PART VI

ANTIRABIES SERUM AND IMMUNOGLOBULIN

PRODUCTION OF ANTIRABIES SERUM OF ANIMAL ORIGIN

P. LÉPINE [1] & P. ATANASIU [2]

Pasteur Institute Method

A therapeutic antirabies serum is produced at the Pasteur Institute by immunization of horses or mules with the Pasteur strain of fixed rabies virus. Immunization is begun with β-propiolactone-inactivated vaccine, injection of which is continued for a period of 2 months. This is followed by a series of injections of increasing doses of live virus. All the injections are made subcutaneously. When the volume becomes too large for injection at a single site, multiple injections are made in different parts of the body.

The animals used must be carefully selected, as even for the same breed of horse the suitability of any particular animal for serum production varies according to its age, state of health, nutrition, and history. From time to time, samples of serum are taken from the animals undergoing immunization and the antibody titre is determined. Animals showing an inadequate response are eliminated and only those likely to yield a high-titre serum are retained.

The immunization schedule is as follows :

Day 1 to day 60 : 20 ml of β-propiolactone-inactivated vaccine every other day (30 injections).

Day 61 to day 72 : 4 injections, each of a quarter of a rabbit brain inoculated with fixed virus, at intervals of 3 days.

Day 73 to day 88 : 4 injections, each of half a rabbit brain inoculated with fixed virus, at intervals of 4 days.

Day 89 to day 98 : 2 injections, each of a whole rabbit brain inoculated with fixed virus, with an interval of 5 days.

Day 106 : first bleeding.

Day 136 : 1 injection of a whole rabbit brain inoculated with fixed virus.

Day 144 : second bleeding.

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[2] Head, Rabies and Rhabdovirus Research Laboratory, Pasteur Institute, Paris, France.

The immunization period thus lasts 98 days and the first bleeding is made 8 days later. A booster injection is given after another 30 days, followed by a second bleeding after 8 days. The booster injections and bleedings are repeated at these intervals, but the horses are allowed a rest period of 2 months each year.

Method of the Istituto Sieroterapico e Vaccinogeno, Siena

As originally described by d'Antona & Falchetti in the first edition of this monograph, the Siena method also makes use of horses for the production of a hyperimmune serum. Mirchamsy (1963), at the Razi Institute, Hessarek, Iran, slightly modified the method for the immunization of adult mules.

The vaccine used by Mirchamsy is prepared from sheep inoculated intracerebrally with Sassari fixed virus and consists of a 5% brain-tissue suspension containing 0.5% of phenol and inactivated at 37°C for 24–48 hours. The mules used for production of the serum are simultaneously immunized against tetanus by several injections of tetanus toxoid adsorbed on aluminium phosphate (PATT).

The immunization schedule is as follows :

Day 1 : 20 ml of phenolized vaccine + 10 ml of PATT.
Day 2 to day 20 : 20 ml of phenolized vaccine daily (19 injections).
Day 21 : 30 ml of phenolized vaccine + 25 ml of PATT.
Day 22 to day 40 : 30 ml of phenolized vaccine daily (19 injections).
Day 41 : 45 ml of phenolized vaccine + 40 ml of PATT.
Day 42 to day 56 : 45 ml of phenolized vaccine daily (15 injections).
Day 57 to day 115 : resting period.
Day 116 : 30 ml of phenolized vaccine + 30 ml of PATT.
Day 117 to day 126 : 30 ml of phenolized vaccine daily (10 injections).
Day 127 : 45 ml of phenolized vaccine + 45 ml of PATT.
Day 128 to day 136 : 45 ml of phenolized vaccine daily (9 injections).
Day 137 : 30 ml of a 5% suspension of live virus (Sassari fixed strain) + 60 ml of PATT.
Day 138 to day 144 : 30 ml of a 5% suspension of live Sassari virus daily (6 injections).
Day 156 : first bleeding.

Accelerated Method

Fuenzalida & Palacios (1964) developed an accelerated method of hyperimmunizing horses. The animals are first given a series of subcutaneous injections of vaccine in increasing concentrations and then simul-

taneous subcutaneous, intraperitoneal and intradermal injections of pure virus suspended in Freund's adjuvant without mycobacteria.

The horses are bled 20 days after the completion of the immunization schedule, which is as follows :

Day 1 : 40 ml of 1% inactivated vaccine subcutaneously.
Day 7 : 40 ml of 2% inactivated vaccine subcutaneously.
Day 14 : 40 ml of 5% inactivated vaccine subcutaneously.
Day 34 : 3 injections of an 8% suspension of live virus (brain tissue) in Freund's adjuvant — 5 ml intraperitoneally, 4 ml subcutaneously, 1 ml intradermally.
Day 41 : 3 injections of 16% live virus suspension in Freund's adjuvant — 5 ml intraperitoneally, 4 ml subcutaneously, 1 ml intradermally.
Day 48 : 3 injections of 20% live virus suspension in Freund's adjuvant — 8 ml intraperitoneally, 6 ml subcutaneously, 1 ml intradermally.
Day 68 : bleeding.

The vaccine used for the initial stages of immunization is prepared by the method previously described by Fuenzalida & Palacios (1955). Suckling mice are inoculated with fixed rabies virus and the vaccine prepared from the brain tissue is inactivated by irradiation with ultraviolet light. The live-virus suspensions are also prepared from the brains of suckling mice, with the addition of Freund's adjuvant without mycobacteria.

This rapid method seems to produce very satisfactory immunization and Fuenzalida & Palacios report neutralizing antibody titres that compare favourably with those obtained by other methods of immunization.

Immunization of Equines with Cultured Rabies Antigen

Antigens used

Two types of antigen—live and inactivated—are used. Both are prepared from the Pasteur strain of rabies virus cultured on BHK-S13 cells and maintained by weekly passages.

(*a*) *Inactivated antigen*

Cultures from the 20th passage are pooled to give a total volume of about 10 litres. 0.03 ml of this pool usually gives a titre of $10^{-5.1}$ in the mouse and $10^{-5.5}$ in cell cultures.

The antigen is inactivated with β-propiolactone, using 0.1 ml for 1000 ml of antigen ; inactivation is confirmed by intracerebral inoculation of groups of 10 mice, all of which must survive.

(*b*) *Live antigen*

Cultures from the 20th passage are pooled with an equal volume of cultures from the 23rd passage. 0.03 ml of this pool usually gives a titre of $10^{-5.1}$ in the mouse and $10^{-5.5}$ in cell cultures.

Both antigens are stored in the frozen state at −30°C.

Immunization schedule

Either horses or mules may be used for immunization. Before commencing the immunization schedule, the animals are bled to test for possible cytopathogens in the serum. Provided that no cytopathogenic activity is demonstrated, immunization is carried out as follows:

First stage, using inactivated antigen

(*a*) Weekly intramuscular injections for a period of 3 weeks with the following doses:

1st injection	20 ml
2nd injection	50 ml
3rd injection	100 ml
4th injection	100 ml.

(*b*) Two intramuscular injections of 100 ml weekly for 3 weeks, i.e., a total of 6 injections, followed by the first control bleeding.

(*c*) Weekly intramuscular injections of 100 ml for 2 months, i.e., a total of 8 injections, followed by the second control bleeding.

Second stage, using live antigen

Injections once a week of the following doses:

1st injection	20 ml
2nd injection	50 ml
3rd injection	100 ml
4th injection	100 ml

followed by the third control bleeding.

This schedule is continued, with weekly injections of 100 ml and periodic bleedings, until a satisfactory titre is achieved.

Determination of protective titre

At each control bleeding, a steady rise in the antibody titre should be observed. Present experience indicates that the following titres can be expected at the first 3 bleedings.

First bleeding: The protective titre determined on cell cultures against 100 LD_{50} of virus should reach approximately 1 : 300, corresponding to about 30 IU/ml.

Second bleeding : The protective titre is determined in mice in comparison with the International Standard containing 86.6 IU of rabies antibody per vial.[1] Using 100 LD_{50} of standard challenge virus (CVS), the reference serum should give a titre of about 1 : 2800 and the serum under test a titre above 1 : 3000, corresponding to more than 90 IU/ml. Using 300 LD_{50} of CVS, the reference serum should give a titre of approximately 1 : 1300 and the serum under test a titre of 1 : 2800, corresponding to about 170 IU/ml. When the titre is determined in cell cultures using 300 LD_{50} of virus, a value above 1 : 3000 should be obtained.

Third bleeding : The protective titre determined in mice infected with 300 LD_{50} of CVS should be about 1 : 6500, or 450 IU/ml.

Concentration and Purification of Antirabies Serum

The relatively large amounts of antirabies serum necessary for the protection of exposed persons, as well as the risk of anaphylactic accidents and other reactions, have led to the development of a number of methods for preparing a purified, concentrated serum.

Protein fractionation was first attempted by Habel (1945) using ammonium sulfate, and various other methods have since been described.[2] The method of fractionation and purification adopted at the Pasteur Institute consists of 2 stages : first, enzymatic digestion of the proteins followed by precipitation with ammonium sulfate ; second, removal of the excess proteins by thermocoagulation. (See also chapters 38, 39 and 40.)

Whichever method is adopted, it is advisable to determine the final protein content of the purified serum and relate this to its protective titre (see chapter 40, page 314). Paper electrophoresis should also be performed to check the fractionation of the proteins. In general, a concentrated purified serum with a titre of 120 IU/ml should not contain more than 5% of total serum proteins.

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[1] See chapter 40, pages 316 & 317. A reference serum may be obtained from WHO reference centres for rabies (see Appendix 7).

[2] See Koprowski et al. (1950), Pope & Stevens (1951), Delsal & Mirchamsy (1953), Dev et al. (1956), Wang & Lin (1957).

PREPARATION OF ANTIRABIES IMMUNOGLOBULIN OF ANIMAL ORIGIN: METHOD USED IN THE USSR

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Antirabies immunoglobulin is manufactured in the USSR from the hyperimmune serum of horses immunized with a 10% sheep-brain suspension of fixed rabies virus (Moscow strain, a variant of the Pasteur strain). The vaccine is of the Fermi-type and contains 0.5% of phenol. It is partially inactivated by keeping at 22°C for 8 days and is then injected into the subcutaneous tissue in various parts of the body. The schedule for immunization of the animals used for globulin production is set out in the following table:

IMMUNIZATION SCHEDULE FOR HORSES USED IN THE PRODUCTION OF ANTIRABIES IMMUNOGLOBULIN

Immunization stage	Day	Antigen dose
1	1 7 14	40 ml 80 ml 120 ml
2	21 28 35	50 ml 100 ml 150 ml
3	42 49 56 63	50 ml 100 ml 150 ml Test bleeding
4	73 80	150 ml Test bleeding
5	96 103	150 ml Bleeding

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When the titre of virus-neutralizing antibodies exceeds 1 : 500 (measured against 100 LD_{50} of fixed rabies virus) the animals are given an injection of a single dose of 150 ml of antigen. After the 5th stage of immunization, horses with an antibody titre of less than 1 : 500 are either immunized intramuscularly (10, 20 or 30 ml of the antigen at weekly intervals) or are not used for immunoglobulin production.

Extraction of the Immunoglobulin Fraction

A mixture of crude antirabies serum with an antibody titre of at least 1 : 1000 measured against an average of 100 LD_{50} of fixed rabies serum (CVS strain) is precipitated with ethanol in the cold by a modification of Cohn's method (1961).

First stage

The serum is diluted with an equal volume of distilled water and agitated in the cold for 2 hours; 352 ml of 96% ethanol are added per litre of the mixture at a rate of 5–6 litres per hour (final concentration of the ethanol: 25%). The precipitate is separated by centrifugation at −5°C.

Second stage

The precipitate is dissolved in distilled water (25 litres per kg of precipitate). 10 ml of 1M sodium chloride solution are added to each litre of the mixture, and the pH is adjusted with acetate buffer to 5.0 ± 0.1. The mixture is then agitated for 3 hours, after which 96% ethanol is added in the proportion of 215 ml per litre of the mixture (final concentration of the ethanol: 17%; temperature of the mixture 0–6°C). After agitation for an hour the mixture is separated in an ASG-3M separator[1] at a rate of 20 litres per hour. The temperature of the mixture on leaving the separator is 4.5–5°C. The centrifugate should be clear.

Third stage

The centrifugate is poured into a reaction vessel and the following are added:

1M sodium chloride solution, 50 ml per litre of centrifugate;

1M sodium bicarbonate solution, 5 ml per litre of centrifugate, pH 7.0–7.2, with continuous mixing;

34% ethanol, in an amount equal to the combined volume of the sodium chloride and sodium bicarbonate solutions;

96% ethanol, in the proportion of 113 ml per litre of centrifugate, to give a final concentration of 25%.

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[1] This is similar in design to a milk separator, but it operates at higher speeds and in a closed system, thus permitting work under aseptic conditions.

The temperature after precipitation should be 7–8°C. After standing for 1 hour the mixture is separated at the rate of 25 litres per hour. One litre of crude antirabies serum yields 43 g of antirabies immunoglobulin precipitate.

In order to remove the ethanol, the crude immunoglobulin precipitate is placed in sterile muslin and then in filter cloth, and filtered under mechanical pressure for 24 hours at 8–10°C. The crude precipitate is used to prepare a 10% ± 1% solution of immunoglobulin in physiological saline (pH 7.0–7.5). Within 24 hours of preparation, the immunoglobulin solution is filtered under pressure through Seitz-type (Sal'nikov) filters fitted with sterilizing plates with a pore diameter of 300 µm.

To achieve stabilization, the immunoglobulin solution is kept for 20–30 days at room temperature and then filtered again.

Control tests of the antirabies immunoglobulin are made at all stages of production. The final preparation is tested for:

(*a*) freedom from bacterial and fungal contaminants;

(*b*) safety;

(*c*) protective activity (the titre should be not less than 580 IU per ml by comparison with the WHO reference serum[1]);

(*d*) electrophoretic homogeneity in a Tiselius apparatus or on paper (immunoglobulin content not less than 97%);

(*e*) protein concentration (10% ± 1%);

(*f*) residual ethanol (not exceeding 4.5%);

(*g*) turbidity (the preparation should be transparent and should not exhibit more than slight opalescence).

The antirabies immunoglobulin is stored at 2–4°C. The expiry date is 2 years from the time at which the protective activity was determined.

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[1] Obtainable from WHO reference centres for rabies serum (see Appendix 7). See also footnote on page 317.

PREPARATION OF ANTIRABIES IMMUNOGLOBULIN OF HUMAN ORIGIN

R. K. SIKES[1]

The use of homologous immunoglobulins for human postexposure treatment virtually eliminates the risk of anaphylaxis and serum sickness normally associated with heterologous serum products. Approximately 16% of persons treated with antirabies serum of equine origin develop serum sickness. For persons over 15 years of age, the incidence increases to 46% (Karliner & Belaval, 1965).

Efforts have therefore been directed to the production of rabies immunoglobulins of human origin. So far, several lots prepared on a large scale have proved statistically to be as effective as antirabies horse serum in preventing rabies in experimental animals (Winkler et al., 1969 ; Sikes, 1969).

This chapter contains the detailed procedures for the preparation of immunoglobulins of human origin, as applied at the Center for Disease Control, Atlanta, USA.

Two major problems must be overcome :

(1) a supply of donors whose antibody titre is at least 1 : 400 must be obtained ;

(2) the cost of providing such a specialized globulin is still very high.

Formula

Human rabies immune globulin (HRIG) for intramuscular administration is a 16.5% ± 1.5% solution of immunoglobulin in 0.3 M glycine, and preserved with a 1 : 10 000 thiomersal[2] solution.

Source and Shipment of Blood

Donors of plasma for the production of HRIG should have demonstrated high levels of neutralizing antibody following pre-exposure or postexposure immunization with a rabies vaccine. One or more booster immunizations with a licensed rabies vaccine should be given 1–2 weeks before the first collection of blood. In order to prepare HRIG of sufficient potency, and assum-

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[1] Veterinary Director, Office of Veterinary Public Health Services, Center for Disease Control, Atlanta, Ga., USA.

[2] Also known as thimerosal and merthiolate.

ing a 15-fold to 20-fold concentration of antibody during preparation, it is necessary to start with a rabies immune plasma pool containing 8–10 antibody units per ml or having a serum neutralizing antibody titre of 1 : 700 to 1 : 1000. Studies have shown that, following a booster dose of antirabies vaccine, about 10% of subjects who have received pre-exposure antirabies prophylaxis and about 40% of those with a history of post-exposure treatment will develop antibody titres of sufficient levels.

The collecting centre may obtain a unit of plasma by plasmapheresis or by separation of a unit of whole blood aseptically. The plasma is shipped refrigerated to the laboratory and is stored frozen until it is ready to be pooled for fractionation.

Reagents

The preparation and storage of the reagents required are described in the Annex on page 312.

Technique[1]

The immunoglobulins are separated from the plasma by the cold ethanol fractionation technique described below (Cohn et al., 1946).

Precipitation of fraction I

1. Pool the plasma; pour individual units through cheesecloth into a tared vat large enough to contain 1.2 litres per litre of plasma.

2. Determine the pH; if necessary, adjust to the range 7.0–7.4 with acetate buffer (80-fold concentrate) or 0.5M sodium phosphate solution.

3. Freeze three 15-ml samples of pooled plasma.

4. Determine the weight of plasma.

5. Cool the plasma to 0°C and add 163 g of cold 53.3% ethanol per kg of plasma. Adjust the rate so that the addition of ethanol takes about 1 hour. Cool to −2°C during the addition. Stir for half an hour after the addition of ethanol is completed.

6. Centrifuge into a tared vat large enough to contain 1.7 litres per litre of original plasma. Maintain the temperature at −2°C to −3°C during centrifugation. Freeze a 15-ml sample of supernatant I (S1) and weigh it. Maintain at −2°C, and begin precipitation of fraction II-III.

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[1] The procedures used in this chapter are used by the Bureau of Laboratories, Michigan Department of Public Health, East Lansing, Mich., USA; they have been adopted for the fractionation of HRIG at the Center for Disease Control, Atlanta.

Precipitation of fraction II-III (P2)

1. For every kg of supernatant I, add 552 g of 53.3% ethanol to which has been added 1.33 ml of acetate buffer 80-fold concentrate. Adjust the rate so that the addition of the ethanol takes about 1 hour. Cool to −9°C during the addition.

2. Stir at −9°C for half an hour after the addition of ethanol is completed.

3. Centrifuge, collecting the centrifugate in a tared bowl. Maintain the temperature at −6°C to −9°C during centrifugation.

4. Determine the weight of the precipitate, which is fraction II-III (P2). Freeze in the bowl at −20°C, and hold for precipitation of fraction II-IIIw.

Precipitation of fraction II-IIIw (P2w)

1. Remove frozen P2 from the centrifuge bowl and rapidly homogenize to a uniform suspension in a mixture of water and crushed ice. Use 2 g of water-and-ice mixture per gram of P2. Avoid excessive foaming.

2. For every gram of P2 immediately add a mixture containing 0.107 ml of 0.5M sodium hydrogen phosphate (Na_2HPO_4) in 2.89 g of water and crushed ice.

3. Stir in the cold until all the solid is in suspension.

4. Pour into a vat containing 20 g of cold water per gram of P2, and stir at 1°C for 30 minutes or until no ice remains in the mixture. The vat should be large enough to contain 4 litres per 100 g of P2.

5. Remove 5 ml of the mixture from the vat, add 5 ml of 0.15M sodium chloride solution, and determine the pH; if necessary, adjust the pH of the vat mixture to between 7.0 and 7.4 by addition of acetate buffer 80-fold concentrate or 0.5M sodium hydrogen phosphate solution.

6. Freeze a 15-ml sample.

7. Add 14.1 g of 53.3% ethanol per gram of P2; adjust the rate so that the addition of the ethanol requires about 1 hour. Cool to −6°C during addition of the ethanol. Stir at −6°C for 2–4 hours after the addition of ethanol is completed.

8. Centrifuge and collect the centrifugate in a tared bowl. Maintain the temperature at −5°C to −7°C during centrifugation.

9. Freeze a 15-ml sample of supernatant II-IIIw (S2w).

10. Determine the weight of precipitate, which is fraction II-IIIw (P2w). Freeze in the bowl at −20°C and hold for precipitation of fraction III.

Precipitation of fraction III

1. Remove the frozen P2w from the bowl, and rapidly homogenize to a uniform suspension in a mixture of crushed ice and water. Use 2 g of water-and-ice mixture per gram of P2w. Avoid excessive foaming.

2. Immediately add 2 ml of cold 0.175M sodium acetate solution per gram of P2w, and stir in the cold until all the solid is in suspension.

3. Adjust the pH to 5.2 ± 0.1 by addition of acetate buffer 80-fold concentrate diluted 1 : 25 with cold water, and then add more cold water until the total amount added is 1 ml per gram of P2w.

4. Stir in the cold for 1 hour or until no ice remains in the mixture. Determine the pH and adjust, if necessary, to the range 5.1–5.3.

5. Pour into a vat containing 13.5 g of cold water per gram of P2w. The vat should be large enough to contain 2.9 litres per 100 g of P2w.

6. Add 8.1 g of 53.5% ethanol per gram of P2w; adjust the rate so that the addition of ethanol requires about 1 hour. Cool to −6°C during the addition of the ethanol.

7. Stir at −6°C for half an hour after the addition of ethanol is completed.

8. Centrifuge into a cold pressure tank and filter into a tared vat. The vat should be large enough to contain 3.1 litres per 100 g of P2w. Use a 1.2 µm "Versapor" pre-filter[1] over a 0.8-µm membrane. Maintain the temperature at −5°C to −6°C during centrifugation and filtration.

9. Freeze a 15-ml sample of supernatant III (S3).

10. Determine the weight of S3, maintain at −6°C, and proceed with the precipitation of fraction II.

Precipitation of fraction II

1. Stir S3 vigorously at −6°C, and for every kg of this solution slowly add 2 g of sodium hydrogen carbonate.

2. Add 2 ml of the vat mixture to 8 ml of 0.15M saline, and determine the pH; if necessary, add additional sodium hydrogen carbonate to adjust the pH to the range 7.4 ± 0.2.

3. Add 94.7 g of 95% ethanol per kg of solution. Adjust the rate so that the addition of ethanol requires about 1 hour. Cool to −9°C during the addition of the ethanol.

4. Centrifuge, collecting the centrifugate in a tared bowl. Maintain the temperature at −6°C to −9°C during centrifugation.

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[1] An epoxy reinforced glass filter obtainable from Gelman Instrument Co., Ann Arbor, Mich., USA. Type G pre-filters (see next page) are available from the same source.

5. Determine the weight of fraction II, freeze in the bowl at —20°C, and hold for lyophilization.

Fraction II lyophilization and final preparation

1. Remove the frozen fraction II from the bowl, and rapidly homogenize to a uniform suspension in cold 0.3M glycine, using 1 ml per gram of frozen paste.

2. Add 4 g of cold water [1] per gram of paste; stir for 2–4 hours in the cold, and allow to stand overnight at 0°C without stirring.

3. Decant the supernatant, lyophilize, and determine the weight of lyophilized product.

4. For preparation of the final solution of the product, the quantities are calculated as follows:

(*a*) Dry weight of powder = weight of lyophilized powder (g) $\times 0.98$.

(*b*) Volume of powder (ml) = $a \times 0.75$.

(*c*) Volume of water to be added (ml) = ml of glycine added before lyophilization $-b$.

(*d*) Dry weight of globulin (g) = $a - (0.0225 \times$ ml glycine added before lyophilization).

(*e*) Final volume (ml) = $\frac{d}{0.17}$

(*f*) Volume of 0.3M glycine to be added (ml) = $e - b - c$

(*g*) Volume of 10% thiomersal to be added (ml) $e \times 10^{-3}$.

5. Put the required volume of water (*c*) [1] and 0.3M glycine (*f*) in a beaker with a magnetic stirring bar, and add the lyophilized globulin. Stir to solution; avoid excessive foaming.

6. Add the required volume of 10% thiomersal (*g*). A second person should check this calculation and observe the addition. Determine the pH and adjust, if necessary, to 6.8 with acetate buffer 80-fold concentrate.

7. Filter the globulin solution through a 0.8 μm membrane with 1.2 μm "Versapor" and glass type G pre-filters. [2]

8. In the sterile room, sterilize the globulin solution by filtration into the bulk product container.

9. Perform the bulk container sterility test with fluid thioglycolate medium. Use one 2-ml sample.

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[1] Use Water for Injection, see footnote p. 312.

[2] See footnote on previous page.

10. Refrigerate the bulk container material, and hold for final container filling.

Testing

Tests for safety and sterility, pyrogenic substances, heat stability, pH, and turbidity are needed to ensure that the product satisfies the minimum requirements specified in national standards. For potency requirements see chapter 40, p. 314.

Annex

PREPARATION AND STORAGE OF REAGENTS

Unless otherwise specified, reagent grade chemicals are used.

Distilled water

Water is collected from the still directly into a pressure-filtration tank and filtered through a sterile 0.2-µm membrane into a sterile dispensing vessel. Except when pyrogen-free Water for Injection [1] is required, this water is used in all procedures and for making ice and reagents.

95% (v/v) ethanol

95% ethanol is filtered through a 0.2-µm membrane (not sterile) into dispensing vessels and stored under refrigeration (cold room).

53.3% (v/v) ethanol

5060 g of water are added to 4940 g of 95% ethanol. The mixture is filtered through a 0.2-µm membrane (not sterile) and stored under refrigeration (cold room). The specific gravity, measured with a 60°F/60°F hydrometer at 15°C, should be between 0.9275 and 0.9285.

Acetate buffer, 80-fold concentrate

Dissolve 108.9 g of sodium acetate and 240.2 g of glacial acetic acid in water and make up to one litre. This yields an 80-fold concentrate. When this concentrate is diluted with 80 parts of water the pH of the buffer should be 4.00 ± 0.02.

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[1] See *Specifications for the quality control of pharmaceutical preparations—second edition of the International Pharmacopoeia*, Geneva, World Health Organization, 1967, p. 50.

0.5M sodium phosphate

Dissolve 71.0 g of sodium hydrogen phosphate (Na_2HPO_4) or 89.1 g of the dihydrate (Na_2HPO_4, $2H_2O$) in water and make up to one litre. The pH of this solution should be 9.2. Store at room temperature.

0.175M sodium acetate

Dissolve 23.8 g of sodium acetate in water and make up to one litre.

0.3M glycine

Only glycine that has been determined to be pyrogen-free should be used. A 0.3 M solution contains 22.5 g of glycine per litre. Use Water for Injection [1] to prepare this solution. It should always be freshly prepared for use.

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[1] See footnote p. 312.

QUANTITATIVE ASSAY AND POTENCY TEST OF ANTIRABIES SERUM AND IMMUNOGLOBULIN

P. ATANASIU[1]

Principle

The method described below consists in neutralizing a constant dose of the previously titrated challenge virus with a series of different dilutions of the serum. The method is used mainly for the assay and potency testing of therapeutic antirabies serum and immunoglobulin, but it is also applicable to any serum containing rabies antibody. Thus, it can be used to determine the antibody titres of human sera collected during therapeutic trials of different vaccines.

The method comprises the following 3 stages :

1. Preparation and titration of the challenge virus.

2. Serum-virus neutralization—preparation of the serum and of the serum-virus mixtures ; inoculation of mice.

3. Interpretation of the results.

The serum neutralization assay by means of the plaque reduction technique is described in chapter 9, page 113.

1. Preparation and Titration of Challenge Virus

Challenge virus

The strain normally used is the standard challenge virus strain (CVS), as used in the mouse potency test for rabies vaccine (see chapter 33, page 279). The laboratory strain of fixed virus may also be used, provided that its LD_{50} for the mouse is known and remains constant.

Titration of stock virus

The challenge virus is stored as a 20% suspension, which is dispensed into ampoules and deep frozen. When carrying out the test, an ampoule is taken from the stock and thawed rapidly under the tap. Serial tenfold

[1] Head, Rabies and Rhabdovirus Research Laboratory, Pasteur Institute, Paris, France.

dilutions are then prepared, giving concentrations of 2×10^{-2}, 2×10^{-3} etc., up to 2×10^{-7}. The diluent used is double-distilled water, to which has been added 2% of normal horse serum, inactivated for 30 minutes at 56°C.

In each of 5 haemolysis tubes is then placed 0.5 ml of one of the virus dilutions, starting with 2×10^{-3} and ending with 2×10^{-7}. To each tube is then added 0.5 ml of inactivated horse serum diluted 1 : 5, so that the final dilutions in the 5 tubes are 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}.

After shaking, the mixtures are incubated for $1\frac{1}{2}$ hours at 37°C, in order to ensure that the conditions are the same as those of the serum-virus neutralization stage. The tubes are then cooled in ice-water. Mice are inoculated intracerebrally with 0.03 ml of the virus suspension, 5 mice being used for each dilution. Provided that the highest dilution is used first, all the inoculations may be made with the same syringe. A record is made of the number of mice that die between the 6th and 20th day after inoculation.

For the calculation of the virus titre, see Appendix 1, Table 1 (page 323), examples 1–3.

Calculation of challenge doses

As an example of how challenge doses are calculated, let it be assumed that a given CVS stock preparation contains $10^{-5.84}$ LD_{50} per 0.03 ml, i.e., 1 LD_{50} is contained in 0.03 ml of a $10^{-5.84}$ dilution of the stock virus. For sera of high titre, between 100 and 300 LD_{50} are used in the serum-virus neutralization assay. To find the dilution of CVS that will contain 300 LD_{50} in 0.03 ml the logarithm of 300 is subtracted from the logarithm of the assumed titre of the stock preparation :[1]

log dilution of challenge preparation containing 300 LD_{50} of virus per 0.03 ml $= \log 10^{-5.84} - \log 300 = 5.84 - 2.48 = 3.36$

Since $3.36 = \log 2300$, this means that the CVS stock preparation will have to be diluted 1 : 2300. For this purpose, first make serial tenfold dilutions of the stock CVS until a 10^{-3} dilution is obtained, then add 1.3 ml of diluent for every 1 ml of the 10^{-3} dilution. The final dilution will then contain 300 LD_{50} of virus per 0.03 ml.

For sera of low titre (e.g., postvaccinal human sera), only 50 LD_{50} may be needed. The calculation then becomes :

$$\log 10^{-5.84} - \log 50 = 5.84 - 1.70 = 4.14$$

Since $4.14 = \log 14\,000$, the CVS stock preparation will have to be diluted 1 : 14 000 by making tenfold dilutions down to a dilution of 10^{-4} and then adding a further 0.4 ml of diluent for every 1 ml of this dilution. This gives a final dilution containing 50 LD_{50} of virus per 0.03 ml.

[1] In making these calculations, the minus signs preceding the logarithms are disregarded.

2. Serum-Virus Neutralization

Inactivation

The sera to be tested are inactivated for 30 minutes at 56°C.

Neutralization

The following serial dilutions of the serum or immunoglobulin under test are prepared : 1 : 500, 1 : 1000, 1 : 2000, 1 : 4000, etc., so that the final dilution will show no neutralization.[1] From each of these dilutions, 0.5 ml is transferred to one of a series of test tubes. Next, 0.5 ml of the virus dilution corresponding to 300 LD_{50} (in the example given above, a dilution of 1 : 2300) is added to each tube. This results in a twofold dilution of both the virus and the serum, so that the final virus dilution is 1 : 4600, or $10^{-3.66}$, and the final serum dilutions are 1 : 1000, 1 : 2000, 1 : 4000, 1 : 8000, etc.

It is advisable to include in the test a reference serum, which is titrated at the same time as the unknown sera. This reference serum is a serum of known titre that has previously been titrated against the International Standard[2] and then stored in the laboratory with the usual precautions. If the results of the test are to be expressed in international units (IU), the inclusion of such a reference serum is mandatory.

Titration of virus control

It is essential to determine the actual quantity of virus used in the test. For this purpose, 0.5 ml of the challenge virus (dilution 1 : 2300 = 300 LD_{50} per 0.03 ml) is mixed with 0.5 ml of diluent containing 20% of inactivated normal horse serum. Thus, a dilution of challenge virus is obtained that contains 150 LD_{50} per 0.03 ml. This tube is marked "O" and 3 serial tenfold dilutions are prepared and marked —1, —2, and —3. After shaking, the tubes containing the sera under test and the control tubes are incubated at 37°C for 1½ hours.

Inoculation of mice

After incubation at 37°C for 1½ hours, all the tubes are placed in a vessel filled with ice-water. Batches of mice weighing 14–16 g each are then inoculated intracerebrally with 0.03 ml of each dilution, using 5 mice per dilution. This is done both for the sera under test and for the virus control. When a large number of sera are being tested, it is important to make the injections of the virus control after having injected half the sera ;

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[1] If the levels of antibody are expected to be low, as in vaccinated human subjects, serum dilutions starting with 1 : 5, 1 : 25, 1 : 125 are employed and the final virus dilution should be 20–50 LD_{50}.

[2] See footnote on next page.

this ensures that the dilutions of the virus control and the sera under test are kept for the same average length of time before making the injections. The different groups of mice are placed separately in labelled jars and kept under observation; a record is made of those dying between the 6th and 20th day.

3. Calculation and Interpretation of Results

The following example is based on the titration of an experimental batch of therapeutic serum with dilutions of the stock virus referred to in the example on page 315.

Determination of LD_{50} actually used in the test

Tube	*Deaths*	*Survivals*
0	5	0
−1	5	0
−2	4	1
−3	0	5

Applying the same calculation as in Example 2 in Appendix 1 (Table 1, page 323) and Table 5, page 326), the 50% endpoint dilution of the virus is found to be $10^{-2.3}$. The antilog of 2.3 (= 200) is the actual number of LD_{50} used in the reaction mixtures for neutralization.

Calculation of the ED_{50} of the serum under test

This calculation is illustrated in Appendix 1 by Example 7, Table 2, page 323; dilution factor = 1 : 2. In this case, the total number of survivors is determined and the 50% endpoint dilution is estimated by using Table 3 (page 324). For the serum under test the ED_{50} is found to be $10^{-3.21}$ or 1 : 1620. The ED_{50} of the reference serum is similarly estimated from the tables.

Example 8 in Appendix 1, page 323, illustrates the calculation of the ED_{50} for a low-titre serum diluted 1 : 5, 1 : 25, 1 : 125, and 1 : 625. This range of dilutions is frequently used for the postvaccinal antibody assay of human sera (see footnote on p. 316). In the example used the ED_{50} (or antibody titre) found is $10^{-2.10}$ or 1 : 125.

The number of international units (IU) contained in the International Standard for Antirabies Serum is fixed arbitrarily at 80 per ml.[1] To express

[1] This is a round figure in order to simplify the calculations. Actually, the International Standard for Antirabies Serum contains 86.6 mg of dried serum per ampoule, which is equivalent to 86.6 IU. This serum is available to national laboratories, on request, from the International Laboratory for Biological Standards, Statens Seruminstitut, Copenhagen, Denmark.

the potency of a serum under test in IU, its neutralizing power must be compared with that of the International Standard or with that of a reference serum that has been calibrated against the International Standard. This is done by calculating the difference between the logarithms of the 50% endpoint dilutions of the two sera. In example 7 referred to above, the 50% endpoint dilution of the serum under test is $10^{-3.21}$. Assuming the 50% endpoint dilution of the reference serum to be $10^{-3.19}$, the calculation becomes :

$$-3.19-(-3.21) = 0.02.$$

The serum under test is therefore $10^{0.02} = 1.05$ times as potent as the reference serum, so that its potency is $1.05 \times 80 = 84$ IU per ml.

Therapeutic potency test

An antirabies serum should satisfy the requirements laid down in the fourth report of the WHO Expert Committee on Rabies : [1]

> A serum shall pass the test for sufficient therapeutic potency if, in a single comparative assay, it is revealed to be equal to or better than the International Standard Serum. In case a serum fails the test, two more similar tests may be carried out. If the serum proves equal to or better than the International Standard Serum in both these additional tests, it shall pass. The outcome is " equal " or " better " if the total survivor fraction (survivors/total number of mice) for the serum under test is equal to or larger than that for the standard.

These requirements are also applicable to immunoglobulin.

[1] *Wld Hlth Org. techn. Rep. Ser.*, 1960, **201**, 10.