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(57) Abstract

Endotoxin sorbent comprising a sorbent material selected from the group consisting of polymyxin B and salts, immobilised on a solid phase support, the solid phase-polymyxin B sorbent having been treated with an anticoagulant capable of preventing or limiting fibrin deposition, whereby free anticoagulant binding sites are blocked which may be used to remove endotoxin from endotoxin-containing fluids e.g. from blood in the treatment of gram-negative bacterial shock.

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Endotoxin Removal

The present invention relates to the removal of endotoxin from endotoxin-containing fluids.

Endotoxin, which is part of the cell wall of gram negative bacteria, is implicated in gram-negative bacteraemia. This occurs with a frequency of from 4 to 12 per 1000 hospital admissions, and shock as a complication is seen in 16-44 % of these cases. The precise pathogenesis of gram-negative bacterial shock, also called septic shock, is unclear, but it is widely held that endotoxin is responsible for many of its features. The overall mortality from septic shock is approximately 60% and has remained at this level for many years, despite improvements in supportive care, more effective antimicrobial agents, the use of corticosteroids and other pharmacological approaches.

Endotoxin is also implicated in various other serious conditions including chronic and acute liver disease, chronic and acute renal disease, radiation sickness and heat stroke.

Polymyxin B is a cyclic polypeptide antibiotic which, in addition to its antimicrobial activity, has been known for many years to have potent anti-endotoxin properties. Polymyxin B (PB) binds stoichiometrically to endotoxin, and will neutralize many endotoxin-induced phenomena including mouse lethality, intravascular coagulation, and the Shwartzman phenomenon. The use of polymyxin B for its anti-endotoxin (rather than anti-microbial) properties has been proposed but the drug is unsuitable for repeated parenteral administration because it is both neurotoxic and nephrotoxic.

An alternative approach that has been proposed is to link polymyxin B to a solid phase support and to use

this as a specific sorbent. Polymyxin B has been linked to Sepharose (Trade Mark) and also to polystyrene fibres, and such systems have been found to be capable in vitro of removing endotoxin from endotoxin-containing solutions. The use of fibre-linked polymyxin B has also been described in haemoperfusion of endotoxin-treated dogs, with improved survival in the treated group. There are, however, a number of problems associated with haemoperfusion which reduce its clinical applicability, in particular biocompatibility problems resulting from destruction of cellular elements in the blood (including platelets) during the passage of the blood through the sorbent. The smaller the size of the sorbent particles, the greater is the problem of cell destruction.

The present invention is based on the surprising observation that plasmapheresis of endotoxin-containing blood can be carried out successfully if a solid phase support carrying immobilised polymyxin B is treated before use with an anticoagulant. This observation is surprising because we have attempted to use polymyxin B linked to a solid phase support to remove endotoxin in vivo using the technique of plasmapheresis, but such attempts have failed because of fibrin deposition. This deposition generally starts in the sorbent column within 5 to 10 minutes of starting plasmapheresis and within 15 to 20 minutes the plasmapheresis apparatus as a whole becomes clogged to such an extent that it can no longer function effectively. The fibrin is deposited throughout the plasmapheresis system, not just in the sorption column itself, and the deposition occurs despite the presence of the heparin present conventionally in the circulating plasma.

The present invention provides an endotoxin sorbent comprising polymyxin B immobilised on a solid

phase support, the solid phase-polymyxin B sorbent having been treated with an anticoagulant capable of preventing or limiting the deposition of fibrin to block free anticoagulant binding sites.

Polymyxin B may be used as such or in the form of a salt for example, the sulphate. Also there may be used a mixture of any two or more components selected from polymyxin B and salts thereof. Unless otherwise specified, the term "polymyxin B" is used herein to encompass all such possibilities.

The solid phase support may comprise any substance capable of immobilizing polymyxin B. It is thought that in some cases at least polymyxin B can be bound to a support via an amino group, and on this basis the support may be, for example, a solid material capable of forming a covalent bond with an amino group. The polymyxin B may be immobilised on a solid support either directly or via a coupling group. Accordingly, other suitable solid phase support materials are those capable of accepting coupling groups suitable for the immobilisation of polymyxin B. Examples of solid support materials are agarose and agarose derivatives, other carbohydrates, polystyrene and regenerated cellulose.

The solid phase support may be used in any of a variety of physical forms, such as, for example, those proposed for affinity chromatography in general, for example, beads, fibres, webs, membranes and hollow fibres. Beads, for example, agarose beads, may be used in columns. The size of beads preferred is generally in the range of from 45 to 165μ, but larger or smaller beads may be used, longer columns generally being required with larger diameter beads. Such bead size/column length parameters will be well-known to those skilled in the art of affinity chromatography.

Other physical forms of solid support, for example, fibres, for example, polystyrene fibres, webs, membranes and hollow fibres may be used analogously to their use in affinity chromatography generally.

The anticoagulant used to treat the polymyxin B sorbent must be capable of preventing or limiting the deposition of fibrin, either directly, that is to say, by influencing the conversion of fibrinogen to fibrin, or indirectly, that is to say, by influencing any one or more of the other steps involved in the intrinsic and extrinsic pathways of blood coagulation. Heparin is widely used and known to be effective in preventing fibrin deposition. It is readily available, relatively inexpensive, and substantially free from side effects. Accordingly, heparin is a preferred anticoagulant for the present invention, but any other anticoagulant having the property of interfering with fibrin deposition may be used.

In the heparin-type category are, for example, low molecular weight heparin derivatives and other heparin derivatives. Such derivatives may have advantages over native heparin, for example, with regard to the specificity of their anti-fibrin-deposition activity. Thus, for example, lower molecular weight derivatives may affect platelets less than native heparin does (see, for example, Salzman E.W., Rosenberg R.D., Smith M.H., Linden J.N., and Savreau L., J. Clinical Investigation (1981) 65 64-67). Other strongly acidic substances having a heparin-like anticoagulant effect may be used, for example, dextran sulphate. Hirudin may also be used.

Antithrombotic agents directed towards different aspects of the haemostatic process may be used as the anticoagulant, for example, inhibitors of intrinsic coagulation; and also platelet aggregation inhibitors

(antiplatelet agents), for example, adenyl compounds, for example, AMP adenosine and 2-chloroadenosine; dipyridamole and lidoflazine; prostacyclin and prostaglandin E; anti-inflammatory drugs, for example, aspirin, phenylbutazone, indomethacin, meclofenamate and hydroxychloroquine; membrane stabilizers, for example, local anaesthetics and many antihistamines; serotonin antagonists; sulphydryl inhibitors, for example, N-ethylmaleimide and p-hydroxymercuribenzoate; arginine esters and other guanidino compounds; various miscellaneous drugs which inhibit platelet aggregation, for example, clofibrate, methylxanthines, and monoamine oxidase inhibitors; and also plasminogen activators which enhance fibrinolysis, for example, plasmin, streptokinase, and urokinase.

The present invention further provides a process for producing anticoagulant-treated, solid phase-immobilised polymyxin B, which comprises immobilising polymyxin B on a solid phase support and subsequently treating the solid phase support carrying polymyxin B with an anticoagulant capable of preventing or limiting fibrin deposition under conditions such that free anticoagulant binding sites are blocked.

The anticoagulant treatment of the solid component may be carried out by incubating a sample of the polymyxin B-bearing solid phase support with a pre-determined volume of a solution comprising the anticoagulant, or an anticoagulant-containing solution may be passed over and/or through a sample of the polymyxin B-bearing solid phase component.

As indicated above, a wide variety of substances may be used as the solid phase support, and polymyxin B may be immobilised on the support in a conventional manner. In the case of agarose, available commercially in bead form as "Sepharose", the agarose is activated,

for example, using cyanogen bromide, and then reacted with polymyxin B, for example, according to the method of Issekutz (J. Immunological Methods 61 (1983) 275-281). According to this method, all reactions should be carried out in a pyrogen-free environment, that is to say, the buffers and reagents should be prepared in a sterile fashion using pyrogen-free water, glass-ware should be rendered as pyrogen-free as possible, and sterile plasticware should be used whenever practicable. Materials of the "Sepharose" type can be activated with cyanogen bromide in a conventional manner, or Sepharose itself can be obtained in activated form from the manufacturers (Pharmacia, Uppsala, Sweden). The activated material is generally swollen, washed, for example, in 0.1 M HCl, and suspended in a suitable buffer, for example, a sodium bicarbonate buffer, for example, 0.1 M $NaHCO_3$ containing 0.5 M NaCl, final pH 8.3. The resulting gel is preferably allowed to settle, excess buffer removed, and then polymyxin B, generally in the form of polymyxin B sulphate, is added to the gel, generally in the form of a solution in a buffer, for example, as described above. The mixture of polymyxin B and the agarose material, for example, Sepharose, is allowed to react for a suitable time, for example, overnight, at a temperature generally between $0^{o}C$ and room temperature, for example, in the range of from $2^{o}C$ to $18^{o}C$, for example, $4^{o}C$. Excess buffer is then generally discarded. At this stage it is conventional to incubate the gel with an agent capable of blocking remaining reactive groups, for example, ethanolamine.

In the case of other solid phase support materials, the polymyxin B may be immobilised in an analogous or conventional manner. The immobilisation of polymyxin B on polystyrene fibres has been described by

Endo et al (Abstracts of the 14th International Congress of Chemotherapy 1985, Kyoto, Japan, Abstract P-40-9, International Society of Chemotherapy, active halogen method), and also by Hanasawa et al (Therapeutic Apheresis: A critical look. Nose Y, Malchesky P.S., Smith JW, ISAO Press, Cleveland 1984 pp 167-170).

As indicated above treatment of the solid phase support on which polymyxin B has been immobilised with the anticoagulant may be carried out by incubating the solid component with a solution comprising the anti-coagulant. A range of temperatures may be used, for example, from 2°C to 25°C, but room temperature is generally suitable.

The amount of anticoagulant used and the time of incubation are such that free anticoagulant binding sites on the solid component are blocked. Clearly, the number of free anticoagulant binding sites will vary depending on the chemical constitution and physical form of the solid, and on the loading with polymyxin B. Having appreciated that the sites, and preferably as many as possible, should be blocked, it is a matter of simple trial and error to determine appropriate amounts of anticoagulant and incubation times. It is generally convenient to use an excess, preferably a considerable excess, of the anticoagulant compared with the amount generally incorporated in saline to prime plasmapheresis apparatus before use. In the case of heparin, this priming amount is generally within the range of from 5 to 20 units of heparin per ml of saline, for example, 10 units of heparin per ml.

As a guide, a suitable amount of anticoagulant in a plasmapheresis system for removing endotoxin from blood where the anticoagulant is heparin, the solid phase is Sepharose 4B beads having a diameter within

the range of from 45 to 165μ, and the amount of polymyxin B incubated with the Sepharose is 50 mg per 1.6 g dry weight, is 1,000 or more units of heparin per ml of Sepharose gel. Amounts of heparin as low as 100 units per ml of Sepharose gel will give satisfactory results for a short time but after 30 to 40 minutes, fibrin deposition in the extracorporeal circulation is observed. Accordingly, amounts of heparin in excess of 100 units per ml Sepharose gel are recommended, preferably 500 units or more, and especially 1,000 units or more, for example, 5,000 units or more, for example, 10,000 units.

The amounts of heparin required for other systems may be determined readily by simple trial and error but, as indicated above, it is generally convenient to use an excess, and preferably a large excess, and these considerations apply, mutatis mutandis , to other chosen anticoagulants.

As indicated above, instead of treating a batch of polymyxin B-bearing solid phase support with a predetermined volume of anticoagulant-containing solution, the solution may be passed over and/or through the batch of solid component. Again, the amount of anticoagulant to be used may be determined by simple trial and error and the considerations given above with regard to the amount of anticoagulant to be used also apply to the present embodiment of the invention.

There is preferably used an excess, especially a large excess, of anticoagulants especially heparin compared with the amount generally incorporated in saline for the purpose of priming plasmapheresis apparatus before use. The guide values given above for heparin may be followed. The perfusion of the polymyxin B-bearing solid phase support with anticoagulant-containing solution may be carried out as

a separate step before incorporation of the treated material in plasmapheresis apparatus, or the perfusion may be carried out in situ in plasmapheresis apparatus before use, that is to say, before connection to the subject to be treated.

The resulting anticoagulant-treated endotoxin sorbent may be prepared in advance and stored before use, for example, in pyrogen-free water containing a suitable preservative, for example, 25% (v/v) ethanol, or 0.2% (w/v) sodium azide, or it may be prepared as required, for example, in situ as described above.

As indicated above, the resulting anticoagulant-treated endotoxin sorbent may be incorporated in suitable plasmapheresis apparatus, and the present invention also provides an endotoxin sorbent according to the present invention in a form suitable for use in plasmapheresis apparatus, for example, in a chromatography column, and the present invention further provides plasmapheresis apparatus including an anticoagulant-treated polymyxin B-carrying solid phase support according to the present invention.

Surgical techniques for the insertion into a body of lines suitable for the removal and return of blood to be treated by plasmapheresis are known. The blood removed is generally pumped through a plasma separator within which the plasma is separated from the cellular components of the blood. In one method of plasmapheresis, the separated plasma is discarded, and the cellular components of the blood are returned to the body with replacement plasma or plasma substitute. In another method, the separated plasma is passed through sorbent means comprising a sorbent material to adsorb or absorb certain materials either non-specifically or specifically. The treated plasma is then reunited with the cellular blood components and

returned to the body. The sorbent material may be a non-specific sorbent material, for example, powdered charcoal, or may comprise material suitable for affinity chromatography. The sorbent material is conventionally present in the form of a column. Before carrying out plasmapheresis, the extracorporeal circuit should be primed with anticoagulant-containing solution, for example, heparin-containing saline, for example, saline containing 10 units of heparin per ml, or donor plasma containing 10 units of heparin per ml.

The present invention provides a method of removing endotoxin from endotoxin-containing blood, which comprises separating plasma from the cellular components of the blood, and treating the plasma with an endotoxin sorbent according to the present invention.

The separation of the plasma and its subsequent treatment with the endotoxin sorbent may be carried out in the same apparatus or the separated plasma may be treated in a separate endotoxin sorbent-containing apparatus.

The method of removing endotoxin from endotoxin-containing blood comprises, for example, subjecting the blood to plasmapheresis in apparatus comprising anticoagulant-treated, polymyxin B-bearing solid phase support according to the present invention as a sorbent means.

The second method of plasmapheresis described above may be used in the present invention, that is to say, the method whereby the separated plasma is treated with sorbent means, reunited with the cellular components and returned to the body. In this case, separated plasma is treated "on line" with the sorbent means.

Alternatively, plasma may be separated from

endotoxin-containing whole blood in a plasma separator and then treated on separate apparatus, for example, a column comprising an endotoxin sorbent according to the invention. The treated plasma may then if desired, be reunited with the cellular components of the blood, before return to the body. The method of the present invention includes both of these embodiments.

In plasmapheresis apparatus comprising sorbent means, the sorbent means may be any of the forms of anticoagulant-treated, polymyxin B-bearing solid phase support described above. The sorbent means is preferably in the form of a column, and the solid support is especially agarose or polystyrene. The anticoagulant is especially heparin.

The sorbent means may be pre-treated with the same anticoagulant as is used to prime the extra-corporeal circuit, or a different anticoagulant may be used. It is preferable to use the same agent both for pre-treatment of the solid support and for priming the extra-corporeal circuit. Heparin is preferably used.

Alternatively, as indicated above, the sorbent means incorporated in the plasmapheresis apparatus may be polymyxin B-bearing solid phase support that is treated <u>in situ</u> with anticoagulant. The amount of anticoagulant to be circulated through the solid component is indicated above, and should be an excess, especially a large excess compared with the amount of anticoagulant used conventionally for priming plasmapheresis apparatus, for example, in the case of heparin with agarose beads (in the form of Sepharose 4B), amounts of heparin in excess of 100 units per ml agarose gel are recommended, preferably 500 units or more, and especially 1,000 units or more, for example, 5,000 units or more, for example, 10,000 units.

The endotoxin-removal component of the

plasmapheresis apparatus may be used in conjunction with one or more other components suitable for removing substances from plasma, either specifically or non-specifically, that is to say, one or more other sorbent means. These other components may be used in series or in parallel with the endotoxin-removing component.

Examples of components for removing other substances from plasma are activated charcoal, broad-based sorbents used in the treatment of acute poisoning, chronic and acute renal failure; particles suitable for the removal of non-polar solutes from polar media for example, plasma; non-ionic macroreticular resins; sorbents with a particular attraction for lipid-soluble molecules; anionic exchange resins; sorbents suitable for the removal of protein-bound solutes, for example, bound antibodies having a selective removal action; artificial cells; and immobilised or encapsulated enzymes.

In some cases, it may be possible to immobilise another sorbent, for example, certain of those described above, on the same solid phase support as the polymyxin B. The present invention includes such mixed sorbents.

After use, some or all of the endotoxin may be removed from the sorbent using certain buffer systems, for example, a solution containing deoxycholate, for example, 1% (w/v) deoxycholate, thus regenerating the sorbent. For clinical use, however, it is advisable to use fresh rather than regenerated sorbent material.

The efficacy of the anticoagulant-treated polymyxin B bearing solid phase support as sorbent in the removal of endotoxin can be tested in vitro. There are various assays which can be used for determining endotoxin levels, for example, those using Limulus amoebocyte lysate. There are various

modifications of this assay, (see for example Cohen and McConnell (J. Infect. Dis 1984 50 916-924). Assay kits for Limulus amoebocyte lysate gelation tests are available commercially.

Polymyxin B may be assayed in a conventional manner, for example, using the standard bioassay which employs Bordetella bronchiseptica ATCC 4617 (see Sullman S.C. Polymyxins In: Reeves D.S., Philips I., Williams J.D. and Wise R., Laboratory methods in antimicrobial chemotherapy, 1st Ed. Churchill-Livingstone, 1978, 232-234).

The assay methods described above may also be used in connection with plasmapheresis.

It has been found that the polymyxin B remains substantially immobilised on the solid phase support and is not eluted off during treatment of plasma. This is an important advantage, as polymyxin B is neurotoxic and nephrotoxic.

It will be appreciated that, although the invention has been described above in terms of polymyxin B, it is applicable to all endotoxin sorbents. Accordingly, the present invention also provides endotoxin sorbent immobilised on a solid phase support, the solid phase-endotoxin sorbent having been treated with an anticoagulant capable of preventing or limiting fibrin deposition, whereby free anticoagulant binding sites are blocked, apparatus comprising the sorbent and a method of removing endtoxin from blood using the sorbent.

The following Examples illustrate the present invention. In the Examples, percentages are calculated as w/v, unless otherwise specified.

Example 1

a. Preparation of PB-Sepharose. (Polymyxin B-Sepharose).

The method described by Issekutz (loc cit) was followed. Sterile pyrogen-free plasticware was used whenever practicable. Glassware was washed thoroughly and heated at 160°C overnight before use. All buffers and reagents were prepared in a sterile fashion and were pyrogen-free. 1.6 g of cyanogen bromide activated Sepharose 4B (Pharmacia, Uppsala, Sweden) was washed extensively in 1 mM HCl and then resuspended in coupling buffer (0.1 M $NaHCO_3$ containing 0.5 M NaCl, final pH 8.3.) The gel was allowed to settle, and excess buffer was removed and replaced with 2.5 ml of the above coupling buffer containing 50 mg of PB (Calmic Medical, London England). (For control columns, PB was omitted). The tube containing the Sepharose was then sealed and incubated at 4°C, for 18 hours on a mechanical rotator. Next, the gel was centrifuged at 80 g and excess buffer removed. Ten ml of 1 M ethanolamine pH7 were added and the tube incubated for 2 hours at room temperature. After further gentle centrifugation the supernatant was removed and the gel washed in three alternating cycles of acetate buffer (0.1 M sodium acetate with 0.5 M NaCl final pH 4.0) and borate buffer (0.1 M sodium tetraborate with 0.5 M NaCl final pH 8.0). Finally, the gel was incubated on a rotary mixer for 1.5 hours at room temperature with heparinized sterile 0.9% saline using 10,000 units of heparin per ml of gel. The treated gel was washed with 0.9% saline and was stored at 4°C in 0.9% saline containing 0.02% sodium azide.

b. Endotoxin assay.

There was used a modified quantitative chromogenic Limulus amoebocyte lysate (LAL) assay, as described previously (Cohen and McConnell, loc cit). This method was further modified: the lysate was used at a 1 in 10 dilution in pyrogen-free water of the strength recommended by the manufacturers. (MA Bioproducts, Walkersville, Md, USA). The chromogenic substrate was S2423 (Kabi Diagnostica, Stockholm, Sweden).

c. Polymyxin B assay.

A standard bioassay which employs Bordetella bronchiseptica ATCC 4617 (Sullmann S.C. et al, loc. cit) was used.

d. Experimental procedure.

One millilitre of PB-Sepharose (or antibiotic-free control Sepharose: CON-Sepharose) was washed with 20 ml of 0.9% saline and incubated for 30 minutes at room temperature with 5000 mg of endotoxin derived from Escherichia coli 0127: B8 (Sigma, Poole, Dorset UK). The suspension was placed in a 2 ml sterile plastic syringe pre-packed with a small wad of glass wool. The column was washed with sterile 0.9% saline and six sequential 1 ml fractions collected over 30 minutes. The amount of endotoxin in each fraction was determined as described above. The experiment was carried out five times for both PB-Sepharose and CON-Sepharose.

Additionally, 10 ml of 0.9% saline were run three times through PB-Sepharose or CON-Sepharose columns, then bioassayed for PB.

Example 2

a. Plasmapheresis.

The surgical technique for insertion of indwelling carotid arterial and jugular venous lines, allowing perfusion in the unrestrained and conscious state was as previously described (Ryan C.J., Pusey C.D., Aslam M, Gaylor J.D., Maini R., and Courtney J.M., Artificial Organs Vol. 10, No. 2, 135-144 (1986).

Plasmapheresis apparatus is described, by way of example only, with reference to Figure 1, which is a diagrammatic representation. A rat 1 to which indwelling carotid arterial and jugular venous lines 2 and 3 respectively had been inserted was kept in a container 4 and given food and water ad libitum . The arterial blood is pumped, by a pump 5, through a specially designed plasma separator 6 into a reservoir 7. The filtered plasma is pumped by a pump 8 through a column 9 containing the sorbent material. The treated plasma is then reunited with the cellular blood components in a mixing means 10 and returned, pumped by pump 11, to the animal via jugular vein lines. Using a 3 pump system trans-membrane pressure can be maintained at less than 50 mm Hg with a filtration rate of 0.2-0.22 ml/min. By these means, one complete plasma volume (approximately 11-12 ml) can be treated in 50-60 minutes. The extracorporeal circuit is primed with approximately 3 ml of heparinized saline, obviating the need for donor blood or plasma. Previous studies in 15 normal animals have established that the system can be used repeatedly with no apparent side effects, nor marked effects on red cell, leucocyte or platelet counts. A sieving coefficient of >98% for the plasma membrane was obtained for C3, IgC and albumen.

b. Clearance of endotoxin.

Two groups of 3 rats were studied. Twenty-one hours following cannulation, lead acetate 50 mg/kg was

given intravenously (Selye H., Tuchweber B., and Bertok L., J. Bact. (1966) 91 884-890), and 45 minutes later the animals received endotoxin 10 µg/kg intra-arterially. After a further 15 minutes plasmapheresis as described in (a) above was begun and continued for 90 minutes, in one group using PB-Sepharose columns and in the other, CON-Sepharose. Plasma samples for endotoxin measurement were obtained simultaneously from 3-way taps [10 and 11] placed immediately beneath ('pre') and above ('post') the column, respectively.

To determine the effect of the lead acetate, two rats were studied as described above, except that they received 1 ml of 0.9% saline instead of endotoxin. Serial blood samples were obtained, and the animals were observed for 24 hours. In addition, an aliquot of each plasma sample was mixed in vitro with endotoxin to give a fixed final concentration of 50 ng/ml. These samples were assayed to determine if plasma containing lead acetate inhibited the detection of endotoxin in the LAL assay.

c. Effects of endotoxin removal.

An additional two groups of 4 rats received lead acetate and endotoxin as described above and then underwent plasmapheresis as described above using either PB-Sepharose or CON-Sepharose columns. Blood samples were obtained prior to injection of endotoxin, immediately before plasmapheresis, and at intervals thereafter for estimation of haemoglobin, packed cell volume and leucocyte count using a Coulter Counter model 2F; platelet counts were determined manually. The animals were observed for 24 hours.

Plasma from four PB-Sepharose perfused animals was used to determine if PB was eluted from the column in vivo . Samples obtained at 5, 10 and 15 minutes and at

90, 120 and 240 minutes were pooled and assayed for PB as described.

d. Statistical methods

In vitro clearance of endotoxin on PB-Sepharose or CON-Sepharose colums was compared by Students' T-test. The effect of plasmapheresis on leucocyte and platelet counts was examined by analysis of variance.

e. Results

1). In vitro studies.

Figure 2 illustrates the cumulative recovery of endotoxin in 6 x 1 fractions collected from CON-Sepharose and PB-Sepharose columns. Twenty-four per cent of the challenge dose (5,000 ng) remained on the control column, compared to 94% on the PB-Sepharose column ($p=0.05$). There was no detectable anti-microbial activity in the 0.9% saline washed through the PB-Sepharose column. The B. bronchiseptica assay could detect 10 µ/ml of PB.

2). In vivo studies.

Plasma endotoxin levels measured 'pre' and 'post' the PB-Sepharose and CON-Sepharose columns after injection of 10mg/g endotoxin followed by plasmapheresis are shown in Figure 3. In the control animals, the peak level reached at 20 minutes was 260 ng/ml. The 'pre' and 'post' column endotoxin levels were the same, indicating that there was no significant endotoxin clearance across the CON-Sepharose column. In animals perfused over PB-Sepharose, the peak 'pre' column level, which occurred at 30 minutes, was 50 ng/ml. Moreover, the peak 'post' column endotoxin concentration was 13 ng/ml, and for most of the

remainder of the experiment was undetectable. It is of interest that endotoxin was present continually in 'pre' column samples from PB-Sepharose treated animals, despite the fact that endotoxin was undetectable in 'post' column samples for most of the experiment. Thus, the absorbent was effectively removing the endotoxin presented to it, but it appeared that further endotoxin (albeit at a low level) was being generated from the animal. The most plausible explanation is that this 'fresh' endotoxin is derived from the intestinal microflora.

Control animals that received lead acetate alone remained well throughout 24 hours of observation, and serial blood samples revealed no change in haemoglobin, leucocyte or platelet counts. The presence of lead acetate in the plasma did not impair endotoxin detection in the LAL assay (data not shown). Pooled plasma samples from 4 PB-Sepharose animals contained no detectable PB activity.

The effect of PB-Sepharose perfusion on endotoxin-induced leucopenia is shown in Figure 4. The total leucocyte count fell from $7.15 \pm 3.9 \times 10^9$/L immediately prior to plasmapheresis, to $6.25 \pm 1.5 \times 10^9$/L after 4 hours. In animals perfused over CON-Sepharose, the leucocyte count fell from $5.7 \pm 1.3 \times 10^9$/1 to $2.5 \pm 1.0 \times 10^9$/L (p 0.01).

Figure 5 illustrates the effect of the procedure on the platelet count. Animals perfused over the PB-Sepharose were protected substantially from thrombocytopenia: the initial count was $550 \pm 37 \times 10^9$/L, and it fell to $430 \pm 29 \times 10^9$/L. In control animals, the count fell from $546 \pm 130 \times 10^9$/L to $108 \pm 45 \times 10^9$/L (p 0.001)

All four control animals died 5-10 hours after the procedure began. In contrast, all four animals perfused

over PB-Sepharose survived the 24 hour observation period.

Unequivocal evidence was obtained to show that the PB-Sepharose-treated animals were protected from endotoxin-induced leucopenia, thrombocytopenia and death.

It was important to establish if these observations were indeed the result of selective endotoxin removal or whether there could be an alternative explanation, in particular, whether PB was leaking from the column and neutralizing the endotoxin in the fluid phase. Using a sensitive bioassay for PB, there was found no detectable antimicrobial activity either in eluates obtained after extensive in vitro washing, or in pooled plasma samples from animals perfused over PB-Sepharose. The possibility cannot be excluded that very small amounts of PB were eluted from the column, but if so the antibiotic would be present at concentrations considerably less than the typical peak serum level of 2-8 mg/l (20-80,000 µ/l).

Claims

1. Endotoxin sorbent comprising a sorbent material selected from the group consisting of polymyxin B and salts thereof, immobilised on a solid phase support, the solid phase-polymyxin B sorbent having been treated with an anticoagulant capable of preventing or limiting fibrin deposition, whereby free anticoagulant binding sites are blocked.

2. Endotoxin sorbent according to claim 1, wherein the anticoagulant is heparin or a low molecular weight heparin derivative.

3. Endotoxin sorbent according to claim 1, wherein the solid phase support is selected from the group consisting of agarose, agarose derivatives, and polystyrene.

4. Endotoxin sorbent according to claim 1, wherein the solid phase support is agarose and the anticoagulant is heparin.

5. Process for producing an endotoxin sorbent, which comprises immobilising a sorbent material selected from the group consisting of polymyxin B and salts thereof on a solid phase support, and subsequently treating the solid phase support carrying polymyxin B or a salt thereof with an anticoagulant capable of preventing or limiting fibrin deposition, whereby free anticoagulant binding sites are blocked.

6. Process according to claim 5, wherein the anti-coagulant is heparin or a low molecular weight heparin derivative.

7. Process according to claim 6, wherein the solid phase support is selected from the group consisting of agarose, agarose derivatives, and polystyrene.

8. Process according to claim 5, wherein the solid phase support is agarose and the anticoagulant is heparin.

9. In a plasmapheresis apparatus the improvement which comprises providing sorbent means for the removal of endotoxin from plasma, wherein the endotoxin sorbent means comprises a sorbent material selected from the group consisting of polymyxin B and salts thereof, immobilised on a solid phase support, the solid phase-polymyxin B sorbent having been treated with an anticoagulant capable of preventing or limiting fibrin deposition, whereby free anticoagulant binding sites are blocked.

10. Plasmapheresis apparatus according to claim 9, wherein the solid phase support is agarose and the anticoagulant is heparin.

11. Plasmapheresis apparatus according to claim 9, which also comprises one or more other sorbents.

12. Plasmapheresis apparatus according to claim 9, which comprises one or more other sorbent means selected from the group comprising of activated charcoal; broad-based sorbents used in the treatment of actur poisoning or chronic or acute renal or liver disease; particles suitable for the removal of non-polar solutes from plasma; non-ionic macroreticular resins; sorbents having a particular attraction for

lipid-soluble molecules; anionic exchange resins; sorbents suitable for the removal of protein-bound solutes; immunoadsorbents; and immobilised encapsulated enzymes.

13. Method of removing endotoxin from endotoxin-containing blood, which comprises subjecting the blood to plasmapheresis in plasmapheresis apparatus including sorbent means for the removal of endotoxin from plasma, wherein the endotoxin sorbent means comprises a sorbent material selected from the group consisting of polymyxin B and salts thereof immobilised on a solid phase support, the solid phase-polymyxin B sorbent having been treated with an anticoagulant capable of preventing or limiting fibrin deposition, whereby free anticoagulant binding sites are blocked.

14. Method of removing endotoxin from endotoxin-containing blood according to claim 13, wherein the solid phase support is agarose, an agarose derviative or polystyrene.

15. Method of removing endotoxin from endotoxin-containing blood according to claim 13, wherein the anticoagulants heparin or a low molecular weight heparin derivative.

16. Method of removing endotoxin from endotoxin-containing blood according to claim 13, wherein the solid phase support is agarose and the anticoagulant is heparin.

17. Method of removing endotoxin from endotoxin-containing blood, which comprises separating plasma from the blood, and treating the plasma with an

endotoxin sorbent comprising polymyxin B or a salt thereof immobilised on a solid phase support, the solid phase-polymyxin B sorbent having been treated with an anticoagulant capable of preventing or limiting the deposition of fibrin whereby free anticoagulant binding sites are blocked.

18. Endotoxin sorbent immobilised on a solid phase support, the solid phase-endotoxin sorbent having been treated with an anticoagulant capable of preventing or limiting fibrin deposition, whereby free anticoagulant binding sites are blocked.

FIGURE 1

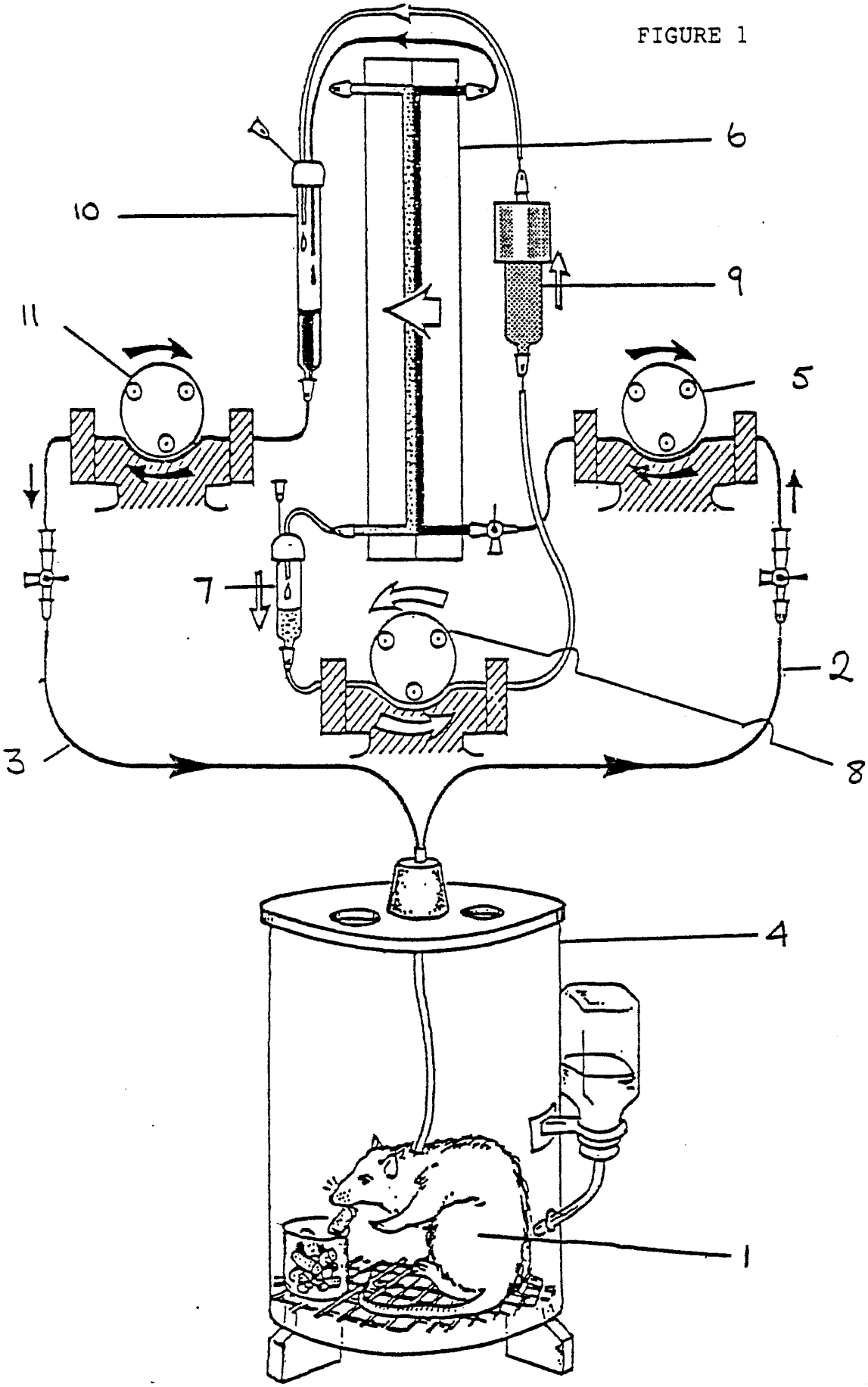


FIGURE 2

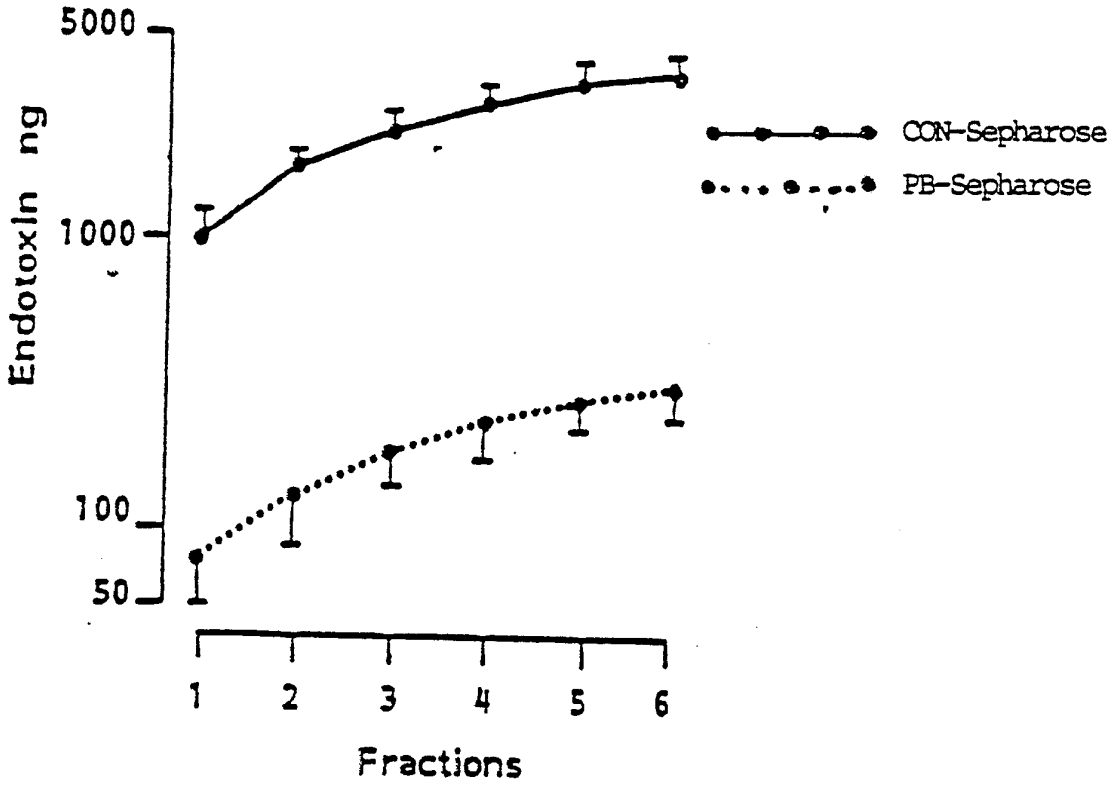

5000
1000
100
50
Endotoxin ng
1
2
3
4
5
6
Fractions
CON-Sepharose
PB-Sepharose

FIGURE 3

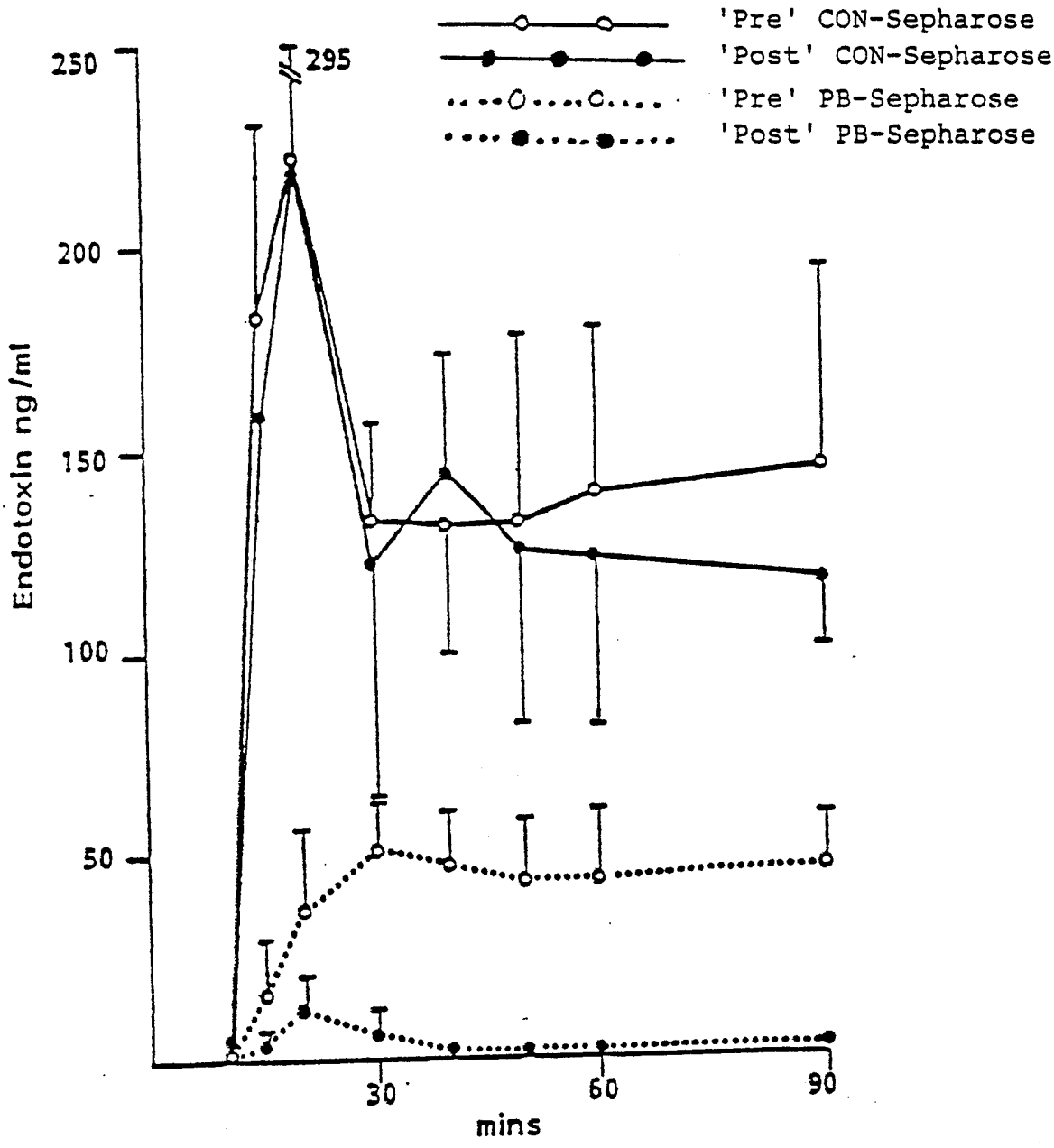

'Pre' CON-Sepharose
'Post' CON-Sepharose
'Pre' PB-Sepharose
'Post' PB-Sepharose
250
295
200
150
100
50
Endotoxin ng/ml
30
60
90
mins

FIGURE 4

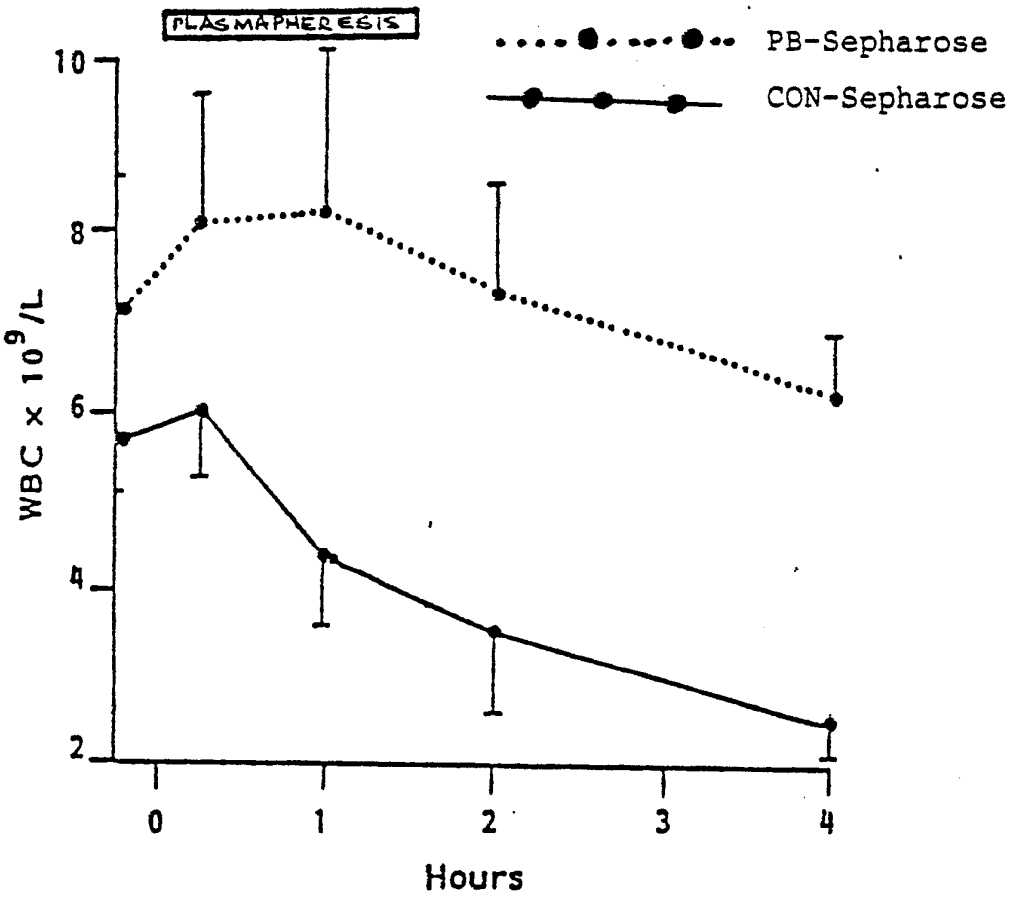
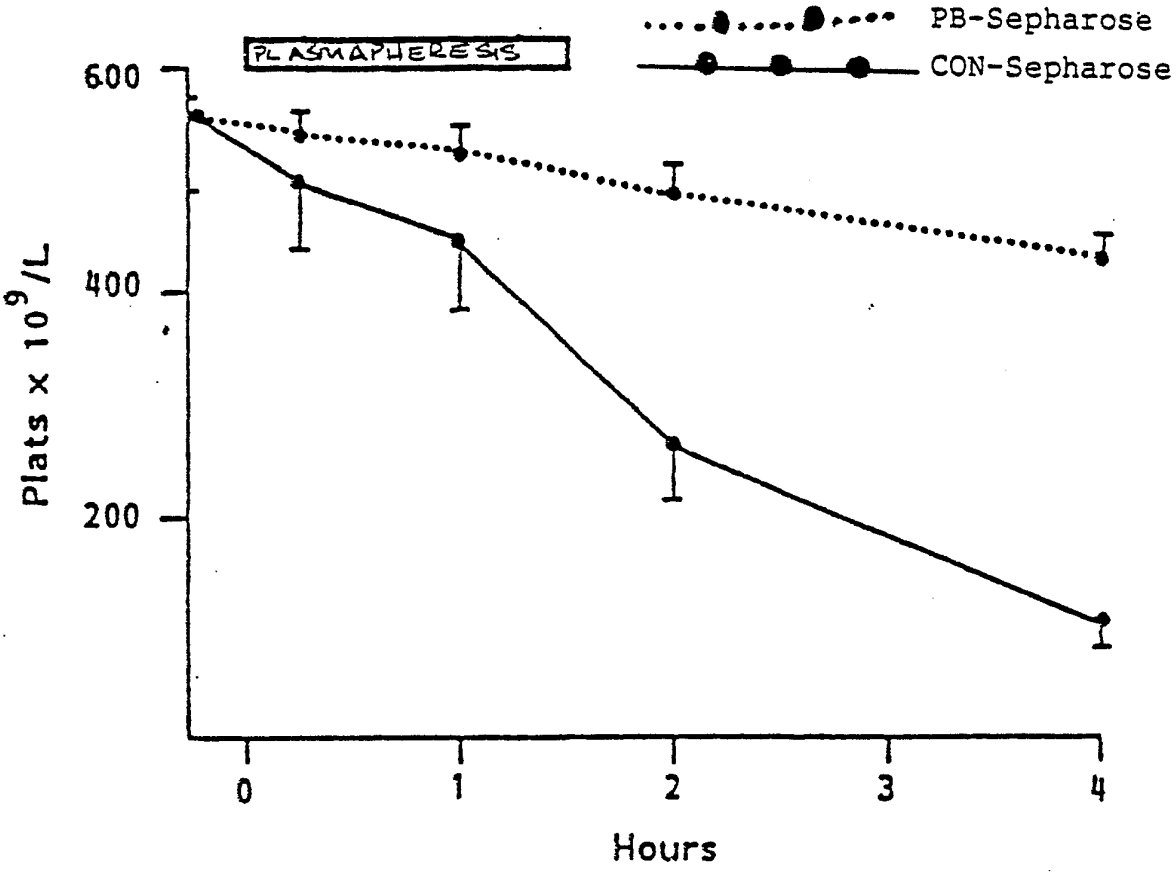

PLASMAPHERESIS
PB-Sepharose
CON-Sepharose
WBC x 10^9/L
10
8
6
4
2
0
1
2
3
4
Hours

FIGURE 5


PB-Sepharose
CON-Sepharose
PLASMAPHERESIS
600
400
200
Plats x 10^9/L
0
1
2
3
4
Hours

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 87/00406

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: B 01 J 20/32; A 61 M 1/36 // A 61 K 35/14

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	A 61 K; B 01 J; A 61 M

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	EP, A, 0110409 (KANEGAFUCHI KAGAKU KOGYO KABUSHIKI KAISHA) 13 June 1984 see page 5, line 10 - page 6, line 10; page 8, lines 3-19; page 9, lines 1-23; page 15, example 5; page 19, example 18; page 20, example 19; page 23, example 35; test example 1; claims 1,6-8	18
A		1,2,9
	--	
A	EP, A, 0129786 (TORAY INDUSTRIES, INC.) 2 January 1985 see page 2, line 14 - page 3, line 14; page 3, line 25 - page 4, line 17; page 4, line 32 - page 5, line 4; page 9, example 4; claim 1	1,3
	--	
A	Chemical Abstracts, vol. 106, no. 6, 9 February 1987 (Columbus, Ohio, US) J. Cohen et al.: "Protection from endotoxemia: a rat model of plasma-pheresis and specific adsorption	./.

[10] Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
10th September 1987	13 OCT 1987
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. VAN MOL

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	with polymyxin B.", see page 354, abstract no. 38403d, & Recent Adv. Chemother., Proc. Int. Congr. Chemother., 14th 1985 (Antimicrobial Sect. 3), 2073-4 (Eng)	1,3
	--	
A	Chemical Abstracts, vol. 105, 21 July 1986 (Columbus, Ohio, US) M.J. Rogers et al.: "Comparison of the binding of gram-negative bacterial endotoxin by polymyxin B sulfate, colistin sulfate and colistin sulfomethate sodium", see page 22, abstract no. 17881x, & Infection (Munich) 1986, 14(2), 79-81 (Eng)	1,18

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/GB 87/00406 (SA 17436)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 23/09/87

The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A- 0110409	13/06/84	JP-A-	59102436	13/06/84
		AU-A-	2183283	07/06/84
		US-A-	4576928	18/03/86
		US-A-	4637994	20/01/87
		EP-A-	0225867	16/06/87
		JP-A-	59156431	05/09/84
		JP-A-	59193135	01/11/84
		JP-A-	59196738	08/11/84
		JP-A-	60077769	02/05/85
EP-A- 0129786	02/01/85	JP-A-	60005166	11/01/85
		US-A-	4661260	28/04/87