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(54) IMMUNOSTIMULATING AGENT

(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:-

This invention relates to an agent for the stimulation of the immunological response, in particular antibody formation which is inducible by antigens.

It is well known that the immunological response of an organism which has been provoked by the application of antigens can be influenced by the addition of certain substances. A substance which increases the response is designated an adjuvant. Adjuvants have no particular composition or structure, however, and their mechanism of action is still the subject of speculation. Their activity can be tested by comparative tests only, so that it is extremely difficult if not impossible to predict adjuvant activity in any substance not yet tested.

In view of the great economic importance of immunizing agents in the prophylaxis and therapy of many diseases, the discovery of adjuvant activity in a substance is of great economic interest.

The present invention is based on the surprising observation that the enzyme neuraminidase (glycoprotein-N-acetylneuraminyl -hydrolase, classified under EC. 3.2.1.18) is a valuable immunostimulating agent, that is to say, a so-called adjuvant.

The invention accordingly provides an agent for stimulating an immune response, which comprises an antigen and neuraminidase.

Neuraminidase is used in these preparations generally in an amount of 0.01 - 150, preferably 0.5 - 50, in many cases 5 - 50, units per immunizing dose.

The agents may be in unit dosage form and may comprise a pharmaceutically suitable carrier or may be in lyophilized form.

The antigen is any substance capable of provoking an immune response and may be soluble or particulate. A microbial antigen may be alive, dead or attenuated, and cells and cell material obtained from higher animals, for example, tumour cells and tumour antigens may be used. The adjuvant of the invention is particularly useful in combination with antigens used in conventional vaccines but, as mentioned above, may be used in conjunction with *any* antigen.

The invention also provides a method of stimulating an immune response, which comprises administering to a non-human mammal, simultaneously or one immediately after the other, an antigen and neuraminidase. The nature of the antigen and the preferred dose of neuraminidase are as indicated above.

This method is particularly useful in the preparation of antisera, because the response to administration of a particular antigen is both faster and more pronounced, that is to say, more antibodies are produced per unit of antigen than is the case on administration of the antigen alone. The invention accordingly provides a method of preparing an antiserum, which comprises administering to a host animal an antigen and neuraminidase, either together or one immediately after the other, withdrawing blood after the formation of antibodies, and isolating the antiserum therefrom.

The term "neuraminidase" encompasses a series of enzymes which are capable of splitting off the neuraminic acid from neuraminic acid-containing glycoproteins. They may be

isolated and purified according to methods in the literature. The neuraminidases utilizable as adjuvants in the present invention are characterised by their activity to hydrolyse the α-ketoside linkage between neuraminic acid and a sugar partner. Preferably, the neuraminidases used are those which hydrolyse one or more or the linkages 2→3, 2→4, 2→6, and 2→8. Thus, particularly effective is the neuraminidase obtained from *vibrio cholarae* which is capable of hydrolyzing all four linkages. The neuraminidase of *Clostridium perfringens* has a similar activity. Moreover viral neuraminidases may be used according to the invention, even though they do not generally have the broad specificity of the bacterial enzymes. In addition to the neuraminidases which can be isolated from microorganisms, there may also be used neuraminidases which are obtainable from body fluids, for example, plasma, and organs of vertebrate animals.

The neuraminidase preparations used as adjuvants should be prepared with all the care required in the production of pharmaceuticals.

The quantity of the adjuvant neuraminidase to be added per dose of an antigen is suitably expressed in units of the activity of the enzyme. 1 neuraminidase unit as used according to this invention is the quantity of the enzyme which is required to set free in 15 minutes at 37°C 1 microgram of N-acetylneuraminic acid from human α_1-glycoprotein (Orosomucoid) in 0.05 molar sodium acetate buffer having a pH-value of 5.5 with the addition of 9 mg/ml of sodium chloride and 1 mg/ml of calcium chloride (E. Mohr and G. Schramm, Z. Naturf. 15 b, page 568, (1960), and Schultze *et al*. Biochem. Zschr. *329*, p 429, (1958)). A comparison of the enzymatic activity as defined according to Schramm and Mohr, Nature *183*, 1677, (1959), and other definitions of the enzymatic activity of neuraminidase is given by Schick and Zilg, International symposium on biological preparations in the treatment of cancer, London, 1977. Develop. biol. Standard, *38* pp. 81-85 (1978)

It is known that the immunogenicity of glycoproteins can be increased significantly by the removal of the N-acetylneuraminic acid with the aid of the enzyme neuraminidase. In these cases, however, the increase in the immunogenicity is clearly explained by the action of the neuraminidase on the antigen which sets free new antigenic determinants by the enzymatic attack on the glycoside portion of the glycoprotein. The modified glycoprotein was not previously administered with the neuraminidase: after separation of the N-acetyl neuraminic acid radicals, the desialo-glycoprotein was generally washed several times to remove the neuraminidase before it was used for immunisation.

In view of the possibility, however, that the adjuvant activity of neuraminidase may result from its antigen-modifying ability, tests were carried out using antigens that do not contain neuraminic acid residues i.e. that are not susceptible to the enzymic activity of neuraminidase, and also on neuraminic acid-containing antigens. The antigens were pre-incubated with neuraminidase, washed thoroughly to remove the enzyme, and then administered. None of the results obtained indicate that the adjuvant activity of neuraminidase lies in its ability to split neuraminic acid from a neuraminic acid-containing antigen, so neuraminidase may be used as an adjuvant with any antigen regardless of its chemical composition.

This was demonstrated by the following test:

Mice were immunized intraperitoneally with 2×10^8 killed *E. coli*/mouse together with various amounts of *Vibrio cholerae* neuraminidase. For one group of the mice the neuraminidase was first added to the killed *E. coli,* then the neuraminidase was removed from the bacteria by a centrifugation process in which the suspension of bacteria was centrifuged, the microorganisms were re-suspended in a suitable buffer medium and this measure was repeated three times. Another group of mice received the *E. coli* together with *Vibrio Cholera* neuraminidase.

Table 1 shows the antibody titres in the blood of the various groups of mice on the 5th and 20th day after the immunization had been effected.

Table 1: *Immunization of mice with E. coli.*

Antigen: *E. coli* 2 x 10^8/mouse	Adjuvant: *Vibrio cholerae* Neuraminidase	Average antibody titre * ± standard deviation on the day	
		5	20
yes	without	10 ± 10	160 ± 120
yes	0.5 U	60 ± 40	640 ± 240
yes	0.5 U washed	10 ± 10	130 ± 60
yes	0.5 U	10 ± 10	320 ± 140
yes	5 U	160 ± 120	640 ± 320
yes	5 U washed	10 ± 10	160 ± 100
yes	50 U	80 ± 40	320 ± 160
yes	50 U washed	10 ± 10	160 ± 80

* measured by the test for bactericidal activity.

These results show that the administration of an antigen (*E. coli*) which has been pre-incubated with neuraminidase and subsequently, before administration, freed from neuraminidase, does not lead to a significant stimulation of the immune response to that antigen. The results also show that the concommitant administration of the antigen and neuraminidase leads to an earlier reaction and higher serum antibody levels than is found on administration of untreated antigen alone.

The stimulation of the immunological response was found to be effective independently of the route of administration of the antigen together with the neuraminidase, for example, it was effective on intraperitoneal, subcutaneous, intradermal and intramuscular administration. The indicated antibodies were measured by the test for bactericidal activity.

Similar results, analogous to those in Table 1, were obtained in an immunization test with *Vibrio cholerae*, using the test for indirect hemagglutination with the aid of *Vibrio cholerae* lipopolysaccharide.

The activity of neuraminidase as an adjuvant was also demonstrated for virus antigens, for example, the rubella virus (cf. Table 2).

Table 2: *Immunization of mice with rubella virus.*

Antigen: Rubella virus	Adjuvant *Vibrio cholerae* Neuraminidase	Mean hemagglutination-inhibition titre ± Standard deviation
yes	0.0	82 ± 98
yes	0.5 U per mouse	105 ± 83
yes	5 U per mouse	461 ± 246
yes	50 U per mouse	89 ± 47
no	5 U per mouse	8

With the same system it was demonstrated that a second immunization (booster immunization) can be carried out with very good success and that, here too, the virus suspensions in combination with neuraminidase provoke an increased immuno-response.

The above tests demonstrate the activity of neuraminidase as adjuvant with particulate antigens.

The activity of neuraminidase as an adjuvant may also be demonstrated with soluble antigens. When, for example, an immunization of mice was carried out with bovine serum

albumin, an increase in the immunological response was found with the antigen preparations to which neuraminidase had been added. This was shown with the aid of indirect hemagglutination of wether erythrocytes charged with bovine serum albumin.

In addition, neuraminidase is capable of breaking through an immunologic tolerance. For this purpose, quantities of neuraminidase per dose of about 0.5 - 5 units have been found to be effective, whereas distinctly higher doses result in less distinct reactions.

With neuraminidase as adjuvant, an antibody response against sheep erythrocytes was experimentally increased. This can be demonstrated with the aid of the method, known as the Jerne technique of proving "plaque forming cells" in the spleens of the immunized animals. This test method was also used to demonstrate that a subsequent washing of the erythrocytes incubated with neuraminidase does not stimulate the immunologic response.

In addition to the previously described tests which demonstrate the increase in antibody formation with the aid of neuraminidase, tests have also been carried out to show that the adjuvant of the present invention produces an improved resistance against infections.

Table 3 shows the results of a test in which mice were immunized with *S. typhimurium,* with and without addition of an adjuvant. For this purpose, the mice were each given on the 1st and 14th day 4×10^7 killed *S. typhimurium*. On the 28th day after the first injection, the mice were infected intravenously with 2×10^5 of virulent *S. typhimurium*. In order to determine the sucess of the immunization, some animals from the individual groups were sacrificed on the 1st, 3rd and 8th day after the infection, the liver and the spleen were removed and the live germs contained in them were determined. The following Table shows the number of live germs. The remaining animals of the individual groups were observed until the 28th day and the number of the surviving animals was determined. The percentage survival rate of the animals is indicated in Table 3.

Table 3:

Antigen	Adjuvant	Live germs in liver and spleen on the day indicated ± standard deviation			Survival rate
		1	3	8	
none	none	$8 \times 10^3 \pm 1 \times 10^3$	$6 \times 10^4 \pm 2 \times 10^4$	$4 \times 10^7 \pm 3 \times 10^7$	30%
S. typhi.	none	$8 \times 10^3 \pm 3 \times 10^3$	$2.5 \times 10^5 \pm 1 \times 10^5$	$6 \times 10^5 \pm 3 \times 10^5$	45%
S. typhi.	Neuraminidase 6 U per dose	$1.5 \times 10^4 \pm 0.2 \times 10^4$	$2.5 \times 10^4 \pm 2.2 \times 10^4$	$2.4 \times 10^4 \pm 2.3 \times 10^4$	70%

The antigen *S. typhimurium* in combination with neuraminidase leads to a distinctly reduced number of live germs in the organs and to an improved survival rate of the animals.

As has been shown by the tests described above, neuraminidase is utilisable as an adjuvant for the most various antigen preparations, with soluble as well as particulate, live or dead antigens which can be isolated from microorganisms or from higher animals. It is an essential feature of the invention that the neuraminidase is administered either simultaneously with the antigen against which a specifically increased immunological response of the host treated is to be obtained, or that the two substances are administered immediately one after another, in either order.

The adjuvant has a particular practical imprtance, among others, in the increase of the resistance to infections, but also in the increase of the immunologic response upon administration of tissue cells, for example, those of tumour tissue, and of antigens extracted therefrom. Neuraminidase is used with particular advantage as an adjuvant in the preparation of antisera against defined antigens, since in this case the same amount of the required antigen results in an important increase of the yield of the antibody to be isolated from the serum of the immunized animals.

WHAT WE CLAIM IS:

1. An agent for stimulating an immune response, which comprises an antigen and neuraminidase.
2. An agent as claimed in claim 1, which comprises from 0.01 to 150 units of neuraminidase per dose of antigen.
3. An agent as claimed in claim 2, which comprises from 0.5 to 50 units of neuraminidase per dose of antigen.
4. An agent as claimed in claim 3, which comprises from 5 to 50 units of neuraminidase per dose of antigen.
5. An agent as claimed in any one of claims 1 to 4, wherein the neuraminidase is capable of hydrolysing one or more of the linkages $2 \rightarrow 3$, $2 \rightarrow 4$, $2 \rightarrow 6$, and $2 \rightarrow 8$ between neuraminic acid and a sugar.
6. An agent as claimed in claim 5, wherein the neuraminidase is the neuraminidase of *Vibrio cholerae*.
7. An agent as claimed in any one of claims 1 to 6, in unit dosage form.
8. An agent as claimed in any one of claims 1 to 7, wherein the antigen is a microbial antigen.
9. An agent as claimed in any one of claims 1 to 7, wherein the antigen is a tissue cell or an antigen obtained therefrom.
10. An agent as claimed in claim 9, wherein the tissue cell is a tumour cell.
11. A method of stimulating an immune response, which comprises administering to a non-human mammal, simultaneously or immediately one after another and in either order, an antigen and neuraminidase.
12. A method of producing an antiserum, which comprises administering to a host animal, simultaneously or immediately one after another, in either order, an antigen and neuraminidase, withdrawing blood after the formation of antibodies and isolating the antiserum therefrom.
13. A method as claimed in claim 11 or claim 12, wherein the neuraminidase and the antigen are in the form of an agent as claimed in any one of claims 1 to 10.
14. A method as claimed in claim 11 or claim 12, wherein the neuraminidase is as defined in any one of claims 2 to 6 and the antigen is as defined in any one of claims 8 to 10.

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