

## Organ-Protective Solutions

### Description

The invention relates to the use of poly-sulfatized glycosamino-glycans with a sulfur content of at least 12.5% for the manufacture of pharmaceutical preparations for inhibiting interferon  $\gamma$ -induced up-regulation of MHC Class I, MHC Class II proteins and ICAM 1. The invention furthermore relates to organ-protective solutions containing poly-sulfatized glycosamino-glycans and a process for ex vivo protection of transplant organs.

The use of glycosamino-glycans and particularly of heparins and heparinoids for the manufacture of pharmaceutical preparations for treatment of circulatory disturbances is well known.

More recently, the use of glycosamino-glycans for a series of additional ailments has been described. Thus US 5,236,910 claims the use of glycosamino-glycans in treatment of diabetic nephropathy and neuropathy. The use of low-molecular heparins for the same indication has been described by van der Pijl et al (J Americ Soc Nephrol, 1997, 8: 456-462).

US 5,032,679 claims the use of glycosamino-glycans for inhibiting the proliferation of smooth muscle cells and related diseases.

In US 4,966,894 poly-sulfatized heparins are claimed for treatment of diseases caused by retro viruses.

Gralinski et al have described modulation of the complement system with poly-sulfatized heparins.

In Clin Exp Immunol (1997, 107: 578-584) the antagonisation of the inflammation-promoting effect of interferon  $\gamma$  with heparin, heparan sulfate or heparin-like

molecules has been studied by Douglas et al. Heparin is in a position to influence the immunogenic effect of interferon  $\gamma$ .

When organs are transplanted, undesirable rejection reactions are a frequent occurrence. To prevent such rejection reactions, a number of different paths have been tread. Thus and foremost, the histo-compatibility antigens of donor and recipient have been compared. Only those organs are transplanted where donor and recipient are maximally identical or who have very similar histo-compatible antigens.

Nonetheless, undesirable organ rejection persistently results. Here, for instance, acute renal allograft rejection occurs where recognition of the allo-MHC antigens by T lymphocytes is the main effect which entails lysis of the tubular cells. It further entails with the so-called "graft versus host reaction" a strong reaction to the recipient from the donor's immune cells, transplanted with the organ. Cytotoxic T cells and antibodies are formed against the host organism.

In order to further reduce the risk of transplant rejection, the organs are immediately refrigerated after their removal and stored under an organ protection solution. Moreover, the recipient is given medication suppressing the recipient's immune defence.

In the literature, a whole series of organ-protective solutions have been described. Thus Collins et al (Lancet, vol 2, 1969: 1219) describe intracellular electrolyte solutions for conserving organs. Sacks SA (Lancet, vol 1, 1973: 1024) described solutions which have an osmotically stabilising effect. ATP-MgCl<sub>2</sub>, AMP-MgCl<sub>2</sub> and inosine have been described as propitious agents in such solutions (Siegel, NJ et al, Am J Physiol, 254: F530, 1983; Belzer et al, Transpl Proc 16: 161, 1984). US 4,920,004 claims a solution containing mannitol, adenosine and ATP-MgCl<sub>2</sub>. US 4,798,824 and US 4,873,230 claim an organ-protective solution containing hydroxy-ethyl starch. In US 4,879,283 a solution is claimed containing KH<sub>2</sub>PO<sub>4</sub>, MgSO<sub>4</sub>, adenosine, allopurinol, raffinose and hydroxy-ethyl cellulose. It is also known as the University of Wisconsin solution (= UW solution). This solution was described for the successful conservation of liver, kidney and heart (Jamieson et al, Transplantation, vol 46, 1988: 517; Ploeg et al, Transplantation, vol 46, 1988: 191; and Wicomb WN,

Transplantation, vol 47, 1988: 733). In US 5,200,398 an additional additive in such protection solutions is described with glucuronic acid, its salts and esters.

Despite the success attained in protecting organs for transplantation and suppression of undesirable organ rejection, there still remains a need for further improvements.

The object therefore was to achieve further improvement both in organ protection before transplantation as well as during it as well as further reducing the danger of organ rejection.

This object has been accomplished by using poly-sulfatized glycosamino-glycans with a sulfur content of at least 12.5% (by weight) for manufacture of pharmaceutical preparations for inhibiting interferon  $\gamma$ -induced up-regulation of MHC Class I, MHC Class II proteins and ICAM 1.

The invention relates in addition to organ-protective solutions containing a quantity of a poly-sulfatized glycosamino-glycane with a sulfur content of at least 12.5% for appropriate pharmaceutical preparations effectively maintaining cell integrity and vitality.

The pharmaceutical preparations manufactured for use according to the invention, including for instance organ-protective solutions as well, can contain the above-cited compounds as free compounds in the form of their physiologically active salts or esters, their tautomer and/or isomer forms or in the form of the combination of free compounds and various salts. Notable as propitious physiologically effective salts are, for instance, the Na, Ca or Mg salts. Likewise, salts with organic bases like diethylamine, triethylamine or triethanolamine are suitable. The pharmaceutical preparations can advantageously contain at least one free substance or at least one compound in the form of its salt or mixtures of the same.

The poly-sulfatized glycosamino-glycans (= muco-poly-saccharides) used according to the invention are negatively charged poly-saccharides (= glycans) consisting of variously linked units of di-saccharides in which, for instance, one molecule of a so-called uronic acid like D glucuronic acid or L iduronic acid is

glycosidically combined with 3rd or 4th position of an amino-sugar like glucosamine or galactosamine. At least one of the sugars in the di-saccharide possesses a negatively charged carboxylate or sulfate group which can be bound by means of an oxygen or nitrogen atom. With the uronic acids as well as with sulfuric acid ester groups the glycosamino-glycanes react strongly acidic. These acidic reacting groups are in part already present in nature but are valuable in order to attain the sulfatization degree required by the invention by synthetically introducing, for instance sulfatization into the compound. As sulfatization methods, literature describes, as examples, sulfatization with sulfuric acid and sulfur-chloric acid (US 4,727,063, US 4,948,881), sulfatization with sulfur-chloric acid in pyridine (Wolfson et al, J Am Chem Soc, 1953, 75, 1519) or sulfatization with nitrous acid (Shively et al, Biochemistry, vol 15, no 18, 1976: 3932). Further methods are well known to those versed in the art. Examples are the use, for sulfatization, of natural glycosamino-glycanes like heparin, heparan sulfate, keratan sulfate, dermatan sulfate, chondroitin or chondroitin sulfate. The structure of heparan sulfate corresponds to that of heparin with the difference that by contrast to the latter it possess fewer N and O sulfate groups and more n acetyl groups.

Glycosamino-glycanes can easily be isolated from animal tissue such as intestinal mucosa or from the ears of pigs and cattle. The tissue used for isolation of the glycosamino-glycanes is, for instance, autolysed and extracted with alkali. Next the protein is left to coagulate and is precipitated, e.g. by acidifying it. After administering the precipitate in a polar non-aqueous solution like ethanol or acetone, the fats are removed by extraction with an organic solvent. By means of proteolytic digestion, the proteins are ultimately removed and thus the glycosamino-glycanes recovered. Charles et al (Biochem J, vol 30, 1936: 1927-1933) and Coyne E in Chemistry and Biology of Heparin (Elsevier Publishers, North Holland, NY, Lunblad RL, ed 1981) describe methods for isolating heparin, for example.

These glycosamino-glycanes isolated from natural sources can preferably be given another derivation by poly-sulfatizing them as described, by way of example, in US 5,013,724 or as described above. By means of such poly-sulfatization, the glycosamino-glycanes then show a sulfur content of 6-15% (by weight). For use according to the invention or for pharmaceutical preparations, poly-sulfatized glycosamino-glycanes are selected having a sulfur content of at least 12.5% (by

weight). Preferably, such poly-sulfatised glycosamino-glycanes have a sulfur content of 13-16% (by weight), preferably of 13-15% (by weight), particularly good is from 13.5-14.5% (by weight). These substances are used for manufacture of the pharmaceutical preparations which are suitable for inhibiting interferon  $\gamma$ -induced up-regulation of the MHC Class I, MHC Class II proteins and ICAM 1. Preference is given to using such substances in a physiologically effective quantity for treatment and prevention of diseases associated with interferon  $\gamma$ -induced up-regulation of MHC Class I, MHC Class II proteins and ICAM 1. Among derivatives of the substances one also includes compounds which improve the application properties of the poly-sulfatised glycosamino-glycanes used in regard to their effect, their stability and their elimination, particularly from the body.

By preference, heparins and/or dermatan sulfate with average molecular weight of some 1000 to 2000 Dalton should be used, preferably between 1500 and 9000 Dalton and particularly between 2000 and 9000 Dalton and, optimally, between 2000 and 6000 Dalton. Particularly advantageous are low-molecular poly-sulfatised heparins and/or dermatan sulfates in the form of free acid or in the form of a salt with physiologically tolerable bases or mixtures made from such compounds. Such substances have a slight anti-coagulating effect and are therefore particularly suitable for treatment and prevention when used according to the invention. Preferred salts of poly-sulfatised glycosamino-glycanes are, for instance, sodium, calcium and magnesium salts.

Low-molecular glycosamino-glycanes, for instance, low-molecular heparins and/or dermatan sulfates can be manufactured by a series of methods. The production of low-molecular heparins via de-polymerisation with the aid of nitrous acid is described, for instance, in EP-B-0 037 319 or in Biochemistry, vol 15, 1976: 3932. The manufacture of low-molecular heparin or low-molecular glycosamino-glycanes can also be accomplished with enzymes (Biochem J, vol 108, 1968: 647), with sulfuric acid and sulfuric chloric acid (FR No 2,538,404, simultaneous sulfatisation), with periodate or with physical methods such as  $\gamma$  radiation (EP-A-0 269 937) or ultra sound (Fuchs et al, Lebensm Unters Forsch, vol 198, 1994: 486-490).

Additional uses according to the invention lie in organ-protective solutions. By means of propitious administration of the poly-sulfatised glycosamino-glycanes to

organ-protective solutions the storage of the organs after removal from the donor organism, i.e. ex vivo can be further enhanced by inhibiting the interferon  $\gamma$ -induced up-regulation of the MHC Class I, MHC Class II proteins and ICAM 1. Preferably, the organs should be refrigerated, as is known to those versed in the art.

A series of such solutions as described above are known from literature. The solutions generally contain salts, buffers, substances supposed to stabilise the organs osmotically or which are supposed to prevent oxidation such as sugar or sugar alcohols, proteins, amino acids, lower carboxylic acids, purines, pyrimidines or pharmaceutical agents. As examples for such substances, the following might be mentioned: raffinose, glucose, potassium di-hydrogen phosphate, di-potassium hydrogen phosphate, potassium chloride, potassium hydrogen carbonate, sodium hydrogen carbonate, magnesium sulfate, magnesium chloride, adenosine, albumin, mannitol, citrate, verapamil, allopurinol, insulin, dexamethason, hydroxy-ethyl starch, glutathion or glucuronic acid.

The invention's intended use in the organ-protective solutions leads to making it possible to transplant the organs in better condition or store them for a longer period of time than has been usual so that rejection reactions can be reduced.

In order to further reduce the risk of organ rejection, the poly-sulfatised glycosamino-glycanes can be administered to transplant patients or transplant donors, where possible, orally or parenterally prior to transplantation. Post-operative treatment of the patients with the substances is also feasible.

The poly-sulfatised glycosamino-glycanes are contained in the organ-protective solutions or in other pharmaceutical preparations in quantities of 10 mg/l to 10,000 mg/l, preferably in quantities of 10 mg/l to 5000 mg/l, preferably still in quantities of 50 mg/l to 3000 mg/l and most preferably in quantities of 100 mg/l to 3000 mg/l. Additionally, 5 to 100 g/l of an osmotically stabilising substance containing hydroxy-ethyl starch are an advantage.

Further advantageous organ-protective solutions have the following composition:

- a) 10 mg/l to 10,000 mg/l poly-sulfatised glycosamino-glycanes with a sulfur content of at least 12.5% (by weight), 5 to 100 g/l of hydroxy-ethyl starch and 5 to 100 mmol

raffinose or b) 10 mg/l to 10,000 mg/l poly-sulfatised glycosamino-glycanes with a sulfur content of at least 12.5% (by weight), 5 to 100 g/l of hydroxy-ethyl starch, 5 to 100 mmol raffinose, 5 to 40 mmol of  $\text{KH}_2\text{PO}_4$ , 1 to 50 mmol  $\text{MgSO}_4$ , 1 to 50 mmol adenosine, 0.5 to 5 mmol allopurinol or 1 to 10 mmol glutathion.

For treatment of patients, poly-sulfatised glycosamino-glycanes can be used together with usual formula process substances.

The pharmaceutical preparations intended for use according to the invention can be administered in the usual way orally or parenterally (subcutaneously, intravenously, intramuscularly, intraperitoneally) with oral or intravenous applications being preferred.

Dosage depends on the patient's age, condition and weight and on the type of application.

The glycosamino-glycanes are most profitably applied in a dosage of 0.1 to 500 mg/kg of body weight per day. In case of parenteral application, the glycosamino-glycanes are best administered in a dosage of 0.1 to 30 mg/kg of body weight per day, in case of oral application in a dosage from 0.2 to 500 mg/kg of body weight per day where the dosage administered can be applied in one dose or in several doses. Also mixtures of, for instance, at least one low-molecular heparin and/or its poly-sulfatised derivative and/or at least one low-molecular dermatan sulfate and/or its poly-sulfatised derivative are administered in a dosage of 0.1 to 30 mg/kg of body weight per day in parenteral application or in a dosage of 0.2 to 500 mg/kg of body weight per day in case of oral application.

Among pharmaceutical preparations containing the poly-sulfatised glycosamino-glycanes for treatment and prevention of diseases in connection with organ transplantation one in principle includes the usual galenic application forms for oral or parenteral application, whether solid or liquid, such as tablets, coated tables, capsules, powder, granulate, dragees, suppositories, solutions or suspensions. They are manufactured in the usual way. The active agents can be processed with the usual galenic process materials like tablet binders, filling, preservatives, tablet explosives, flow regulator substances, softeners, moisturisers, dispersion agents,

emulsifiers, solvents, retardants, anti-oxidants and/or propellant gases (cf H Sucker et al: *Pharmazeutische Technologie*, Thieme-Verlag, Stuttgart, 1991). The application forms thus obtained contain the active agent normally in a quantity of 0.1% to 90% by weight.

For production of tablets, coated tablets, dragees and hard gel capsules, polysulfated glycosamino-glycanes can also be processed with pharmaceutically inert, inorganic or organic excipients. As such excipients for tablets, dragees and hard gel capsules one can use lactose, maize starch, or derivatives thereof, talcum, stearic acid or their salts. For soft gel capsules, vegetable oils, waxes, fats, semi-solid and liquid polyols are suitable as excipients.

For manufacture of solutions and syrups, suitable excipients are, for instance, water, polyols, sucrose, invert sugar, glucose and so forth. For injection solutions, water, alcohols, polyols, glycerine, vegetable oils are suitable as excipients. For suppositories, suitable excipients are natural or hardened oils, waxes, fats, semi-liquid or liquid polyols and so forth.

The pharmaceutical preparations can additionally contain preservatives, solvent agents, stabilisers, moisturisers, emulsifiers, sweeteners, colouring, aromatic agents, salts for modification of osmotic pressure, buffers, protective layers and/or antioxidants.

## Examples

For experiments, PTEC (= proximal tubular epithelial cells) and HUVEC cells (= human umbilical vein endothelial cells) can be used. The PTEC cells were cultivated with the method described by Detrisac et al (*Kidney Int* 25, 1984: 383). The PTEC cells were settled in tissue culture bottles coated with Collagen I (Sigma, St Louis MO) and foetal calf serum (= FCS, Gibco BRL). The culture medium consisted of "Eagle's Medium" modified according to Dulbecco and Ham's F12 Medium (both from Gibco BRL) in a 1:1 ratio, supplemented with insulin (5 µg/ml), transferrin (5 µg/ml), selenium (5 ng/ml), hydrocortisone (36 ng/ml), tri-iodothyronin (4 pg/ml), epidermal growth factor (10 ng/ml) and penicillin/streptomycin [(5IU/ml, 5 µg/ml) all from Sigma]. The cell lines were obtained from various sources such as biopsies of

pre-transplant tissue, allografts not usable for transplants and from normal surgical kidney samples. The experiments were carried out with cells from the passages 1 through 4. PTEC was characterised by means of a positive marking with an epithelial membrane antigen (= EMA, Dako Glostrup, Denmark) and with adenosine-deaminase-binding protein (graciously supplied by Dr Dinjens, University Hospital, Maastricht, Netherlands).

The HUVEC cells (= endothelial cells of human umbilical cord veins) were recovered from fresh umbilical cords with the method described by Jaffe et al (Culture of Human Endothelial Cells Derived from Umbilical Veins. Identification by Morphologic and Immunologic Criteria. J Clin Invest 52, 1973: 2745-2756). The procedure is briefly described as follows:

The endothelial cells were isolated from umbilical cord veins by digestion with Collagenase V (Sigma, St Louis MO, 20 minutes at 37°C). Following, the veins were rinsed with sterile culture medium and the endothelial cells were collected. The culture medium consists of Medium 199 (Gibco BRL) supplemented by 15% foetal calf serum (= FCS), endothelial cell growth factor and the antibiotics penicillin and streptomycin. The cells were cultivated in 25 cm<sup>2</sup> bottles coated with 1% gelatin (Sigma, St Louis MO).

All experiments were carried out with cells from the third to sixth cell culture passage. The HUVEC cells were characterised by their positive colouring with the Factor VIII-related antigen (Dako, High Wycombe, UK) and the endothelial marker EN4 (CD31).

#### IFN $\gamma$ stimulation, heparin and sodium chlorate treatment

Confluent mono-layers of PTEC and HUVEC cells were treated with trypsin and disseminated in 24-well plates. When confluence was attained, the cells were stimulated with IFN  $\gamma$  (Sigma, St Louis MO) for 72 hours in the presence or absence of different heparinoids in varying concentrations contained in different heparins such as Heparin-Braun® from Braun-Melsungen, Melsungen, Germany, low-molecular Heparin Fragmin® P from Pfrimmer Kabi, Erlangen, Germany and modified low-molecular heparin from Knoll AG, Ludwigshafen, Germany.

In some experiments, the culture medium was supplemented with sodium chlorate in order to inhibit sulfatation with cell-bound heparan sulfate proteoglycan (= HSPG). The chlorate was used in concentrations of 50-150 mM. It was added to the medium 24 hours before the administration of IFN  $\gamma$ . The stimulation with IFN  $\gamma$  was carried out in the presence of chlorate. Sodium chloride was used as osmolar control. The cultivated cells were after cultivation recovered with a trypsin EDTA treatment for flow cytometry.

#### Flow cytometry

The cells of the various deposits were brought together and subsequently split up in two test tubes and washed. Antibodies that do not bond to cell isotopes and which were linked with RPE and FITC (from Dako, Glostrup, Denmark) and Cy-5 (from Dianova, Hamburg Germany) were administered to the first test tube. This deposit was used as negative control for the FACS background. The cells in the second deposit were marked with antibodies against MHC Class I (RPE conjugated, W6/32, Dako), against MHC Class II (Cy-5 conjugated, CR3/43, Dianova) and against ICAM 1 (FITC conjugated, Dianova) in concentrations as indicated by the manufacturers. After incubating the cells for 30 minutes at 4°C, the latter were washed and analysed with flow cytometry (FACSscan, Becton Dickinson). At least 10,000 positive events were analysed. The results were expressed as mean fluorescence intensity (= mean fluorescence intensity = MFI).

#### Dot-blot analyses

For dot-blot analyses, narrow strips of a nitro-cellulose membrane were prepared to which 1  $\mu$ l heparin, Fragmin and various other N de-sulfated N acetylated glycosamino-glycans (= GAGs, all in a concentration of 1 mg/l) were applied. After drying, the strips were fixed with 1% glutaraldehyde + 0.5% cetyl-pridinium chloride in order to prevent GAG losses and subsequently washed with Tris buffer. The finished strips were finally exposed on a Kodak film.

#### Statistical analysis

The significance of the changes in the antigen expression were determined with the aid of Student's T test. P values < 0.05 were considered as being significant.

## Results

The expression of the MHC Class I, Class II and ICAM 1 proteins was modulated in PTEC by the IFN  $\gamma$  depending on dosage. The MHC Class I and ICAM 1 expression was regulated up by a concentration of 50 ng/ml of IFN  $\gamma$ . In addition, with the same concentration of IFN  $\gamma$ , the MHC Class II expression in PTEC was raised (see Figure 1). Corresponding results were obtained with the cultivated HUVEC cells.

In order to study heparin's influence on IFN  $\gamma$ 's ability to modulate the MHC and ICAM 1 expression, both the HUVEC and the PTEC cultures were stimulated with IFN  $\gamma$  in the presence or absence of heparin. The administration of heparin in concentrations of 0.03 to 3 mg/ml to HUVEC cultures completely prevents the up-regulation of MHC Class I and ICAM 1 proteins caused by the 100 ng/ml IFN  $\gamma$ . Likewise, the induction of MHC Class I proteins was suppressed in these cells by the administration of heparin. Heparin itself had in the absence of IFN  $\gamma$  no influence on the expression of the three antigens studied (Figure 2).

In comparable experiments with PTEC it could likewise be shown that with 100 ng/ml of IFN  $\gamma$  induced "up-regulation" of ICAM 1 and induction of MHC Class II proteins can be inhibited by heparin. The up-regulation of MHC Class I proteins with IFN  $\gamma$  could not, however, be influenced with heparin. In order to test whether up-regulation of the MHC Class I proteins induced by negligible IFN  $\gamma$  concentration can be blocked by heparin, the same experiments were carried out with 10 ng/ml IFN  $\gamma$ -stimulated cells. It turned out that heparin itself in concentrations of 3 mg/ml only has a marginal influence of up-regulation of MHC Class I with IFN  $\gamma$  (see Figure 3a). By contrast, both induction of MHC Class II as well as up-regulation of ICAM 1 are significantly inhibited by 0.03 mg/ml heparin ( $p < 0.01$ ) (see Figures 3b and c). In order to study the influence of the sulfatization degree of the heparin on inhibition of the MHC and ICAM 1 expression after stimulation with IFN  $\gamma$ , various heparinoids were investigated in this test system (Table 1). The heparinoids were all tested in a concentration of 3 mg/ml for their ability to inhibit MHC Class I and ICAM 1 after

stimulation with IFN  $\gamma$ . The MHC Class II tests were carried out with a stimulation with 10 ng/ml IFN  $\gamma$ . Comparable to the normal and low-molecular heparins, the super-sulfatized GAG (GAG 6-8) inhibit expression of MHC and ICAM 1 both in HUVEC as well as in PTEC after stimulation with IFN  $\gamma$ . By contrast, the de-sulfatized N acetylated GAG (GAG 1-5) could not influence MHC and ICAM 1 expression in these cells after stimulation with  $\gamma$  (Figures 4a-c and 5a-c). A clear tendency was evident that super-sulfatized GAGs were clearly more effective in inhibition than normal and low-molecular heparins were. This was more pronounced with the PTEC cultures (Figure 5a). Further dosage-related trials with low-molecular heparins and GAG 6-8 and with PTEC cells after stimulation with 10 ng/ml of IFN  $\gamma$  showed that with the administration of GAG 6-8 in concentrations of 0.03 mg/ml to IFN  $\gamma$ -stimulated cultures significantly inhibited MHC and ICAM 1 expression ( $p < 0.05$ ). Heparin showed under these conditions no significant impact on the MHC Class I and II expression, ICAM 1 was likewise inhibited by heparin under these conditions; however, significantly less so than with super-sulfatized GAG (Figure 6a-c). These results showed clearly that super-sulfatized GAGs are more effective than a comparable heparin in inhibiting the MHC and ICAM 1 expression.

In order to study whether the sulfatization degree of glycosamino-glycans has an important influence on the inhibiting effect of GAG on MHC and ICAM 1 expression after stimulation with IFN  $\gamma$ , PTEC cells were incubated in the presence of  $\text{NaClO}_3$ . In this way, the sulfatization of HSPG was supposed to be blocked. As a control, the cells were treated with equimolar concentrations of NaCl. Then both deposits were stimulated with IFN  $\gamma$  in concentrations of 0 to 10 ng/ml. Although IFN  $\gamma$  was still in a position to modulate the MHC and ICAM 1 expression in the PTEC cells treated with  $\text{NaClO}_3$ , however such modulation was considerably less than with the cells treated with NaCl (see Figures 7a-c). This means that the sulfatization degree of HSPG plays an important role in modulating the expression of these antigens with IFN  $\gamma$ .

To clarify whether GAGs must be sulfatized for IFN  $\gamma$  bonding, bonding studies were carried out with  $^{125}\text{I}$ IFN  $\gamma$ . The results showed that both heparin as well as super-sulfatized GAGs can bond  $^{125}\text{I}$ IFN  $\gamma$ . This can no longer be done by de-sulfatized N acetylated GAGs showing only a few sulfate groups (GAG 3-5). De-sulfatized N acetylated GAGs with a higher portion of sulfate (GAG 1 and 2) still bond  $^{125}\text{I}$ IFN  $\gamma$ ,

but markedly less than heparin or super-sulfatized GAGs. The bonding of  $^{125}\text{I}$ Fn  $\gamma$  to heparin or GAG bound to nitrocellulose filters can be blocked by a 3000-fold excess of heparin in the bonding solution. Under such conditions, no more bonding took place to GAG 1 and 2 while bonding to heparin, Fragmin and super-sulfatized GAGs was markedly reduced (Figure 8).

Table 1: Properties of the various heparins and glycosamino-glycanes\*) used in this study

| Name                                         |      | Sulfate Fxa<br>[%] | FIIa<br>[IU/mg] | M <sub>p</sub> | M <sub>r</sub> |
|----------------------------------------------|------|--------------------|-----------------|----------------|----------------|
| De-sulfatised N<br>acetylated<br>heparinoids |      |                    |                 |                |                |
| GAG 1                                        | 7.3  | 0                  | 0               | 3289           | 3802           |
| GAG 2                                        | 6.4  | 0                  | 0               | 2992           | 3548           |
| GAG 3                                        | 5.5  | 0                  | 0               | 2869           | 3382           |
| GAG 4                                        | 3.4  | 0                  | 0               | 2364           | 2800           |
| GAG 5                                        | 1.2  | 0                  | 0               | 1618           | 2074           |
| Sulfatised<br>heparinoids                    |      |                    |                 |                |                |
| GAG 6                                        | 14.2 | 26.1               | 39              | 7800           | 8360           |
| GAG 7                                        | 14.2 | 21.5               | 30              | 6000           | 7700           |
| GAG 8                                        | 13.7 | 25.8               | 28              | 5500           | 6300           |
| Commercially<br>available<br>heparinoids     |      |                    |                 |                |                |
| Braun                                        | ND   | ND                 | ND              | 14,000         | 18,000         |
| Fragmin P                                    | ND   | 160                | 70              | 4900           | 6000           |

\*) Properties of the various heparins and glycosamino-glycanes used in this study: M<sub>p</sub>: total weight divided by the number of molecules; M<sub>w</sub> molecular weight, ND - not determined, FIIa and Fxa activity was determined according to the method described by Handeland et al (Assay of Unfractionated and LMW Heparin with Chromogenic Substrates: Twin Methods with Factor Xa and Thrombin, Thrombosis Res 1984, 35: 627) with the first international standard for a low-molecular heparin (introduced in 1987, code no 85/600).

Figure 1

Dosage-related MHC Class I, Class II and ICAM 1 expression in PTEC after stimulation with IFN  $\gamma$ . The cells were stimulated for over 72 hours with various IFN  $\gamma$  concentrations. Thereafter, the expressions of MHC Class I (left scale), MHC Class II (right scale) and ICAM 1 (left scale) were determined with the FACS (= flow cytometry). The results are given in the Figure as mean fluorescence intensity of a representative experiment.

Figure 2

Effect of heparin on MHC and ICAM 1 expression of IFN  $\gamma$ -stimulated HUVEC cells. IFN  $\gamma$ -stimulated (100 ng/ml, for 72 hours, grey bar) or non-stimulated (white bar) HUVEC were incubated during stimulation with various heparin concentrations. The MHC and ICAM 1 expression was thereafter determined with the FACS. The results are given in the Figure as mean fluorescence intensity of a representative experiment.

Figure 3

Effect of heparin on the MHC and ICAM 1 expression of IFN  $\gamma$ -stimulated PTEC cells. IFN  $\gamma$ -stimulated (10 ng/ml, for 72 hours) or non-stimulated PTEC were incubated during stimulation with various heparin concentrations. The MHC and ICAM 1 expressions of three replicated cultures were then determined with the FACS. Figure 3a shows the MHC Class I expression. Figure 3b shows the MHC Class II expression. Figure 3c shows the ICAM 1 expression. The results are given in the figure as mean fluorescence intensity  $\pm$  2 SD.

Figure 4

Effect of various heparins and glycosamino-glycanes on the MCH and ICAM 1 expression of IFN  $\gamma$ -stimulated cells. IFN  $\gamma$ -stimulated (10 ng/ml, for 72 hours, solid bar) or non-stimulated (hatched bar) HUVEC cells were incubated with various heparins or glycosamino-glycanes in a concentration of 3 mg/ml during the stimulation period. The MHC and ICAM 1 expression was thereafter determined with the FACS. Figure 4a shows the MHC Class I expression, Figure 4b shows the MHC

Class II expression, and Figure 4c shows the ICAM 1 expression. The results are given in the Figure as mean fluorescence intensity  $\pm$  2 SD.

#### Figure 5

Effect of various heparins and glycosamino-glycanes on the MHC and ICAM 1 expression of IFN  $\gamma$ -stimulated PTEC cells. IFN  $\gamma$ -stimulated (10 ng/ml, for 72 hours, solid bar) or non-stimulated (hatched bar) PTEC cells were incubated with various heparins or glycosamino-glycanes in a concentration of 3 mg/ml during the stimulation period. The MHC and ICAM 1 expression was thereafter determined with the FACS. Figure 5a shows the MHC Class I expression, Figure 5b shows the MHC Class II expression and Figure 5c shows the ICAM 1 expression. The results are given in the Figure as mean fluorescence intensity of a representative experiment.

#### Figure 6

Comparison of GAG 6-8 and heparin in their effect of inhibiting the MHC and ICAM 1 expression of IFN  $\gamma$ -stimulated (10 ng/ml, for 72 hours) PTEC cells. The PTEC cells were incubated with various concentrations of GAG 6 ( ), GAG 7 ( ), GAG 8 ( ) and heparin ( ) during the stimulation period. The MHC and ICAM 1 expression was thereafter determined with the FACS. The expression of these antigens was also determined in the absence of GAG (-GAG) or in the absence of IFN  $\gamma$  (-IFN). Figure 6a shows the MHC Class I expression, Figure 6b shows the MHC Class II expression and Figure 6c shows the ICAM 1 expression. The results are given in the Figure as mean fluorescence intensity  $\pm$  2 SD.

#### Figure 7

Effect of  $\text{NaClO}_3$  on the extent of the stimulation of MHC and ICAM 1 expression in PTEC cells brought about by IFN  $\gamma$ . The PTEC cells were treated one day prior to IFN  $\gamma$  stimulation with 150 mM  $\text{NaClO}_3$  (solid bar) or 150 mM of NaCl (hatched bar) as osmolar control. Thereafter, the cells with the various IFN  $\gamma$  concentrations were stimulated for 72 hours in the presence of the same concentration of  $\text{NaClO}_3$  or NaCl. The MHC and ICAM 1 expressions of three replica cultures were then determined with the FACS. Figure 7a shows the MHC Class I expression, Figure 7b

shows the MHC Class II expression. Figure 7c shows the ICAM 1 expression. The results are given in the Figure as mean fluorescence intensity  $\pm$  2 SD. An asterisk signifies  $p < 0.05$  and two asterisks signify  $p < 0.01$  in the Student T test.

#### Figure 8

Bonding of  $^{125}$ IFN  $\gamma$  to heparin, Fragmin and various other glycosamino-glycanes. Heparin (Hep), glycosamino-glycane (GAG) 1 through 8 and Fragmin (Fragm) were blotted on a nitrocellulose filter which was produced as described above. The nitrocellulose strips were incubated with  $^{125}$ IFN  $\gamma$  in the presence or absence of a 3000-fold excess of heparin.



#### Patent claims

1. Organ-protective solutions containing a quantity of the following components effective for maintaining cell integrity and cell vitality:
  - a) 10 mg/l to 10,000 mg/l poly-sulfatized glycosamino-glycanes with a sulfur content of at least 12.5% by weight
  - b) 5 to 100 g/l hydroxy-ethyl starch
2. Organ-protective solution according to Claim 1 containing
  - a) 10 mg/l to 10,000 mg/l poly-sulfatized glycosamino-glycanes with a sulfur content of at least 12.5% by weight
  - b) 5 to 100 g/l hydroxy-ethyl starch
  - c) 5 to 100 mmol raffinose
3. Organ-protective solution according to Claims 1 and 2 containing
  - a) 10 mg/l to 10,000 mg/l poly-sulfatized glycosamino-glycanes with a sulfur content of at least 12.5% by weight
  - b) 5 to 100 g/l hydroxy-ethyl starch
  - c) 5 to 100 mmol raffinose
  - d) 5 to 40 mmol  $\text{KH}_2\text{PO}_4$



- e) 1 to 50 mmol  $\text{MgSO}_4$
  - f) 1 to 50 mmol adenosine
  - g) 0.5 to 5 mmol allopurinol
  - h) 1 to 10 mmol glutathion
4. Application of organ-protective solutions according to Claims 1 through 3 for manufacture of pharmaceutical preparations for inhibiting  $\gamma$  interferon-induced up-regulation of MHC Class I and MHC Class II proteins and ICAM 1.
5. Application of organ-protective solutions according to Claim 4 for treatment and prevention of diseases associated with  $\gamma$  interferon-induced up-regulation of MHC Class I and MHC Class II proteins and ICAM 1.

#### Drawing

6. Application of organ-protective solutions according to Claim 4 or Claim 5 for treatment of transplant patients.
7. Application of organ-protective solutions according to Claim 4 or Claim 5 for protection and storage of organs that are to be used for transplants.

#### Organ-protective solutions

#### Summary

The invention relates to organ-protective solutions containing poly-sulfatized glycosamino-glycans.