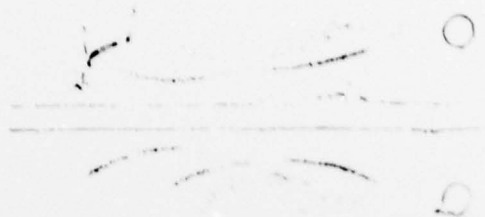


Fig. 3 Immune electropherogram of Type E globulin fraction against goat anti-rabbit full serum. Time of travel: 180 min.

3. Osmolarity measurements

Only buffer solutions were used whose osmolarity was between 270 mosm/1000 g H₂O and 295 mosm/1000 g H₂O.

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of 10-45 min. at 37°C had no effect on PHA, while on the other hand, tannin treatment at 56°C proved to be unsuitable since erythrocytes treated in this manner showed spontaneous agglutination even before sensitization. In the main experiments, therefore, tannin treatment was carried out for 10 min. at 37°C. With Type A and Type B antitoxins, with the same sensitivity of detection (see Table 2), the PHA reactions using a tannin concentration of 1:40,000 could be more clearly evaluated, while for experiments with Type E antitoxin, a tannin concentration of 1:20,000 was more suitable (see Table 2).

Table 2. Results of PHA with various globulin and tannin concentrations.

Globulin concentration (Anti-toxin A, B, E) mg protein/ml	Titer ¹					
	Toxin A		Toxin B		Toxin E	
	1:20,000 ²	1:40,000	1:20,000	1:40,000	1:20,000	1:40,000
0.0125	- ³	-	-	-	-	-
0.025	-	-	-9	-9	-	-
0.05	-9	-9	-9	-9	-	-
0.1	+ ⁴	+	+	+	-	-
0.2	+	+	+	+	-	-
1.0					-7	-
2.0					-7	-
4.0					-8	-
8.0					+	+

¹ Diluted in 2ⁿ steps. The exponent of the degree of dilution at base 2 is given. The toxins were prediluted 1:10.

² Tannin concentrations used.

³ All reactions negative.

⁴ Spontaneous agglutinations.

4.2. Sensitization

Table 2 shows the optimal globulin concentrations determined in preliminary experiments for the sensitization of erythrocytes.

The optimal globulin concentrations differ upon several repetitions of the same experimental procedure at the most by a factor of 2 from the values shown in Table 2.

If sensitization was carried out with an incubation period of 30 or 45 min, spontaneous agglutination occurred more frequently than with a 15 min incubation at 30°C. Furthermore, the longer period of incubation did not lead to greater sensitivity of the PHA.

Use of the various buffer solutions with addition of 1% inactivated rabbit serum also caused no change in the sensitivity of PHA, while buffering with 0.5% or 0.15 M NaCl solution with addition of 0.5% or 1% rabbit serum always led to spontaneous agglutinations

4.3. Specific detection of toxin with PHA

The smallest toxin quantities detected with PHA using anti-toxins of *C. botulinum* types A, B and E produced by us can be seen from Table 3, while the cross reactions occurring with this system of detection can be seen from Table 4.

Table 3. Smallest toxin quantity demonstrated with PHA

Antitoxin	Toxin quantity ng toxin-N	Titer ¹
A	0.246	-9
B	0.28	-9
E	0.383	-8

¹ Diluted in 2ⁿ steps. The exponent of the degree of dilution at base 2 is given. Toxins were prediluted 1:10. Average values from 5 experimental series are given.

Table 4. Titer of cross reactions with *C. botulinum* antitoxins

Strain (type)	Titer ¹ Antitoxin			Strain (type)	Titer ¹ Antitoxin		
	A	B	E		A	B	E
<i>C. botulinum</i> (A)	-8	-	-	<i>C. sporogenes</i>	-1	-	-1
<i>C. botulinum</i> (B)	- ²	-8	-	<i>C. perfringens</i> (A)	-	-	-
<i>C. botulinum</i> (C)	-	-	-	<i>C. perfringens</i> (C)	-3	-3	-2
<i>C. botulinum</i> (D)	-	-	-	<i>C. perfringens</i> (E)	-	-	-
<i>C. botulinum</i> (E)	-	-	-6	<i>C. perfringens</i> (F)	-	-	-
<i>C. tetani</i>	-	-	-	<i>Bacillus cereus</i>	-	-	-
<i>C. butyricum</i>	-2	-1	-1	<i>Bacillus subtilis</i>	-	-	-
<i>C. putrificum</i>	-	-	-	<i>Bacillus polymyxa</i>	-	-	-

¹Diluted in 2ⁿ steps. The exponent of the degree of dilution at base 2 is given. Homologous filtrates of *C. botulinum* were prediluted 1:10, all other toxin or culture filtrates were used undiluted. Average values from 5 experimental series are given.

²All reactions negative.

The formalinized erythrocytes could be used for up to 4 months without loss of titer in PHA (a longer period cannot at the moment definitely be encompassed). We do not as yet have sufficient knowledge of the storage qualities of already sensitized erythrocytes.

Discussion

Toxin production in protein-free culture media of standard composition is essential - together with absorption of the hemagglutinin components following the purification of the toxin - for the production of highly specific antitoxins. Instead of the difficult absorption with sheep erythrocytes, the considerably more elaborate column chromatography can be used for the separation of the hemagglutinin components - and for toxin purification

in general. However, with this method of purification we must anticipate a very low stability of the purified toxins.

In toxin production with liver bouillon (Franke 1974), unspecific protein components may find their way into the purified toxins from the medium as a result of the precipitation reaction during purification. This is indicated by the high nitrogen content of these purified toxins (0.12-0.55 mg N/ml) compared to those used here (0.05-0.07 mg N/ml).

The low number of injections of small toxoid or toxin quantities described here in the immunization of rabbits also increases the specificity of the antitoxins obtained. For immunization, both the antigen preparation with complete Freund's adjuvant and the application of small toxoid quantities to the foot pads of the rabbit (at the first application) have proved satisfactory. Four rabbits per group was not sufficient to interpret the titers.

Osmolarity measurements with the buffers used in PHA and in the preparation of the different reaction partners showed that buffers with an osmolarity of less than 270 mosm/1000 g water and more than 295 mosm/1000 g water gave rise to spontaneous agglutinations.

The range of 270-295 mosm/1000 g water roughly corresponds to the osmolarity of human serum (281-297 mosm/1000 g water).

Although, for instance, Rycaj (1956), Sinitsyn (1960), Yafaev and Chepelev (1961), Sakaguchi et al. (1971) and Evancho et al. (1973) used sheep erythrocytes in PHA for the detection of

C. botulinum toxin, in the present investigations spontaneous agglutinations took place in all experimental runs with formalinized sheep erythrocytes. This may be attributable to the absorption of the purified toxin with sheep erythrocytes. Since the appearance of hemolysis during absorption could only be roughly estimated, it is not impossible that the rabbits were also immunized with components of these erythrocytes.

In establishing the optimal globulin concentration for sensitization of the erythrocytes it is apparent that compared to Type A and B globulins, in Type E globulin a 40 to 160-fold quantity of globulins is required, even though this globulin fraction has a 10 times higher antitoxin content. Upon repeated sensitization with the same globulin fraction the optimal globulin concentration for Types A, B and E varied by a maximum of 2 dilution stages (factor of 2^{-1}). These divergences are within the range of "pipette error".

The inactivated rabbit serum (1%) added to the buffer used after sensitization of the erythrocytes prevents the appearance of spontaneous agglutinations in PHA when using optimal globulin concentrations.

The sensitivity of detection of PHA in the experiments described, in agreement with other authors (Evancho et al. 1973, Franke 1974, and others), is within the capacity limit of this reaction. The results are estimated very cautiously, since + reactions were no longer considered in determining the lower

limit of detection of PHA. Only in the case of the purified Type A toxin ($LD_{50} 3.2 \times 10^7 / ml$) is the mouse lethality test superior to PHA, while in the case of toxins Type B and E, lower toxin quantities can be detected with PHA than with the animal experiment. The increase in the sensitivity of detection of PHA by 2 titer stages described by Evancho et al. (1973) by using chromium chloride in sensitization could not so far be tested in our own experiments.

All reactions with PHA could be estimated with adequate certainty within 1 hour. Thus the serologic detection of toxin is superior to the animal experiment with regard to the duration of detection.

In testing the specificity of PHA there were no cross reactions with toxin filtrates of toxin Types A-E. So far, this has only been described by Evancho et al. (1973) in erythrocytes sensitized with Type A antitoxin. Slight cross reactions (up to the third titer stage) appeared between the erythrocytes sensitized with Type A, B and E antitoxin and culture filtrates from *C. butyricum*, *C. sporogenes* (only with Type A and E antitoxin) and *C. perfringens* Type C. The specific mixtures simultaneously revealed titers of $\geq 1:640$. The cross reaction of Type A and B antitoxin with culture filtrates of *C. sporogenes* described by Bulatova (1964) can thus be confirmed for Type A antitoxin, while the cross reaction described by the same author with culture filtrates of *C. putrificum* could not be observed. A cross reaction with *C. perfringens* Type C - culture filtrates was also described by Franke (1974).

The final assessment of the effectiveness of the method described for the detection of *C. botulinum* toxins A, B and E can only be made after use on toxin-containing foods or fodders. Due to the sometimes high protein content of such samples, there is a possibility of unspecific positive reactions of PHA.

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