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(19)

(54) FISSION PRODUCT OF RABIES VIRUS, PROCESS FOR ITS PREPARATION AND VACCINE CONTAINING SAME

(71) We, BEHRINGWERKE AKTIENGESELLSCHAFT, a body corporate organised according to the laws of the Federal Republic of Germany, of D—3550 Marburg/Lahn, Federal Republic of Germany, do hereby declare the invention for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement: —

The present invention relates to fission products of the rabies virus, to a process for its preparation and to a vaccine containing it.

It has been proposed previously to subject the rabies virus to a fission process in which a series of fission products are formed. The fission is preferably carried out with the use of a nonionic surface active substance (detergent), for example, Triton X—100 (the reaction product of 1 mole *tert.* octylphenol and 9 to 10 mols ethylene oxide, "Triton" being a Trade Mark). The most important products resulting from the fission and which can be isolated by electrophoresis are a nucleoprotein fraction (N), a glycoprotein fraction (G) and two fractions essentially consisting of membrane proteins (M_1 and M_2). It has been found that the immunizing capacity of the virus appears in the glycoprotein fraction (G), but due to its electrophoretic behaviour, this fraction was hitherto considered uniform.

The present invention is based on the observation that the glycoprotein fraction can be further fractionated and that the immunizing capacity can be isolated in one of the further fractions.

The present invention provides a process for the preparation of an immunologically active fission product of the rabies virus, which comprises separating the glycoprotein fraction of the rabies virus fission products into fractions having different isoelectric points, and isolating the fraction having an isoelectric point within the range of from 6.8 to 7.4, preferably 7.0.

The present invention also provides an immunologically active fission product of the rabies virus, which product is contained in that fraction of the glycoprotein fraction of the rabies virus fission products having an isoelectric point within the range of from 6.8 to 7.4, preferably 7.0.

The present invention further provides a vaccine comprising as the active ingredient the fission product of the invention.

To produce the fission product there is generally used a rabies virus grown in a cell culture. The character of the virus strain is of minor importance, and the nature of the cell culture used for the virus production is also of minor importance. A number of experiments, however, have shown that the growth of rabies virus of the ERA strain in a culture of BHK—21—cells is most advantageous. The production of rabies virus in cell cultures is known, that is to say, in actual use in the art or described in the literature of the art.

The fission of the virus by means of a nonionic surface active substance is likewise known. To obtain the glycoprotein fraction, which is further split according to the invention, the conditions under which the fission is carried out are not critical provided that the virus is split to a sufficient extent. Splitting is preferably effected by the action of a solution containing from about 0.2 to 5% (wt/vol) of a nonionic surface active substance at a temperature of from 5 to 35°C during a period of from 10 minutes to 6 hours.

The glycoprotein fraction is isolated from the resulting mixture, for example, by electrophoresis. Alternatively, the nucleoprotein can be separated by centrifugation at high g-values and the supernatant, which comprises the glycoprotein fraction, can be submitted directly to the subsequent fractionation according to isoelectric peaks. It has proved advantageous in the latter case to subject the supernatant to a dialysis after centrifugation in order to remove constituents of low molecular weight.

The glycoprotein fraction, which is generally in the form of an about 0.05 to 0.4% (wt/vol) aqueous dispersion, is then separated into

fractions having different isoelectric points. To this end, there may be used any process which enables polypeptide mixtures to be separated into fractions according to their isoelectric points. It is especially advantageous to use a method known as "isoelectric focusing" in which in a liquid column a pH gradient is built up with the use of appropriate dipolar ions, for example, polyaminopolycarboxy-alkanes (ampholites), and in the gradient the mixture is fractionated.

The column used for isoelectric focusing is, for example, charged with a solution comprising about 0.2 to 5% (wt/vol) of a compound containing dipolar ions, advantageously in a density gradient, for example, a gradient of from 0 to 50% (wt/nt) of a nonionic water-soluble compound, for example, a polyhydroxy compound, preferably a polysaccharide. In such a system focusing is effected under an electrical potential corresponding to the optimum potential of the column, during a period of from about 24 hours to about 8 days.

After isoelectric focusing, the contents of the column are separated into fractions. The constituents of the glycoprotein fraction are found to be concentrated in 3 bands at pH values of about 4.5, 7.0 and 8.0. The fraction corresponding to a pH value of around 7.0 (6.8 to 7.4) is collected separately. It contains the fission product according to the invention free from other high molecular weight constituents. In order to remove auxiliaries used in the isoelectric focusing and in the preceding operations, the fission product can be subjected to further purification, for example, to one or more of the following procedures: dialysis, sedimentation, chromatography and centrifugation.

The fission product according to the invention can be used, in admixture with a pharmaceutically suitable carrier, as a vaccine against rabies. In some cases, the other constituents of the isolated fraction may constitute the carrier. Compared with vaccines obtained in known manner from the intact virus, the vaccine according to the invention has about the same antigenic activity but reduced side effects.

In order to improve the stability and/or efficiency of the fission product of the invention *per se*, in the form of the isolated fraction or as a vaccine, known additives and stabilizers may be added and/or the fission product or the vaccine may be lyophillized.

The invention also provides a method of immunizing animals other than humans against rabies, which comprises administering to the animals a vaccine of the invention. The animals to be immunized are especially those most at risk in an outbreak of rabies, for example, dogs, cats and foxes.

The following Example illustrates the invention.

EXAMPLE

A clone of rabies virus (ERA strain) was plaque cloned in BHK 21-cells.

The virus was propagated in roller cultures of BHK—21—C—13 cells. Culture conditions have been described in L.G. Schneider, M. Horzinek and H.D. Matheka, 1971 "Purification of rabies virus from tissue culture", Arch.ges. Virusforsch., 34: pages 351—359. Infectious cell culture fluids and infected cells were harvested after pronounced cytopathological effect (CPE) was evident.

The viruses from the clarified supernatant were centrifuged in a Beckman R 19 rotor at 19,000 rpm for 120 minutes at 4°C. ("Beckman" is a Trade Mark). The supernatant was discarded and the pellet eluted overnight in a small volume of 0.15 M NaCl, 0.01 M tris-HCL, 0.001 M EDTA (STE) buffer of pH 7.5. The eluate was clarified by low speed centrifugation and layered on a preformed 10 to 50% (wt/vol) sucrose gradient, and was then centrifuged in a Beckman SW 27 rotor at 25,000 rpm for 90 minutes at 4°C. The virus band was collected and dialysed extensively against STE buffer.

Treatment of rabies virus with Triton X—100 was carried out substantially as described by Helenius and Söderlund in "Stepwise dissociation of the Semliki forest virus membrane with Triton X—100", Biochem.Biophys. Acta 307: pages 287 to 300: To a suspension of 5 mg rabies virus in 5 ml ST-buffer, Triton—X—100 was added to a final concentration of 1% (protein: Triton ratio was 1 : 10). The mixture was kept for 20 minutes at room temperature and thereafter chilled in an ice bath. The still turbid solution was centrifuged in a Beckman SW 65-rotor at 45,000 rpm for 60 minutes at 4°C. The supernatant was retained and the pellet was resuspended in ST-buffer containing 2 M LiCl and 5 mg/ml digitonin. The suspension was incubated for 30 minutes at room temperature and centrifuged in a Beckman SW 65 rotor at 45,000 rpm for 60 minutes at 4°C. The supernatant was retained and the pellet discarded.

The supernatant of the Triton X—100 treated virus, obtained after centrifugation at 120,000 g, was extensively dialysed against 1% (wt/vol) glycine and 1% (wt/vol) glycerol in distilled water. After addition of 1% Ampholine, pH 3.5 to 10 (Trade Mark of LKB, Uppsala, Sweden) the Triton X—100 extract was clarified at 5,000 g for 10 minutes and a 5 ml sample was layered on a 5 to 40% (wt/vol) sucrose gradient containing 0.1% Triton X—100 and 1% Ampholine in pH 3.5 to 10. The preparation was electrofocused for 72 hours in a 110 ml LKB column as described by Friesen *et al* (A.D. Friesen, J.C. Jamieson and F.E. Ashton 1971 "Effect of nonionic detergent on fractionation of proteins by isoelectric focusing", Anal.Biochem.

41: pages 149 to 157). Thereafter, the gradient was fractionated and the pH 7 fraction was collected and dialysed extensively for 2 days against distilled water to reduce the Triton X—100 concentration and to remove the Ampholine and the sucrose, and was then lyophilized.

Alternatively, after the final dialysis, the fraction was brought into the form of a vaccine in conventional manner, and lyophilized.

WHAT WE CLAIM IS:—

1. An immunologically active fission product of the rabies virus, which product is contained in that fraction of the glycoprotein fraction of the rabies virus fission products having an isoelectric point within the range of from 6.8 to 7.4.

2. A product as claimed in claim 1, wherein the fraction has an isoelectric point of 7.0.

3. A process for preparing an immunologically active fission product of the rabies virus, which comprises separating the glycoprotein fraction of the rabies virus fission products into fractions having different isoelectric points, and isolating the fraction having an isoelectric point within the range of from 6.8 to 7.4.

4. A process as claimed in claim 3, wherein there is isolated the fraction having an isoelectric point of 7.0.

5. A process as claimed in claim 3 or claim 4, wherein the glycoprotein fraction is separated by isoelectric focusing.

6. A process as claimed in claim 5, wherein in the isoelectric focusing a pH gradient is set up by means of polyamino-polycarboxyalkanes as the dipolar ions.

7. A process as claimed in claim 5 or claim 6, wherein a solution comprising from 0.2 to 5% (wt/vol) of the compound containing dipolar ions is used to set up the pH gradient.

8. A process as claimed in any one of claims 5 to 7, wherein in the isoelectric focusing utilises a density gradient.

9. A process as claimed in claim 8, wherein the density gradient is a gradient of from 0 to 50% (wt/nt) of a non-ionic water-soluble compound.

10. A process as claimed in claim 8 or claim 9, wherein the density gradient is a gradient of a polyhydroxy compound.

11. A process as claimed in claim 10, wherein the polyhydroxy compound is a polysaccharide.

12. A process as claimed in any one of claims 3 to 11, wherein the isolated fraction is subjected to further purification.

13. A process as claimed in claim 12, wherein the further purification is any one or more of the following: dialysis, sedimentation, chromatography and centrifugation.

14. A process as claimed in any one of claims 3 to 13, wherein the rabies virus is the ERA strain.

15. A process as claimed in claim 14, wherein the rabies virus has been cultured on BHK—21—cells.

16. A process as claimed in any one of claims 3 to 15, wherein the rabies virus fission products have been obtained by treating the rabies virus with a solution comprising from 0.2 to 5% (wt/vol) of a nonionic surface active substance at a temperature of from 5 to 35°C.

17. A process as claimed in any one of claims 3 to 16, wherein the glycoprotein fraction of the rabies virus fission products is isolated by electrophoresis of the fission products.

18. A process as claimed in any one of claims 3 to 16, wherein the glycoprotein fractions of the rabies virus fission products is obtained by centrifuging the fission products at high g-values to separate the nucleoprotein fraction in the centrifugate and the glycoprotein fraction in the supernatant.

19. A process as claimed in any one of claims 3 to 18, wherein the resulting fraction having an isoelectric point within the range of from 6.8 to 7.4 is lyophilized.

20. A process as claimed in claim 3, carried out substantially as described in the Example herein.

21. An immunologically active fission product of rabies virus, whenever prepared by a process as claimed in any one of claims 3 to 20.

22. A vaccine which comprises an immunologically active fission product of the rabies virus as claimed in claim 1, claim 2 or claim 21, in admixture with a pharmaceutically suitable carrier.

23. A vaccine as claimed in claim 22, wherein the other components of the isolated fraction constitute the carrier.

24. A vaccine as claimed in claim 22 or claim 23, in lyophilized form.

25. A method of immunizing an animal other than a human being against rabies,

which comprises administering to the animal a vaccine as claimed in any one of claims 22 to 24.

26. A method as claimed in claim 25, wherein the animal is a dog or a cat.

27. A method as claimed in claim 25, wherein the animal is a fox.

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