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(54) Title: LEUKAEMIA INHIBITORY FACTOR-BINDING PROTEIN		
(57) Abstract <p>The present invention relates to an isolated leukaemia inhibitory factor (LIF)-binding protein (LBP) in soluble form and obtainable from a first mammalian species, said LBP capable of inhibiting the ability of LIF from a second mammalian species to induce differentiation of M1 myeloid leukaemic cells <i>in vitro</i> to a greater extent when compared to its ability to inhibit LIF from said first mammalian species.</p>		

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LEUKAEMIA INHIBITORY FACTOR-BINDING PROTEIN

- 5 The present invention relates to a Leukaemia Inhibitory Factor-Binding Protein (LBP) and more particularly to a soluble LBP, uses thereof and compositions containing same.

10 Leukaemia Inhibitory Factor (LIF) is a polyfunctional glycoprotein with actions on a broad range of tissue and cell types, including induction of differentiation in a number of myeloid leukaemic cell lines, suppression of differentiation in normal embryonic stem cells, stimulation of proliferation of osteoblasts and DA-1 haemopoietic cells and potentiation of the proliferative action of interleukin-3 (IL-3) on megakaryocyte precursors. Functionally, it is able to switch autonomic
15 nerve signalling from adrenergic to cholinergic mode, stimulate calcium release from bones, stimulate the production of acute phase proteins by hepatocytes and induce loss of fat deposits by inhibiting lipoprotein lipase-mediated lipid transport into adipocytes¹.

- 20 This array of actions is puzzling since it is difficult to conceive of any situation that would require a co-ordinated response in all the known target tissues of LIF. Actions of LIF, therefore, are probably designed to be restricted by co-localisation of LIF-producing cells and LIF-responsive cells, with tight regulation of LIF production. However, such an arrangement is likely to result in some release of
25 LIF into the circulatory system including blood and other bodily fluids.

In work leading up to the present invention, the inventors discovered a LIF-binding protein in serum which is capable of inhibiting the biological activity of LIF. The identification of this LIF antagonist will now permit greater control in
30 LIF therapy and to prevent any systemic effects of locally administered LIF which are not therapeutically desirable. It also provides a new agent useful in the treatment of LIF associated diseases or conditions. In a particular embodiment,

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the inventors have discovered that the inhibitory effect of the LIF-binding protein may be more pronounced in heterologous systems, i.e. where the LIF-binding protein from one mammal is used to inhibit LIF in another mammal.

- 5 Accordingly, one aspect of the present invention provides a LIF-binding protein (LBP) in soluble form and isolatable from a mammal.

More particularly, the present invention is directed to an isolated LBP in soluble form and obtainable from a first mammalian species, said LBP capable of
10 inhibiting the ability of LIF from a second mammalian species to induce differentiation of M1 myeloid leukaemic cells *in vitro* to a greater extent when compared to its ability to inhibit LIF from said first mammalian species.

The isolated LBP is preferably biologically pure meaning that it represents at
15 least 20%, preferably at least 50%, even more preferably at least 70% and still more preferably at least 85% of the molecule in a solution or composition as determined by weight, biological activity or other convenient means of measurement. Notwithstanding that the LBP is isolated, it may also be in the form of a composition. According to this aspect of the present invention there is
20 contemplated a composition comprising an LBP in soluble form and obtainable from a first mammalian species, said LBP capable of inhibiting the ability of LIF from a second mammalian species to induce differentiation of M1 myeloid leukaemic cells *in vitro* to a greater extent when compared to its ability to inhibit LIF from said first mammalian species, said composition substantially free of
25 protein molecules not having LBP properties.

The isolated LBP in soluble form and obtainable from the first mammalian species is further characterised in that the LBP has at least a 100 fold higher binding affinity for a LIF from the second mammalian species compared to the
30 binding affinity for a LIF from said first mammalian species.

- In accordance with the present invention, the first mammal is preferably a human, mouse or rat or other rodent, pig, cow, sheep or other ruminant, goat, horse or primate. The second mammal may also be a human, mouse or rat or other rodent, pig, cow, sheep or other ruminant, goat, horse or primate. Preferably, the first mammal is a non-human mammal and the second mammal is a human. Most preferably, the first mammal is a mouse and the second mammal is a human.
- 10 Accordingly, in a preferred embodiment, the present invention is directed to a LBP in soluble form isolatable from a murine animal. More particularly, the present invention provides a LBP in soluble form isolatable from a murine animal, said LBP capable of greater inhibition of human LIF compared to murine LIF.
- 15 The isolated LBP may be the naturally occurring molecule, a naturally occurring derivative, part or fragment thereof or may be a recombinant or synthetic form of the molecule including any recombinant or synthetic derivatives, parts or fragments thereof. The LBP may be naturally glycosylated, partially glycosylated or unglycosylated or may have an altered glycosylation pattern from the naturally occurring molecule. The molecule may, for example, undergo treatment with N-glycanase resulting in a deglycosylated or substantially deglycosylated molecule. A "derivative" of LBP is considered herein to generally comprise a single or multiple amino acid insertion, deletion and/or substitution of amino acid residues relative to the naturally occurring sequence or an insertion, deletion and/or substitution of molecules associated with LBP such as carbohydrate moieties. A "derivative" is also considered to be a molecule with at least 45% amino acid sequence similarity to the amino acid sequence of the LBP.
- 20
- 25
- 30 Amino acid insertional derivatives of LBP include amino and/or carboxyl terminal fusions as well as intra-sequence insertions of single or multiple amino acids. Insertional amino acid sequence variants are those in which one or more

Where the LBP is derivatised by amino acid substitution, the amino acids are generally replaced by other amino acids having like properties, such as hydrophobicity, hydrophilicity, electronegativity, bulky side chains and the like.

- 5 Amino acid substitutions are typically of single residues. Amino acid insertions will usually be in the order of about 1-10 amino acid residues and deletions will range from about 1-20 residues. Preferably, deletions or insertions are made in adjacent pairs, i.e. a deletion of two residues or insertion of two residues.
- 10 The amino acid variants referred to above may readily be made using peptide synthetic techniques well known in the art, such as solid phase peptide synthesis⁽¹⁴⁾ and the like, or by recombinant DNA manipulations. Techniques for making substitution mutations at predetermined sites in DNA having known or partially known sequence are well known and include, for example, M13
- 15 mutagenesis. The manipulation of DNA sequences to produce variant proteins which manifest as substitutional, insertional or deletional variants are conveniently described, for example, in Maniatis *et al*⁽¹⁵⁾.

Other examples of recombinant or synthetic mutants and derivatives of the LBP

20 of the present invention include single or multiple substitutions, deletions and/or additions of any molecule associated with the LBP such as carbohydrates, lipids and/or proteins or polypeptides.

In one embodiment, the LBP is truncated at its carboxy terminal end portion to

25 render said LBP soluble.

In another embodiment, the LBP is a fusion molecule between LBPs from said first and second mammalian species.

According to this embodiment, there is provided a fusion polypeptide defining an LBP, said fusion polypeptide comprising first and second amino acid sequences wherein said first amino acid sequence is derivable from an LBP from a first mammalian species and said second amino acid sequence is derivable from an LBP from a second mammalian species wherein the LBP from said first mammalian species is capable of inhibiting the ability of LIF from said second mammalian species to induce differentiation of M1 myeloid leukaemic cells *in vitro* to a greater extent when compared to its ability to inhibit LIF from said first mammalian species such that said fusion polypeptide retains the ability to inhibit LIF from said second mammalian species to a greater extent than LIF from said first mammalian species.

In a preferred embodiment, the first mammalian species is a mouse, rat or other rodent, pig, cow, sheep or other ruminant, goat, horse or primate and said second mammalian species is a human. Most preferably, the first mammalian species is a mouse and the fusion polypeptide is referred to as a "humanised" form of the mouse LBP. Such molecules are particularly advantageous in avoiding or reducing possible induction of an antigenic immune response by administering to said first mammal, an LBP from said second mammal.

According to this most preferred aspect of the present invention, there is provided a fusion polypeptide defining an LBP, said fusion polypeptide comprising first and second amino acid sequences wherein said first amino acid sequence is derivable from an LBP from a mouse and said second amino acid sequence is derivable from an LBP from a human wherein said fusion polypeptide is capable of inhibiting the ability of LIF from a human to induce differentiation of M1 myeloid leukaemic cells *in vitro* to a greater extent when compared to its ability to inhibit mouse LIF and wherein administration of said fusion polypeptides into a human results in a substantially reduced immune response against said fusion polypeptide compared to the administration to said human of native or recombinant mouse LBP. Conveniently, an "immune response" is measured by

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titre of antibodies specific to a molecule and/or involve extent of a cellular immune response.

The fusion polypeptides can be prepared by a range of suitable methods but conveniently is by a method similar to the method employed by the inventors to map the site on the hLIF molecule that confers both binding to the hLIF receptor α -chain and the unusual high affinity binding to the mouse LIF receptor α -chain (mLBP) similar to that described in the experiment summarised in Figure 9. In particular, a hLBP molecular frame work is used to construct a series of mouse-human mLBP chimaeric molecules in order to determine the minimum number of hLIF amino acid residues that is necessary to substitute into the hLBP sequence in order to create a molecule that has the properties that are peculiar to hLBP.

Reference in the specification and claims herein to "LBP" includes reference to a fusion polypeptide defining an LBP as defined above.

The terms "analogues" and "derivatives" also extend to any functional chemical equivalent of the LBP characterised by its increased stability and/or efficacy *in vivo* or *in vitro*. The terms "analogues" and "derivatives" also extend to any amino acid derivative of the LBP as described above.

Analogues of LBP contemplated herein include, but are not limited to, modifications to side chains, incorporation of unnatural amino acids and/or derivatising the molecule and the use of crosslinkers and other methods which impose conformational constraints on the LBP molecule or its analogues.

Examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with NaBH_4 ; amidiation with methylacetimidate; acylation with acetic anhydride; carbamoylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS); acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with

pyridoxal-5'-phosphate followed by reduction with NaBH_4 .

The guanidino group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3- butanedione,
5 phenylglyoxal and glyoxal.

The carboxyl group may be modified by carbodiimide activation via O-acylisourea formation followed by subsequent derivitisation, for example, to a corresponding amide.

10

Sulphydryl groups may be modified by methods such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; formation of
15 mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulphonic acid, phenylmercury chloride, 2-chloromercuri-4-nitrophenol and other mercurials; carbamoylation with cyanate at alkaline pH.

Tryptophan residues may be modified by, for example, oxidation with N-
20 bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphenyl halides. Tyrosine residues on the other hand, may be altered by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

Modification of the imidazole ring of a histidine residue may be accomplished by
25 alkylation with iodoacetic acid derivatives or N-carbonylation with diethylpyrocarbonate. Other types of modifications include iodination of tyrosine and biotinylation of lysine.

Examples of incorporating unnatural amino acids and derivatives during protein
30 synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-

methylheptanoic acid, 2-thienyl alanine and/or D-isomers of amino acids.

- Crosslinkers can be used, for example, to stabilise 3D conformations, using homo-bifunctional crosslinkers such as the bifunctional imido esters having $(\text{CH}_2)_n$ spacer groups with $n = 1$ to $n = 6$, glutaraldehyde, N-hydroxysuccinimide esters and hetero-bifunctional reagents which usually contain an amino-reactive moiety such as N-hydroxysuccinimide and another group-specific reactive moiety such as maleimido or dithio moiety (SH) or carbodiimide (COOH). In addition, peptides could be conformationally constrained by, for example, incorporation of C_α and N_α -methylamino acids, introduction of double bonds between C_α and C_β atoms of amino acids and the formation of cyclic peptides or analogues by introducing covalent bonds such as forming an amide bond between the N and C termini, between two side chains or between a side chain and the N or C terminus.
- 15 The LBP molecule of the present invention may also be pegolated using polyethylene glycol (PEG) or other equivalent or similar fatty acid to aid in increasing stability and/or the half life of the protein. In this regard, reference can conveniently be made, for example, to US Patent No. 5,089,261 which describes the use of PEG to increase the *in vivo* half life of interleukin 1.
- 20 Conveniently, the LBP may be in a purified or semi-purified form from blood, serum or other biological fluid sample. More conveniently, the LBP can be purified or semi-purified by sequential fractionation using affinity chromatography on an immobilised LIF column, anion exchange chromatography, size exclusion chromatography and preparative native polyacrylamide gel electrophoresis. One or more of the foregoing steps may be altered or a similar or equivalent step substituted therefor without departing from the scope of the present invention provided the LBP is enriched from a particular biological sample.
- 25
- 30 The LIF used in the LIF affinity column is generally of first mammalian origin, i.e. same mammalian origin for both LBP and LIF although any LIF capable of binding the LBP to be purified can be used in the affinity column.

Conveniently, the purification of the LBP is monitored by binding to labelled LIF and preferably radioactively labelled LIF, such as using known ^{125}I -LIF binding assays. Other means of monitoring LBP activity can also be used, such as specific antibody binding or inhibition of LIF activity through competitive assays.

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The murine LBP (mLBP) in accordance with the preferred aspects of the present invention when purified as generally described above has an apparant molecular weight as determined on SDS-PAGE of approximately $90,000 \pm 10,000$ daltons in glycosylated form and $65,000 \pm 10,000$ daltons in another glycosylated form and specifically binds ^{125}I -murine LIF (mLIF) with an equilibrium dissociation constant of about 0.5-2nM. Furthermore, mLIF is approximately 1,000-10,000-fold less effective than human LIF (hLIF) in competing with ^{125}I -hLIF for binding to mLBP. More significantly, however, as shown by the *in vitro* effects herein described, mLBP is at least 100-fold and is generally about 1,000-fold more active as an inhibitor of hLIF than of mLIF. In addition, the direct binding affinity of mLBP for hLIF is approximately 100 times higher than that for mLIF.

In the most preferred embodiment of the present invention, the mLBP has an amino acid sequence in the N-terminal region comprising Gly-Val-Gln-Asp-Leu-Lys-Cys-Thr-Thr-Asn-Asn-Met-Arg-Val-Trp-Asp-Cys-Thr-Trp-Pro-Ala-Pro-Leu (SEQ ID No. 1), is soluble, particularly in aqueous buffered solutions and has an apparant molecular weight in the glycosylated form of $90,000 \pm 10,000$ daltons, and preferably $90,000 \pm 5,000$ daltons as determined by SDS-PAGE. On the basis of treatment with N-glycanase, one deglycosylated form has a molecular weight of approximately $65,000 \pm 15,000$ daltons and preferably $65,000 \pm 10,000$ daltons and another deglycosylated form has a molecular weight of approximately $50,000 \pm 10,000$ daltons as determined by SDS-PAGE. The present invention extends to LBP molecules having an N-terminal amino acid sequence with at least 45%, preferably at least 55%, more preferably at least 65% and still more preferably at least 75-85% and even more preferably greater than 90% similarity to the amino acid sequence: Gly-Val-Gln-Asp-Leu-Lys-Cys-Thr-Thr-Asn-Asn-Met-Arg-Val-Trp-Asp-Cys-Thr-Trp-Pro-Ala-Pro-Leu (SEQ ID No. 1).

The present invention extends to nucleic acid molecules and preferably isolated nucleic acid molecules comprising a sequence of nucleotides encoding or complementary to a sequence encoding an LBP as hereinbefore described including a fusion polypeptide defining an LBP. The nucleotide sequence may
5 correspond to the naturally occurring amino acid sequence of the LBP or may contain single or multiple nucleotide substitutions, deletions and/or additions thereto. Preferably, the nucleic acid encodes an LBP with an N-terminal amino acid sequence comprising Gly-Val-Gln-Asp-Leu-Lys-Cys-Thr-Thr-Asn-Asn-Met-Arg-Val-Trp-Asp-Cys-Thr-Trp-Pro-Ala-Pro-Leu (SEQ ID No. 1) or contains a
10 nucleotide sequence capable of hybridising under low, preferably under medium and more preferably under high stringency conditions to a nucleotide sequence encoding the above stated amino acid sequence. Put in alternative terms the hybridising nucleotide sequence is at least 45%, preferably at least 55%, more preferably at least 65-75% and even more preferably greater than 85% similar to
15 the nucleotide sequence encoding the above-stated amino acid sequence.

For the purposes of defining the levels of stringency, reference can conveniently be made to Sambrook *et al*⁽¹⁵⁾ at pages 387-389 where the washing step at paragraph 11 is considered herein to be high stringency. A low stringency wash is
20 defined herein to be 0.1%-0.5% w/v SDS at 37-45 °C for 2-3 hours and a medium level of stringency is considered herein to be 0.25%-0.5% w/v SDS at ≥ 45 °C for 2-3 hours. The alternative conditions are applicable depending on concentration, purity and source of nucleic acid molecules.

25 In a particularly preferred embodiment, the nucleic acid molecule of the present invention is in an expression vector capable of replication and expression in eukaryotic organisms (e.g. CHO cells or other mammalian cells, yeast cells, insects cells) and/or in prokaryotic organisms (e.g. *E. coli*). Such expression vectors and cells transformed with same are convenient sources of the
30 recombinant LBP molecules of the present invention.

The LBP of the present invention will be particularly useful as an inhibitor of the systemic effects of locally produced or administered LIF. Where the use of a heterologous LBP relative to the mammal to be treated is significantly more active than homologous LBP (i.e. LBP from the same species of mammal), then
5 this high activity is particularly advantageous in reducing, for example, the immunological consequences of introducing the heterologous protein into a mammal. The high activity will also enable the administration of as little LBP as possible to ensure that the LBP can be localised to a particular site and cannot disseminate to other areas of the mammal. It may also be important in
10 conjunction with LIF therapy to maintain effective levels of LBP in the circulatory fluids including serum so as to prevent dissemination of LIF administered in the course of the therapy, such as where LIF is locally administered.

Accordingly, another aspect of the present invention contemplates a method of
15 inhibiting the activity of LIF in a mammal comprising administering to said mammal, an effective amount of a soluble LBP.

More particularly, the present invention contemplates a method of inhibiting the activity of LIF in a mammal comprising administering to said mammal, an
20 effective amount of a soluble heterologous LBP wherein said heterologous LBP is capable of greater inhibition of the LIF in the mammal to be treated when compared to a LIF of same mammalian origin to the LBP.

Preferably, the mammal to be treated is a human and the LBP is mLBP.
25

Preferably, this method is used in the inhibition of the systemic effects of locally produced LIF which are therapeutically undesirable, unintended or unwanted.

Administration may be by any convenient means applicable to the condition being
30 treated but is particularly conveniently administered locally to the site where LIF is to be inhibited. Alternatively, the LBP may be administered to elevate serum levels while LIF is administered locally.

The effective amount of LBP will vary depending on the mammal and condition to be treated but can range from serum levels of 0.001 $\mu\text{g/ml}$ to 100 $\mu\text{g/ml}$, preferably 0.01 $\mu\text{g/ml}$ to 50 $\mu\text{g/ml}$, more preferably 0.1 $\mu\text{g/ml}$ to 20 $\mu\text{g/ml}$ and most preferably 0.5 $\mu\text{g/ml}$ to 10 $\mu\text{g/ml}$. The amount required will be that
5 amount required to completely or partially inhibit LIF activity or at least reduce it to a clinically acceptable level. Furthermore, the "LIF activity" may be all activities associated with LIF or only some of these activities and may be measured in any number of convenient ways such as in a bioassay⁽⁹⁾ or a receptor-binding assay.

10

The LBP may be administered alone or in combination with other active compounds such as, but not limited to, cytokines, antibiotics, anti-cancer agents or immuno-stimulatory or reducing compounds. Administration of the LBP and other active compounds may be by simultaneous or sequential administration.
15 Furthermore, whether the LBP is administered alone or in combination with other compounds, a single dose of LBP may be sufficient or multiple doses or continuous infusion may be required depending on the condition and mammal to be treated and whether any adverse clinical reactions appear.

20 Yet a further aspect of the present invention provides a pharmaceutical composition comprising LBP as hereinbefore defined and one or more pharmaceutically acceptable carriers and/or diluents. The preparation of pharmaceutical composition is discussed generally in Remington's Pharmaceutical Sciences, 17th ed. Mach Publishing Co., Easton, Pennsylvania, USA.

25 Alternatively, the LBP may be administered genetically using transgenic animal cells or microbial cells.

The active ingredients of the pharmaceutical composition comprising an LBP as herein described are contemplated to exhibit excellent activity when administered
30 in a dosage regimen adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily, weekly, monthly or in other suitable time intervals or the dose may be proportionally reduced as

indicated by the exigencies of the situation. The active compound may be administered in a convenient manner such as by the oral, topical, intravenous, intramuscular, subcutaneous, intranasal, intradermal or suppository routes or implanting (eg using slow release molecules). Depending on the route of
5 administration, the active ingredient which comprises an LBP may be required to be coated in a material to protect said ingredient from the action of enzymes, acids and other natural conditions which may inactivate said ingredients. In order to administer the vaccine by other than parenteral administration, the LBP may be coated by, or administered with, a material to prevent its inactivation. For
10 example, the LBP may be administered in an adjuvant, co-administered with enzyme inhibitors or in liposomes.

The active compound may also be administered in dispersions prepared in glycerol, liquid polyethylene glycols, and/or mixtures thereof and in oils. Under
15 ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the
20 extemporaneous preparation of sterile injectable solutions or dispersion. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or
25 dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The
30 prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thormerosal, and the like. In many cases, it will be preferable to

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include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by, for example, the use in the compositions of agents delaying absorption.

- 5 Sterile injectable solutions are prepared by incorporating the active compound in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredient(s) into a sterile vehicle which contains the basic dispersion medium
- 10 and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

- 15
- When the LBP is suitably protected as described above, the molecule may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food
- 20 of the diet. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course,
- 25 be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in the vaccine compositions is such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.5 ug and 20 mg of active compound.

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The tablets, troches, pills, capsules and the like may also contain the following: a binder such as gum tragacanth, acacia, corn starch or gelatin; excipients such as

dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavoring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring such as cherry or orange flavor. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and formulations.

As used herein, pharmaceutically acceptable carriers and/or diluents include any and all solvents, dispersion media, aqueous solutions, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the composition is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

To reduce the potentially disadvantageous effects of administering a heterologous LBP to a mammal, it may be possible to derivatise or otherwise alter the heterologous LBP to reduce its antigenicity in the mammal to be treated. This can be accomplished by rendering the LBP more like a protein from the mammal to be treated. For example, the LBP can be coupled or masked with a protein or polypeptide or other suitable molecule from the species of mammal to be treated or coupling or fusing the heterologous LBP or parts thereof to the LBP from the target species. In a particularly preferred embodiment, the murine LBP is rendered non-immunogenic in a human and has been derivativatised more like a

human molecule.

In yet another aspect of the present invention, the LBP of first mammalian origin is used to detect LIF of second mammalian origin.

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In one embodiment, the method contemplated for detecting LIF in a biological sample said method comprising contacting said biological sample with an immobilised LBP from a mammal wherein the LBP is capable of inhibiting the ability of the first mentioned LIF to induce differentiation of M1 myeloid
10 leukaemic cells *in vitro* to a greater extent when compared to its ability to inhibit LIF of same mammalian origin as LBP and/or wherein said LBP has at least a 100-fold higher binding affinity for said first mentioned LIF compared to the binding affinity of LIF of same mammalian origin as said LBP wherein said contact is for a time and under conditions sufficient for a complex to form
15 between the immobilised LBP and the LIF in the sample; contacting the LBP-LIF complex with an antibody specific for said LIF and labelled with a reporter molecule capable of providing a signal; determining the presence of bound LIF on the basis of the signal produced by said reporter molecule.

20 In an alternative embodiment, the LBP-LIF complex is contacted with an unlabelled antibody specific to said LIF and then LIF is detected by a second antibody labelled with a reporter molecule and specific to said first antibody.

In yet another alternative embodiment, the LIF in the sample is immobilised (e.g.
25 by an immobilised antibody) and then bound LIF detected by labelled LBP or first by unlabelled LBP followed by a labelled antibody specific to said LBP.

An embodiment of this aspect of the present invention is described hereinafter with reference to the preferred embodiment of using mLBP to detect hLIF. The
30 present invention, however, is not so limited and extends to the use of LBP and LIF from other mammals.

According to this embodiment, there is contemplated a method of detecting hLIF in a biological sample, said method comprising contacting said sample to mLBP as hereinbefore defined, immobilised to a solid support for a time and under conditions sufficient for a mLBP-hLIF complex to form and then detecting for the presence of said mLBP-hLIF complex.

In a particularly preferred method, the mLBP-hLIF complex is detected by contacting the complex with an antibody for hLIF with the antibody itself being labelled with a reporter molecule or with an additional step of contacting the mLBP-hLIF-antibody complex with a labelled second antibody capable of binding to the first antibody.

The antibodies may be polyclonal or monoclonal and both are obtainable by immunization of a suitable animal with hLIF and either type is utilizable in the LIF assay. The methods of obtaining both types of sera are well known in the art. Polyclonal sera are less preferred but are relatively easily prepared by injection of a suitable laboratory animal with an effective amount of hLIF, or antigenic parts thereof, collecting serum from the animal, and isolating specific sera by any of the known immunoabsorbent techniques. Although antibodies produced by this method are utilizable in virtually any type of LIF assay, they are generally less favoured because of the potential heterogeneity of the product.

The use of monoclonal antibodies in an immunoassay is particularly preferred because of the ability to produce them in large quantities and the homogeneity of the product. The preparation of hybridoma cell lines for monoclonal antibody production derived by fusing an immortal cell line and lymphocytes sensitized against the immunogenic preparation can be done by techniques which are well known to those who are skilled in the art. (See, for example Douillard and Hoffman, *Basic Facts about Hybridomas*, in *Compendium of Immunology* Vol II, ed. by Schwartz, 1981; Kohler and Milstein, *Nature* 256: 495-499, 1975; *European Journal of Immunology* 6: 511-519, 1976).

The presence of a hLIF may be accomplished in a number of ways such as by Western blotting and ELISA procedures. A wide range of immunoassay techniques are available as can be seen by reference to US Patent Nos. 4,016,043, 4,424,279 and 4,018,653. These, of course, include both single-site and two-site
5 or "sandwich" assays of the non-competitive types, as well as in the traditional competitive binding assays. These assays also include direct binding of a labelled antibody to a target.

Sandwich assays are among the most useful and commonly used assays and are
10 favoured for use in the present invention. A number of variations of the sandwich assay technique exist, and all are intended to be encompassed by the present invention. Briefly, in a typical forward assay, mLBP is immobilized on a solid substrate and the sample to be tested brought into contact with the bound molecule. After a suitable period of incubation, for a period of time sufficient to
15 allow formation of an mLBP-hLIF complex, an antibody specific to the hLIF, labelled with a reporter molecule capable of producing a detectable signal, is then added and incubated allowing sufficient time for the formation of a complex of mLBP-hLIF-labelled antibody. Any unreacted material is washed away, and the presence of the hLIF is determined by observation of a signal produced by the
20 reporter molecule. The results may either be qualitative, by simple observation of the visible signal, or may be quantitated by comparing with a control sample containing known amounts of hapten. Variations on the forward assay include a simultaneous assay, in which both sample and labelled antibody are added simultaneously to the bound mLBP. In accordance with the present invention the
25 sample is one which might contain hLIF and includes biological fluid (e.g. blood, serum, tissue extract) fermentation fluid and supernatant fluid such as from a cell culture.

In the typical forward sandwich assay, mLBP or a hLIF-binding part thereof is
30 either covalently or passively bound to a solid surface. The solid surface is typically glass or a polymer, the most commonly used polymers being cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene. The solid

supports may be in the form of tubes, beads, discs of microplates, or any other surface suitable for conducting an immunoassay. The binding processes are well-known in the art and generally consist of cross-linking covalently binding or physically adsorbing mLBP to the polymer. After immobilising the mLBP, the polymer-mLBP complex is washed in preparation for the test sample. An aliquot of the sample to be tested is then added to the solid phase complex and incubated for a period of time sufficient (e.g. 2-40 minutes) and under suitable conditions (e.g. 25 °C) to allow binding of hLIF in the sample to the immobilised mLBP. Following the incubation period, the complex is washed and optionally dried and then incubated with an antibody specific for hLIF. The antibody is linked to a reporter molecule which is used to indicate the binding of hLIF to mLBP. Alternatively, a second antibody conjugated to a reporter molecule and capable of binding to the first antibody may be used.

An alternative method involves immobilizing the target molecules (i.e. hLIF) in the biological sample and then exposing the immobilized target to mLBP which may or may not be labelled with a reporter molecule. Depending on the amount of target and the strength of the reporter molecule signal, a bound target may be detectable by direct labelling of mLBP. Alternatively, a labelled antibody, specific to mLBP is exposed to the complex to form a tertiary complex. The complex is detected by the signal emitted by the reporter molecule.

By "reporter molecule" as used in the present specification, is meant a molecule which, by its chemical nature, provides an analytically identifiable signal which allows the detection of antigen-bound antibody. Detection may be either qualitative or quantitative. The most commonly used reporter molecules in this type of assay are either enzymes, fluorophores or radionuclide containing molecules (i.e. radioisotopes) and chemiluminescent molecules.

In the case of an enzyme immunoassay, an enzyme is conjugated to the hLIF-specific antibody, generally by means of glutaraldehyde or periodate. As will be readily recognized, however, a wide variety of different conjugation techniques

exist, which are readily available to the skilled artisan. Commonly used enzymes include horseradish peroxidase, glucose oxidase, beta-galactosidase and alkaline phosphatase, amongst others. The substrates to be used with the specific enzymes are generally chosen for the production, upon hydrolysis by the corresponding
5 enzyme, of a detectable color change. Examples of suitable enzymes include alkaline phosphatase and peroxidase. It is also possible to employ fluorogenic substrates, which yield a fluorescent product rather than the chromogenic substrates noted above. Generally, the enzyme-labelled antibody is added to the mLBP-hLIF complex, allowed to bind, and then the excess reagent is washed
10 away. A solution containing the appropriate substrate is then added to the tertiary complex. The substrate will react with the enzyme linked to the antibody, giving a qualitative visual signal, which may be further quantitated, usually spectrophotometrically, to give an indication of the amount of hapten which was present in the sample. "Reporter molecule" also extends to use of cell
15 agglutination or inhibition of agglutination such as red blood cells on latex beads, and the like.

Alternately, fluorescent compounds, such as fluorecein and rhodamine, may be chemically coupled to antibodies without altering their binding capacity. When
20 activated by illumination with light of a particular wavelength, the fluorochrome-labelled antibody adsorbs the light energy, inducing a state to excitability in the molecule, followed by emission of the light at a characteristic color visually detectable with a light microscope. As in the EIA, the fluorescent labelled antibody is allowed to bind to the mLBP-hLIF. After washing off the unbound
25 reagent, the tertiary complex is then exposed to the light of the appropriate wavelength and the fluorescence observed indicates the presence of the hapten of interest. Immunofluoresence and EIA techniques are both very well established in the art and are particularly preferred for the present method. However, other reporter molecules, such as radioisotope, chemiluminescent or bioluminescent
30 molecules, may also be employed. The above considerations apply where the antibody is an anti-immunoglobulin and is labelled so that a quaternary complex is obtained.

Furthermore, the mLBP of the present invention may be packaged in kit form, for example, to conduct an assay for LIF. The kit is in compartmental form adapted to contain mLBP and may further comprise in the same or different compartments the reagents for the LIF assay.

5

The foregoing description is equally applicable to the use of LBP from a first mammalian species to detect LIF from a second mammalian species as hereinbefore defined. Furthermore, variations such as binding LIF using immobilised antibodies and then detecting the bound LIF with LBP conjugated to a reporter molecule or using first LBP then LBP-binding antibody conjugated to a reporter molecule.

10

The present invention is further described by reference to the following non-limiting Figures and/or Example.

15

In the Figures:

Figure 1 is a graphical representation showing size exclusion profile of ^{125}I -mLIF in the presence and absence of normal mouse serum. The Ultrogel AcA44 (20 x 1 cm) column was equilibrated and run in 20 mM Na phosphate buffer pH 7.4, 150 mM NaCl (PBS) at 0.2 ml/min, collecting 1 min fractions that were counted in a gamma counter. (A) 100,000 cpm ^{125}I -mLIF in 100 μl PBS (B) 100,000 cpm ^{125}I -mLIF preincubated with 100 μl normal mouse serum for 16 hr at room temperature.

20

Figure 2 is a graphical representation showing representative fractionations of normal mouse serum by LIF affinity chromatography, anion exchange chromatography and size exclusion chromatography. Absorbance and specific ^{125}I -mLIF binding of fractions in a Con-A Sepharose binding assay are shown. (A) 50 ml mouse serum was applied to a column of LIF-pABAE-Sepharose 4B in PBS and eluted with guanidine-HCl as described in the Example. (B) Active fractions from affinity chromatography were pooled, applied to a Mono-Q HR 5/5 and

30

eluted with a salt gradient as described in the Example. (C) Active fractions from anion-exchange chromatography were pooled, concentrated to 100 μ l and injected onto a Superose-12 10/30 column equilibrated in PBS, and elution was carried out isocratically using the same buffer.

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Figure 3 is a photographic and graphical representation of preparative native gel electrophoresis of LBP. Upper panel: Fractions from size-exclusion chromatography containing LIF-binding activity were pooled and applied to a 20ml 7.5% w/v polyacrylamide native gel and electrophoresed in a BioRad Model 10 491 Prep Cell as described in the Example. Specific 125 I-mLIF binding of eluted fractions in a Con-A Sepharose binding assay is shown. Lower panel: NaDodSO₄-PAGE of 20 μ l aliquots of pooled fractions containing LBP activity from various stages of purification: s, low molecular weight standards (Pharmacia) (MWx10⁻³); a, normal mouse serum (1/50); b & c, 1st LIF-pABAE 15 Sepharose 4B column; d, 2nd LIF-pABAE Sepharose 4B column; e, Mono-Q HR 5/5 column; f, Superose-12 10/30 column; g, preparative native gel electrophoresis, arrow shows band of interest.

Figure 4 is a graphical representation showing Scatchard analysis of 125 I-mLIF 20 binding to LBP, solubilized murine liver membranes and 3T3-L1 cells. (A) Saturation isotherm for 125 I-mLIF binding to mouse serum (5 μ l in 210 μ l total) showing total cpm bound (\blacktriangle), non-specific cpm bound (\bullet) and specific cpm bound (o). (B) Scatchard transformation of 125 I-mLIF binding to LBP in mouse serum (5 μ l in 210 μ l total) (o) and purified LBP (1 μ l in 90 μ l total) (\bullet). (C) Scatchard 25 transformation of 125 I-mLIF binding to solubilized murine liver membranes (25 μ l in 175 μ l total) (o) and 3T3-L1 cells (0.6×10^6 in 80 μ l total) (\bullet).

Figure 5 is a graphical representation of displacement curves for unlabelled mLIF (o) and hLIF (\bullet) competing with the binding of (A) 125 I-mLIF and (B) 125 I-hLIF 30 for purified LBP (100 μ l total volume).

Figure 6 is a graphical representation showing blocking of induction of

differentiation of mLIF stimulated colonies of M1 leukemic cells by affinity-purified LBP. (A) Titration of LBP in the presence of constant amounts of mLIF as indicated. (B) Titration of mLIF with constant levels of LBP as indicated.

- 5 Figure 7 is a graphical representation showing the blocking of induction of mLIF and hLIF stimulated colonies of M1 leukaemic cells by normal mouse serum. mLIF and hLIF have an equal ability to stimulate the differentiation of murine M1 colonies. A. Effect on 100 U *E. coli* derived murine LIF (emLIF) and 800 U *E. coli* derived human LIF (ehLIF). B. Effect on 400 U emLIF and 200 U each
- 10 of ehLIF, CHO cells derived human LIF (chohLIF) and yeast derived human LIF (YhLIF). As in Figure 6A, a 1:8 dilution of normal mouse serum (equivalent to 125 ng/ml LBP) was able to inhibit 50% of activity stimulated by 100U/ml mLIF. In contrast, as little as 1:512 dilution of normal mouse serum (equivalent to ~ 1.5 ng/ml LBP) was able to inhibit 50% of the activity stimulated by 800 U/ml hLIF.

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- Figure 8 is a graphical representation showing specific binding of ^{125}I -mLIF (A) and ^{125}I -hLIF (B) to mLBP immobilized onto 96-well PVC plates. Affinity purified mLBP was diluted in various buffers (PBS, 0.1M NaHCO_3 pH9.5 and 0.1M NaHCO_3 pH9.5 containing 4 $\mu\text{g}/\text{ml}$ bovine serum albumin (BSA)) and 100 μl
- 20 aliquots incubated in 96-well PVC plates (Costar) for 16 hours at room temperature. The plates were then washed with PBS containing 0.05% v/v Tween-20 (wash buffer) and blocked with HEPES-buffered RPMI medium containing 10% v/v Foetal calf serum (HRF) for 1 hour at room temperature. The plates were again washed with wash buffer then incubated with 50 μl HRF
- 25 containing ^{125}I -mLIF or ^{125}I -hLIF for 16 hours at room temperature. Non-specific binding was determined from incubations containing in addition 20 μl of 50 $\mu\text{g}/\text{ml}$ unlabelled mLIF or hLIF. Bound and free labelled ligand were separated by washing the plates three times in wash buffer. The plates were dried and the assay plates containing the bound labelled ligand were exposed to a
- 30 phosphorimager screen (Molecular Dynamics) for 24 hours. Results were analysed using Imagequant version 3 (Molecular Dynamics) software. The alphabetically labelled bars are as follows:

- 5 a) 5.76 $\mu\text{g/ml}$ mLBP in PBS
 b) 2.88 $\mu\text{g/ml}$ mLBP in 0.1M Na bicarbonate pH9.5
 c) 1.44 $\mu\text{g/ml}$ mLBP in 0.1M Na bicarbonate pH9.5
 d) 0.72 $\mu\text{g/ml}$ mLBP in 0.1M Na bicarbonate pH9.5
 e) 0.36 $\mu\text{g/ml}$ mLBP in 0.1M Na bicarbonate pH9.5
 f) 0.18 $\mu\text{g/ml}$ mLBP in 0.1M Na bicarbonate pH9.5
 g) 5.76 $\mu\text{g/ml}$ mLBP in PBS + 4 $\mu\text{g/ml}$ BSA
 h) 2.88 $\mu\text{g/ml}$ mLBP in 0.1M Na bicarbonate pH9.5 + 4 $\mu\text{g/ml}$ BSA
 10 i) 1.44 $\mu\text{g/ml}$ mLBP in 0.1M Na bicarbonate pH9.5 + 4 $\mu\text{g/ml}$ BSA
 j) 0.72 $\mu\text{g/ml}$ mLBP in 0.1M Na bicarbonate pH9.5 + 4 $\mu\text{g/ml}$ BSA
 k) 0.36 $\mu\text{g/ml}$ mLBP in 0.1M Na bicarbonate pH9.5 + 4 $\mu\text{g/ml}$ BSA
 l) 0.18 $\mu\text{g/ml}$ mLBP in 0.1M Na bicarbonate pH9.5 + 4 $\mu\text{g/ml}$ BSA
 m) 5.76 $\mu\text{g/ml}$ mLBP in PBS
 15 n) 2.88 $\mu\text{g/ml}$ mLBP in 0.1M Na bicarbonate pH9.5
 o) 1.44 $\mu\text{g/ml}$ mLBP in 0.1M Na bicarbonate pH9.5
 p) 0.72 $\mu\text{g/ml}$ mLBP in 0.1M Na bicarbonate pH9.5
 q) 0.36 $\mu\text{g/ml}$ mLBP in 0.1M Na bicarbonate pH9.5
 r) 0.18 $\mu\text{g/ml}$ mLBP in 0.1M Na bicarbonate pH9.5
 20 s) 5.76 $\mu\text{g/ml}$ mLBP in PBS + 4 $\mu\text{g/ml}$ BSA
 t) 2.88 $\mu\text{g/ml}$ mLBP in 0.1M Na Bicarbonate pH9.5 + 4 $\mu\text{g/ml}$ BSA
 u) 1.44 $\mu\text{g/ml}$ mLBP in 0.1M Na Bicarbonate pH9.5 + 4 $\mu\text{g/ml}$ BSA
 v) 0.72 $\mu\text{g/ml}$ mLBP in 0.1M Na Bicarbonate pH9.5 + 4 $\mu\text{g/ml}$ BSA
 w) 0.36 $\mu\text{g/ml}$ mLBP in 0.1M Na Bicarbonate pH9.5 + 4 $\mu\text{g/ml}$ BSA
 25 x) 0.18 $\mu\text{g/ml}$ mLBP in 0.1M Na Bicarbonate pH9.5 + 4 $\mu\text{g/ml}$ BSA

Figure 9 is a graphical representation showing competitive binding of mouse LIF (•), human LIF (◊), porcine LIF (▲) and a series of mouse-human LIF chimeric molecules (■, □, ◆, ◇). Aliquots of 20 μl of normal mouse serum diluted 1 in 20
 30 in PBS were added to 96-well filtration assay plates containing a 0.65 micron Durapore membrane (Millipore) with 10 μl ^{125}I -hLIF, 50 μl unlabelled ligand diluted in PBS and 25 μl Concanavalin-A Sepharose 4B (diluted 1 in 4 in 0.1M

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sodium acetate pH6.0 containing 1 mM each $MgCl_2$, $MnCl_2$ and $CaCl_2$) and incubated at room temperature, overnight, with agitation. Bound and free radioactivity were separated by vacuum filtration of the supernatant, and the Concanavalin-A Sepharose pellet was washed once with cold PBS. Assay plates
5 containing the Concanavalin-A Sepharose pellet were exposed to a phosphorimager screen (Molecular Dynamics) for 24 hours. Results were analysed using Imagequant version 3 (Molecular Dynamics) software.

Figure 10 is a graphical representation showing comparative binding data for
10 mLIF and hLIF to mLBP and recombinant hLIF receptor α -chain.

(A) Scatchard analysis of ^{125}I -mLIF binding to mLBP (normal mouse serum diluted 1 in 20) (\bullet , $K_d = 1-4nM$), ^{125}I -hLIF binding to mLBP (normal mouse serum diluted 1 in 1000) (\circ , $K_d = 17pM$), ^{125}I -hLIF binding to conditioned medium collected 4 days after transfection of COS cells with a plasmid encoding a soluble
15 truncated form of the hLIF receptor α -chain (\blacktriangle , $K_d = 300-400pM$) and ^{125}I -hLIF binding to 44×10^6 cells/ml Allen1 cells, which express a high affinity hLIF receptor (\blacktriangle , $K_d = 80pM$).

(B) Competition of unlabelled mouse LIF (\bullet , $ID_{50} = 500nM$) and human LIF (\circ , $ID_{50} = 0.1nM$) with ^{125}I -hLIF for binding to mLBP. Experimental conditions
20 were as described in Figure 5.

(C) Competition of unlabelled mouse LIF (\bullet , $ID_{50} > 10,000nM$) and human LIF (\circ , $ID_{50} = 2nM$) with ^{125}I -hLIF for binding to conditioned medium collected 4 days after transfection of COS cells with a plasmid encoding a soluble truncated form of the hLIF receptor α -chain. Experimental conditions were as described in
25 Figure 5.

Figure 11A is a photographic representation showing Coomassie Blue stained gel of elution fractions from hLIF-Affigel-10 column. The hLIF-Affigel column was synthesized using 5ml Affigel-10 (BioRad), 3.5mg hLIF and 5mg ovalbumin as a
30 filler protein according to manufacturer's instructions. An amount of 60ml of normal C_3H/HeJ mouse serum (as a source of mLBP) was applied to the 5ml hLIF-Affigel-10 column equilibrated in PBS. The column was eluted with 85ml

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equilibration buffer and a 30ml linear gradient to 6M guanidine-HCl in the same buffer. Fractions of 2.5ml were collected throughout at a flow rate of 0.5ml/min, exchanged where necessary into equilibration buffer using PD10 columns (Pharmacia) and concentrated to 1ml using Centricon microconcentrators (Amicon). SDS-PAGE was performed according to the method of Laemmli⁽⁴⁾. Aliquots of 15 μ l of each fraction were diluted 1 in 2 in SDS sample buffer and electrophoresed in 13% w/v polyacrylamide gels in a Mini-Protean II system (BioRad) and stained with Coomassie Brilliant Blue. The positions of the molecular weight markers are shown.

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Figure 11B is a graphical representation showing monitoring of mLBP amounts by direct ¹²⁵I-mLIF binding assays of elution fractions from hLIF-Affigel-10 column.

Figure 12 is a photographic representation showing deglycosylation of mLBP using N-glycanase. Aliquots of ¹²⁵I-mLBP in 50mM sodium phosphate pH7.5 were incubated in the presence of 0.25 units of N-glycanase (Genzyme) in the same buffer (lanes a and b), buffer alone (lane c) or 0.25 units of N-glycanase, 0.1% w/v SDS and 1% v/v 2-mercaptoethanol in the same buffer (lanes d and e) at 37 °C for 24 hours. The incubation mixtures were diluted 1 in 2 in SDS sample buffer and electrophoresed in 10% w/v polyacrylamide gels in a Mini-Protean II system (BioRad), stained with Coomassie Brilliant Blue, dried and exposed to a phosphorimager screen (Molecular Dynamics) for 24 hours. Results were analysed using Imagequant version 3 (molecular Dynamics) software. The positions of the molecular weight markers are shown.

25

EXAMPLE 1

1. Materials and Methods5 **Collection of mouse sera.**

Blood from C57BL/6J, C3H/HeJ, CBA f/Ca H, DBA/2J and BALB/C/An Bradley mice was collected by exsanguination. Red cells were separated from the serum by centrifugation at 3000g for 10 min, and the serum stored at -20°C for up to 6 months.

10

Affinity Chromatography.

Leukaemia Inhibitory Factor p-aminobenzamidoethyl-Sepharose 4B (LIF-pABAE Sepharose 4B) was prepared according to the method of Cuatrecasas and Anfinsen². Briefly, 8.4g CNBr-Activated Sepharose 4B (Pharmacia, Uppsala) was
15 washed in 1mM HCl, reacted with an equal volume of 2M ethylene diamine at pH10 for 16 hr at 4°C. The p-nitrobenzamido-ethyl Sepharose 4B was washed extensively with 50% v/v dimethyl formamide, then reduced with 0.2M sodium dithionite in 0.5M sodium bicarbonate pH8.5 for 60 min. at 40°C. The p-aminobenzamidoethyl Sepharose 4B was washed in 0.5M HCl then diazotised
20 with 0.1M sodium nitrite for 7 min at 4°C. Aliquots of 7ml of this diazonium-Sepharose derivative were then reacted with 8mg each of *E. coli*-derived murine LIF³ and chicken ovalbumin (Sigma, MO) as a non-specific filler protein in 10ml 0.2M sodium tetraborate pH9.2 for 16 hr at 4°C. The LIF-pABAE-Sepharose 4B was washed extensively with 20mM sodium phosphate pH7.4 containing 150mM
25 sodium chloride (PBS) and 6M guanidine-HCl in PBS before use.

Aliquots of 50 ml mouse serum or pooled fractions containing LIF-binding protein (LBP) activity in PBS were applied to a 13 x 0.7 cm column of LIF-pABAE-Sepharose 4B equilibrated in PBS (this and all succeeding buffers
30 contained 0.02% v/v Tween 20 and 0.02% w/v sodium azide) and eluted with 20 ml equilibration buffer followed by a 15 ml linear gradient to 6M guanidine-HCl in the same buffer. Fractions of 2.5ml were collected at a flow rate of 0.5ml/min

and exchanged where necessary into equilibration buffer using a prepacked Sephadex G-25 column (PD10, Pharmacia, Uppsala).

Anion-exchange Chromatography

- 5 Fractions from affinity chromatography containing LIF-binding activity were pooled and diluted 20-fold with 20mM Tris pH7.0, then applied to a Mono-Q HR 5/5 (Pharmacia, Uppsala) column equilibrated in the same buffer. Elution was carried out using 25ml of equilibration buffer, followed by a 30ml linear gradient to 1M NaCl in equilibration buffer. The 0.5ml fractions were collected at a flow rate of 0.5ml/min.

Size-exclusion Chromatography

- Fractions from anion-exchange chromatography containing LIF-binding activity were pooled and concentrated to 100 μ l using a Centricon-10 microconcentrator (Amicon, MA). The sample was injected onto a Superose-12 10/30 (Pharmacia, Uppsala) column equilibrated in PBS, and elution was carried out isocratically using the same buffer. The 0.2ml fractions were collected at a flow rate of 0.2ml/min.

20 Preparative Native Polyacrylamide Gel Electrophoresis

- Fractions from size-exclusion chromatography containing LIF-binding activity were diluted 2-fold in 0.062M Tris pH6.8, 12.5% v/v glycerol, 0.02% w/v bromophenol blue, applied to a 20ml 0.375M Tris pH8.8, 7.5% w/v polyacrylamide/0.2% w/v Bis separating gel with a 10ml 0.125M Tris pH6.8, 4% w/v acrylamide/0.11% w/v stacking gel, containing no sodium dodecyl sulfate (NaDodSO₄). The sample was electrophoresed in a Model 491 Prep Cell (BioRad, CA) using a 0.025M Tris, 0.19M glycine buffer pH8.3 as an electrode buffer at 40mA for approximately 6 hr. Fractions of 2.5ml were collected at a flow rate of 1ml/min after the elution of the bromophenol blue dye front from the gel.

Analytical sodium dodecyl sulfate polyacrylamide gel electrophoresis

NaDodSO₄-PAGE was performed according to the method of Laemmli⁴.

Aliquots of samples to be analysed were diluted in NaDodSO₄ sample buffer and
5 electrophoresed in 12.5% w/v polyacrylamide gels in a Mini-Protean II system
(BioRad, CA) and silver-stained⁵.

Iodination of LIF

Amounts of 1-2µg recombinant murine or human LIF produced in *E. coli* were
10 iodinated and purified as previously described⁽³⁾. The iodinated materials
retained biological activity and had specific activities of 3-5x10⁵ cpm/pmole for
¹²⁵I-mLIF and 6-10x10⁵ cpm/pmole for ¹²⁵I-hLIF.

Binding Assays

15 Aliquots of samples containing the LIF-binding activity were added to Eppendorf
tubes containing 10µl ¹²⁵I-LIF (5-10 x 10⁴ cpm) and 50-100µl concanavalin-A
Sephrose (Pharmacia, Uppsala) (diluted four-fold in 100mM sodium acetate
pH6.0 containing 1mM each MgCl₂, MnCl₂ and CaCl₂). Non-specific binding was
determined from incubations containing in addition 10µl of 50µg/ml unlabelled
20 LIF. Assay tubes were incubated at room temperature for 16 hr with agitation.
Bound and free label were separated by resuspending the incubation mixture,
layering it over 150µl fetal calf serum (FCS) in a tapered microcentrifuge tube
and spinning at 13,000rpm for 1 min. The tip of the tube, containing the
Sephrose pellet, was then cut off using a scalpel blade and both the pellet and
25 supernatant were counted for 1 min in a gamma counter (Packard Crystal
Multidetector, Packard Instruments).

For competitive binding experiments, LBP and Concanavalin-A Sepharose were
incubated with between 1x10⁻⁴ and 2x10⁻⁴ ng unlabelled ligand and a fixed
30 concentration (2nM) of labelled ligand and then processed as described above.

Scatchard analyses

3T3-L1 cells were maintained as previously described⁶ and harvested using HEPES buffered RPMI medium with 10% v/v fetal calf serum (HRF) containing 40mM EDTA and 200µg/ml chondroitin sulfate. Cells resuspended in 60µl HRF were placed in Falcon 2054 tubes (Becton Dickinson, NJ) with 10µl ¹²⁵I-mLIF, then incubated at 4°C for 3 hr. Non-specific binding was determined as above. Mouse liver membranes were prepared essentially as described⁷, solubilised in 1% v/v Triton X-100 (Pierce, IL) and assayed as for LBP. Saturation binding experiments were carried out and Scatchard analysis of the binding isotherm was determined using the curve-fitting program, LIGAND⁸, after correction for the bindable fraction of radioligand as previously described⁽³⁾. Scatchard analysis allows the derivation of the affinity of a labelled ligand for its binding site and the concentration of these binding sites, thereby permitting the quantitation of LBP in samples of known volume, assuming one binding site per LBP molecule and a molecular weight of 90kDa for LBP.

Bioassay

Samples for bioassay were exchanged into normal saline using prepacked Sephadex G25M (PD10 columns, Pharmacia, Uppsala), concentrated using Centricon-10 microconcentrators (Amicon, MA) and sterilised by passage through a 0.45µm filter (Millipore, MA). M1 differentiation assays were performed as previously described⁹.

25 Protein Assay

Protein was estimated by the method of Bradford⁽¹³⁾ using a Coomassie blue based reagent (Pierce, IL) according to the manufacturers instructions.

Amino acid sequence analysis

30 Amino acid sequence analysis was carried out by Edman degradation of proteins blotted onto a polyvinylidene difluoride (PVDF) membrane as previously described¹⁰.

2. Analysis of LIF Binding Protein

Identification of LIF-binding activity in mouse serum

LIF-binding protein (LBP) was detected in normal mouse sera by its ability to
5 inhibit the binding of ^{125}I -mLIF to a neutralising monoclonal antibody in a
competition radioimmunoassay or to its cellular receptor on 3T3-L1 cells in a
competition radioreceptor assay. It was detected by either of these assays in
normal mouse sera at a dilution of 1/4 to 1/8, but at a 20 to 30-fold greater
dilution in the sera of pregnant mice and a 2-4 fold lower dilution in the sera of
10 neonatal mice (Table 2). Because LIF is a very basic protein, it was important to
determine whether this LIF-binding activity was due to a non-specific association
of LIF with an acidic serum protein, however a variety of other basic proteins,
including lysozyme, α -chymotrypsinogen-A and cytochrome-C, were unable to
compete for ^{125}I -mLIF binding to LBP. In addition, other acute-phase proteins,
15 such as IL-1 and IL-6, were also unable to compete for binding.

Direct binding of ^{125}I -mLIF to LBP was detected in two different ways. By size-
exclusion chromatography, ^{125}I -mLIF exhibits a molecular weight of approximately
20kDa. When ^{125}I -mLIF was chromatographed in the presence of normal mouse
20 serum, under non-dissociating conditions, a labelled complex was detected with an
apparent molecular weight of 110kDa (Fig. 1), signifying that the LIF-binding
protein in serum had a molecular weight of about 90kDa. Concanavalin-A
Sephrose was demonstrated to have the ability to precipitate the ^{125}I -mLIF-LBP
complex from serum, implying that LBP was a glycoprotein, since the
25 recombinant LIF labelled for these studies was produced in E. coli and so
contained no carbohydrate groups. This property was used to separate bound
from free ^{125}I -LIF as recently described for solubilised receptor assays¹¹.

Isolation of LBP

The purification of mLBP from normal mouse sera was achieved by sequential fractionation using affinity chromatography on an immobilised LIF column, anion
5 exchange chromatography, size exclusion chromatography and preparative native polyacrylamide gel electrophoresis (PAGE), as detailed in the methods section, and monitoring LBP by direct ^{125}I -mLIF binding assays.

LBP was found to be a very unstable protein that was completely inactivated by
10 acidic conditions and in the presence of many of the chemicals usually used in protein purification procedures, such as acetonitrile, methanol, NaDodSO₄ and ammonium sulfate. This proved the major difficulty in the isolation of mLBP as it precluded the use of many high-resolution purification techniques, and may be the basis for the relatively low yields obtained during fractionation (Table 3).

15 Chemical modification of lysine residues in the LIF molecule has been shown to destroy its biological activity whereas iodination of tyrosine residues is not detrimental to LIF activity⁽³⁾. A method of linking protein to Sepharose beads through tyrosine residues was therefore chosen to ensure that the LIF molecule
20 would be active when attached to an affinity column matrix. Affinity chromatography (Figure 2A) on an mLIF-pABAE Sepharose 4B affinity matrix removed a large proportion of the contaminating serum proteins, although the guanidine used as an eluent had some denaturing effects on LBP activity. A second round of affinity chromatography resulted in a significant additional
25 purification. In contrast, LBP did not bind to a control ovalbumin-pABAE Sepharose 4B column. Anion exchange (Figure 2B) and size exclusion chromatography (Figure 2C) confirmed the acidic nature of the protein and its molecular weight of approximately 90kDa, and achieved a further 1.6-fold purification. Analytical NaDodSO₄-PAGE of fractions from the size exclusion
30 column revealed two bands that were barely separated from each other on a 12.5% w/v polyacrylamide gel, but were well separated from other contaminating bands. Both had an apparent molecular weight of approximately 90kDa and their

distributions in size exclusion column fractions and native preparative gel fractions as determined by silver staining of analytical NaDodSO₄-PAGE gels exactly matched that of LBP activity by direct ¹²⁵I-mLIF binding assay. The two peak fractions were electrophoresed on a 7% w/v NaDodSO₄-PAGE gel, blotted
5 onto a PVDF membrane and the two bands of interest were subjected to N-terminal sequencing. The major band had a slightly lower molecular weight and yielded amino acid sequence at the 5 picomole level (23 amino acid residues), while the higher molecular weight band yielded sequence at the 3 picomole level (8 amino acid residues). Both revealed an N-terminal sequence consistent with
10 the following sequence: Gly-Val-Gln-Asp-Leu-Lys-Cys-Thr-Thr-Asn-Asn-Met-Arg-Val-Trp-Asp-Cys-Thr-Trp-Pro-Ala-Pro-Leu (SEQ ID No. 1). The two forms of mLBP were not seen in every preparation and were presumed to be glycosylation variants, although the C-terminus of mLBP is yet to be identified and differences at this end of the molecule could account for the slight size
15 heterogeneity.

Preparative gel electrophoresis using native conditions (Fig 3A) allowed high resolution separation of LBP from contaminating proteins with only two major bands of 90 and 67kDa revealed by analytical NaDodSO₄-PAGE after pooling
20 active fractions from this step (Fig 3B). Fractions pooled from this step were used in assays to determine the binding characteristics of LBP.

Binding properties of LBP

The specific binding of ¹²⁵I-mLIF to purified LBP is shown in Figure 4A.
25 Scatchard analysis of the saturation binding isotherm of ¹²⁵I-mLIF to normal mouse sera or purified LBP showed that LBP contained a single class of mLIF binding site (K_D 500pM - 3nM) and indicated that normal mouse serum contains approximately 1-5 µg/ml of LBP (Figure 4B). The affinity of LBP for mLIF was comparable to that of the low affinity LIF receptor solubilised by detergent from
30 mouse liver membranes (K_D = 680pM) and was significantly lower than that of the high affinity cellular receptor on 3T3-L1 cells (K_D = 57pM) (Figure 4C).

Although hLIF was able to bind to mLBP, its binding characteristics were rather different. Unlabelled mLIF and hLIF showed a similar ability to compete with ¹²⁵I-mLIF for binding to mLBP (Figure 5A), but mLIF was consistently 10,000-fold less effective than hLIF in competing with ¹²⁵I-hLIF for binding to mLBP
5 (Figure 5B).

Blocking activity of LBP

When affinity purified mLBP was combined with mouse LIF in cultures of murine M1 myeloid leukemic cells, it blocked the ability of LIF to induce differentiation
10 of M1 colonies in a dose dependent manner (50% inhibition of 90U/ml LIF at 62-125ng/ml LBP). Intermediate doses of mLIF (stimulating 50% differentiation of M1 colonies) revealed inhibition by high doses of LBP, but possibly augmentation at low doses of LBP (2-4ng/ml) (Figure 6A). The presence of
125ng/ml LBP in the culture was able to shift a mLIF titration curve 2-fold
15 towards higher mLIF doses (Figure 6B).

Figure 7 shows the blocking of induction of mLIF and hLIF stimulated colonies of M1 leukaemic cells by normal mouse serum. In 1ml cultures of 300 murine M1 myeloid leukaemic cells, 0.1 ml of serum from normal adult (8 week) C57BL
20 mice was able to block the differentiation-inducing effects after 7 days of incubation of 100 Units of purified recombinant *E. coli*-derived murine LIF (50% blocking with a serum dilution of 1:8) (Figure 7A). This serum had a greater capacity to block the effects of 800 Units of purified recombinant *E. coli*-derived human LIF (50% blocking with a serum dilution of 1:512). A similar heightened
25 activity in blocking human LIF was seen using purified recombinant human LIF expressed in *E. coli*, CHO cells or in yeast (Figure 7B).

EXAMPLE 2**m-LIF and hLIF binding to mLBP**

5 Figure 8 is a graphical representation showing specific binding of ^{125}I -m-LIF (A) and ^{125}I -LIF (B) to mLBP immobilised onto 96 well PVC plates. The results show that mLIF immobilised to a solid support provides a ligand for capturing hLIF in a sample. Figure 8 shows that hLIF bound to the mLBP at least 20-30 fold better than mLIF as measured by specific binding. Apart from the
10 therapeutic implications on such a finding, these results identify mLBP as a suitable ligand for assaying the presence of hLIF in a biological sample.

EXAMPLE 3**Fusion LBP Polypeptides**

15 Administration of a mouse protein to a human subject may induce an antigenic immune response with therapeutically undesirable consequences. In order to avoid or reduce this potential problem, a protein that is antigenically very similar to a human LBP, but which retains the high-affinity for hLIF that is the particular
20 property of mLBP is constructed. The method used is similar to the method the inventors have previously used to map the site on the hLIF molecule that confers both binding to the hLIF receptor α -chain and the unusual high-affinity binding to the mouse LIF receptor α -chain (mLBP). Using a mLIF molecular framework, the inventors constructed a series of mouse-human LIF chimaeric molecules in
25 order to determine the minimum number of hLIF amino acid residues that it is necessary to substitute into the mLIF sequence in order to create a molecule that has the properties that are peculiar to hLIF (see Figure 9).

By constructing a series of mouse-human LBP chimaeric molecules using
30 recombinant DNA technology, the minimum number of mLBP amino acid residues is determined that it is necessary to substitute into a hLBP (soluble human LIF receptor α -chain) sequence in order to create a molecule that binds

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to hLIF with high affinity, as mLBP does, rather than a molecule that binds hLIF with low affinity, as does hLBP (Figure 10). Recombinant mLBP molecule is anticipated to have identical binding and inhibition properties to the native form of mLBP isolated from normal mouse serum, as all forms of the mouse LIF receptor tested, whether cellular or soluble, have shared the binding properties of mLBP. The ultimate aim is to synthesize a recombinant molecule that is based on the hLIF receptor α -chain amino acid sequence, but is C-terminally truncated so that it is a soluble rather than cell membrane-bound molecule, is of the minimum size that retains LIF-binding properties and is easily expressed in large quantities. In addition, this molecule will contain approximately 5-15 amino acid residues that are substituted for the amino acid residue that is in the identical position in the mouse LIF receptor α -chain amino acid sequence. These substitutions will specifically induce the hLBP molecule to bind hLIF with high affinity, and thus act as an effective hLIF antagonist *in vitro* or *in vivo*, however, these few amino acid substitutions would be unlikely to cause the human LBP molecule to become antigenic to the human immune system *in vivo*.

EXAMPLE 4

Digestion of mLBP with N-glycanase

N-terminal amino acid sequencing of mLBP suggests that it is a truncated form of the cellular LIF receptor α -chain. It is possible to estimate the size of a protein by SDS-PAGE, and correlate this with the predicted molecular weight of the protein, calculated by adding up the individual molecular weights of all the amino acid residues in the amino acid sequence. The mLBP isolated from mouse serum has the ability to bind to a carbohydrate-binding lectin, Concanavalin-A, and so must be a glycosylated molecule. Techniques are not readily available to estimate the proportion of carbohydrate versus protein in a glycosylated protein, so the apparent 90-95 kDa molecular weight of mLBP, as estimated by SDS-PAGE, cannot be correlated with the amino acid sequence.

A second form of mLBP has become apparent in purified preparations of mLBP over time, and is presumed to be formed by proteolytic degradation. This form of mLBP is capable of binding both mLIF and hLIF, and has a molecular weight of approximately 65 kDa as estimated by SDS-PAGE (Figure 11A,B).

In order to estimate the position of the C-terminus of both the 90-95 kDa and 65 kDa forms of mLBP, preparations containing both these forms were subjected to digestion by N-glycanase, an enzyme that specifically cleaves the bond between an asparagine residue and an N-linked carbohydrate. Digestion was allowed to go to completion and the molecular weights of the deglycosylated proteins were estimated by SDS-PAGE (Figure 12). The two bands produced by N-glycanase digestion had molecular weights of approximately 63 kDa and 50 kDa, respectively. These bands corresponded to the molecular weights of the protein cores of the two forms of mLBP, without the N-linked carbohydrate, and so could be correlated with the amino acid sequence.

The cellular mLIF receptor comprises two haemopoietin domains, three fibronectin III-like domains, a transmembrane domain and a cytoplasmic domain, and has a glycosylated molecular weight of 190 kDa⁽¹²⁾. The molecular weight of the reported form of the soluble mLIF receptor which consists of two haemopoietin domains and two out of three fibronectin III-like domains is 130 kDa when glycosylated⁽¹²⁾ and 75 kDa for the core protein. The predicted molecular weight for the core protein for consisting of two haemopoietin domains and one out of three fibronectin III-like domains is 63 kDa, while for the two haemopoietin domains alone, the predicted molecular weight is 53 kDa. These molecular weights correspond well to the molecular weights of the two deglycosylated forms of mLBP as estimated by SDS-PAGE, which suggests that a variety of forms of mLBP are created by sequential proteolytic removal of fibronectin III-like domains and that each of these forms retains the binding activity described for mLBP.

EXAMPLE 5

Determination of the Amino Acid Sequence of the C-terminus of mLBP

Further information about the C-terminal amino acid sequence of mLBP is
5 obtainable by sequencing proteolytically-produced peptide fragments of mLBP.
In order that sequence data can be gained from the different molecular weight
forms of mLBP using as little protein as possible, the proteolytic digestion is
carried out within an SDS-PAGE gel and the peptides extracted from the gel and
separated by reversed-phase HPLC for microsequence analysis.

10

Briefly, the Coomassie Blue stained band of the correct molecular weight is cut
out of the gel and destained with 50% v/v n-propanol containing 3% w/v SDS.
The gel slice is then washed extensively with water and dried by centrifugal
lyophilization. The gel slice is then rehydrated in 100 μ l 0.1M NaHCO₃
15 containing 0.02% v/v Tween-20 and 1-2 μ g of protease (for example, trypsin or
LysC). Peptides are then extracted from the gel slices with 100 μ l 1% v/v
trifluoroacetic acid (TFA) for 4 hours, 100 μ l 70% v/v TFA for 4 hours, 100 μ l
70% v/v TFA for 16 hours and 100 μ l 50% v/v TFA/50% acetonitrile for two
times 4 hours. The combined eluents are evaporated to near dryness then diluted
20 to 1ml with 0.1% v/v TFA. The peptides are then separated by reversed-phase
HPLC and subjected to amino acid sequence analysis.

TABLE 2
LEVELS OF LBP IN SERUM¹

SERUM TYPE	STRAIN	SEX	AGE (wks)	DILUTION GIVING 50% INHIBITION IN RIA	LBP CONC (μ g/ml)
MOUSE					
ADULT	MIXED	MIXED	N/A ³	1/4 - 1/8	1
"	C57	M	6	1/4	1
"	C57	F	6	1/4	1
"	CBA	F	12	1/4	1
NEONATAL	CBA	F	1	1/2	0.5
"	CBA	M	1	1/1	0.25
"	CBA	M	2	1/4	1
PREGNANT	CBA ²	F	12	1/128	32
HUMAN	N/A ³	MIXED	N/A ³	N/D ⁴	0
RAT	N/A ³	MIXED	N/A ³	1/4	1

¹ Levels of LBP detected in serum expressed as the dilution of serum required to inhibit 50% of specific binding of ¹²⁵I-mLIF to a neutralising monoclonal antibody in a competition RIA. The number of ¹²⁵I-mLIF binding sites in normal mouse serum was determined by Scatchard analysis to be 1 μ g/ml and used as a standard to convert 50% inhibition in RIA to LBP concentration.

² 14 days pregnant

³ Not Applicable

⁴ Not Detectable

TABLE 3
 REPRESENTATIVE PURIFICATION OF LBP FROM NORMAL
 MOUSE SERUM¹

FRACTIONATION PROCEDURE	VOL (ml)	TOTAL LBP (μ g)	TOTAL PROTEIN (μ g)	YIELD (%)	p ² (fold)
Normal Serum	85	102	2.24×10^6	-	-
LIF Sepharose 1	78	41	1.54×10^4	39.8	58
LIF Sepharose 2	38	18	2.73×10^3	19.4	147
Anion Exchange	8.9	18	2.45×10^3	19.9	158
Size Exclusion	3.2	6.0	573	7.8	232
Native Gel	0.8	1.4	11.6	1.4	2620

¹ Representative purification of LBP from normal mouse serum. Total LBP was calculated from the concentration of ¹²⁵I-mLIF binding sites derived from Scatchard analysis, assuming one binding site per LBP molecule and a molecular weight of 90 kDa for LBP.

² Purification (fold)

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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 - (D) SOFTWARE: WordPerfect 5.1
 - (vi) CURRENT APPLICATION DATA
 - (A) APPLICATION NUMBER: International application
 - (B) FILING DATE: 1 July, 1993
 - (C) CLASSIFICATION:
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INFORMATION FOR SEQ ID NO. 1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 amino acid residues
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (v) FRAGMENT TYPE: N-terminal fragment
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO. 1:

Gly Val Gln Asp Leu Lys Cys Thr Thr Asn 10
Asn Met Arg Val Trp Asp Cys Thr Trp Pro 20
Ala Pro Leu

CLAIMS:

1. An isolated leukaemia inhibitory factor (LIF)-binding protein (LBP) in soluble form and obtainable from a first mammalian species, said LBP capable of inhibiting the ability of LIF from a second mammalian species to induce differentiation of M1 myeloid leukaemic cells *in vitro* to a greater extent when compared to its ability to inhibit LIF from said first mammalian species.
2. An isolated LBP in soluble form and obtainable from a first mammalian species characterized in that said LBP has at least a 100-fold higher binding affinity for a LIF from a second mammalian species compared to the binding affinity for a LIF from said first mammalian species.
3. An isolated LBP according to claim 1 or claim 2 wherein said first mammalian species is selected from the group consisting of a human, mouse, rat, pig, cow, sheep, goat, horse or primate.
4. An isolated LBP according to claim 1 or 2 wherein said second mammalian species is selected from the group consisting of a human, mouse, rat, pig, cow, sheep, goat, horse or primate.
5. An isolated LBP according to claim 1 or 2 wherein the first mammalian species is a mouse and the second mammalian species is a human.
6. An isolated LBP according to claim 5 possessing one or more of the following properties:
 - (i) an apparent molecular weight in one glycosylated form of approximately 90,000 \pm 10,000 daltons as determined by SDS-PAGE;

(ii) an apparent molecular weight after N-glycanase treatment of one unglycosylated form of approximately $65,000 \pm 15,000$ daltons as determined by SDS-PAGE; and

(iii) an amino acid sequence in its N-terminal portion substantially comprising: Gly-Val-Gln-Asp-Leu-Lys-Cys-Thr-Thr-Asn-Asn-Met-Arg-Val-Trp-Asp-Cys-Thr-Trp-Pro-Ala-Pro-Leu (SEQ ID No. 1) or having at least 55% amino acid similarity thereto;

or a functional mutant, derivative or part thereof.

7. A fusion polypeptide defining an LBP, said fusion polypeptide comprising first and second amino acid sequences wherein said first amino acid sequence is derivable from an LBP from a first mammalian species and said second amino acid sequence is derivable from an LBP from a second mammalian species wherein the LBP from said first mammalian species is capable of inhibiting the ability of LIF from said second mammalian species to induce differentiation of M1 myeloid leukaemic cells *in vitro* to a greater extent when compared to its ability to inhibit LIF from said first mammalian species such that said fusion polypeptide retains the ability to inhibit LIF from said second mammalian species to a greater extent than LIF from said first mammalian species.

8. A fusion polypeptide according to claim 7 characterised in that said fusion polypeptide has at least a 100-fold higher binding affinity for LIF from said second mammalian species compared to the affinity for LIF from said first mammalian species.

9. A fusion polypeptide according to claim 7 or 8 wherein said first mammalian species is a mouse and second said mammalian species is a human.

10. A fusion polypeptide defining an LBP, said fusion polypeptide defining an LBP, said fusion polypeptide comprising first and second amino acid sequences wherein said first amino acid sequence is derivable from an LBP from a mouse and said second amino acid sequence is derivable from an LBP from a human wherein said fusion polypeptide is capable of inhibiting the ability of LIF from a human to induce differentiation of M1 myeloid leukaemic cells *in vitro* to a greater extent when compared to its ability to inhibit mouse LIF and wherein administration of said fusion polypeptide into a human results in a substantially reduced immune response against said fusion polypeptide compared to the administration to said human of native or recombinant mouse LBP.
11. A composition comprising an LBP in soluble form and obtainable from a first mammalian species, said LBP capable of inhibiting the ability of LIF from a second mammalian species to induce differentiation of M1 myeloid leukaemic cells *in vitro* to a greater extent when compared to its ability to inhibit LIF from said first mammalian species, said composition substantially free of protein molecules not having LBP properties.
12. A composition comprising an LBP in soluble form and obtainable from a first mammalian species characterized in that said LBP has at least 100-fold higher binding affinity for a LIF from a second mammalian species compared to the binding affinity for a LIF from said first mammalian species, said composition substantially free of protein molecules not having LBP properties.
13. A composition according to claim 11 or 12 wherein said first mammalian species is selected from the group consisting of a human, mouse, rat, pig, cow, sheep, goat, horse or primate.
14. A composition according to claim 11 or 12 wherein said second mammalian species is selected from the group consisting of a human, mouse, rat, pig, cow, sheep, goat, horse or primate.

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15. A composition according to claim 11 or 12 wherein the first mammalian species is a mouse and the second mammalian species is a human.
16. A composition according to claim 11 wherein the LBP possesses one or more of the following properties:
 - (i) an apparent molecular weight in one glycosylated form of approximately $90,000 \pm 10,000$ daltons as determined by SDS-PAGE;
 - (ii) an apparent molecular weight after N-glycanase treatment of one unglycosylated form of approximately $65,000 \pm 15,000$ daltons as determined by
SDS-PAGE; and
 - (iii) an amino acid sequence in its N-terminal portion substantially comprising:
Gly-Val-Gln-Asp-Leu-Lys-Cys-Thr-Thr-Asn-Asn-Met-Arg-Val-Trp-Asp-Cys-
Thr-Trp-Pro-Ala-Pro-Leu (SEQ ID No.1) or having at least 55% amino acid similarity thereto;or a functional mutant, derivative or part thereof.
17. A nucleic acid isolate comprising a sequence of nucleotides encoding or complementary to a sequence encoding the LBP according to claim 1 or 2 or 6 or 7 or comprising a sequence of nucleotide encoding an LBP and capable of hybridising under low stringency conditions to a nucleic acid molecule encoding the amino acid sequence: Gly-Val-Gln-Asp-Leu-Lys-Cys-Thr-Thr-Asn-Asn-Met-Arg-Val-Trp-Asp-Cys-Thr-Trp-Pro-Ala-Pro-Leu (SEQ ID No. 1).
18. A nucleic acid isolate capable of hybridizing to a contiguous series of at least 10 nucleotides in the nucleotide sequence according to claim 17.
19. A vector comprising the nucleic acid isolate according to claim 17.

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20. A method of inhibiting the activity of LIF in a mammal said method comprising administering to said mammal an effective amount of a soluble heterologous LBP wherein said heterologous LBP is capable of greater inhibition of the LIF in the mammal to be treated when compared to a LIF of same mammalian origin to the LBP.

21. A method according to claim 20 wherein the LBP possesses one or more of the following properties:

(i) an apparent molecular weight of one glycosylated form of approximately 90,000 \pm 10,000 daltons as determined by SDS-PAGE;

(ii) an apparent molecular weight after N-glycanase treatment of one unglycosylated form of approximately 65,000 \pm 15,000 daltons as determined by SDS-PAGE; and

(iii) an amino acid sequence in its N-terminal portion substantially comprising: Gly-Val-Gln-Asp-Leu-Lys-Cys-Thr-Thr-Asn-Asn-Met-Arg-Val-Trp-Asp-Cys-Thr-Trp-Pro-Ala-Pro-Leu (SEQ ID No. 1) or having at least 55% amino acid similarity thereto;

or a functional mutant, derivative or part thereof.

22. A method according to claim 20 wherein the LBP is a fusion polypeptide, said fusion polypeptide comprising first and second amino acid sequences wherein said first amino acid sequence is derivable from an LBP from a first mammalian species and said second amino acid sequence is derivable from an LBP from a second mammalian species wherein the LBP from said first mammalian species is capable of inhibiting the ability of LIF from said second mammalian species to induce differentiation of M1 myeloid leukaemic cells *in vitro* to a greater extent when compared to its ability to inhibit LIF from said first mammalian species

such that said fusion polypeptide retains the ability to inhibit LIF from said second mammalian species to a greater extent than LIF from said first mammalian species.

23. A method according to claim 20 wherein the LBP is a fusion polypeptide, said fusion polypeptide defining an LBP, said fusion polypeptide comprising first and second amino acid sequences wherein said first amino acid sequence is derivable from an LBP from a mouse and said second amino acid sequence is derivable from an LBP from a human wherein said fusion polypeptide is capable of inhibiting the ability of LIF from a human to induce differentiation of M1 myeloid leukaemic cells *in vitro* to a greater extent when compared to its ability to inhibit mouse LIF and wherein administration of said fusion polypeptide into a human results in a substantially reduced immune response against said fusion polypeptide compared to the administration to said human of native or recombinant mouse LBP.
24. A method according to claim 21 or 22 wherein the mammal to be treated is a human and the LBP is of mouse origin.
25. A method according to claim 24 wherein the effective amount of heterologous LBP in serum of the mammal is from 0.001 µg/ml to 100 µg/ml of serum.
26. A method according to claim 20 further comprising the sequential or simultaneous administration of one or more active compounds selected from the group consisting of a cytokine, antibiotic, anti-cancer agent and immunomodulating compound.
27. A pharmaceutical composition comprising LBP according to claim 1 or 2 or 6 or 7 together with one or more pharmaceutical carriers and/or diluents.

28. A method for detecting LIF in a biological sample said method comprising contacting said biological sample with an immobilised LBP from a mammal wherein the LBP is capable of inhibiting the ability of the first mentioned LIF to induce differentiation of M1 myeloid leukaemic cells *in vitro* to a greater extent when compared to its ability to inhibit LIF of same mammalian origin as LBP and/or wherein said LBP has at least a 100-fold higher binding affinity for said first mentioned LIF compared to the binding affinity of LIF of same mammalian origin as said LBP wherein said contact is for a time and under conditions sufficient for a complex to form between the immobilised LBP and the LIF in the sample; contacting the LBP-LIF complex with an antibody specific for said LIF and labelled with a reporter molecule capable of providing a signal; determining the presence of bound LIF on the basis of the signal produced by said reporter molecule.

29. A method according to claim 28 alternatively comprising contacting the LBP-LIF complex with an unlabelled antibody specific to said LIF and then detecting LIF bound antibody using a second antibody specific to said first antibody and labelled with a reporter molecule.

30. A method according to claim 28 or 29 wherein said LBP is of mammalian origin selected from the group consisting of human, mouse, rat, pig, cow, sheep, goat, horse or primate.

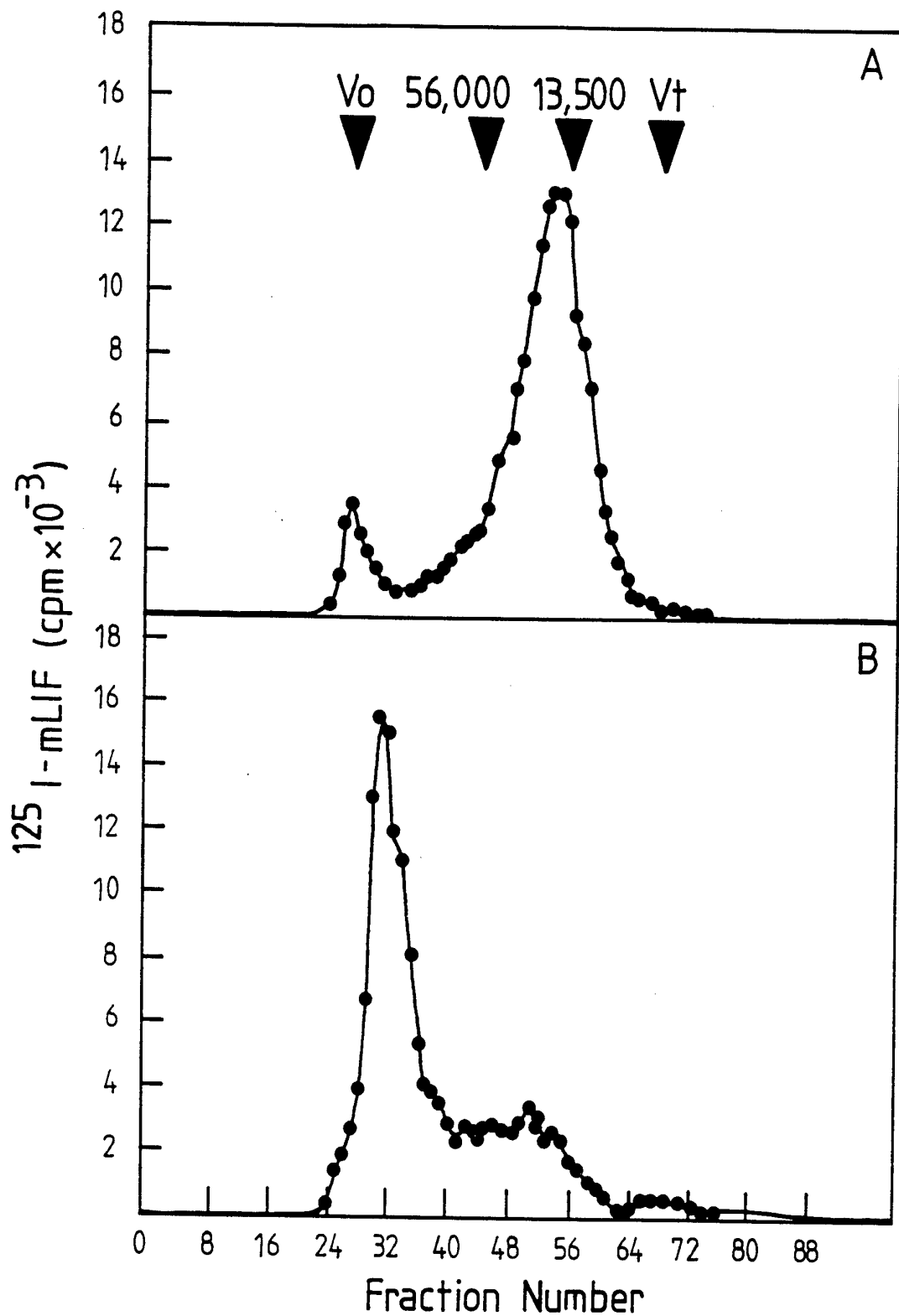
31. A method according to claim 28 or 29 wherein said LIF is of mammalian origin selected from the group consisting of human, mouse, rat, pig, cow, sheep, goat, horse or primate.

32. A method according to claim 28 or 29 wherein the LBP is of mouse origin and the LIF is of human origin.

33. A method according to claim 32 wherein the LBP possesses one or more of the following properties:

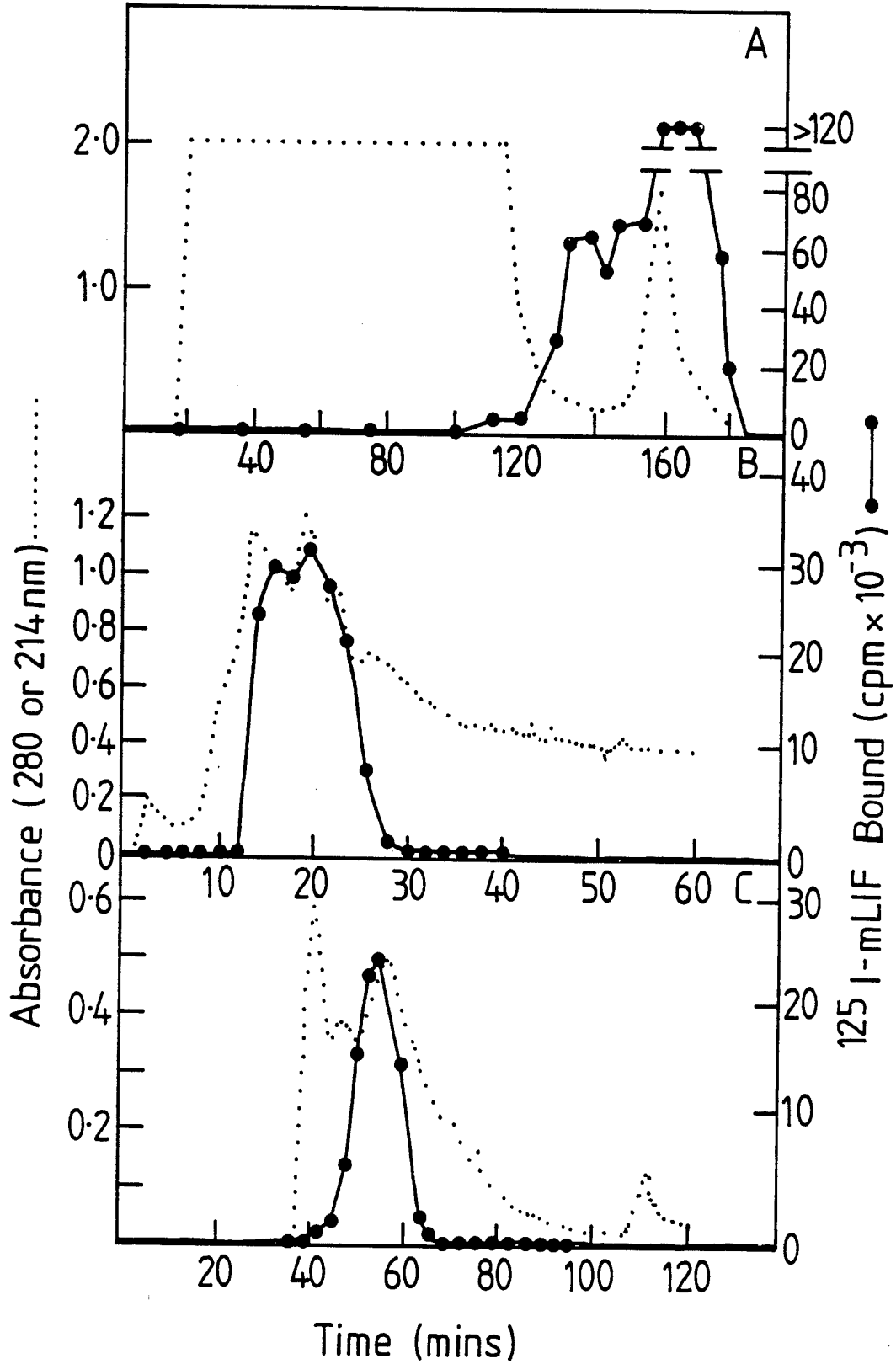
- (i) an apparent molecular weight of one glycosylated form of approximately 90,000 \pm 10,000 daltons as determined by SDS-PAGE;
 - (ii) an apparent molecular weight after N-glycanase treatment of one unglycosylated form of approximately 65,000 \pm 15,000 daltons as determined by SDS-PAGE; and
 - (iii) an amino acid sequence in its N-terminal portion substantially comprising: Gly-Val-Gln-Asp-Leu-Lys-Cys-Thr-Thr-Asn-Asn-Met-Arg-Val-Trp-Asp-Cys-Thr-Trp-Pro-Ala-Pro-Leu (SEQ ID No. 1) or having at least 55% amino acid similarity thereto;
- or a functional mutant, derivative or part thereof.

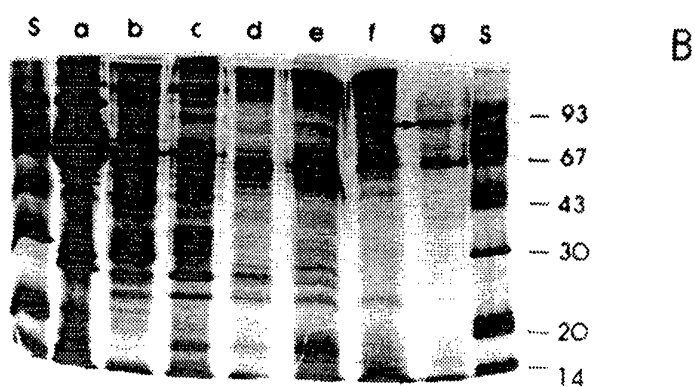
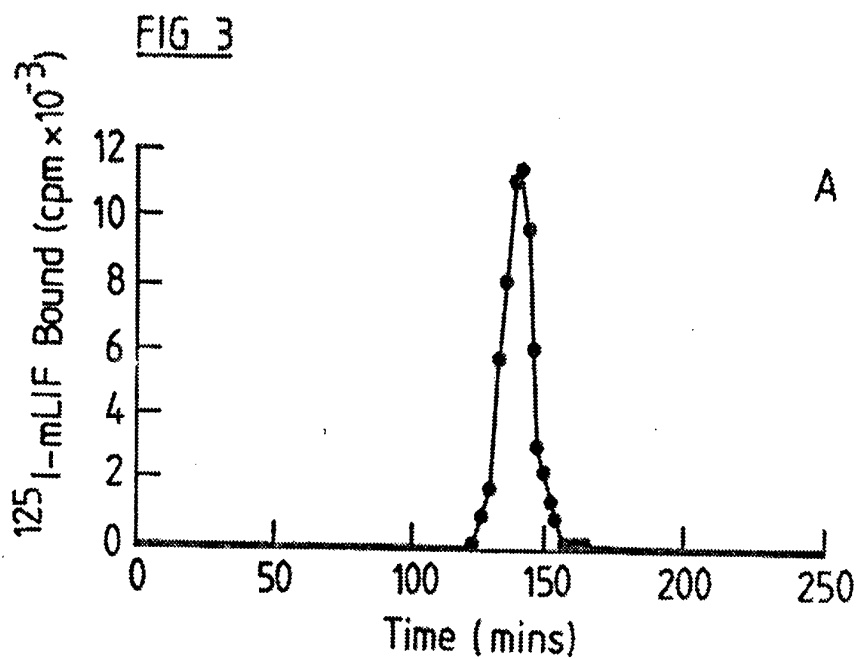
FIG 1



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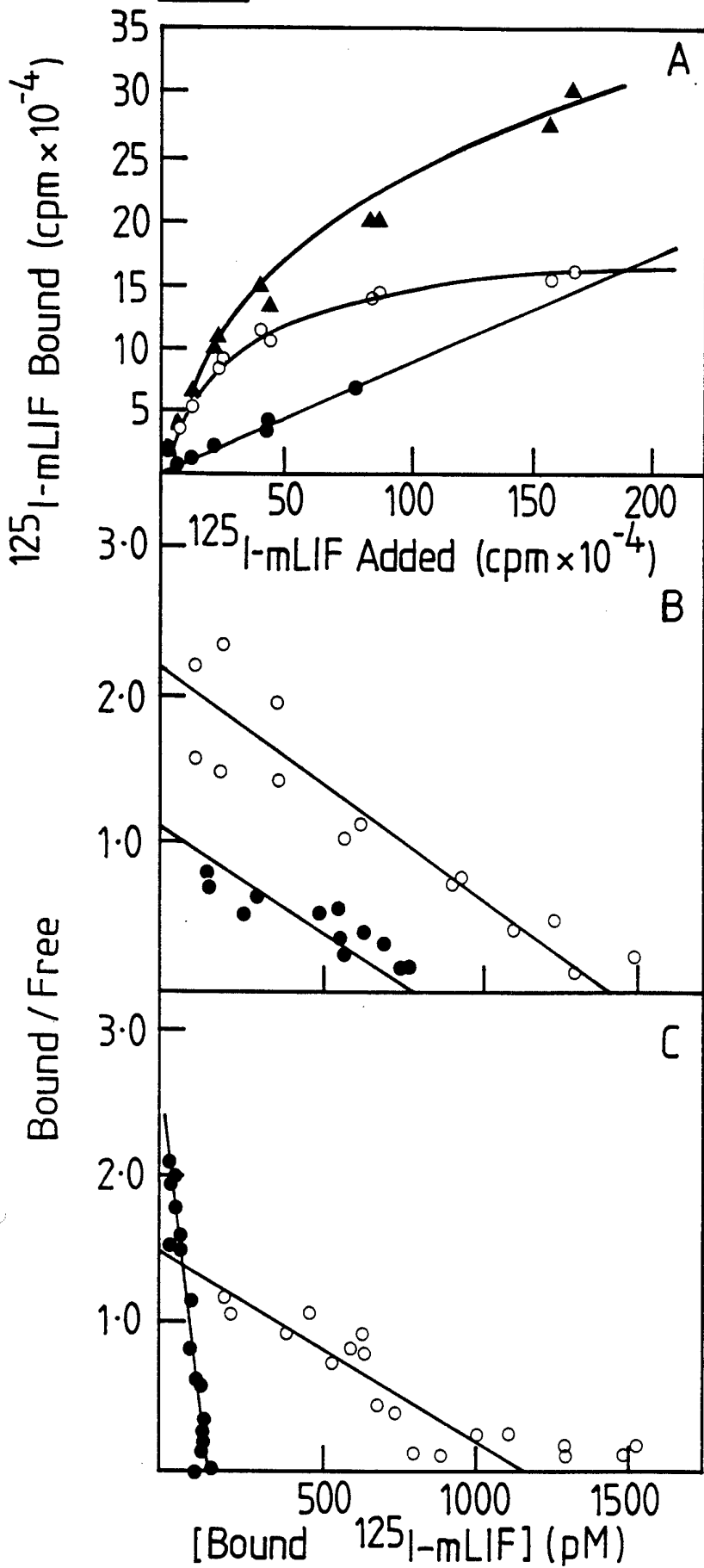
FIG 2





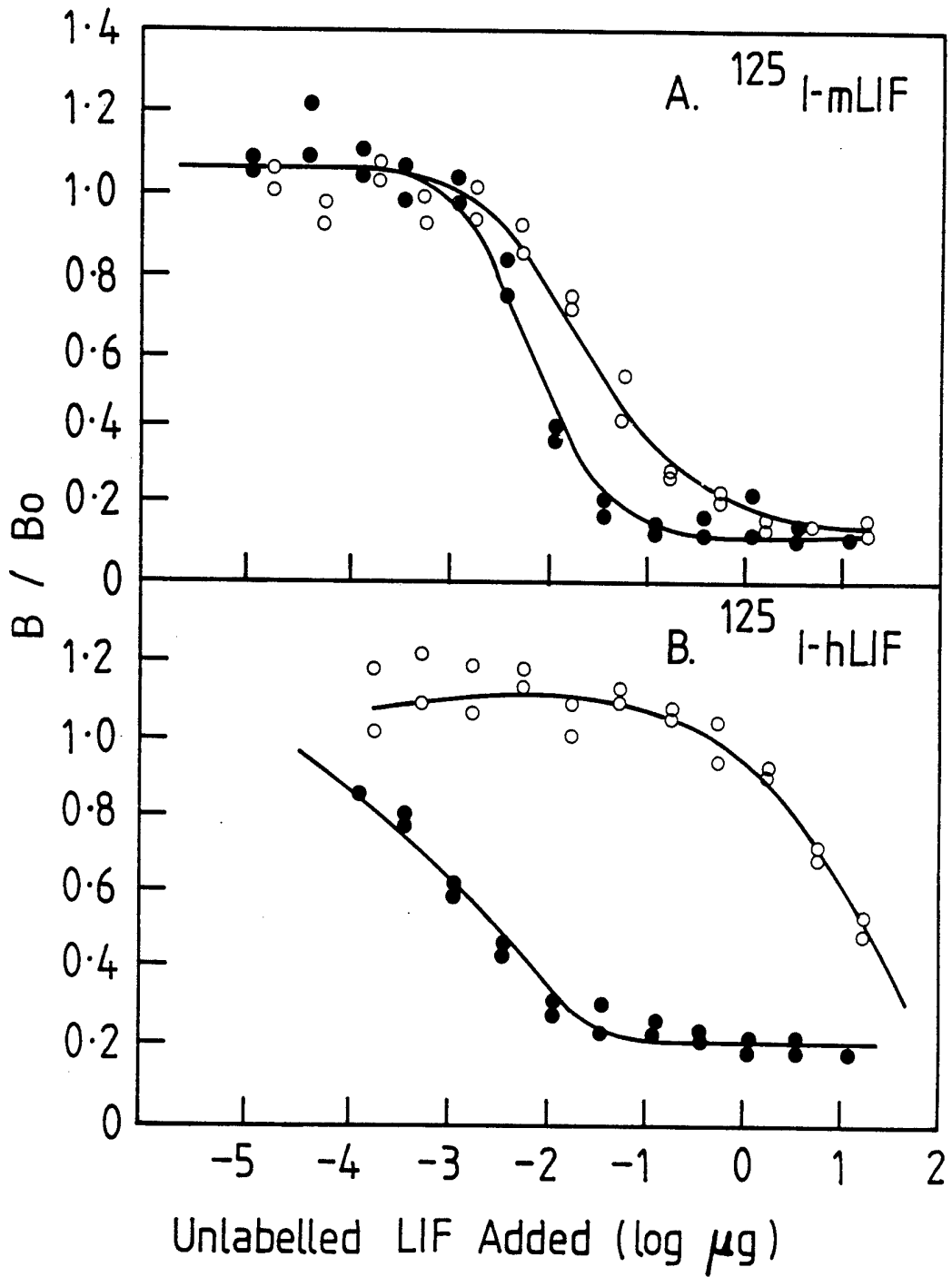
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FIG 4



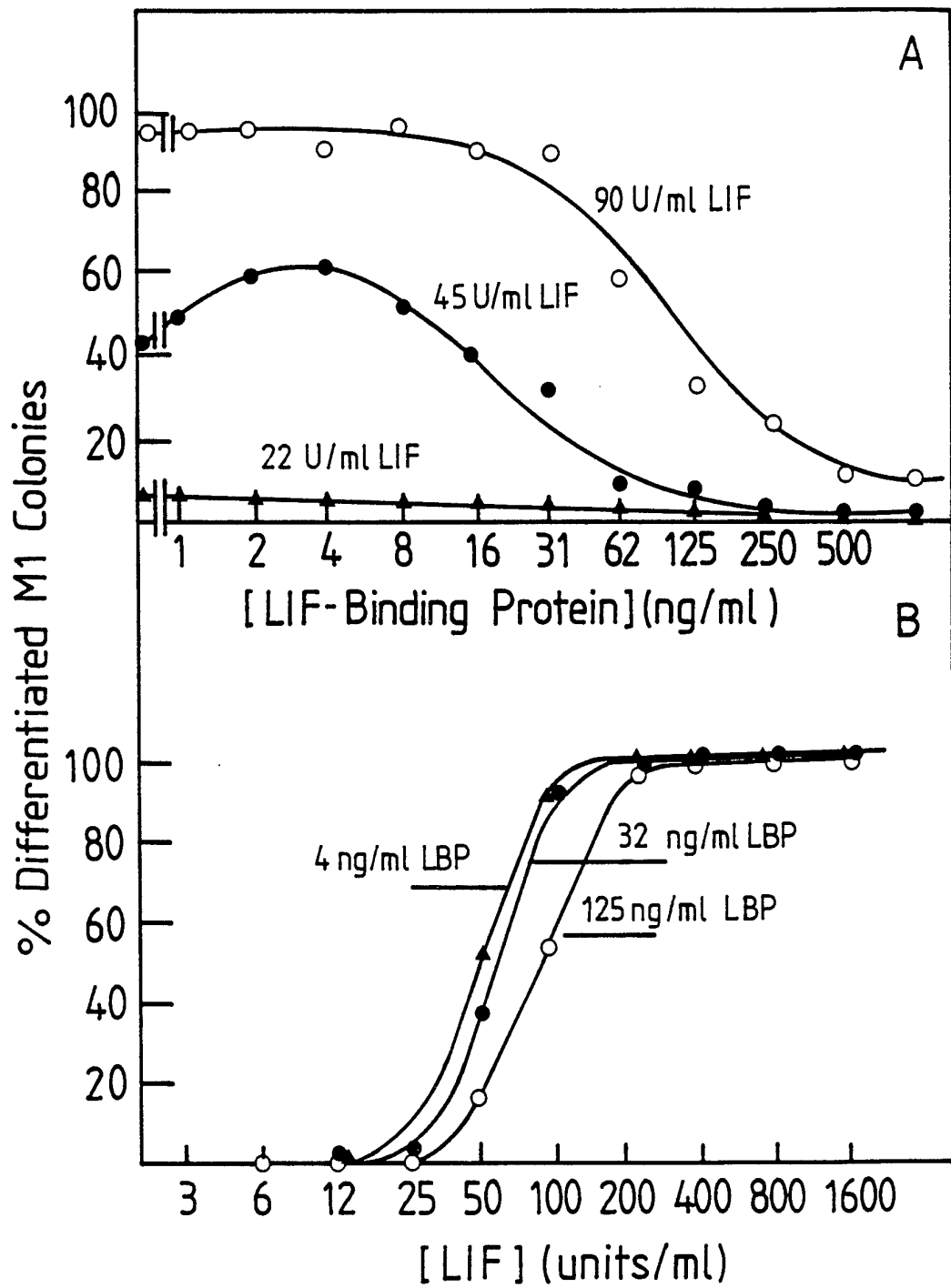
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FIG 5



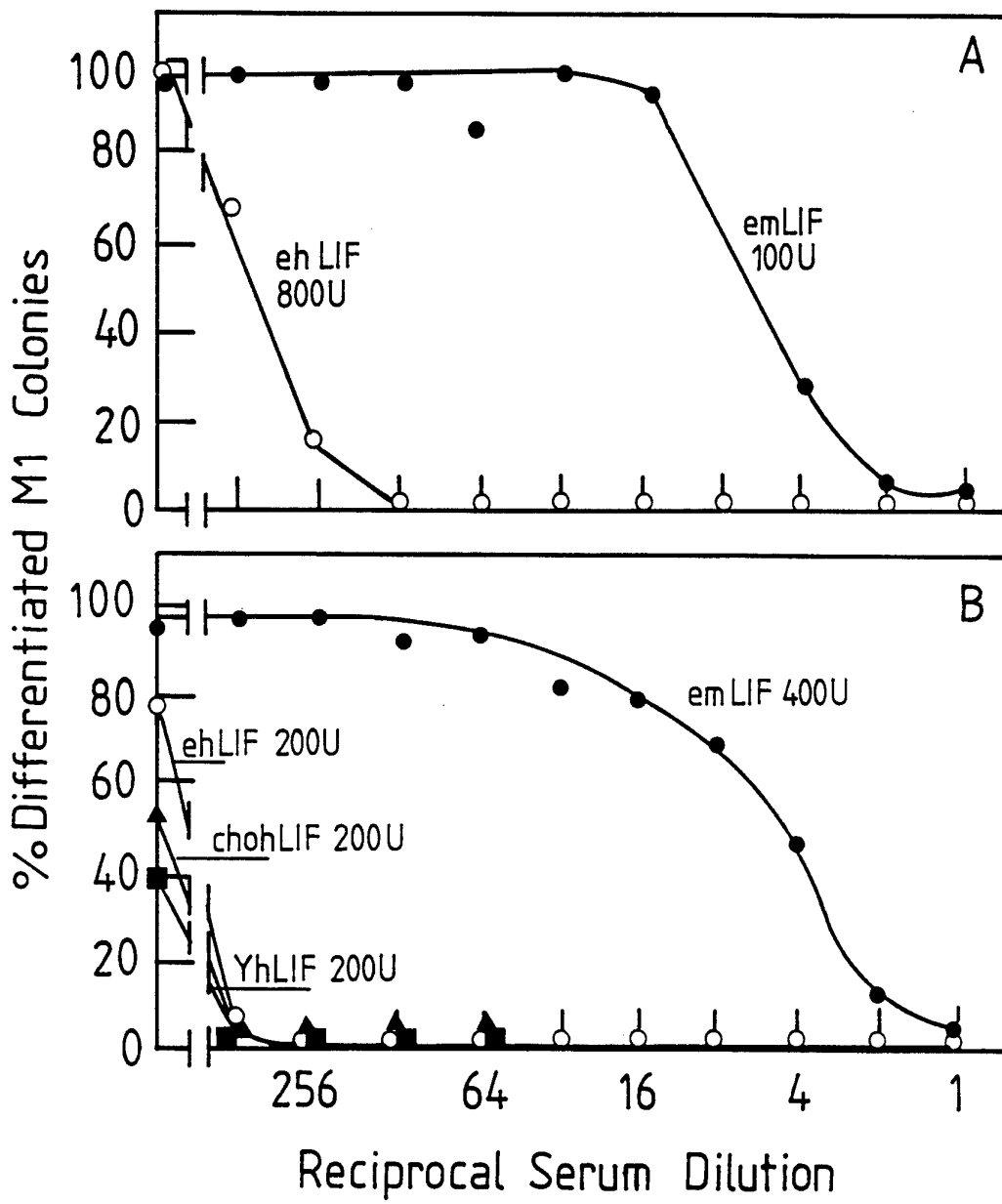
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FIG 6



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FIG 7



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FIG 8A

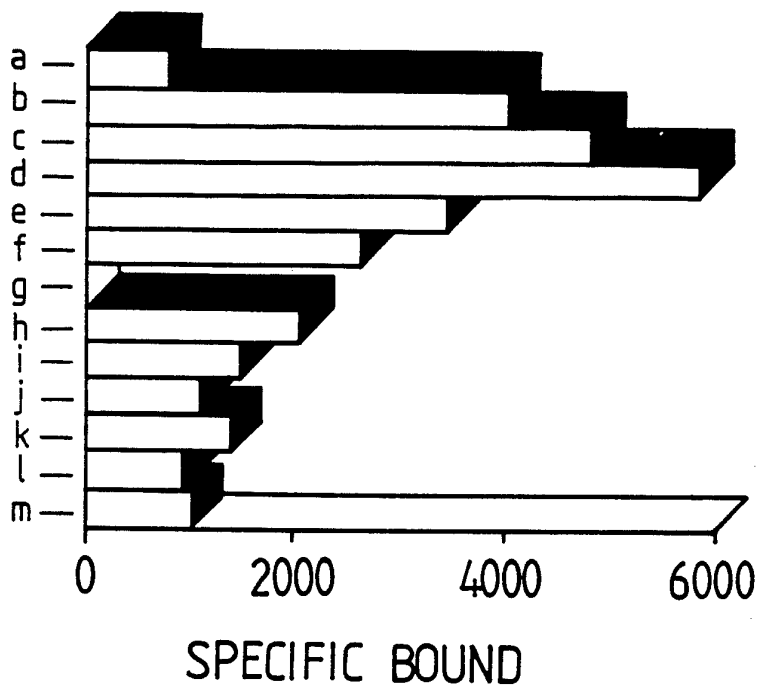
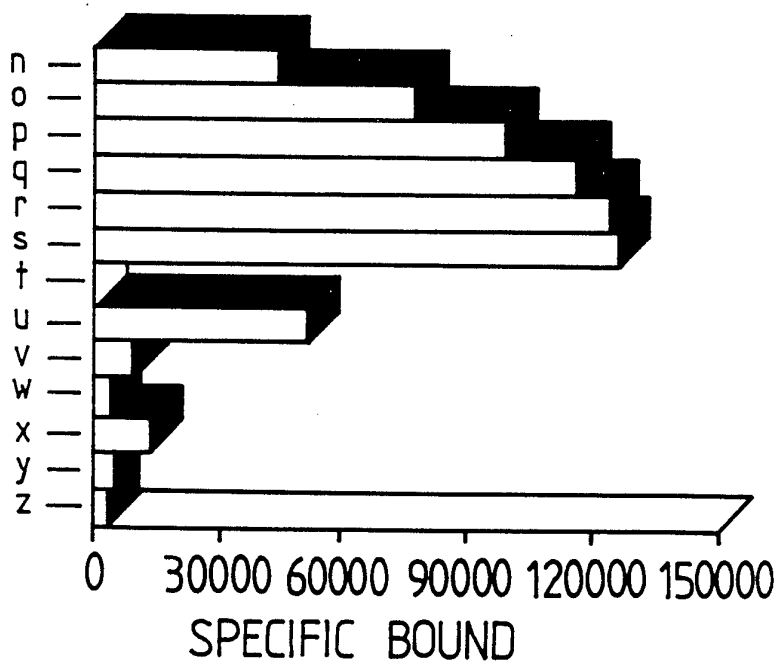


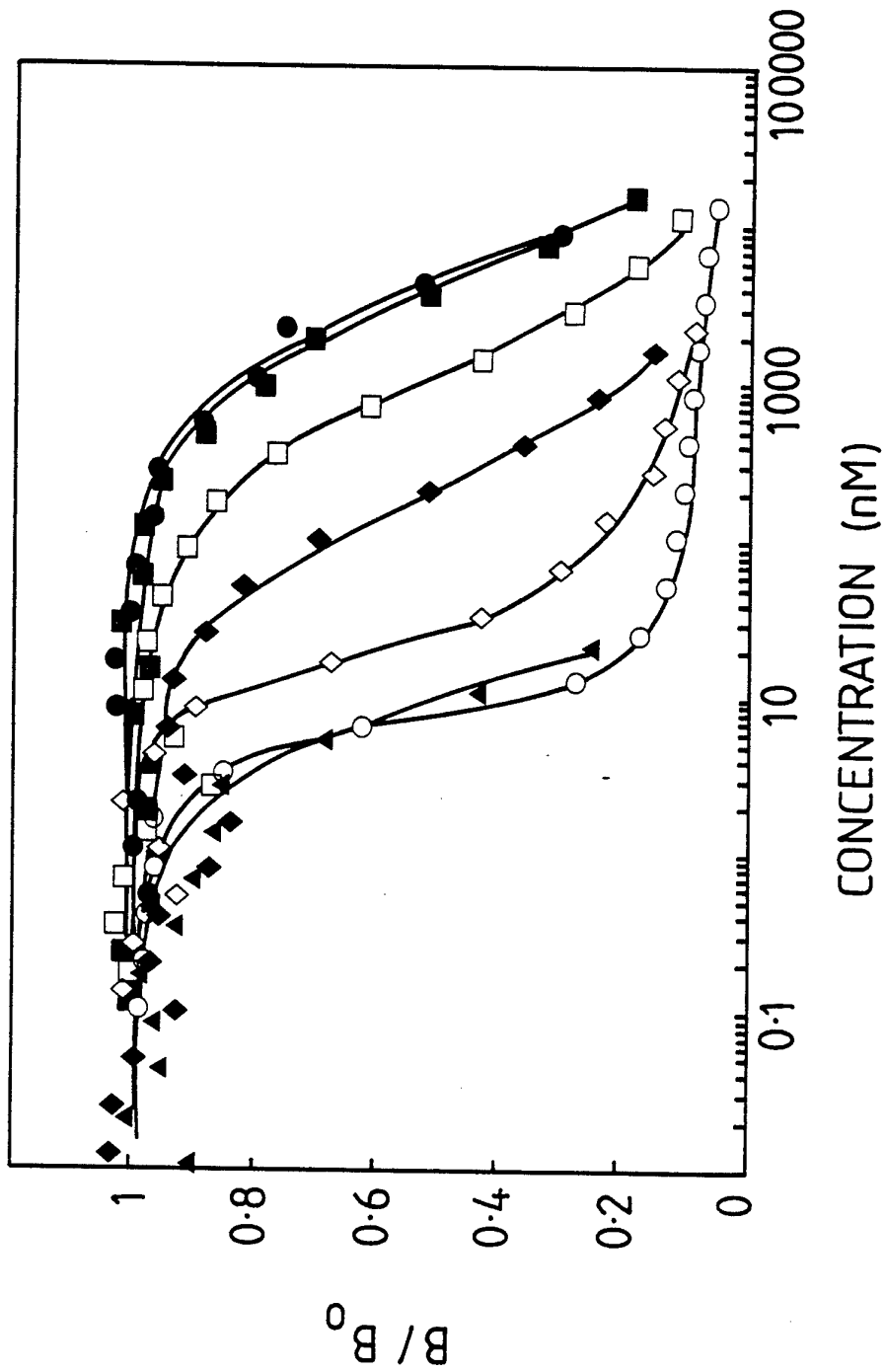
FIG 8B



SUBSTITUTE SHEET

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FIG 9



SUBSTITUTE SHEET

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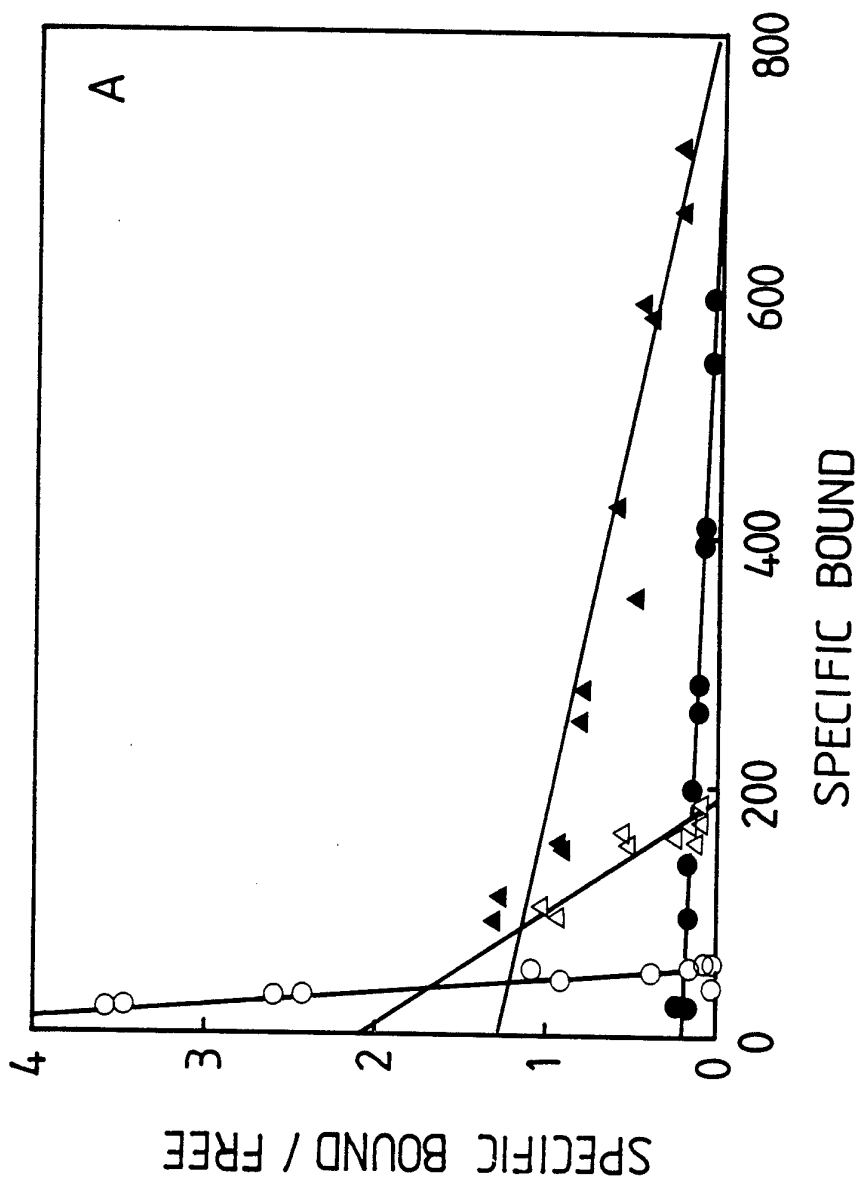
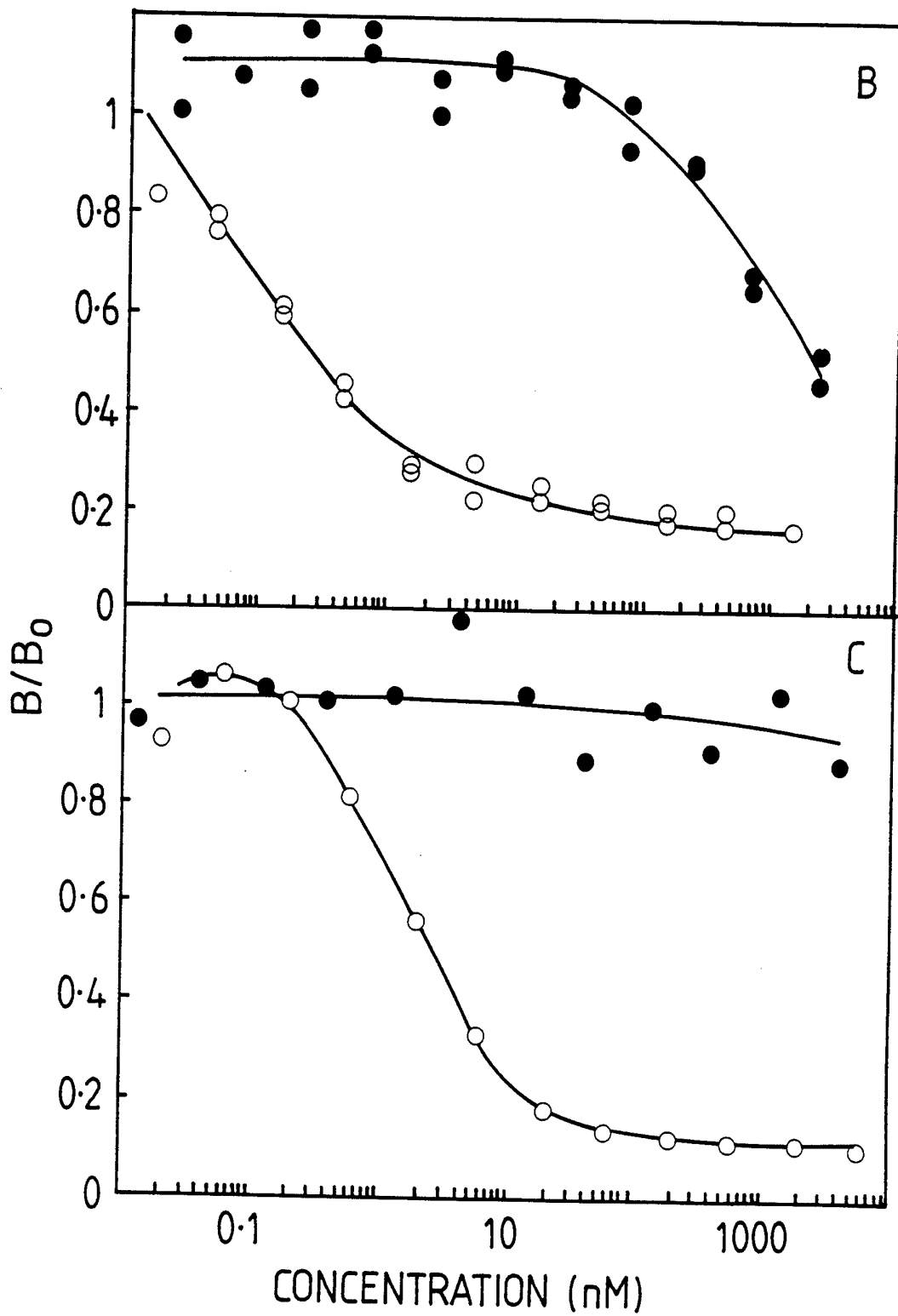


FIG 10A

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FIG 10B,C



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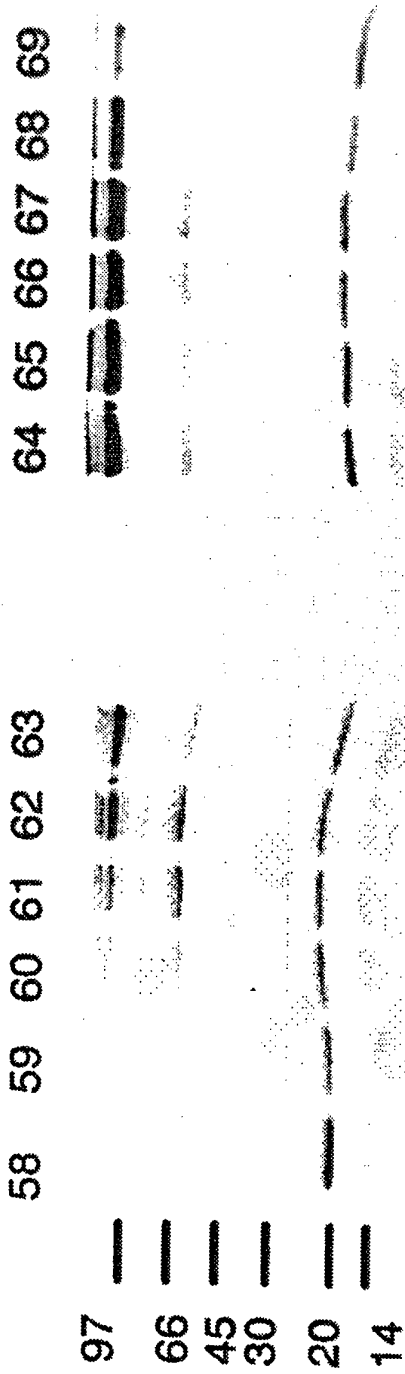


FIGURE 11A

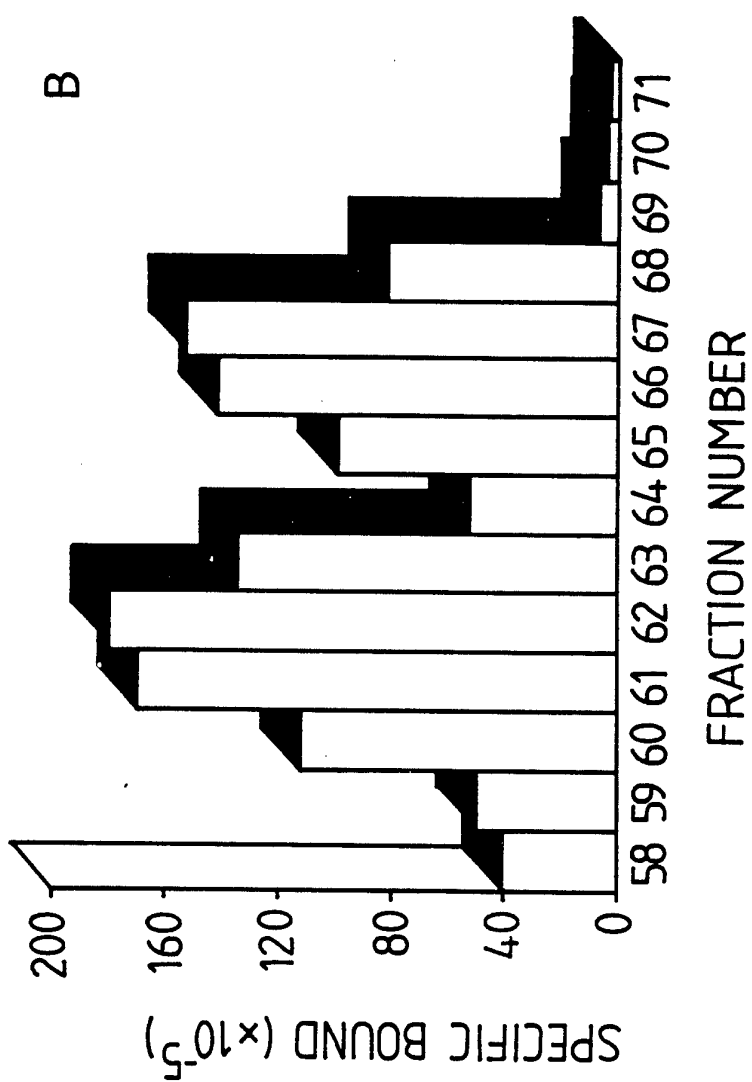


FIG 11B

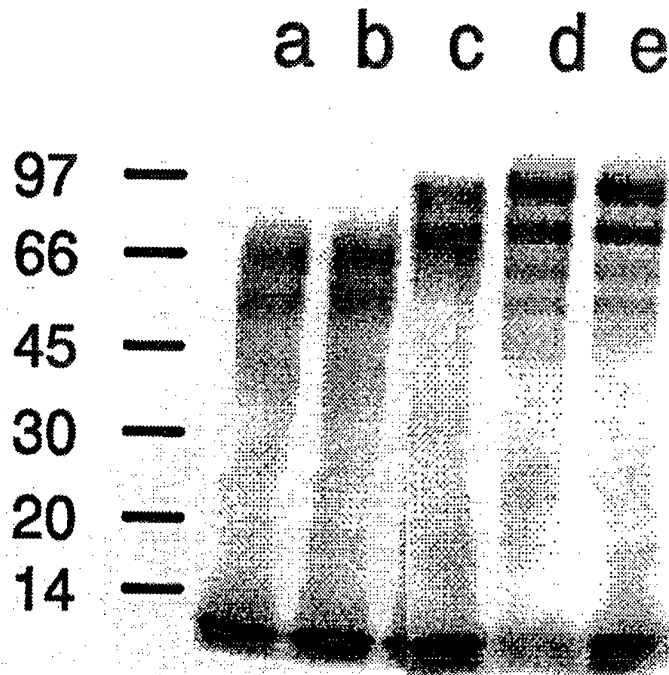
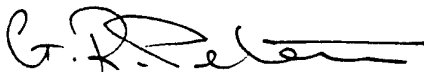


FIGURE 12

A. CLASSIFICATION OF SUBJECT MATTER Int. Cl. ⁵ C07K 15/00, A61K 37/02, C12N 15/12 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC: C07K 15/00, C12N 15/12 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched AU: IPC C07K 15/00, C12N 15/12 Electronic data base consulted during the international search (name of data base, and where practicable, search terms used) DERWENT: LEUKAEMIA (W) INHIBITORY (W) FACTOR # <u>OR</u> LEUKAEMIA (W) INHIBITORY (W) FACTOR # <u>AND</u> BINDING <u>AND</u> PROTEIN # <u>OR</u> PEPTIDE # <u>OR</u> POLYPEPTIDE # <u>OR</u> FACTOR #		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	Proc. Natl Acad. Sci. USA, Volume 89, issued 1992, September (Washington D.C. USA), M Layton et al.,: "A major binding protein for leukemai inhibitory factor in normal mouse serum: Identification as a soluble form of the cellular receptor", see pages 8616-8620.	
A	The EMBO Journal, Volume 10 no 10 issued 1991 (IRL Press Ltd), (Oxford England), D. Gearing et al, : "Leukaemia Inhibitory Factor Receptor is structurally related to the IL-6 signal transducer, gp 130", see pages 2839-2848.	
A	AU,A,91229/91 (IMMUNEX CORPORATION, D GEARING) 8 July 1992 (08.07.92), see whole document.	
<input type="checkbox"/> Further documents are listed in the continuation of Box C.		
<input checked="" type="checkbox"/> See patent family annex.		
* Special categories of cited documents :		
"A"	document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier document but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed	
Date of the actual completion of the international search 11 October 1993 (11.10.93)		Date of mailing of the international search report 15 OCT 1993 (15.10.93)
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. 06 2853929		Authorized officer  G. PETERS Telephone No. (06) 2832244

INTERNATIONAL SEARCH REPORT
Information on patent family mem

International application No.

PCT/AU 93/00325

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
AU	91229/91	CA	2097291	EP	561960	FI	9312477
		NO	931955	WO	9210570		
END OF ANNEX							