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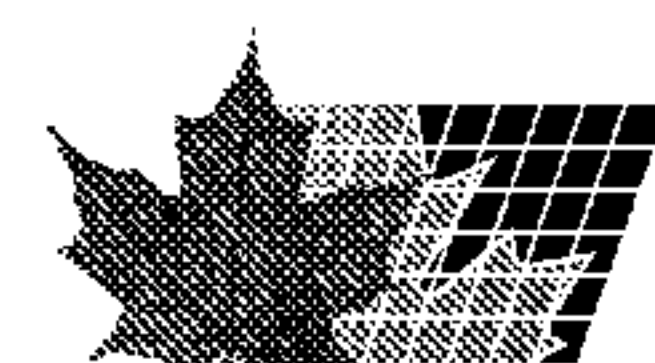
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(54) Titre : UTILISATION DE LA PROTEINE C OU DE LA PROTEINE C ACTIVEE

(54) Title: USE OF PROTEIN C OR ACTIVATED PROTEIN C

(57) **Abrégé/Abstract:**

There is disclosed the use of protein C or activated protein C for preparing a drug useful for the maintenance and improvement of the microcirculation in patients suffering from circulatory disturbances, in particular with the proviso that the drug does not contain any immunoglobulin G.



ABSTRACT OF THE DISCLOSURE:

There is disclosed the use of protein C or activated protein C for preparing a drug useful for the maintenance and improvement of the microcirculation in patients suffering from circulatory disturbances, in particular with the proviso that the drug does not contain any immunoglobulin G.

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The invention relates to a new use of protein C or activated protein C for the preparation of a drug.

Protein C is a vitamin K-dependent glycoprotein that is synthesized in the liver and circulates in
5 plasma as an inactive zymogen at a concentration of 4 $\mu\text{g/ml}$. It is converted into the active serine protease (activated protein C) by the thrombin-thrombomodulin complex on the surface of the vessel wall (endothelium). It is known that activated protein C has
10 profibrinolytic properties. It also has an anticoagulant effect, because it proteolytically degrades both factor Va, the cofactor for the factor Xa-induced prothrombin activation (thrombin formation), and factor VIIIa, the cofactor for the factor IXa-
15 induced factor X activation.

The activation of protein C in vivo constitutes a negative feedback reaction in the generation of thrombin. A process for the preparation of activated protein C in vitro is described, for instance, in EP-A-
20 0 416 890.

It is known that activated protein C has a favorable effect on sepsis and septic shock.

Septic shock is the systemic reaction to the release of endotoxins, i.e., lipopolysaccharide
25 components of the cell walls of gram negative bacteria, into the circulation. Septic shock is a major cause of death of hospitalized patients. Frequently,

gram negative bacteremia proves to be the cause of sepsis.

Septic shock and the Shwartzman reaction show comparable pathophysiologic elements. The Shwartzman reaction is an inflammatory hemorrhagic and thrombotically necrotizing lesion of the skin elicited by the local and subsequent intravenous administration of endotoxin of gram negative bacteria. Specific syndromes in man, which are described in the following, are to be attributed to the Shwartzman reaction.

A skin lesion macroscopically occurring in the Shwartzman reaction very much resembles the syndrome of purpura fulminans in septic conditions (e.g., meningococcus sepsis). Purpura fulminans is a potentially fatal syndrome that induces extensive skin necroses and autoamputations of the extremities. Despite conventional therapeutical attempts, for example, by means of antibiotics or intensive care procedure, the death rate is very high.

Likewise, chronic ulcerative diseases of the intestines constitute an example of the Shwartzman reaction in man. Again, circulating endotoxins induce the infection of the wall of the intestines, a local lesion corresponding to the Shwartzman reaction.

The effects of septic shock elicited by an infection with *E. coli* in baboons are summarized in Critical Care Medicine (1988). Primarily, an

inflammatory reaction is caused and, secondarily,
coagulopathic reactions and cell injury occur. It is
mentioned that the infusion of activated protein C
protects against the secondary consequences of the
5 infection.

In PCT application WO 90/08556, combined
preparations for the treatment and prophylaxis of
sepsis and septic shock are described, which comprise a
bactericidal effective amount of immunoglobulin G as
10 well as a blood clot-dissolving effective amount of
activated protein C. It is recommended not to use the
zymogen protein C because of potential difficulties
with in vivo activation.

Likewise, it is known from J. Clin. Invest. (1987)
15 that the effect of an infection with E. coli in baboons
will be aggravated by blocking in vivo protein C
activation. This even leads to the death of the test
animals in case of infections with sublethal doses of
E. coli. However, the death may be prevented by
20 coinfusion of activated protein C. Nothing is said
about a preventive or therapeutic effect of the
zymogen.

Furthermore, it is known that many patients
suffering from protein C deficiency exhibit purpura
25 fulminans syndrome, which has been successfully treated
with a protein C concentrate (Blood 10, Suppl. 1: 2070,
1990). However, the triggering mechanism of

this disease substantially differs from purpura fulminans elicited by a microbial infection. Equivalent treatment would therefore not be an obvious option in the case of sepsis.

5 In addition to purpura fulminans, dermatitis ulcerosa also may be induced by endotoxins. So far, no remedy has been known for the prophylaxis and therapy of these syndromes.

10 It is the object of the invention to widen the field of therapeutic application of protein C or activated protein C.

15 The invention consists in the use of protein C or activated protein C for the preparation of a drug intended for the maintenance and improvement of microcirculation in patients suffering from circulatory disturbances, as well as for the prophylaxis and therapy of the clinical equivalents to the local and generalized Shwartzman reactions, in particular, with the proviso that the drug does not contain any

20 immunoglobulin G. Both protein C and activated protein C have proved to exhibit excellent activities in the above-mentioned cases without being combined with bactericidal effective substances.

25 Protein C or activated protein C has proved particularly useful for the treatment of circulatory disturbances associated with malign diseases,

autoimmune diseases, immunocomplex diseases, infectious diseases and with shock syndromes.

Other preferred indications are:

- reduction of inflammatory processes caused by an
5 impaired microcirculation;
- prophylaxis and therapy of purpura fulminans
caused by microbial infections; and
- prophylaxis and therapy of clinical equivalents to
local and generalized Shwartzman reactions.

10 In the same manner, protein C, according to the invention, may be used for the preparation of drugs intended for the prophylaxis and therapy of chronic inflammatory diseases of the intestines and for the prophylaxis and therapy of dermatitis ulcerosa.

15 It was demonstrated in an animal model that both the zymogen protein C and activated protein C attenuate the local Shwartzman reaction. The therapeutic mechanism is presumed to be associated with the inhibition of the stimulation of leukocytes and of the
20 synthesis and release of cytokins caused by endotoxin. In addition, it is assumed that the endothelial stimulation and expression of leukocyte adhesion molecules are prevented and leukocytic adhesion is suppressed. It was demonstrated that protein C or
25 activated protein C prevents the microcirculatory clot-forming activation and thrombosis elicited by microbiologic infections.

The preparation of protein C and of activated protein C as well as the investigation as to their influence on the local Shwartzman reaction will be described in the following.

5 Preparation of Protein C

Highly purified protein C was recovered from a crude protein C fraction obtained from commercially available prothrombin complex concentrate. Purification was effected by affinity chromatography by means of
10 monoclonal antibodies. Monoclonal anti-protein C antibodies were produced as follows:

BALB/C mice were immunized with 100 µg human protein C by intraperitoneal injection at two-week intervals. After six weeks, another 50 µg of human
15 protein C were injected and fusion was carried out three days later. The myeloma cell line (P3-X-63-AG8-653, 1.5×10^7 cells) was mixed with 1.7×10^8 mouse spleen cells and fused according to the modified method of Köhler & Milestein by using PEG 1500 (Köhler G.,
20 Milestein C., Nature 256 (1975), 495-497).

Positive clones, assayed by means of ELISA, were subcloned twice. Ascites production was effected by injection of 5×10^6 hybridoma cells per BALB/C mouse two weeks after Pristan treatment.

25 The immunoglobulin was purified from ascites by means of ammonia sulfate precipitation and subsequent chromatography on QAE-Sephadex (Pharmacia) and,

further, by chromatography on Sephadex G200 (Pharmacia). To reduce the risk of transmission of murine viruses, the antibody was subjected to a further virus inactivation step prior to immobilization. The monoclonal protein C antibodies thus obtained were coupled to CNBr-activated Sepharose 4B (Pharmacia). The following buffers were used for the purification of protein C by means of affinity chromatography:

Adsorption buffer: 20 mM Tris, 2 mM EDTA, 0.25 mM NaCl and 5 mM benzamidine;

Washing buffer: 20 mM Tris, 1 M NaCl, 2 mM benzamidine, 2 mM EDTA, pH 7.4;

Elution buffer: 3 M NaSCN, 20 mM Tris, 1 M NaCl, 0.5 mM benzamidine, 2 mM EDTA.

In detail: The prothrombin complex concentrate was dissolved in the adsorption buffer, with approximately 10 g of the prothrombin complex concentrate being employed for a 20 ml monoclonal antibody column. Subsequently, the dissolved prothrombin complex concentrate was filtered, centrifuged at 20,000 r.p.m. for 15 min and sterile filtered through a 0.8 μ m filter. The sterile filtered and dissolved prothrombin complex concentrate was applied to the column at a flow rate of 10 ml/h. Subsequently, the column was washed free of non-bound protein with the washing buffer, and finally the bound protein C was eluted by means of the elution buffer at a flow rate of 5 ml/h and the

fractions were collected. The eluted protein C was dialyzed against a buffer (0.2 mol/l Tris, 0.15 M glycine and 1 mM EDTA, pH 8.3). Protein C antigen concentration was determined using the method described
5 by Laurell, and protein C activity was determined using Protac activation.

The protein C eluate thus obtained was then finished to a pharmaceutically applicable preparation in the following manner:

10 The eluate was first subjected to ultrafiltration and diafiltration steps. Diafiltration was carried out with a buffer containing 150 mmol NaCl and 15 mmol trisodium citrate.2H₂O per liter, at a pH of 7.4. The obtained filtrate was then freeze-dried and virus
15 inactivated by a one-hour vapor treatment at 80°C \pm 5°C and at 1375 \pm 35 mbar.

The lyophilized, virus inactivated material was then dissolved in a sterile isotonic NaCl solution and potentially present antibodies or serum amyloid P were
20 eliminated by means of ion exchange chromatography on Q-Sepharose® (Pharmacia). The purified solution was concentrated by means of an additional ultrafiltration and diafiltration stage. After this stage, 10 g albumin, 150 mmol NaCl and 15 mmol trisodium citrate
25 per liter were added to the solution obtained. The pH of the solution was 7.5. Neither murine immunoglobulin nor factors II, VII, IX and X could be detected.

Subsequently, the solution was sterile filtered, filled in containers and lyophilized. The specific activity was 14 units protein C per mg of protein. One unit of protein C activity is defined as the protein C activity in 1 ml normal plasma and is calibrated against the first international standard of protein C. An amidolytic assay was used as the activity test, wherein protein C is activated by means of Protac (from Pentapharm).

10 Preparation of Activated Protein C

Activation of the purified protein C was effected by coupling 70 ml thrombin (500 NIH units/ml corresponding to approximately 2000 NIH units/mg protein) to CNBr-activated Sepharose 4 B (Pharmacia), whereupon protein C was mixed with the thrombin gel at a ratio of about 6 units protein C to 1 unit thrombin at 37°C and allowed to react for 3 hours under continuous shaking. The protein C activity was then determined by means of chromogenic substrate (S 2366). The activated protein C subsequently was sterile filtered and finished to a pharmaceutical preparation as described above.

20 Influence on the Local Shwartzman Reaction in Rabbit by Protein C

Method: The assays were carried out in (White New Zealand) rabbits of both sexes weighing 2 to 3 kg.

Under brief anesthesia (20 mg/kg Ketamin® + 5 mg/kg Rombun® i.m.), the abdominal skin of rabbits was

depilated by shaving and by using Depilan®. After this, six wheals were intradermally raised in each animal with 0.2 ml endotoxin of Salmonella typhimurium (Sigma L 7261) each. The endotoxin doses employed were 6.25, 12.5, 25, 50, 100 and 200 µg (preparative doses). 24 hours later, the rabbits received 20 µg/kg of the endotoxin i.v. in an ear vein (0.2 ml/kg; provocative dose).

There were assayed:

- 10 A) Activated protein C (n = 5 animals) intravenously according to the following scheme: 250 U/kg immediately upon the provocative dose and 50 U/kg after 1, 2 and 3 hours each. The injection volume was 0.25 ml/50 U.
- 15 B) Protein C (n = 7 animals), 500 U/kg i.v. immediately upon the provocative dose and 100 U/kg after 1, 2 and 3 hours each. The injection volume was 0.8 ml/100 U.
- 20 C) A separate control group (n = 6 and 5 animals, respectively) was established for each assayed group (A, B). Apart from the preparative endotoxin doses and the provocative dose, these animals received no other treatment.

25

The assessment of the changes of the skin took place 6, 24 and 48 hours after the provocative dose. The parameters "thickening and swelling" (0 - 3), "size" (0 - 4), "color" (0 - 4) and "ring formation" (0 - 1) were assessed (the numbers in parentheses indicate the scores by aid of which each individual parameter was quantified). The scores of the individual parameters were added to a total score characterizing the extent of the respective change of the skin.

10 From the total scores of each dose group, the mean values were calculated both for the animals of the assayed groups and for the control animals and are depicted in the Table. These results are based on 6-hour values.

15 Results: Lesions of the skin were observed, reaching from petechial rubor to high-grade intradermal hemorrhage with surface crust formation. The degree of the skin lesions depended on the dose of the preparative toxin injection and on the treatment with
20 the test substances. As is apparent from the Table, the changes in untreated control animals are severe, while those of treated rabbits (activated protein C, protein C) are only moderate.

TABLE

Shwartzman Reaction in Rabbit

5	Treatment group	n	Skin lesion, score (mean value), on the sites of preparative toxin injection (μ g)					
			6.25	12.5	25	50	100	200
	A	5	0.4	0.2	2.8	4.6	5.0	5.8
	C _A	6	2.5	4.7	5.4	8.3	8.7	9.7
10	B	7	1.0	1.1	2.0	3.3	3.6	5.4
	C _B	5	1.2	2.2	4.8	7.0	7.8	8.4

15 Activated protein C (400 U/kg i.v.) and protein C
(800 U/kg i.v.) may be compared as to their protective
effects illustrated. If the mean values of the scores
(cf. Table) are plotted against the doses on a
logarithmic scale, parallel dose-response curves will
20 result.

Pretreatment with activated protein C and with
protein C shift the dose-response curves established
for the respective controls to the right each by a
factor 4, towards higher doses.

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CLAIMS:

1. A pharmaceutical composition for maintaining and improving the microcirculation in a patient suffering from circulatory disturbances comprising protein C or activated
5 protein C and a pharmaceutically acceptable carrier or diluent.
2. A pharmaceutical composition for treating and preventing clinical equivalents to local and generalized Shwartzman reactions comprising protein C or activated
10 protein C, and a pharmaceutically acceptable carrier or diluent.
3. A pharmaceutical composition for treating circulatory disturbances associated with a malign disease, an autoimmune disease, an immunocomplex disease, an
15 infectious disease or with shock syndrome comprising protein C or activated protein C and a pharmaceutically acceptable carrier or diluent.
4. A pharmaceutical composition for reducing inflammatory processes caused by an impaired
20 microcirculation in a patient comprising protein C or activated protein C and a pharmaceutically acceptable carrier or diluent.
5. A pharmaceutical composition for treating and preventing purpura fulminans caused by microbial infections
25 comprising protein C or activated protein C and a pharmaceutically acceptable carrier or diluent.
6. A pharmaceutical composition for treating and preventing chronic inflammatory diseases of the intestines comprising protein C or activated protein C and a
30 pharmaceutically acceptable carrier or diluent.

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7. A pharmaceutical composition for treating and preventing dermatitis ulcerosa comprising protein C or activated protein C and a pharmaceutically acceptable carrier or diluent.

5 8. The pharmaceutical composition of any one of claims 1 to 7, with the proviso that the pharmaceutical composition is free of immunoglobulin G.

9. A use of protein C or activated protein C for maintaining and improving the microcirculation in a patient
10 suffering from circulatory disturbances.

10. A use of protein C or activated protein C for treating and preventing clinical equivalents to local and generalized Schwartzman reactions.

11. A use of protein C or activated protein C for
15 treating circulatory disturbances associated with a malign disease, an autoimmune disease, an immunocomplex disease, an infectious disease, or with shock syndrome.

12. A use of protein C or activated protein C for reducing inflammatory processes caused by an impaired
20 microcirculation in a patient.

13. A use of protein C or activated protein C for treating and preventing purpura fulminans caused by microbial infections.

14. A use of protein C or activated protein C for
25 treating and preventing chronic inflammatory diseases of the intestines.

15. A use of protein C or activated protein C for treating and preventing dermatitis ulcerosa.

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16. The use according to any one of claims 9 to 15, wherein the protein C or activated protein C is free of immoglobulin G.

17. A use of protein C or activated protein C in the
5 preparation of a medicament for maintaining and improving the microcirculation in a patient suffering from circulatory disturbances.

18. A use of protein C or activated protein C in the preparation of a medicament for maintaining and preventing
10 clinical equivalents to local and generalized Schwartzman reactions.

19. A use of protein C or activated protein C in the preparation of a medicament for maintaining circulatory disturbances associated with a malign disease, an autoimmune
15 disease, an immunocomplex disease, an infectious disease, or with shock syndrome.

20. A use of protein C or activated protein C in the preparation of a medicament for maintaining inflammatory processes caused by an impaired microcirculation in a
20 patient.

21. A use of protein C or activated protein C in the preparation of a medicament for maintaining and preventing purpura fulminans caused by microbial infections.

22. A use of protein C or activated protein C in the
25 preparation of a medicament for maintaining and preventing chronic inflammatory diseases of the intestines.

23. A use of protein C or activated protein C in the preparation of a medicament for maintaining and preventing dermatitis ulcerosa.

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24. The use according to any one of claims 17 to 23,
wherein the protein C or activated protein C is free of
immoglobulin G.

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