**Title:** REDUCING THE CONTENT OF CELLS IN A BIOLOGICAL SAMPLE

**Abstract:** An adsorbent for reducing the content of circulating cells and/or platelets in a biological sample, e.g. reducing the content of activated leukocytes and/or malarial-infected erythrocytes in a biological sample, the adsorbent comprising: (a) a support; and (b) an affinity agent immobilized on the support; wherein the affinity agent has a binding affinity for a molecule expressed on the surface of the circulating cells and/or platelets, e.g. for a molecule expressed on the surface of activated leukocytes and/or malarial-infected erythrocytes.
Published:
— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.
Reducing the content of cells in a biological sample

The present invention relates to materials, apparatus and methods for reducing the content of particular types of cells in a biological sample, more particularly for reducing the content of circulating cells and/or platelets, e.g. activated leukocytes and/or malarial-infected erythrocytes. The invention may be applied to the treatment and/or prevention of inflammation, e.g. to the treatment and/or prevention of inflammatory conditions such as rheumatoid arthritis, multiple sclerosis, atopic dermatitis, type 1 diabetes, septicemic shock, allograft rejection, HIV infection, hepatitis infection, burns, reperfusion injury following focal ischemia, and chronic infection in haemodialysis patients. The invention may be applied to the treatment of malaria.

The process of inflammation is typically associated with the tissue infiltration of activated leukocytes and their release of endogenous pro-inflammatory cytokines.

Rheumatoid arthritis is a cryptogenic generalized inflammatory disease. The cardinal symptom is polyarthritis but patients may also present with extra-articular manifestations such as subcutaneous nodules, pleurisy, pericarditis, lymphadenopathy, splenomegaly with leukopenia, vasculitis, angiitis and diffuse interstitial pneumonia. The pathology of the disease is thought to involve a rheumatoid factor, an IgM autoantibody which is directed against the Fc fragment of IgG and which is present in the serum and synovia of more than 75% of patients. There is evidence that a therapeutic benefit may be achieved in animals using an antagonist to the pro-inflammatory cytokines involved, namely to tumor necrosis factor and interleukin-1 [Dustin et al (1986)]. Several technological and biological restraints have precluded the application of this methodology to humans; these include an adverse reaction to the antagonist.

Allotransplantation of e.g. kidney, liver and heart is complicated by the threat of acute and chronic rejection. Much information has accumulated on the underlying genetic factors, but the immunological mechanisms involved are thought to be
associated with inflammation. Arterial and endocapillary glomerulitis of the renal allotransplant is a reliable parameter of its acute rejection [Anderson et al].

Multiple sclerosis is an inflammatory demyelinating disease of the central nervous system. It is the most common neurological disease affecting young adults and is thought to entail an autoimmune reaction against myelin proteins. The autoimmunity may be triggered by bacteria and/or viral toxins which have a structural homology with such proteins. The activation of autoreactive \( T_{H2} \)-lymphocytes is a crucial part of the disease pathology [Van Noort et al]. The T-lymphocytes migrate to the inflamed tissues using intercellular adhesion molecules, the expression of which favours the passage of these cells through the blood brain barrier [Franciotta et al].

Inflammatory conditions are usually treated by drug therapy, but such therapy is often problematic due to its limited efficacy and/or severity of side-effects. This has led to an alternative suggestion that inflammatory diseases may be treated by the removal of pro-inflammatory cytokines and/or by reducing the content of total leukocytes in the blood.

Plasmapheresis involves the extracorporeal removal of pro-inflammatory agonists, e.g. by filtration. Itakura et al have reported an extra-corporeal double filtration system for the removal of pro-inflammatory substances in the treatment of rheumatoid arthritis [Itakura et al].

EP 0 478 914 relates to an extracorporeal method for reducing the content of leukocytes in the blood. The technique employs a fibrous filter. US 5 997 496 describes a related device in which an extra-corporeal platelet activation is combined with a leukocyte trapping effect. The leukocyte trapping is again achieved using a fibrous filter material.

Malaria is an infection by \textit{Plasmodium}, a genus of protozoa that live inside the erythrocytes of the blood. The recurrent fever occurs in one of several forms
according to the species of *Plasmodium* involved. The infection of erythrocytes causes a change in their expression of cell-surface molecules.

An object of the present invention is to provide materials and methods for reducing the content of circulating cells and/or platelets in a biological sample, e.g. for reducing the content of activated leukocytes and/or malarial-infected erythrocytes. The invention may be employed in the amelioration and/or prevention of inflammation, and in the treatment of malaria.

In a first aspect, the present invention provides an adsorbent for reducing the content of circulating cells and/or platelets in a biological sample, e.g. for reducing the content of activated leukocytes and/or malarial-infected erythrocytes. The adsorbent comprises: (a) a support; and (b) an affinity agent immobilized on the support; the affinity agent has a binding affinity for a molecule expressed on the surface of the circulating cells and/or platelets, e.g. for a molecule expressed on the surface of activated leukocytes and/or malarial-infected erythrocytes.

By "biological sample" is meant any composition containing a circulating cell and/or platelet. The sample may contain an activated leukocyte and/or a malarial-infected erythrocyte. It may have been prepared *in vitro* (e.g. from a cell line or cell culture) or it may have been removed (either permanently or temporarily) from an organism, e.g. from a mammal, bird or fish, e.g. from a mammal (such as a human) suffering from inflammation or malaria. The biological sample may be blood, plasma, synovial fluid, lymph or tissue fluid.

The adsorbent may be capable of reducing the content of only a particular group of cells in the biological sample, e.g. only leukocytes or erythrocytes: the amount of these cells in the biological sample (as a percentage of total cells) will be reduced. The adsorbent may be capable of extracting only platelets.
In certain embodiments of the invention, the adsorbent is capable of selectively reducing the content of activated leukocytes and/or malarial-infected erythrocytes in the biological sample.

By “selectively reducing the content of activated leukocytes in a biological sample” is meant that the ratio of activated leukocytes to non-activated leukocytes in the biological sample after its contact with the adsorbent is less than the ratio of activated leukocytes to non-activated leukocytes in the biological sample before its contact with the adsorbent.

By “selectively reducing the content of malarial-infected erythrocytes in a biological sample” is meant that the ratio of malarial-infected erythrocytes to non-infected erythrocytes in the biological sample after its contact with the adsorbent is less than that ratio of malarial-infected erythrocytes to non-infected erythrocytes in the biological sample before its contact with the adsorbent.

In embodiments of the invention where a selective reduction in the content of activated leukocytes and/or malarial-infected erythrocytes is required, the molecule which is expressed on the surface of activated leukocytes and/or malarial-infected erythrocytes and for which the affinity agent has a binding affinity may not be expressed on the surface of non-activated leukocytes and non-infected erythrocytes. Expression on the surface of non-activated leukocytes and/or non-infected erythrocytes may however be tolerated, even where such selective reduction is required.

By way of example, if the invention is being used to selectively reduce the content of activated leukocytes in a biological sample, then expression of the molecule on non-infected and/or infected erythrocytes may be tolerated: removal of erythrocytes per se has no effect on the ratio of activated leukocytes to non-activated leukocytes in the biological sample. Expression of the molecule on non-activated leukocytes may even be tolerated, provided that a selective reduction in the content of activated leukocytes in the biological sample occurs, e.g. because the
level of expression on non-activated leukocytes is less than the level of expression on activated leukocytes. Similar considerations apply to a selective reduction in the content of malarial-infected erythrocytes in a biological sample.

In certain embodiments of the invention, the affinity agent will have little or no binding affinity for any molecule expressed on the surface of cells which the user positively requires to remain in the biological sample. By way of example, if the invention is being used to reduce only the content of leukocytes in the biological sample, then the affinity agent may have little or no binding affinity for the molecules expressed on the surface of erythrocytes and/or platelets. If the invention is being used to selectively reduce the content of activated leukocytes in a biological sample, then the affinity agent may have little or no binding affinity for any of the molecules expressed on the surface of non-activated leukocytes, and optionally, erythrocytes and/or platelets.

In relation to the structure of the adsorbent, the affinity agent may be immobilized on the support, either: (i) directly; or (ii) indirectly via one or more spacers.

A spacer may function to increase the separation between the affinity agent and the part of the support on which the affinity agent is immobilized. An increased separation may facilitate the binding of a circulating cell or platelet, e.g. an activated leukocyte or malarial-infected erythrocyte, to the affinity agent, e.g. by reducing or avoiding a steric and/or electrostatic interaction between the cell or platelet and the support. Additionally or alternatively, a spacer may serve to orientate the affinity agent such that a binding portion thereof is directed away from the part of the support onto which the affinity agent is immobilized. A binding portion of the affinity agent is a portion of the affinity agent which provides a binding affinity for the molecule expressed on the surface of the circulating cells and/or platelets, e.g. on the surface of the activated leukocytes and/or malarial-infected erythrocytes. This may facilitate an interaction between the binding portion of the affinity agent and the circulating cell or platelet, e.g. activated leukocyte or malarial-infected erythrocyte.
Where a spacer is provided, the affinity agent may be bound to it either covalently or non-covalently. The spacer will in turn be either covalently or non-covalently bound either to the support, or to a further spacer. In cases where more than one spacer is used, each spacer will be attached either covalently or non-covalently to each of its adjacent components in the adsorbent structure, i.e. to each of the components with which it is directly connected in order to present the affinity agent to the target cells. A series of spacers may be termed a "scaffold". If a spacer is not provided, the affinity agent will be directly immobilized on the support. It will be bound either covalently or non-covalently to the support.

A covalent bonding between any two components of the adsorbent may occur via any conventional chemical coupling, e.g. via an amide, ester, ether, or disulphide bond. Non-covalent bonding may occur through e.g. electrostatic bonds (e.g. ionic and/or hydrogen bonds) or hydrophobic bonds.

Irrespective of what method of attachment is used, the affinity agent should be immobilized on the support such that at least a portion of its binding affinity for the molecule expressed on the circulating cells and/or platelets, e.g. on the activated leukocytes and/or malarial-infected erythrocytes, is retained.

In certain embodiments, the affinity agent will be immobilized on the support (either directly or via one or more spacers) such that a binding portion thereof is directed away from the part of the support onto which the affinity agent is attached (the use of a spacer to achieve this property is discussed elsewhere herein). This may facilitate an interaction between the affinity agent and a circulating cell or platelet, e.g. an activated leukocyte or malarial-infected erythrocyte. By "directed away" is meant that the binding portion of the affinity agent is (on a time average) spatially further away from the surface of the support than those portions of the affinity agent through which it is connected to the support.

In certain embodiments of the invention, the affinity agent and/or spacer(s) may be bound to their adjacent components in the adsorbent structure such that after a
use of the adsorbent, the circulating cells and/or platelets that are bound to the affinity agent (e.g. the bound activated leukocytes and/or malarial-infected erythrocytes) can be eluted from the adsorbent whilst substantially maintaining the level of affinity agent that is immobilized on the support. In this way it will be possible to regenerate the adsorbent for another round of cell removal. Serial repetitions of cell removal from the same biological sample may be conducted. By “substantially maintaining the level of affinity agent that is immobilized on the support” is meant that more than 50%, more than 60%, more than 70%, more than 80%, more than 85%, more than 90%, or more than 95% of the affinity agent remains immobilized on the support. Having said this, the potential for regeneration is not essential: disposable adsorbents are within the scope of the present invention.

As noted above, an affinity agent may be bound to the support either directly or indirectly via one or more spacers. Examples of spacers include: (i) antibodies, fragments of antibodies, or molecules including antibodies/fragments of antibodies, which have a binding affinity for the affinity agent (and/or for an adjacent spacer in the scaffold); (ii) proteins or polypeptides which bind to the affinity agent and/or to an adjacent spacer to form a dimer or other polymeric structure (e.g. the spacer and adjacent component may form a leucine zipper); (iii) carbohydrate recognition proteins or polypeptides which have a binding affinity for a carbohydrate moiety on the affinity agent and/or adjacent spacer; (iv) carbohydrates which are bound by a carbohydrate recognition sequence in the affinity agent and/or adjacent spacer, or glycoproteins containing such carbohydrates; and (v) other conventional linkage couples well known those skilled in the art, e.g. the streptavidin/biotin couple.

Where the spacer comprises an antibody or binding fragment thereof, it may have a binding affinity for a “tag” portion of the affinity agent or adjacent spacer. The tag may be a natural part of the affinity agent or spacer; alternatively it may have been artificially attached to it, e.g. by chemical linking or by recombinant techniques. The tag may be a short stretch of polypeptide residues, e.g. of 5, 6, 7, 8, 9, 10, 10-15, 15-20, or 20-25 amino acids. A polyhistidine tag may for
example be used. Alternatively, the tag may consist of a larger sequence, e.g. it may include an entire domain of a polypeptide. The tag may comprise or consist of an immunoglobulin Fc portion and the spacer may be e.g. an antibody having a binding affinity for the Fc portion (or one or more domains thereof).

As used herein, fragments of antibodies include the Fab fragment consisting of the \( V_L \), \( V_H \), \( C_L \) and \( C_H 1 \) domains; (ii) the Fd fragment consisting of the \( V_H \) and \( C_H 1 \) domains; (iii) the Fv fragment consisting of the \( V_L \) and \( V_H \) domains of a single antibody; (iv) the dAb fragment consisting of a VH domain [Ward et al.]; (v) isolated CDR regions; (vi) \( F(ab')_2 \) fragments: a bivalent fragment comprising two linked Fab fragments; (vii) single chain Fv molecules (scFv): a \( V_H \) domain and a \( V_L \) domain linked by a peptide linker which allows the two domains to associate to form an antigen binding site [Bird et al.; Huston et al.]; (viii) bispecific single chain Fv dimers [PCT/US92/09965]; and (ix) "diabodies": multivalent or multispecific fragments constructed by gene fusion [WO94/13804; Holliger et al.].

As exemplified by e.g. antibody and \( F(ab')_2 \) spacers, certain embodiments of the invention may employ a spacer which is capable of simultaneously binding to two or more affinity agents. The two or more affinity agents may be identical to one another or they may be different. The use of a plurality of affinity agents on a single spacer may increase the total avidity of the support for circulating cells and/or platelets, e.g. for activated leukocytes and/or malarial-infected erythrocytes. Bispecific spacers may be used where two different affinity agents are to be connected to the same spacer: such spacers have two arms of different binding specificity. Two different affinity agents permit the removal of a wider range of cells from the biological sample.

A spacer may comprise an anchor portion. As used herein, an "anchor" is a part of a molecule which facilitates its direct attachment to the support. The anchor may comprise a polypeptide sequence which has a binding affinity for the support, e.g. it may comprise a series of histidine residues (polyhistidine) which has a binding affinity for a nickel coating component or nickel containing outer layer of
the support. The anchor may comprise a hydrophobic element, e.g. a sequence of hydrophobic amino acids or a fatty acid hydrocarbon chain (e.g. a GPI anchor). The hydrophobic portion may insert into a lipid coating of the support, e.g. into a lipid bilayer.

In any embodiment of the present invention, the affinity agent (and, if used, the spacer(s)) may be protein, glycoprotein or carbohydrate in nature. A protein or glycoprotein may be bound to the support or (where used, to an adjacent spacer), by either its N- or C-terminus. Where an affinity agent (or spacer) is a protein or glycoprotein it may have a natural, i.e. wild-type sequence. Alternatively, it may be a variant of such a sequence, e.g. having a sequence which differs from the wild-type sequence by one or more modifications, e.g. by one or more additions, insertions, substitutions and/or deletions. The additions, substitutions, insertions and/or deletions may be of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 10-15, 15-20, 20-25, 25-30, 30-35, 35-40, 40-45, 45-50, or more than 50 amino acids. The variant may be artificially created.

Variants may have an amino acid sequence which shares at least about 30%, or 40%, or 50%, or 60%, or 70%, or 75%, or 80%, or 85%, or 90%, or 95% or more sequence homology with a mature wild-type sequence. As is well-understood in the art, homology at the amino acid level is generally in terms of amino acid similarity or identity. Similarity allows for "conservative variation", i.e. substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as arginine for lysine, glutamic for aspartic acid, or glutamine for asparagine. Homology may be taken over the full-length sequence or over a part of the sequence, e.g. over 20, 30, 40, 50, 60, 70, 80, 90, 100, 120, 150, or over 200 contiguous amino acids. That two amino acid sequences are said to share "homology" or be "homologous" is based on sequence comparison: any phylogenetic relationship is irrelevant.

The affinity agent may comprise only a binding fragment of a wild-type molecule, omitting the rest of that molecule. A "binding fragment" is a fragment of the wild-
type sequence which is sufficient to provide a binding affinity for circulating cells and/or platelets, e.g. for activated leukocytes and/or malarial infected erythrocytes.

The affinity agent may be a chimeric fusion protein which is produced in accordance with techniques that are well known to those skilled in the art. The part of the affinity agent which is heterologous to its binding portion may facilitate its attachment to the support or to a spacer.

As discussed elsewhere herein, the affinity agent may comprise a tag to facilitate its attachment to a spacer. Where a spacer is not being used, the affinity agent may comprise an anchor.

The molecule that is expressed on the surface of circulating cells and/or platelets, e.g. on the surface of activated leukocytes and/or malarial-infected erythrocytes, and for which the affinity agent has a binding affinity may be any cell receptor from the family of cell adhesion molecules. It may be a molecule selected from the group consisting of CD2, CD4, CD8, CD22, CD23, CD35, CD36, CD43, CD44, CELL-CAM 105, LFA-1, LFA-3, CD11/CD18, L-Selectin, M-Cadherin, Mac-1, Myelin-associated glycoprotein, NCAM, Neuroglan, Ng-CAM, P-Cadherin, PECAM-1, P-Selectin, Platelet glycoprotein GPIIb-IIIa, P-Cadherin, T-Cadherin, TAG-1, VCAM-1, VLA-1, VLA-2, VLA-3, VLA-4, VLA-5 and VLA-6 (including mutants, alleles and derivatives of those molecules). In certain embodiments of the invention, the molecule is selected from the group consisting of LFA-1, CD11/CD18, Mac-1 and CD43.

The affinity agent may comprise or consist of: (i) a mature wild-type sequence of a cell surface ligand selected from the group consisting of AMOG, B-Cadherin, Contactin, E-Cadherin, E-Selectin, F3, Fasciclin I, II and III, ICAM-1, ICAM-2, Integrin a6b4, Integrin a7b1, and L1; or (ii) a variant of such a sequence, the variant having a binding affinity for a molecule expressed on the surface of circulating cells and/or platelets, e.g. for a molecule expressed on the surface of
activated leukocytes and/or malarial-infected erythrocytes. Such affinity agents are known as “cell surface ligand based affinity agents”.

In certain embodiments of the invention, the affinity agent may comprise or consist of: (i) a mature wild-type sequence of ICAM-1; or (ii) a variant of such a sequence, the variant having a binding affinity for a molecule expressed on the surface of circulating cells and/or platelets, e.g. for a molecule expressed on the surface of activated leukocytes and/or malarial-infected erythrocytes. Such affinity agents are known as “ICAM-1 based affinity agents”.

ICAM-1 has been fully described in the literature [Rothlein et al; Diamond et al; Staundon et al]. It is a glycoprotein of molecular weight 55,212 (on SDS-page it runs at an apparent molecular weight between 90,000 and 115,000 molecular weight). It is expressed on endothelial cells and is known to be capable of binding to activated leukocytes [Haskart et al; Dustin et al (1988)] with a resistance to shear stresses as high as 30dyn/cm². The binding of activated leukocytes to ICAM-1 is thought to play an important role in the pathology of inflammatory diseases [Hibbs et al]. ICAM-1 may be obtained by extraction from lysates of cell lines expressing the ligand, e.g. by chromatography.

The amino acid sequence of ICAM-1 is set forth in the literature [Simons et al (1988)]. The polypeptide contains 532 amino acids. Starting from the N-terminus, the mature wild-type sequence of ICAM-1 includes five homologous extracellular domains, an internal transmembrane domain, and a C-terminal (intracellular) domain (see Figure 1). Not all of these components of ICAM-1 are necessary to achieve a binding affinity for circulating cells and/or platelets, e.g. for activated leukocytes and/or malarial-infected erythrocytes: the affinity agent may therefore comprise or consist of only a binding fragment of the mature wild-type ICAM-1 sequence. A binding fragment may contain only the first one, two, three, four or five N-terminal domains of the ICAM-1 sequence.
Fragments of a wild-type sequence (e.g. of ICAM-1) are not however the only examples of variants of the sequence. In addition to fragments, variants include all those sequences which differ from the wild-type sequence by one or more of addition, insertion, deletion and substitution. Other examples of variants are discussed elsewhere herein.

Like ICAM-1, many cell surface ligands include a hydrophobic amino acid sequence as a transmembrane domain. A cell surface ligand based affinity agent, e.g. an ICAM-1 based affinity agent, may therefore comprise a hydrophobic amino acid sequence; this may be used to attach the affinity agent to the support by means of a lipid coating on the support matrix; such an anchor is described elsewhere herein. The hydrophobic sequence may comprise or consist of the wild-type transmembrane domain sequence, or a variant thereof.

A cell surface ligand based affinity agent may however omit either a part or all of a transmembrane domain contained within the wild-type sequence. A recombinant soluble form of ICAM-1 in which the transmembrane and intracellular domains of the molecule are absent (sICAM-1) is commercially available from Boehringer-Ingelheim, Germany. It is generated by the insertion of a translation stop codon in the amino acid sequence, upstream of the transmembrane domain.

As described elsewhere herein, a cell surface ligand based affinity agent, e.g. an ICAM-1 based affinity agent, may be bound to the support either directly, or indirectly via one or more spacers. Where an ICAM-1 based affinity agent is used, and that affinity agent includes only the first (N-terminal) one, two, three or four domains of ICAM-1 (or variants thereof), a spacer may be advantageous.

An ICAM-1 based affinity agent will preferably be bound to the support, or if used to the spacer, via its C-terminus, e.g. through a covalent linkage. By binding through its C-terminus, the binding portion of the ICAM-1 based affinity agent will be directed away from the part of the support onto which it is immobilized. Such directed binding will facilitate an interaction between the affinity agent and a
circulating cell or platelet, e.g. an activated leukocyte or malarial-infected erythrocyte.

As discussed elsewhere herein, a cell surface ligand based affinity agent, e.g. an ICAM-1 based affinity agent, may comprise a tag or anchor. The tag or anchor may comprise or consist of an amino acid sequence which is heterologous to the wild-type sequence of the cell surface ligand (e.g. ICAM-1), i.e. a sequence which is not expressed in the same polypeptide as the wild-type sequence and in direct connection thereto.

A recombinant chimeric ICAM-1 polypeptide having a heterologous tag is commercially available from Boehringer Ingelheim, Germany. The polypeptide is a fusion protein comprising the C_{H2} and C_{H3} domains of the Ig heavy chain fused to the C-terminus of the sICAM-1 sequence. The molecule may be employed in conjunction with a spacer which has a binding specificity for the C_{H2} and/or C_{H3} domains, e.g. with an anti-Fc antibody or a binding fragment thereof. Alternatively, the affinity agent may be directly bound to the support (or to an adjacent spacer) by a covalent linkage.

Another suitable ICAM-1 based affinity agent is a chimeric polypeptide comprising a polyhistidine tail (e.g. of six histidine residues). Such a molecule may be attached to the support via a spacer having a binding affinity for polyhistidine, e.g. via an anti-polyhistidine antibody or binding fragment thereof: in this case, the polyhistidine tail is used as a tag. Alternatively, the chimeric molecule may be directly immobilized on the support using a nickel coating on the support matrix: in that case the polyhistidine tail is used as an anchor. Despite the applications of the polyhistidine tail, a covalent binding of the chimeric molecule to the support, or where used to the spacer, may be employed. A chimeric ICAM-1 based affinity agent having a polyhistidine tail may be obtained by means of the insect expression system of baculovirus: this entails the culturing of endothelial cell lines infected with baculovirus. The chimeric molecule is expressed as part of the cell membrane and is recovered by membrane extraction and chromatography techniques. See

In certain embodiments of the present invention, the affinity agent (e.g. a cell surface ligand based affinity agent, more particularly an ICAM-1 based affinity agent) comprises a non-polypeptide molecule such as a probe, reporter or label (e.g. a fluorescent probe or spin label).

An affinity agent for use in accordance with the present invention may comprise or consist of an antibody, or a fragment thereof, which has a binding affinity for a molecule expressed on the surface of circulating cells and/or platelets, e.g. for a molecule expressed on the surface of activated leukocytes and/or malarial-infected erythrocytes. The antibody or fragment thereof may have a binding affinity for a molecule selected from the group consisting of CD2, CD4, CD8, CD22, CD23, CD35, CD36, CD43, CD44, CELL-CAM 105, LFA-1, LFA-3, CD11/CD18, L-Selectin, M-Cadherin, Mac-1, Myelin-associated glycoprotein, NCAM, Neuroglian, Ng-CAM, P-Cadherin, PECAM-1, P-Selectin, Platelet glycoprotein GPIIb-IIIa, P-Cadherin, T-Cadherin, TAG-1, VCAM-1, VLA-1, VLA-2, VLA-3, VLA-4, VLA-5 and VLA-6 (including mutants, alleles and derivatives of those molecules).

In certain embodiments, the antibody or fragment thereof has a binding affinity for a molecule selected from the group consisting of LFA-1, CD11/CD18, Mac-1 and CD43 (including mutants, alleles and derivatives of those molecules).

Antibodies or fragments thereof which have a binding affinity for a molecule expressed on the surface of circulating cells and/or platelets, e.g. on the surface of activated leukocytes and/or malarial-infected erythrocytes, (e.g. for LFA-1, CD11/CD18, Mac-1 or CD43) may be obtained by any of the techniques well known to those skilled in the art.
Methods of producing antibodies include immunising a mammal with a polypeptide, e.g. with a polypeptide component of LFA-1, CD11/CD18, Mac-1 or CD43, or with a peptide fragment thereof. Antibodies may be obtained from the immunised animal using techniques well known in the art, and screened for the desired binding affinity, preferably by assessing binding to the molecule of interest. Western blotting techniques and/or immunoprecipitation may be used [Armitage et al]. Isolation of antibodies and/or antibody-producing cells from an animal may involve sacrificing the animal.

As an alternative or supplement to immunising a mammal, an antibody specific for a molecule of interest may be obtained from a recombinantly produced library of expressed immunoglobulin variable domains, e.g. a library using lambda bacteriophage or filamentous bacteriophage which display functional immunoglobulin binding domains on their surfaces (see e.g. WO92/01047). The library may be naïve, i.e. constructed from sequences obtained from an organism which has not been immunised with the relevant molecule of interest (or a polypeptide component or fragment thereof), or it may be a library constructed using sequences obtained from an organism which has been exposed to the molecule of interest (or a polypeptide component or fragment thereof).

Antibodies for use in the present invention may be modified. The invention extends to the use of derivatives, functional equivalents and homologues of antibodies, including synthetic molecules and molecules whose shape mimics that of an antibody enabling it to bind an antigen or epitope.

It will be further understood by the skilled person that a monoclonal antibody can be subjected to recombinant DNA technology to produce other antibodies or chimeric molecules which retain the specificity of the original antibody. Such techniques may involve combining nucleic acid encoding the immunoglobulin variable region, or the complementarity determining regions (CDRs), of an antibody with nucleic acid encoding constant regions, or constant regions plus framework

An affinity agent for use in accordance with the present invention may comprise or consist of (R)-5-(4-bromobenzyl)-3-(3,5-dichlorophenyl)-1,5-dimethylimidazolodine-2,4-dione (also called BIRT 377) [Gahmberg (1997)], or a derivative of that structure which has a binding affinity for a molecule expressed on the surface of circulating cells and/or platelets, e.g. on activated leukocytes and/or malarial-infected erythrocytes.

The support onto which the affinity agent is directly or indirectly immobilized may be a biocompatible material, i.e. one which has negligible effects, if any, on a biological sample with which it is contacted (apart from a reduction in content of circulating cells and/or platelets, e.g. of activated leukocytes and/or malarial infected erythrocytes). The support should ideally have limited effects, if any, on the clotting system and/or on the complement system and/or on cells which the user does not wish to adsorb, e.g. it should not adsorb these cells, and should have little, if any, effect on their activities, e.g. it should not lead to their activation. The support should ideally not induce the activation of leukocytes. Biocompatibility of the support is advantageous where the adsorbent is to be used for therapeutic purposes.

Advantageously, the support may take a physical form which has a large surface area-to-volume ratio. In this way it will be more efficient at adsorbing the target cells and/or platelets, e.g. activated leukocytes and/or malarial-infected erythrocytes. The support may take any conventional form, e.g. as fibres (either hollow or solid), flakes, sheets, tablets or beads.

The size of the support elements may be selected such that the elements are retained on a sieve which permits the passage of the non-adsorbed cells in the biological sample (e.g. cells other than bound activated leukocytes and/or malarial-infected erythrocytes). Beads and fibres may have a diameter of from about
100\mu m to about 800\mu m; they may have a diameter of from about 200\mu m to about 600\mu m (the largest blood cell has a diameter of about 20\mu m).

A 100\mu m bead is large enough to bind to six cells, so a 10ml dry volume of such beads binds an average of $1.2 \times 10^9$ cells. Note that the total number of leukocytes in the circulating blood is about $50 \times 10^9$ cells, and the respective number of activated cells under pathological conditions is less than 2\% (i.e. $1 \times 10^9$ cells).

The support comprises a matrix material which is preferably biocompatible. Where the matrix is not biocompatible, the support as a whole may still be biocompatible. By way of example, the support may comprise a protective external barrier so as to limit or even prevent any effect of the matrix on the biological sample with which the support is contacted.

The matrix material may be porous or non-porous. It may be made of a synthetic material, e.g. a material selected from the group consisting of polyacrylamide, polystyrene, polyamide, polysulphone, polymethylmethacrylate, and mixtures thereof. It may be formed from a non-synthetic material. Sepharose (such as Fracogel\textsuperscript{\textregistered} type 55 or 65) may be employed. A cellulosic material may be used. The matrix may comprise a mixture of synthetic and non-synthetic materials. It may comprise a glass and/or a ceramic material. A polycarbonate material may be used.

The matrix material may contain free amino groups. Such amino groups will facilitate the binding of a polypeptide based affinity agent (or spacer) via its C-terminus. An amide bind is formed.

As discussed elsewhere herein, the support may have a coating or outer layer (over part of all of its exposed surface) to facilitate the attachment of an affinity agent or (where used) a spacer. The coating may function as a protective barrier for non-biocompatible matrix materials. Where a coating is used to facilitate the attachment of an affinity agent or spacer, the agent or spacer may include an anchor that has
a binding affinity for the coating. The anchor may be part of the natural wild-type form of the affinity agent or spacer, or it may have been artificially attached. Artificial attachment of the anchor may be effected through a chemical linkage or by recombinant techniques.

The coating may comprise a nickel layer and the anchor may comprise a polyhistidine sequence. The coating may comprise lipid (e.g. it may comprise a lipid bilayer) and the anchor may include a hydrophobic component. Where the anchor is a hydrophobic component it may be e.g. polypeptide or lipid in nature, e.g. it may comprise a series of amino acids having a predominantly hydrophobic character, or it may be lipid-based, e.g. it may be a fatty acid derived anchor. The anchor may include the GPI anchor well known to those skilled in the art. Where a lipid bilayer is used, the matrix material may be glass.

In a further aspect, the invention provides a method of manufacture of an adsorbent of the invention. The method comprises the step of immobilizing onto a support an affinity agent having a binding affinity for a molecule expressed on the surface of circulating cells and/or platelets, e.g. on the surface of activated leukocytes and/or malarial-infected erythrocytes. The support, affinity agent and method of immobilization, may be any of those described elsewhere herein.

In another aspect, the invention provides a device comprising an adsorbent of the invention. The device may be for use in a method of medical treatment, e.g. in the amelioration and/or prevention of inflammation, or in the treatment of malaria. Inflammatory conditions include rheumatoid arthritis, multiple sclerosis, atopic dermatitis, type 1 diabetes, septicemic shock, allograft rejection, HIV infection, hepatitis infection, burns, reperfusion injury following focal ischemia, and chronic infection in haemodialysis patients.

In a device of the invention, the adsorbent may be disposed within a container. The container may take any suitable form, e.g. it may be provided as a bag, column, box or tube. It may have a volume of 0-250ml, 250-500ml, 500-1000ml,
or 1000ml or more. The container may be made of any material whose properties are suitable for housing the adsorbent. The material may be sterile and/or pyrogen free. It may be biocompatible, as that term is used elsewhere herein.

The adsorbent may be present in the container as e.g. a suspension, slurry, or emulsion, e.g. in water or in an isotonic medium, e.g. in physiological saline. The ratio of the dry volume of the adsorbent to the total volume of the suspension or slurry may be from about 1:10 to about 9:10. It may be from about 1:5 to about 4:5. It may be from about 1:5 to about 2:3. The final volume of a slurry or suspension will depend on the quantity of biological sample to be treated, and the size of the container, but it may range from about 300 to about 500ml. The dry volume of the adsorbent in the slurry may range from about 100 to about 200ml.

The adsorbent, and optionally the container, may be disposable.

The container may have an inlet for conveying the biological sample into contact with the adsorbent. The container may have an outlet. It may have a port for filling the container with, or emptying the container of, the adsorbent.

The inlet may be connected to collection means for collecting a biological sample and conveying it to the inlet. The collection means may be suitable for collecting a biological sample from a human or other animal patient suffering from an inflammation or malaria. It may be capable of providing a fluid-tight connection between the inlet of the container and a biological cavity or vessel of the patient, e.g. a blood vessel of the patient, e.g. an artery or vein. The collection means may comprise a needle.

The outlet may be connected to return means for returning the biological sample to the patient. The return means may provide a fluid-tight connection between the outlet of the container and a biological cavity or vessel of a patient, e.g. a blood vessel of the patient, e.g. an artery or vein. The return means may comprise a needle.
The collection means and/or return means may be biocompatible, as that term is used elsewhere herein.

The device may include drive means for driving the biological sample into the container via the inlet, and where appropriate, out of the container via the outlet. The transportation means may comprise a pump, e.g. an electric or clockwork pump, or a pump driven by manual actuation.

The device may comprise mixing means for contacting the biological sample with one or more effector compounds before, during or after its contact with the adsorbent. The one or more effector compounds may be selected from the group consisting of analgesics, anticoagulants, anaesthetics, diluents, immunosuppressants and other pharmaceutical compounds.

The device may comprise a filter (e.g. a sieve) which allows the passage of cells in the biological sample but prevents the passage of adsorbent support element(s). The filter may be disposed downstream and/or upstream of the adsorbent. It may be mounted at an inlet and/or at an outlet of the container. The filter may have a pore size of 20μm or more, of 40μm or more, or of 60μm or more.

The device may comprise agitation means for agitating an adsorbent while it is in contact with the biological sample. The agitation means may comprise a plate which may be rockable, e.g. in the horizontal plan, by means of a motor (which may be electric or clockwork) or by manual actuation. The plate may be maintained at a pre-determined temperature, e.g. at approximately 35-40°C, e.g. at approximately 36-37°C.

The device may comprise temperature control means for maintaining the temperature of the biological sample as it travels through the device. The temperature control means may comprise a jacket of a thermally insulating material and/or a means for heating and/or cooling the biological sample.
The device may comprise detecting means for detecting the presence of air and/or adsorbent elements downstream of the adsorbent. Such detecting means may be used to prevent the contamination of a biological sample being returned to a patient with air and/or adsorbent elements.

The device may comprise determining means for determining the level of circulating cells and/or platelets, e.g. activated leukocytes and/or malarial-infected erythrocytes, in the biological sample, before and/or after its contact with the adsorbent.

An electric motor, pump, or heating or cooling element that is employed in a device of the present invention may be chemically powered, e.g. by a cell or battery. Additionally or alternatively, it may be powered by solar energy.

The device may comprise eluant means for eluting bound circulating cells and/or platelets, e.g. bound activated leukocytes and/or malarial-infected erythrocytes, from the adsorbent.

In various further aspects, the invention provides a method of reducing the content of circulating cells and/or platelets in a biological sample, e.g. of activated leukocytes and/or malarial-infected erythrocytes, the method comprising contacting the biological sample with an adsorbent of the invention. The invention further provides the use of an adsorbent of the invention for reducing the content of circulating cells and/or platelets, e.g. the content of activated leukocytes and/or malarial-infected erythrocytes, in a biological sample. In certain embodiments of these aspects of the invention (as with other aspects), a selective reduction of activated leukocytes and/or malarial-infected erythrocytes may be preferred. The adsorbent of the invention may form part of a device of the invention, as described elsewhere herein.

Circulating cells include those cells present in the blood, plasma, lymph, tissue fluid or synovial fluid of a biological organism. The circulating cells which are removed
from a biological sample may be erythrocytes, lymphocytes (e.g. B-lymphocytes and/or T lymphocytes, e.g. \( T_h \) and/or \( T_c \) and/or \( T_s \) lymphocytes), granulocytes, myeloid cells, natural killer cells or neutrophils, and mixtures thereof.

Activated leukocytes which are removed from a biological sample may include any of the leukocytes described above, and mixtures thereof.

The biological sample may be a cell culture. It may be blood, plasma, synovial fluid, lymph or tissue fluid.

The method of reducing the content of circulating cells and/or platelets in a biological sample, e.g. the content of activated leukocytes and/or malarial-infected erythrocytes, may be for non-therapeutic purposes, e.g. for experimental or research work. However, an important aspect of the present invention is a method of treating inflammation or malaria, the method comprising collecting a biological sample from a patient suffering from inflammation or malaria, contacting the biological sample with an adsorbent of the invention, and returning the biological sample to the patient. The adsorbent may be extracorporeal. A device according to the present invention may be employed.

The invention further provides an adsorbent for use in treating inflammation or malaria, and the use of an adsorbent of the invention for the manufacture of a medical device for treating inflammation or malaria. The device may comprise any of the features described elsewhere herein. The inflammation may be associated with rheumatoid arthritis, multiple sclerosis, atopic dermatitis, type 1 diabetes, septicemic shock, allograft rejection, HIV infection, hepatitis infection, burns, reperfusion injury following focal ischemia, and chronic infection in haemodialysis patients.

Various further aspects and embodiments of the present invention will be apparent to those skilled in the art in view of the present disclosure. Certain aspects and
embodiments of the invention will now be described by way of example only and with reference to the Figures described below.

All recited documents are incorporated herein by reference.

**BRIEF DESCRIPTION OF THE FIGURES**

Figure 1 is a schematic illustration of various forms of an ICAM-1 based affinity agent which may be used in the present invention. The circles represent the extracellular domains of the ICAM-1 sequence; the ellipse represents the transmembrane and intracellular domains. D1 is the most N-terminal of the extracellular domains.

Figure 2 is a schematic illustration of the adsorbent described in Example 1. A full length ICAM-1 sequence including transmembrane and intracellular domains is immobilized onto glass beads having a phospholipid bilayer coating. The transmembrane domain inserts into the bilayer.

Figure 3 is a schematic illustration of the adsorbent described in Example 2. A recombinant soluble form of ICAM-1 (sICAM-1) is immobilized onto polymer beads. The carboxyl terminus of the sICAM-1 forms a covalent amide linkage with a free amino group of the polymer matrix.

Figure 4 is a schematic illustration of the adsorbent described in Example 3. A recombinant soluble form of ICAM-1 (sICAM-1) is immobilized onto polymer beads using a monoclonal antibody spacer. The antibody has a binding specificity for domain D5 of the sICAM-1 sequence; it is bound to the polymer beads by a covalent amide linkage.

Figure 5 is a schematic illustration of the adsorbent described in Example 4. A chimeric ICAM-1 based affinity agent comprising an sICAM-1 sequence fused at its C-terminus to the Fc fragment of the Ig heavy chain is immobilized on polyacrylamide beads by a covalent amide linkage.
Figure 6 is a schematic illustration of the adsorbent described in Example 5. A chimeric ICAM-1 based affinity agent comprising the sICAM-1 sequence fused to a polyhistidine anchor is immobilized on support beads by means of a nickel coating.

Figure 7 is a schematic illustration of the adsorbent devices described in Examples 6-10. The Figure shows a plastic bag containing a suspension of the adsorbent. The bag has an inlet and an outlet, each of which is covered by a filter which prevents the passage of adsorbent elements (beads) but permits the passage of non-adsorbed cells. As shown in the Figure, the bags have internal walls to increase the contact between the adsorbent and the biological sample. The adsorbent devices are also illustrated as part of an extra-corporeal system for reducing the content of circulating cells and/or platelets, e.g. activated leukocytes and/or malarial-infected erythrocytes, in a patient’s blood: the bags are placed on an oscillating plate which is maintained at a temperature of 37°C.

Figure 8 is a schematic illustration of the adsorbent devices described in Examples 11-15. The Figure shows a plastic column containing a suspension of adsorbent beads. The column has an inlet and an outlet. The outlet is covered by a filter which prevents the passage of the adsorbent but permits the passage of non-adsorbed cells. The adsorbent devices are also illustrated as part of an extra-corporeal system for reducing the content of circulating cells and/or platelets, e.g. activated leukocytes and/or malarial-infected erythrocytes, in a patient’s blood. The column is held substantially vertically and is connected in circuit with a patient’s blood.

Figure 9 is a schematic illustration of an extracorporeal system for reducing the content of circulating cells and/or platelets, e.g. activated leukocytes (e.g. T-lymphocytes and granulocytes) and/or malarial-infected erythrocytes, in a patient’s blood. The device includes a bag type container housing adsorbent beads, an anticoagulant infusion upstream of the adsorbent, a pump to drive the passage of blood around the system, an oscillating plate to support and optionally agitate the
bag, and a detector to sense the presence of air and/or adsorbent beads within the conduit that returns the blood to the patient. An alternative form including a column is also shown.

Figures 10-14 show the results of the experiments described in Experimental procedures 1-5 respectively.

EXPERIMENTAL

Example 1

Example 1 describes an adsorbent of the invention in which an ICAM-1 based affinity agent is bound in a directed manner onto a glass/lipid support. The affinity agent consists of the full length ICAM-1 molecule (including transmembrane and intracellular domains). The support is provided as glass beads (the matrix) coated with a phospholipid bilayer (a mixture of egg phosphatidylcholine and cholesterol). The transmembrane domain of the affinity agent acts as an anchor: it inserts into the phospholipid bilayer to immobilize the affinity agent onto the support.

1-Sterile and pyrogen free glass beads having a diameter of 400μm to 600μm are boiled in detergent (Linbro 7x solution) for 2 hours, rinsed in deionized water for 24 hours, and stored in ethanol.

2-Egg phosphatidylcholine and cholesterol are mixed in a ratio of 7:2 (molar/molar) in chloroform/methanol (of 9:1 volume/volume). The resulting composition is partially dried under a stream of nitrogen. The composition is then further dried by lyophilization: the composition is held under vacuum for 1 hour at -70°C. The dried lipids are solubilized to a concentration of 0.4 mM in phosphate buffered saline (pH 7.2) containing 2% Octyl-β-D-glucopyranoside. The lipid solution is then mixed at a molar ratio of 1000:1 with a sterile and pyrogen free isolate of wild-type ICAM-1 (obtained from a cell lysate by affinity chromatography). The resulting solution is dialysed against phosphate buffered
saline using a hollow fibre membrane. The membrane is based on regenerated cellulose having a cut off below 5000 MW (i.e. less than 5% of a protein of molecular weight greater than 5000 will pass through the membrane). Vesicles of phospholipid bilayer with inserted affinity agent are formed.

3-The vesicles of the phospholipid bilayer/affinity agent are attached to the glass support matrix under aseptic conditions. One volume of the vesicle preparation is incubated with ½ volume of glass beads (dry volume) at a temperature of 37°C for 1-2 hours. The beads become coated with the phospholipid bilayer/affinity agent, thereby forming the adsorbent.

4-The adsorbent is washed five times with sterile and pyrogen free physiological phosphate buffer (pH 7.4, temperature 5°C) without exposing the beads to air. The end product is stored in a sterile plastic container at 5°C.

Example 2

Example 2 describes an adsorbent of the invention in which a sterile and pyrogen free recombinant soluble form of ICAM-1 (sICAM-1) is covalently immobilized on the support. The support comprises polyamide or polyacrylamide beads. The affinity agent is immobilized on the support by a covalent (amide) linkage between the carboxyl terminus of the sICAM-1 molecule and a free amino group of the polymer support matrix.

1-sICAM-1 (obtained from Boehringer-Ingelheim, Germany) is immobilized under aseptic conditions onto sterile polyamide or polyacrylamide beads (of diameter 600-800μm) using a chemical cross-linking procedure (see below). An amide bond is formed between the terminal carboxyl group of the sICAM-1 affinity agent and a free amino group of the support matrix. The affinity agent becomes bound in a directed manner, its most N-terminal domain (D1) being distal to the support. A binding density of 800 bound molecules of sICAM-1 per μm² support is achieved.
The cross-linking procedure employs N-hydroxysuccinimide (NHS) provided by Merck, and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) provided by Sigma. In addition, the following solutions are used:

- Phosphate buffered saline, 0.15M, pH 7.2 (PBS): 1 litre of distilled sterile water containing 8g NaCl, 0.2g KCl, 1.15g Na₂HPO₄·2H₂O and 0.2g KH₂PO₄
- COVA buffer: to 1 litre PBS is added, 119.9g NaCl, 10g Mg₂SO₄·7H₂O and 5ml Tween 20
- Affinity agent stock solution: 780mg affinity agent, 183ml distilled sterile water and 60ml DMSO
- Affinity agent working solution: 100ml affinity agent stock solution, 366ml NHS, Merck Cat.No. 804518, 2000ml distilled sterile water

3000ml of distilled sterile water is mixed with 1000ml of support material. The composition is incubated until the support material swells to reach a saturated maximum wet volume. The excess water is discarded by mild centrifugation (x 50g) and 2000ml of the working solution is added. The resulting composition is incubated for 90 minutes at 20°C. The working solution is discarded by mild centrifugation (x50g) (it can be used for a second binding reaction) and the resultant adsorbent is washed three times with 500ml COVA buffer (mild centrifugation is used to discard each wash solution). The adsorbent is stored at 5°C in phosphate buffer (total wet volume 5000ml).

2-The adsorbent is washed with a sterile and pyrogen free physiological phosphate buffer (pH 7.4, temperature 5°C). It is then dried and stored in a sterile plastic container at 5°C.

Example 3

Example 3 describes an adsorbent of the invention in which a sterile and pyrogen free recombinant soluble form of ICAM-1 (sICAM-1) is immobilized on a support via a monoclonal antibody spacer. The support consists of polyamide or polyacrylamide beads. The spacer is attached to the support by a covalent (amide) linkage between
its terminal carboxyl group and a free amino group of the support matrix. The sICAM-1 affinity agent is attached to the spacer by means of the immunological specificity of the spacer for the affinity agent. Two molecules of the sICAM-1 affinity agent are bound per molecule of spacer. The affinity agent is bound in a directed manner.

1-Monoclonal antibody CL203 is obtained from Boehringer Ingelheim, Germany: it has an immunological binding specificity for the fifth N-terminal domain of ICAM-1 (D5).

2-The CL203 monoclonal antibody is immobilized under aseptic conditions onto sterile and pyrogen free polyamide or polyacrylamide beads (of diameter 600-800 µm) using the chemical cross-linking procedure described in Example 2. An amide bond is formed between the terminal carboxyl group of the monoclonal antibody and a free amino group of the support matrix. A binding density of 600-900 bound antibodies per µm² of support is achieved.

3-The sICAM-1 affinity agent (Boehringer-Ingelheim, Germany) is immobilized on the support by means of immuno-complexing with the antibody spacer. The binding is carried out by incubating the affinity agent with the support/spacer preparation for at least 8 hours under aseptic conditions. One volume of a concentration of 100 µg/ml of the affinity agent dissolved in phosphate buffer (pH 7.4, temperature 5°C) is incubated with 1/3 dry volume of the support/spacer beads. The beads are covered with the phosphate solution.

4-The resulting adsorbent is washed with a sterile and pyrogen free physiological phosphate buffer (pH 7.4, temperature 5°C). It is dried and stored in a sterile plastic container at 5°C.

Example 4
Example 4 describes an adsorbent of the invention in which a chimeric affinity agent is covalently attached to the support. The affinity agent is a chimeric ICAM-1 based affinity agent consisting of the sICAM-1 sequence fused at its C-terminus to the Fc fragment (C\textsubscript{H}2 and C\textsubscript{H}3 domains) of the Ig heavy chain. It is immobilized on the support by a covalent (amide) linkage between the terminal carboxyl group of the affinity agent (the C-terminus of the Fc portion) and a free amino group of the polymer support matrix. The affinity agent has two binding sites for e.g. activated leukocytes and/or malarial-infected erythrocytes.

1-The chimeric ICAM-1 based affinity agent has been fully described in the literature [e.g. in Martin et al] and is commercially available from Boehringer Ingelheim, Germany. It is immobilized under aseptic conditions onto sterile and pyrogen free polyamide or polyacrylamide beads (diameter of 600-800\(\mu\)m) using the chemical cross-linking procedure described in Example 2. An amide bond is formed between the terminal carboxyl group of the affinity agent and a free amino group of the support matrix. A binding density of 500-1000 molecules of affinity agent per \(\mu \text{m}^2\) of support is achieved.

2-The resulting adsorbent is washed with a sterile and pyrogen free physiological phosphate buffer (pH 7.4, temperature 5 °C). It is dried and stored in a sterile plastic container at 5 °C.

Example 5

Example 5 describes an adsorbent of the invention in which a chimeric ICAM-1 based affinity agent is immobilized on a coated support matrix by means of a polyhistidine anchor. The affinity agent consists of the sICAM-1 sequence fused at its C-terminus to a polyhistidine tail of six consecutive histidines. The support comprises an outer layer of nickel charged resin for which the polyhistidine sequence has a binding affinity.
1-A chimeric s-ICAM-1 molecule having a polyhistidine anchor is obtainable by means of an insect expression system (baculovirus expression).

2-Sterile and pyrogen free beads of nickel charged resin of diameter of 60-165 μm (Qiagen Germany (NI-NTA Agarose)) are filtrated to achieve a diameter of 100-165 μm.

3-One volume of a 100 μg/ml solution of the chimeric affinity agent in carbonate buffer (pH 9.2, 5°C) is incubated under aseptic conditions with ½ dry volume of the nickel charged beads for 1 to 8 hours at room temperature (alternatively 24 hours at 5°C). The beads are covered with carbonate solution during the incubation.

4-The resulting adsorbent is washed with a sterile and pyrogen free physiological phosphate buffer (pH 7.4, temperature 5°C). It is dried and stored in a sterile plastic container at 5°C.

**Examples 6 to 10**

Examples 6 to 10 relate to devices of the invention in which the adsorbent is disposed within a flexible plastic bag container. The adsorbents employed are those described in Examples 1 to 5, each being used as a suspension with physiological saline.

The plastic bags are shown in Figure 7. They have an inlet and an outlet and have internal walls to increase the amount of adsorbent through which a biological sample must travel in order to pass between these apertures. The inlet and outlet are adapted for connection to sample collection means and sample return means, respectively. The inlet and outlet are each covered by a filter of average pore size 40 μm. The filters enable the passage of circulating cells but not the passage of adsorbent.
Figure 7 also shows the bags as part of a larger extracorporeal system device for reducing the content of circulating cells and/or platelets, e.g. of activated leukocytes and/or malarial-infected erythrocytes, in a patient's blood. The bags are placed on a plate which is maintained at a temperature of 37°C and which oscillates smoothly in the horizontal plane. The bag is placed in a circuit with the patient's blood. The method of performing blood treatment comprises the steps of:

1. collecting blood from a vein;
2. circulating the blood through the device of the invention; and
3. pumping the blood back to a vein of the patient.

The construction of the bag type adsorbent devices is described below.

1. A sterile suspension of the adsorbent is prepared: 100-200ml (dry volume) of the adsorbent beads described in Examples 1 to 5 is mixed with phosphate buffered saline solution (0.9% NaCl, pH 7.4) to give a total volume of 300-500 ml.

2. A plastic bag of a suitable volume is emptied by a vacuum procedure and hydrostatically filled with adsorbent suspension. 150ml, 200ml and 250ml bags were used in the devices of these examples.

3. A filter is mounted at the inlet and outlet of the bag and both the inlet and the outlet are closed with a cap. The filter material has a pore size of 40μm.

4. The plastic bag is covered with a second bag by welding. The device is sterilized (using ethylene oxide so that the function of the affinity agent is preserved).

Examples 11-15
Examples 11-15 describe devices of the invention in which the adsorbent is disposed within a plastic column. The adsorbents employed are those described in Examples 1 to 5, each being used as a suspension with physiological saline.

The columns are shown in Figure 8. They have an inlet and an outlet, the latter of which is covered by a mounted filter having a pore size of 40 μm. The filter enables the passage of circulating cells and platelets but not the passage of adsorbent.

Figure 8 also shows the bags as part of a larger extracorporeal device for reducing the content of circulating cells and/or platelets, e.g. activated leukocytes (such as T-lymphocytes and granulocytes) and/or malarial-infected erythrocytes, in a patient's blood. The column is placed in a circuit with the patient’s blood. It is maintained at a temperature of 37°C by heating through its walls. The method of performing blood treatment comprises the steps of:

1. collecting blood from a vein;
2. circulating the blood through the device of the invention; and
3. pumping the blood back to a vein of the patient.

The construction of a column type device is described below.

1-A sterile suspension of the adsorbent is prepared: 100-200 ml (dry volume) of the adsorbent beads described in any one of Examples 1 to 5 is mixed with a phosphate buffered saline solution (0.9% NaCl, pH = 7.4) to a total volume of between 200 and 500 ml.

2-A filter is mounted at the outlet of a column of internal diameter 10 cm, column length 13 cm, and volume approximately 1000 ml. (In alternative embodiments of the invention, a column having a volume of 125ml, 250 ml, 300ml or 500 ml may be used - see e.g. Experimental procedure 1). The filter has a pore size of 40μm.
3-The adsorbent suspension is hydrostatically introduced into the column.

4- The rest of the volume of the column is filled with phosphate buffered saline pH 7.4.

5- The column is sterilised with ethylene oxide.

**Experimental procedure 1**

Procedure 1 was designed to evaluate the ability of an adsorbent of the invention to reduce the content of activated leukocytes in whole blood.

Heparinized blood samples from 10 healthy subjects (of identical rhesus factor) were pooled to make a total volume of 2000ml. Leukocytes in the pooled blood were activated by administration of 5 ng/ml endotoxin (lipopolysaccharide from E. Coli (LPS)-Sigma): this treatment is known to induce the surface expression of counter-receptors to ICAM-1 on neutrophils and lymphocytes.

The pooled blood was passed through an extracorporeal system. The blood flow rate was 50ml/min; perfusion occurred for 100 minutes (the blood is recycled through the system).

The adsorbent devices of the invention that were tested were:

A: a 200 ml plastic bag containing 150 ml dry volume of the adsorbent beads of Example 1.

B: a 150 ml plastic bag containing 150 ml dry volume of the adsorbent beads of Example 2.

C: a 150 ml plastic bag containing 150 ml dry volume of the adsorbent beads of Example 3.

D: a 150 ml plastic bag containing 150 ml dry volume of the adsorbent beads of Example 4.
E: a 250 ml plastic bag containing 100 ml dry volume of the adsorbent beads of Example 5.

F: a 150 ml column containing 150 ml dry volume of the adsorbent beads of Example 1.

G: a 150 ml column containing 150 ml dry volume of the adsorbent beads of Example 3.

H: a 150 ml column containing 150 ml dry volume of the adsorbent beads of Example 4.

I: a 300 ml column containing 100 ml dry volume of the adsorbent beads of Example 5.

Following blood perfusion, the adsorbents were washed with saline to remove residual blood. Bound cells per mm² were counted under the microscope.

The results are shown in Figure 10.

**Experimental procedure 2**

Procedure 2 was designed to evaluate the ability of the adsorbents of the invention to reduce the content of activated leukocytes in the whole blood of patients suffering from rheumatoid arthritis.

Heparinized blood samples were obtained from 12 rheumatoid arthritis patients of identical rhesus factor. The samples were pooled to make a total volume of 800 ml. The blood was mounted in an extracorporeal system. The adsorbent devices tested were those described in Experimental procedure 1. The blood flow was adjusted to 40ml/min; the adsorbents were perfused for 20 minutes.

Following blood perfusion, the adsorbents were washed with saline to remove residual blood. Bound cells per mm² were counted under the microscope.

The results are shown in Figure 11.
Experimental procedure 3

Procedure 3 was designed to evaluate the ability of the adsorbents of the invention to eliminate malarial-infected erythrocytes from whole blood.

A total volume of 800 ml of heparinized (10 IU/ml) fresh blood obtained from 10 healthy subjects (of identical rhesus factor) was infused with $6 \times 10^8$ malarial-infected erythrocytes with a parasitemia of 30 to 40% (from Dr Martin-Diabetes Forschungsinstitute an der Heinrich-Heine-Universität, Düsseldorf, Germany).

The blood was incubated at 37°C under 5% CO$_2$ for 24 hours to achieve a transinfection of erythrocytes. Following incubation, the blood was mounted in an extracorporeal system. The adsorbent devices that were tested are those described in Experimental procedure 1. The blood flow rate was adjusted to 20ml/min; the adsorbents were perfused for 40 minutes.

Following blood perfusion, the adsorbents were washed with saline to remove residual blood. Bound cells per mm$^2$ were counted under the microscope.

The results are shown in Figure 12.

Experimental procedure 4

Procedure 4 was designed to evaluate the ability of the adsorbents of the invention to reduce the content of activated leukocytes in the whole blood of patients suffering from type 1 diabetes.

A total volume of 800 ml heparinized fresh blood was obtained from 8 subjects (of identical rhesus factor) who were suffering from type 1 diabetes. The blood was mounted in an extracorporeal system. The adsorbent devices that were tested are those that are described in Experimental procedure 1. The blood flow rate was adjusted to 40ml/min; different adsorbents were perfused for 20 minutes.
Following blood perfusion, the adsorbents were washed with saline to remove residual blood. Bound cells per mm$^2$ were counted under the microscope.

The results are shown in Figure 13.

**Experimental procedure 5**

Procedure 5 was designed to evaluate the ability of the adsorbents of the invention to reduce the content of activated leukocytes in whole blood of patients suffering from multiple sclerosis.

A total volume of 800 ml heparinized fresh blood was obtained from 8 subjects (of identical rhesus factor) who were suffering from multiple sclerosis. The blood was mounted in an extracorporeal system. The adsorbent devices that were used are those described in Experimental procedure 1. The blood flow rate was adjusted to 40ml/min; the adsorbents were perfused for 20 minutes.

Following blood perfusion, the adsorbents were washed with saline to remove residual blood. Bound cells per mm$^2$ were counted under the microscope.

The results are shown in Figure 14.

**LIST OF REFERENCES**


Dustin *et al.* (1988) Lymphocyte function-associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells. *J. Cell Biol.* 107: p. 321


Hibbs *et al.* (1991) Regulation of adhesion of ICAM-1 by the cytoplasmic domain of LFA-1 integrin beta subunit. *Science* 251: p. 1611


CLAIMS

1. An adsorbent for reducing the content of circulating cells and/or platelets in a biological sample, e.g. for reducing the content of activated leukocytes and/or malarial-infected erythrocytes in a biological sample, the adsorbent comprising:

(a) a support; and

(b) an affinity agent immobilized on the support;

wherein the affinity agent has a binding affinity for a molecule expressed on the surface of the circulating cells and/or platelets, e.g. for a molecule expressed on the surface of activated leukocytes and/or malarial-infected erythrocytes.

2. The adsorbent of Claim 1, wherein the affinity agent has a binding affinity for a molecule selected from the group consisting of CD2, CD4, CD8, CD22, CD23, CD35, CD36, CD43, CD44, CELL-CAM 105, LFA-1, LFA-3, CD11/CD18, L-Selectin, M-Cadherin, Mac-1, Myelin-associated glycoprotein, NCAM, Neurogian, Ng-CAM, P-Cadherin, PECAM-1, P-Selectin, Platelet glycoprotein GPIIb-IIIa, P-Cadherin, T-Cadherin, TAG-1, VCAM-1, VLA-1, VLA-2, VLA-3, VLA-4, VLA-5 and VLA-6 (including mutants, alleles and derivatives of those molecules).

3. The adsorbent of Claim 2, wherein the affinity agent has a binding affinity for a molecule selected from the group consisting of LFA-1, CD11/CD18, Mac-1 and CD43.

4. The adsorbent of any one of Claims 1 to 3, wherein the affinity agent is such that the adsorbent is capable of selectively reducing the content of activated leukocytes and/or malarial-infected erythrocytes in the biological sample.

5. The adsorbent of any preceding claim, wherein the affinity agent is selected from (a) a wild-type ICAM-1 sequence or a variant thereof, said variant having a binding affinity for a molecule expressed on the surface of circulating cells and/or platelets, e.g. on the surface of activated leukocytes and/or malarial-
infected erythrocytes, (b) a recombinant ICAM-1 or a recombinant of portion of ICAM-1.

6. The absorbent of Claim 5 wherein the affinity agent comprises Domain D1 to D5, or D1 to D4, or D1 to D3, or D1 to D2 of the ICAM-1 structure.

7. The adsorbent of any one of Claims 1 to 5, wherein the affinity agent comprises an antibody or a binding fragment thereof.

8. The adsorbent of any preceding claim, wherein the affinity agent is bound to the support via one or more spacers.

9. The adsorbent of Claim 8, wherein at least one spacer comprises an antibody molecule or binding fragment thereof, said antibody molecule or binding fragment having a binding affinity for the affinity agent.

10. The adsorbent of any one of Claims 1 to 7, wherein the support comprises a coating and the affinity agent comprises an anchor having a binding affinity for said coating.

11. The adsorbent of Claim 10, wherein the support comprises a nickel coating and the anchor comprises a polyhistidine sequence.

12. The adsorbent of Claim 10, wherein the support comprises a lipid bilayer and the anchor comprises a hydrophobic component.

13. The adsorbent of any preceding claim, wherein the adsorbent is biocompatible.

14. A device for reducing the content of circulating cells and/or platelets in a biological sample, e.g. for reducing the content of activated leukocytes and/or malarial-infected erythrocytes in a biological sample, the device comprising:
(a) a container having an inlet; and
(b) an adsorbent according to any previous claim which is housed within said container.

15. The device according to Claim 14, wherein the device comprises:
   (c) an outlet; and
   (d) driving means for driving the biological sample through the container from the inlet to the outlet.

16. A method of reducing the content of circulating cells and/or platelets in a biological sample, e.g. for reducing the content of activated leukocytes and/or malarial-infected erythrocytes in a biological sample, the method comprising contacting the biological sample with an adsorbent according to any one of Claims 1 to 13.

17. A method of treating a patient suffering from inflammation, the method comprising reducing the content of activated leukocytes in a biological fluid of said patient by contacting said biological fluid with an adsorbent according to any one of Claims 1 to 13.

18. The method of Claim 17, wherein the inflammation is associated with a condition selected from the group consisting of rheumatoid arthritis, multiple sclerosis, atopic dermatitis, type 1 diabetes, septicemic shock, malarial infection of erythrocytes, HIV infection, hepatitis infection, transplant organ rejection, reperfusion injury, burns and chronic infection in haemodialysis patients.

19. A method of treating a patient suffering from malaria, the method comprising reducing the content of malarial-infected erythrocytes in a biological fluid of said patient by contacting said biological fluid with an adsorbent according to any one of Claims 1 to 13.
FIG. 11 Number of activated leukocytes bound per mm² in devices of experimental procedure 2 (mean ± SD of n=7).
Figure 12: Number of the malaria infected erythrocytes bound per mm² in devices described in experimental procedure 3 (mean ± SD of n=7).
FIG. 13 Number of activated leukocytes bound per mm$^2$ of devices from experimental procedure 4 (mean $\pm$ SD of n=7).
FIG. 14. Number of activated leukocytes bound per mm² of devices from experimental procedure 5 (mean ± SD of n=7).