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Methods for the Purification of Equine Rabies Immunoglobulin: Effects on Yield and Biological Activity

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Abstract. Since rabies is still a major cause of human death in many developing countries and the implementation of recommended post-exposure prophylaxis by vaccination and specific immunoglobulin therapy is largely hampered by its high cost, the development of cheap rabies vaccines and immunoglobulin preparation are a high priority in these countries. In this paper various purification methods of equine rabies immunoglobulin based on different principles are compared with respect to their effect on final yield and biological activity. It is shown that a combination of ammonium sulphate (AS) precipitation and DEAE ion exchange chromatography results in an acceptable recovery rate of biological activity and a product of relatively high purity. Although affinity chromatography with protein G in combination with AS precipitation results in a similar recovery rate and a product of considerably higher purity, the cost of this procedure may be prohibitive for routine use in most developing countries. The effects of pepsin digestion time on the biological activity of the product and on the reduction of intact horse Ig are also studied. The desirability of this digestion procedure with respect to reduction of adverse side effects and efficacy of the product for post-exposure treatment is discussed.

Introduction

Rabies is a major public health problem and remains the cause of many thousands of human deaths in several developing countries.[1–4] Post-exposure treatment of rabies includes the prompt use of human or equine rabies immunoglobulin (HRIG or ERIG) in combination with the administration of rabies vaccine, as recommended by the World Health Organisation.[2,3,5] Although the use of ERIG is associated with adverse side effects in one to six percent of the individuals treated,[2,6] the limited availability of HRIG due to its high cost, necessitates the continued use of ERIG in developing countries.[7] Significant differences in adverse reactions caused by the use of ERIG, have been suggested to reflect differences in manufacturing or purification processes and protein content.[1,7] Although limited information is available on the manufacturing and purification procedures of commercially available ERIG and ERIG preparations produced in developing countries, most of these depend on the use of classical methods developed decades ago.[1,4] Therefore we have evaluated the effects of different purification methods on the yield and *in vitro* and *in vivo* biological activity of ERIG preparations in order to define routine production methods that minimize adverse side effects of ERIG products with high biological activity.

Materials and methods

Immunization of horses

Two different protocols were used to immunize horses against rabies virus. The first was according to the method described by Lepine and Atanasiu.[8] In short, horses were first immunized with inactivated rabies virus (Pasteur strain) prepared from brains of infected suckling mouse and subsequently by a series of immunizations with live virus. This resulted in virus neutralizing (VN) serum antibody levels of 100 to 300 IU per ml serum. In the second protocol horses were immunized four times with inactivated rabies vaccine (Pitman-Moore strain) produced in dog kidney cells,[9] which resulted in VN serum antibody levels of 10–30 IU per ml serum. Most of the experiments were carried out with horse serum exhibiting the higher VN antibody titers.

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Non-chromatographic purification

Salt precipitation. Purification of horse immunoglobulin (Ig) by salt precipitation was performed as described by Philips *et al.*[10] In brief, a saturated $(NH_4)_2SO_4$ (AS) or Na_2SO_4 (SS) (40% w/v) solution was added to an equal volume of horse serum under continuous stirring at 4°C or 20°C respectively. After 30 min the precipitate was centrifuged for 30 min at 10 000 ***g***. The pellet was dissolved in distilled water, and the resulting solution was dialysed against PBS pH 7·2 to remove AS or SS, and adjusted to the original volume with the same buffer.

Caprylic acid. Purification of horse Ig by caprylic acid precipitation was carried out according to the method described by McKinney and Parkinson.[11] Briefly, serum was diluted with 4 volumes of 0·01 M acetic buffer pH 4·5. The solution was stirred slowly and a 1:40 volume of caprylic acid (Merck, Darmstadt, Germany) was added dropwise at 20°C. After 30 min the suspension was centrifuged for 25 min at 10 000 ***g*** and the supernatant was filtered through a 0·45 μm filter. Subsequently an AS precipitation was carried out and the resulting solution was adjusted to the original volume as described above.

Chromatographic purification

Ion-exchange. Purification of horse Ig by ion-exchange was performed batchwise essentially as described by Ter Avest *et al.*[12] with minor modifications, using DE-52 cellulose (Whatmann, Maidstone, U.K.) or DEAE CL-6B (Pharmacia, Uppsala, Sweden). One gram DE-52 cellulose in 6 ml 0·01 M phosphate buffer (PB) pH 6·0 was added per ml of serum. The DEAE CL-6B gel was washed twice with 0·5 m HCL, twice with 0·5 M NaOH and twice with PB pH 6·0 prior to use. For the DEAE 1 ml gel per 1 ml serum was used. After stirring for 1 h at 20°C the suspension was centrifuged at 4500 ***g*** for 25 min. The supernatant was concentrated by AS precipitation and adjusted to the original volume as described above.

Affi-T gel. Purification of horse Ig by Affi-T gel KEM-en-TEC (Copenhagen, Denmark)[13] was performed as described by Lihme and Heegaard.[13] Briefly the serum was adjusted to 0·75 M SS and three volumes of Affi-T gel were added under gentle shaking at 20°C. After 1 h the gel was washed three times with three volumes of 0·75 M AS. Bound Ig was eluted three times with one volume 0·05 M Tris pH 9·0. The eluates were pooled, concentrated by AS precipitation and the resulting solution was adjusted to the original volume as described above.

Protein-A. Purification of horse Ig by Protein-A[14,15] was performed by mixing four volumes of serum with one volume of 0·5 M sodiumphosphate buffer pH 8·1 and run through a Protein-A sepharose CL-4B column (Pharmacia, Uppsala, Sweden). Unbound material was washed from the column with 0·1 M sodiumphosphate buffer pH 8·1 until the E280 value reached the baseline level. The bound Ig was eluted with 0·1 M citrate buffer pH 3·8 in fractions to which 0·25 volumes of 1 M Tris pH 8·9 had been added. Fractions with the highest protein concentrations (see below) were pooled, precipitated with AS and the resulting solution was adjusted to the original volume as described above.

Protein G. Purification of horse Ig by Protein-G sepharose 4 fast flow (Pharmacia, Uppsala, Sweden) was performed as described for the Protein-A purification. Instead of a citrate elution buffer a 0·1 M glycine buffer pH 2·7 was used.

Pepsin digestion

F(ab)2 fragments of horse Ig were prepared by adjusting serum sample with 0·1 M HCL to pH 3·8 and adding 2600 units of pepsin (Flow, Worthington, U.S.A.) per mg protein. After incubation at 37°C, samples were taken from 30 min to 48 h at regular intervals and adjusted to pH 7·0 by adding 0·1 M NaOH. The digested samples were AS precipitated and adjusted to the original volume as described above. Protein concentrations of the samples and biological activities were measured as described below. Remaining intact IgG was detected by SDS-PAGE[16] and silverstaining using Phastsystem equipment (Pharmacia, Uppsala, Sweden) and intact rabies virus-specific IgG was quantitated by an indirect ELISA.[17] In short, plates were coated with rabies virus antigen, serial dilutions of samples containing horse anti-rabies virus Ig were added and bound Ig was measured with a Protein-G conjugate.

Measurement of recovery

Protein measurement. Protein concentrations in the samples were measured according to the method described by Bradford.[18]

Inhibition ELISA. The inhibition ELISA to determine rabies virus-specific Ig is a method based on a monoclonal antibody (MoAb) combination used for the detection of rabies virus glycoprotein as previously described.[19,20] In short, ELISA plates were coated with 0·1 μl volumes (250 ng/well) of MoAb 6-15-C4 in PBS pH 7·2 for 2 h at 37°C. After washing,

plates were blocked with ELISA buffer containing 0·1 M Tris pH 7·4, 0·1% Triton X-100, 0·1% Tween 20, 3% NaCl, 0·5% BSA and 10% normal sheep serum (EBT) and incubated for 18 h at 4°C. In separate plates 50 µl of serial twofold dilutions of the samples were incubated with 50 µl of rabies virus antigen strain Pitman Moore (1·5 µg/ml). After 18 h incubation at 4°C the serum antigen mixture was transferred to the MoAb coated plate and incubated for 2 h at 37°C. Plates were washed and remaining binding capacity of rabies virus antigen was detected by incubation with biotin labeled MoAb 2-22-C5 for 1 h at 37°C. Horse radish peroxidase (HRPO) bound streptavidin (Amersham International, Amersham, U.K.) was allowed to bind to biotin for 30 min at 37°C. Plates were developed using tetramethylbenzidine as a substrate.[19]

Rapid fluorescent focus inhibition test (RFFIT). Virus neutralizing antibodies in the IgG preparations were measured by the RFFIT using the CVS-11 strain and expressed in international units (IU) described by Smith *et al.*[21–23]

Specific activity ratio. The specific activity of the Ig preparations was defined in IU per mg protein measured in the RFFIT and presented as the ratio between the specific activity of the sample, divided by the specific activity of the original horse serum sample.

In vivo *protection experiment*

The *in vivo* neutralization test was performed in weanling NIH outbred mice as described by Atanasiu.[24] In short, threefold dilutions of the sample were incubated with 30 LD50 of the CVS-26 strain, for 90 min at 37°C. The mixtures (30 µl) were inoculated intracerebrally and the mice were observed for the development of typical clinical signs for 21 days. Neutralization titers were expressed in IU.

Results

Comparison of different purification methods

Non chromatographic methods. As shown in Fig. 1 SS and AS precipitation of serum from immunized horses, resulted in a recovery of 93 and 75% of biological activities respectively as measured in RFFIT with specific activity ratios of 2·9 and 2·8 respectively, and in a recovery of 94 and 88% of the biological activities as measured in inhibition ELISA respectively. Caprylic acid precipitation of albumin and other non IgG proteins from serum of immunized horses in combination with AS precipitation resulted in a recovery of 58% of the biological activity measured in RFFIT with a specific activity ratio of 4·6 and in a recovery of 45% of the biological activity as measured in inhibition ELISA.

Ion-exchange chromatography. DE-52 and DEAE ion exchange purification followed by AS precipitation of

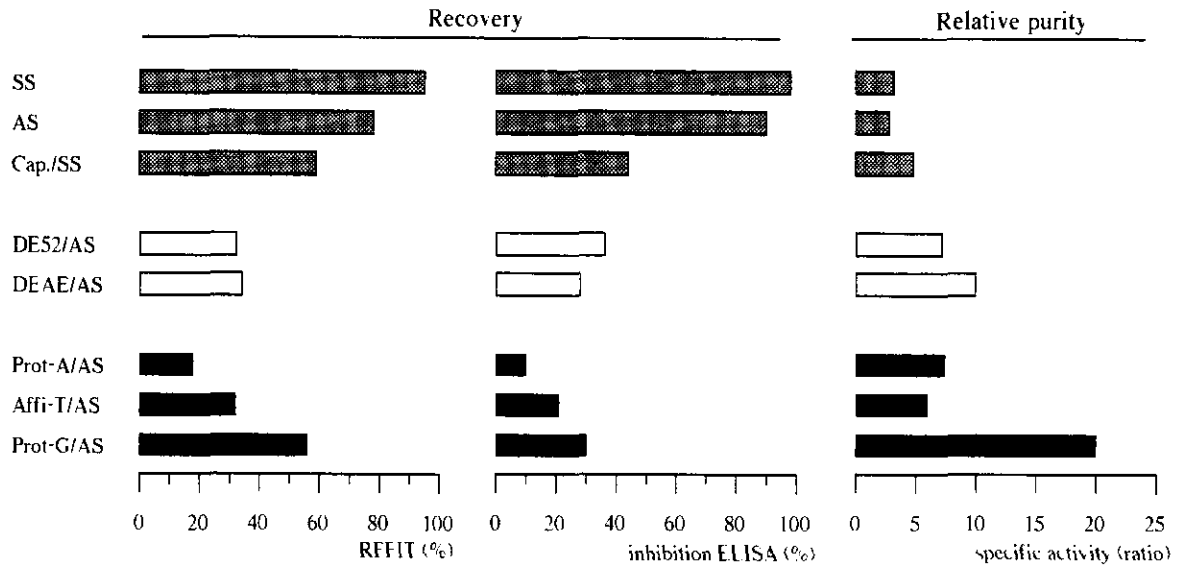

Figure 1. Recovery of biological activity and relative purity of ERIG preparations obtained by different purification methods using serum from a horse immunized with inactivated rabies vaccine prepared on cell-culture (Pitman-Moore strain). SS: Na_2SO_4 precipitation; AS: $(NH_4)_2SO_4$ precipitation; Cap.: caprylic acid precipitation; DE52, DEAE affinity chromatography; Prot-A, affinity chromatography; Affi-T, affinity chromatography and Prot-G, affinity chromatography.

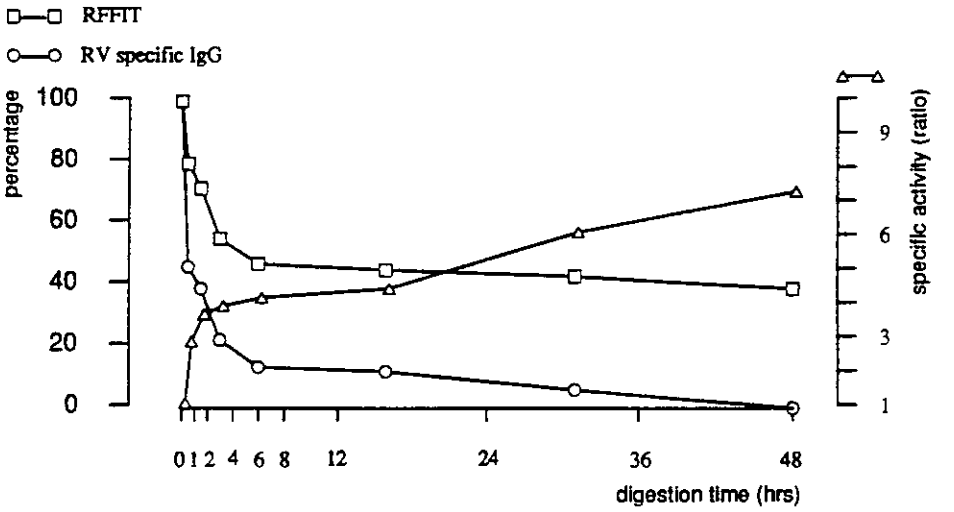

Figure 2. Influence of different incubation times with pepsin (2600 U/ml) at 37°C on biological activities measured by RFFIT and indirect ELISA and on the specific activity of DEAE/AS purified ERIG from a horse immunized with inactivated rabies vaccine (Pitman-Moore strain). RV: rabies virus.

serum from immunized horses resulted in the recovery of 31 and 34% of biological activities determined by RFFIT with specific activity ratios of 6·9 and 10·0 respectively. Analysis of the DE-52/AS and DEAE/AS purified preparations in the inhibition ELISA, resulted in the recovery of 35 and 26% of the biological activities respectively (Fig. 1).

Affinity chromatography

Affinity chromatographic purification of horse serum with Prot-A, Affi-T and Prot-G sepharose gels followed by AS precipitation resulted in the recovery of 15, 31 and 57% of biological activities measured in RFFIT with specific activity ratios of 6·9, 5·4, and 20·0 respectively. Analysis of these preparations by inhibition ELISA showed recoveries of 10·5, 21 and 31% respectively.

Influence of pepsin digestion time

The effects of pepsin digestion time on the *in vitro* biological activity, specific activity and residual levels of intact rabies virus-specific Ig, were determined with the DEAE/AS treated preparation (Fig. 2). During the first 30 min a reduction to about 70% of residual biological activity measured in the RFFIT was observed, which gradually declined to about 55% in the subsequent 5 h. During the following 43 h less than 10% of the original biological activity was subsequently lost. The specific activity ratio increased from 1·0 to 2·8 after 30 min, to 4·0 after 6 h and to 7·2 after 48 h of digestion. Levels of intact rabies virus-specific IgG as measured in the inhibition ELISA decreased about 45% in 30 min, to about 13% in 6 h to finally disappear after 48 h. By SDS-PAGE only traces of intact Ig molecules were detectable after 30 min, which were not detectable after 1 or more h after digestion.

Comparison of RFFIT and inhibition ELISA results

As shown in Fig. 3, there proved to be a good correlation between the results obtained in RFFIT and in the inhibition ELISA, with samples collected from the different purification methods described in the previous sections.

In vivo potency test with ERIG resulting from a selected combination of methods

Based on the results of the *in vitro* analyses and practical considerations, the following combination of methods was selected to be further studied for routine purposes using serum from a horse immunized with rabies virus prepared on dog kidney cells (Pitman Moore): AS precipitation, followed by DEAE chromatography and pepsin digestion for 48 h. This resulted in virus neutralization titers of approximately 12 IU/ml, 6 IU/ml and 4 IU/ml respectively and recoveries of 106, 46 and 39% of the biological activities of the respective products as determined by the *in vivo* mice protection assay.

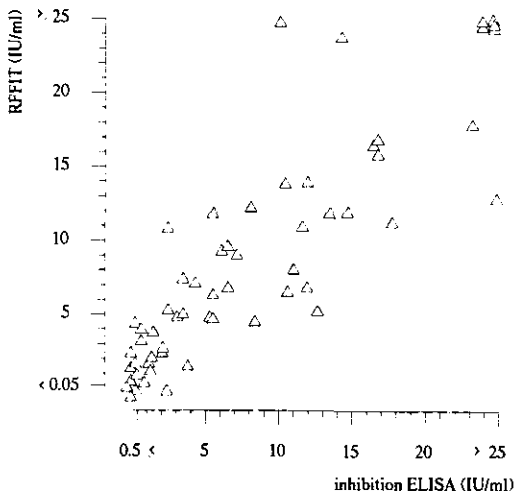

Figure 3. Comparison of the results obtained by RFFIT and inhibition ELISA with samples from the respective purification procedures used in these studies.

Discussion

In the present study we have compared different methods for the purification of ERIG from the serum of horses immunized against rabies virus, with the aim of developing a method yielding ERIG preparations which cause little side effects and can be implemented at low cost in developing countries. In preparing horse immune serum, we confirmed earlier studies,[8] showing that for the induction of high VN titers in horses, booster immunizations with live rabies virus are essential. Three principles of Ig purification from horse serum were compared. Of the non-chromatographic procedures, SS and AS precipitation yielded higher recovery rates (75–95%) than the combination of caprylic acid and AS precipitation. The latter however, yielded a preparation of about twofold higher purity. Since caprylic acid precipitation results in relatively high volumes it was followed by salt precipitation. For routine production procedures the use of AS precipitation, which gives a slightly lower recovery rate of biological activity with about the same purity as SS precipitation, should be preferred over SS precipitation, since it is routinely carried out at 4°C, which limits the effects of possible accidental bacterial contamination. The ion exchange chromatography with DE-52 or DEAE followed by AS precipitation resulted in a relatively pure preparation with a specific activity of about 10 at a recovery rate of about 30%. Of the three methods of affinity chromatography, the use of protein G followed by AS precipitation resulted in a product of the highest specific activity (about 20) at the highest recovery rate (about 50%). Although this method would clearly be the method of choice on basis of these criteria, the relatively high costs involved in this procedure may be prohibitive for large scale production in most developing countries. Therefore we have chosen to further evaluate the DEAE ion-exchange chromatography procedure, in combination with AS precipitation and pepsin digestion.

Anaphylactoid reactions due to treatment with intact Ig preparations may be related to dimers or aggregates of Ig, which activate the complement system and not, or to a lesser extent, to monomers. To minimize anaphylactoid reactions following treatment with horse Ig preparations, pepsin digestion of intact horse Ig levels is generally practised. We have evaluated the effects of different pepsin digestion times, using 2600 units of pepsin per mg of protein at 37°C (a routinely used procedure), on the biological activity and residual intact horse Ig levels. After a relatively sharp decline in biological activity during the first 6 h to about 50%, it only decreased with about another 10% during the subsequent 42 h of digestion. After 3 h of incubation still about 20% of the intact rabies virus-specific Ig as measured in the indirect ELISA was present, which had completely disappeared after 48 h of incubation. The relatively short pepsin digestion time of 0·5 to 3 h, which is generally practised, has been based on the detection of intact Ig by SDS PAGE. It may be expected that the removal of less than 90% of intact horse Ig may still not fully eliminate anaphylactoid reactions after administration. Therefore the question arises, whether the generally used pepsin digestion procedures are indeed sufficiently effective. A more important question to be addressed is whether the *in vivo* effect of ERIG after pepsin digestion is indeed reflected by the *in vitro* and *in vivo* VN tests as recommended by the WHO.[24] We have shown that there is a good correlation between both assays, when applied to the product resulting from an eventually selected procedure, the latter being notoriously more prone to biological variation. It should however be recommended not to use ERIG products after pepsin digestion, until their relative protective activities have been measured in post-exposure treatment experiments, rather than after the advocated pre-exposure neutralization of challenge virus.[3,20] It cannot be ruled out that for optimal *in vivo* activity in a post-exposure setting intact rabies virus-specific Ig molecules would be needed.

By comparing the data generated with the RFFIT and the inhibition ELISA based on MoAbs with VN activity, there proved to be a good correlation (corr. coeff. 0·89) between the results of both assays.

Although the inhibition ELISA does not directly measure VN activity, it may be concluded from these data that it can be used as a cheap alternative for in process controls in the preparation of ERIG products and as a diagnostic tool.

In conclusion, the procedure that we have selected for further evaluation and implementation in a large scale production setting was based on the results of *in vivo* and *in vitro* assays and practical considerations. It consists of AS precipitation followed by DEAE ion exchange chromatography. Whether, in addition to this procedure a pepsin digestion procedure should be included, remains to be decided on basis of a general assessment of the risk of adverse side effects induced by the thus purified preparation and of the results of post-exposure protection experiments.

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References

1. Wilde H, Chutivongse S. Equine rabies immune globulin: a product with an undeserved poor reputation. Am J Trop Med Hyg 1990; 42: 175–178.
2. World Health Organization. WHO Expert Committee on Rabies, Eighth Report 1992.
3. Schumacher CL, Dietzschold B, Erlt HCS, Nin HS, Rupprecht CE, Koprowski H. Use of mouse anti-rabies monoclonal antibodies in post-exposure treatment in developing countries. J Clin Invest 1989; 84: 971–975.
4. Bögel K, Motschwiller E. Incidence of rabies and post-exposure treatment in developing countries. WHO Bull 1986; 64: 883–887.
5. Cabasso VJ. Properties of rabies immune globulin of human origin. J Biol Stand 1974; 2: 43–50.
6. Karliner CJS, Belaval LTCG. Incidence of reaction following administration of anti-rabies serum. JAMA 1965; 193: 109–112.
7. Wilde H, Vhoomkasien P, Hemachudha T, Supich C, Chuntivongse S. Failure of rabies post-exposure treatment in Thailand. Vaccine 1989; 7: 49–52.
8. Lepine P, Atanasiu P. Production of therapeutic antirabies serum. WHO Monogr Ser 1966; 23: 161–166.
9. Van Wezel AL, Van Steenis B, Hannik CA, Cohen H. New approaches to the production of concentrated and purified inactivated polio and rabies tissue culture vaccines. Dev Biol Standard 1978; 41: 159.
10. Phillips AP, Martin KL, Horton WH. The choice of methods for immunoglobulin IgG purification: Yield and purity of antibody activity. J Immunol Meth 1984; 74: 385–393.
11. McKinney MM, Parkinson A. A simple non-chromatography procedure of immunoglobulins from serum and ascites fluid. J Immunol Meth 1987; 96: 271–278.
12. Ter Avest A, Van Zoelen EJJ, Spijkers IEM, Osterhaus ADME, Van Steenis G, Van Kreyl CF. Purification process monitoring in monoclonal antibody preparation: Contamination with viruses, DNA and peptide growth factors. Biologicals 1992; 20: 177–186.
13. Lihme A, Heegaard P. Thiophilic adsorption chromatography; The separation of serum protein. Anal Biochem 1991; 192: 64–69.
14. Lindmark R, Tolling KT, Sjöquist J. Binding of Immunoglobulin to protein A and immunoglobulin levels in mammalian sera. J Immunol Meth 1983; 62: 1–3.
15. Goudswaard J, Van der Donk JA, Noordzij A, Van Dam RH, Vaerman JP. Protein A reactivity of various mammalian immunoglobulins. Scand J Immunol 1987; 8: 21–28.
16. Laemmli HK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227: 680–685.
17. Bunschoten H, Gore M, Claassen IJTM, UytdeHaag FGCM, Dietzschold B, Wunner WH, Osterhaus ADME. Characterization of a new virus-neutralizing epitope that denotes a sequential determination on the rabies virus glycoprotein. J Gen Virol 1989; 70: 291–298.
18. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248–254.
19. Osterhaus ADME, Groen J, UytdeHaag FGCM, Bunschoten EJ, De Groot IGM, Van der Meer R, Van Steenis G. Quantification of rabies virus vaccine glycoprotein ELISA with monoclonal antibodies: correlation with single radial diffusion and the induction of anti-viral antibodies and protection in mice. In: Progress in Rabies Control. Thraenhart O, Koprowski H, Bögel K, Surea P (eds), Staples Printers Rochester Ltd. 1990.
20. Schumacher CL, Erlt HCG, Koprowski H, Dietzschold B. Inhibition of immune response against rabies virus by monoclonal antibodies directed against rabies virus antigens. Vaccine 1992; 10: 754–760.
21. Smith JS, Yager PA, Baer GM. A rapid tissue culture test for determining rabies neutralizing antibody. In: Laboratory Techniques in Rabies (3rd edn). Kaplan MM, Koprowski H (eds), WHO Monogr Ser 1973; 23: 354–357.
22. Zalan E, Wilson C, Pukitis D. A microtest for the quantitation of rabies virus neutralizing antibodies. J Biol Stand 1979; 7: 213–220.
23. Dietzschold B, Gore M, Casali P, Ueki Y, Rupprecht CE, Notkins AL, Koprowski H. Biological characterization of human monoclonal antibodies to rabies virus. J Virol 1990; 19: 3087–3090.
24. Atanasiu P. Quantitative assay and potency test of anti-rabies serum and immunoglobulin. In: Laboratory Techniques in Rabies (3rd edn). Kaplan MM, Koprowski H (eds), WHO Monogr Ser 1973; 23: 314–318.

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