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(54) Title: THE USE OF PASTEURELLA HAEMOLYTICA GLYCOPROTEASE IN A PROCESS FOR RECOVERING CELLS RICH IN O-GLYCOSYLATED SURFACE PORTIONS (57) Abstract A method for recovering viable cells having surface protein determinants having portions rich in O-glycosylated carbohydrates is provided. The proteins have determinants which are substrate sensitive to a <i>P. haemolytica</i> derived neutralmetallo-glycoprotease. The glycoprotease has highly restricted specificity for cleaving solely from the cell surfaces the O-glycosylated protein portions having the determinants while retaining cell viability. The process comprises: i) contacting the cells in solution with affinity matrices which bind specifically to one or more of the determinants which are part of the glycoprotease sensitive substrates, the affinity matrices are allowed to bind to the determinants on the cells, ii) separating the cells to which the affinity matrices are bound from any remaining matter in the solution, the affinity matrices having sufficient binding affinity for the determinants to remain bound to the determinants during the separation, iii) contacting the glycoprotease with the separated cells in sufficient concentration and duration to cleave solely the protein substrate portions having the determinants and bound by the affinity matrices and thereby release the cells from the affinity matrices, the released cells retain viability due to the restricted specificity of the glycoprotease cleaving solely O-glycosylated protein portions having the determinants, and iv) recovering the released cells.		

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THE USE OF PASTEURELLA HAEMOLYTICA GLYCOPROTEASE
IN A PROCESS FOR RECOVERING CELLS RICH IN
O-GLYCOSYLATED SURFACE PORTIONS

This invention relates to the use of *Pasteurella*
5 *haemolytica* glycoprotease in processes for recovering
cells having surfaces rich in O-glycosylated
carbohydrates.

Sugar-linked proteins (glycoproteins) on the
surfaces of living cells are the labels by which cells
10 are recognized by the immune system, as "self" or "non-
self", and as normal or tumor cells. These labels
influence the fate of cells, their development,
association and interaction. In the blood system, the
wide variety of cell types are all thought to be derived
15 from a small pool of primitive precursor bone marrow
cells. The disruption of the normal development of bone
marrow stem cells can give rise to many varieties of
leukaemia, immunodeficiency syndromes and anaemia. A
detailed knowledge of the structure, function and
20 distribution of glycoproteins on blood cell surfaces is
needed to understand the causes of leukaemia. In
particular, there is interest in the use of primitive
stem cell antigens, for the isolation of these cells and
their use in transplantation. Stem-cell bone marrow
25 transplantation is an important surgical procedure for
re-establishing the bone marrow of cancer patients after
radiation treatment and chemotherapy.

In the chemotherapy of many cancers, it is the toxic
effect of the drugs on the haematopoietic bone marrow
30 cells that limits the patient's tolerance to treatment.
This problem can be overcome in some cases by autologous
bone marrow transplantation following marrow-ablative
chemotherapy and radiotherapy. However, in many tumors
such as breast cancer, lymphoma, and neuroblastoma,
35 cancer cells may already have metastasized into the bone
marrow, and autologous transplantation may require
additional strategies. Therefore a variety of ex vivo
techniques ("negative purging") have been developed to
remove the tumor cells prior to reinfusion of the bone marrow.

Potential problems with this general approach of 'negative selection' to provide autografts free of cancer cells include toxicity to primitive haematopoietic progenitor cells, inadequate reduction in tumor load and the frequent requirement for different purging modalities for different types of tumor.

With the development of monoclonal antibodies to CD34, (which represents the only cell-surface antigen expressed on the most primitive haematopoietic progenitor cells of all lineages) alternative techniques have become available. Recent studies with baboons, rhesus monkeys, and humans, suggest that the affinity-purified CD34+ marrow fraction contains the progenitor cells capable of initiating long-term haematopoiesis. 'Positive selection' of haematopoietic stem/progenitor cells thus represents a promising alternative strategy to the purging of autografts of neoplastic cells.

It is difficult to isolate the most primitive (i.e. CD34+) progenitor cells from normal bone marrow populations for effective reconstitution of haematopoietic function in human recipients. Current method generally fail to provide pure populations of CD34+ progenitor cells in the quantities required on a reliable and cost-effective basis. More recently, the proteolytic enzyme chymopapain, has been used in conjunction with magnetic affinity matrix techniques to rapidly effect the release of the purified CD34+ population from the magnetic beads. However, since this enzyme cleaves a large number of cell-surface molecules in addition to the CD34 antigen, its usefulness may be limited in some clinical situations.

We have discovered a new technique for the rapid isolation of bone marrow progenitor cells. The ability to purify haematopoietic stem cells from tumor-infiltrated marrow can have applications in autologous bone marrow transplantation. This successful strategy can also be used in allogeneic stem cell transplantation,

to alleviate immunodeficiency syndromes and bone marrow failures such as aplastic anaemia.

Pasteurella haemolytica serotype A1 is a Gram-negative bacterium commonly found in the nasal passages of cattle and sheep (1-1). It is associated with a severe pneumonia which occurs when the animals are stressed or shipped. It is the principal microorganism associated with bovine pneumonic pasteurellosis, a major cause of sickness and death in cattle in North America (1-2, 1-3). *P. haemolytica* has been divided into sixteen serotypes based on soluble or extractable surface antigens (1-4). Among the sixteen serotypes, serotype A1 is the predominant microorganism isolated from pneumonic lungs (1-5). *P. haemolytica* A1 secretes a number of antigens into the culture supernatant during its growth. These antigens include a glycoprotease specific for sialoglycoproteins (1-6), a heat-labile cytotoxin specific for ruminant leukocytes (1-7), a serotype-specific outer-membrane protein (1-8), and a neuraminidase (1-9).

We have discovered that the glycoprotease of *P. haemolytica* A1 is highly specific for O-glycosylated proteins and that proteins which lack extensive O-sialoglycopeptides residues are not cleaved. This substrate specificity is unique among proteolytic enzymes. The enzyme will cleave only O-sialoglycoproteins, unlike other proteolytic enzymes, which can cleave many proteins, including glycoprotein substrates. The best characterized glycoprotein substrate is glycophorin A, a transmembrane cell surface protein of human erythrocytes (1-10). This enzyme is a neutral metallo-protease and is non-toxic to cultured cells, including human leukocytes, bovine pulmonary macrophages, cultured bovine endothelial cells and erythrocytes.

As described in U.K. application S.N. 9100825.0 filed January 15, 1991, the gene for the bacterial enzyme was isolated and cloned. This enzyme is unique for the

cleavage of certain glycoproteins on the surfaces of living cells. The significant characteristic of the enzyme is that it splits only a few of the many types of cell-surface proteins.

5 The enzyme (P.h. glycoprotease) is derived from the bacterium *Pasteurella haemolytica*. Its restricted substrate specificity can be used in the rapid immuno-selection of haematopoietic stem cells for bone marrow transplantation.

10 We have found that the bacterial enzyme as described in the U.K. application S.N. 9100825.0 can be used to improve the isolation from human bone marrow of primitive blood stem cells that bear a unique glycoprotein label CD34. These stem cells are required for human bone
15 marrow transplantation, and can be isolated by immunoaffinity matrices e.g., magnetic beads coated with antibodies which bind to the CD34 glycoprotein. The enzyme specifically and rapidly releases the isolated cells by cleaving the glycoproteins to which the
20 antibodies are attached. This cleavage releases the stem cells from the magnetic beads, and enables these stem cells to be obtained at high purity and yield. Hence, the invention provides a relatively rapid process for separating and purifying stem cells or other cells having
25 surface glycoproteins which are cleaved by this P.h. glycoprotease. The protease can cleave from the cells any type of conjugate including antibodies which are attached to the substrate glycoproteins. This process does not harm cell viability.

30 The invention therefore provides a simple, rapid and flexible method to isolate primitive haematopoietic progenitor cells from normal bone marrow. These cells, which express the CD34 antigen, were positively selected using, for example, antibodies (to portions of the CD34
35 antigen which are removed by the glycoprotease), and immunomagnetic beads. Prior depletion of naturally adherent cells was not required. After magnetic

selection of CD34+ cells/magnetic beads, the cells were detached from the beads by incubation with the P.h. glycoprotease. The purity of the released cells was assessed using anti-CD34 antibodies which detect the remaining cells-bound fragment of CD34 which is not removed by the glycoprotease. In all experiments, the purity of the enzyme-released cells was high. The yield of CD34+ cells was also high. The purified cells generated normal numbers of haematopoietic colonies and reconstituted haematopoiesis in long-term culture, indicating that the functional competence of CD34+ progenitor cells *in vitro*, was unaffected by P.h. glycoprotease treatment.

The technique according to this invention offers several improvements over previously described procedures for the purification of primitive haematopoietic progenitor cells. Prior enrichment of the progenitor-cell pool by laborious removal of naturally adherent cells is not required. Thus, the potential loss of primitive progenitor cells (which may be crucial for long-term haematopoietic reconstitution), by non-specific adhesion to plastic can be avoided. Negative selection with a panel of monoclonal antibodies to remove mature leukocyte subsets, prior to positive immunomagnetic selection of CD34+ cells, is also avoided because many of the CD34+ progenitor cells also express these structures. This technique therefore allows a more critical assessment of the functional capabilities of the whole CD34+ fraction. Furthermore, potential time-consuming and expensive procedures are obviated.

According to an aspect of the invention, a method for recovering viable cells with surface protein determinants having portions rich in O-glycosylated carbohydrates is provided. The proteins which have the determinants are substrates sensitive to a

P. haemolytica-derived neutral metallo-glycoprotease, and the glycoprotease has highly restricted specificity for cleaving solely from the cell surfaces the O-glycosylated protein portions having the determinants while retaining cell viability. The process comprises:

- i) contacting the cells in solution with affinity matrices which bind specifically to one or more of the determinants which are part of the glycoprotease sensitive substrates and allowing the affinity matrices to bind the determinants on the cells,
- ii) separating the cells to which the affinity matrices are bound from any remaining matter in the solution, the affinity matrices having sufficient binding affinity for the determinants to remain bound to the determinants during the separation,
- iii) contacting the glycoprotease with the separated cells in sufficient concentration and duration to cleave solely the protein substrate portions having the determinants and the affinity matrices and thereby release the cells from the affinity matrices, the released cells retaining viability due to the restricted specificity of the glycoprotease cleaving only O-glycosylated protein portions having the determinants, and,
- iv) recovering the released cells.

According to another aspect of the invention, modified viable haematopoietic progenitor cells have removed therefrom by a glycoprotease, O-glycosylated portions of an antigenic glycoprotein epitope selected from the group of epitopes identified by entities CD34, CD43, CD44 and CD45. The modified cells are viable for long term haematopoiesis.

FIGURE LEGENDS**Figure 1**

Binding of specific antibodies to KG1a cells before or after cleavage with neuraminidase or *P. haemolytica* glycoprotease

Panels A, B and C; cells stained with anti-CD34 antibody B1.3C5 and FITC-conjugated goat anti-mouse Ig: panels D, E, F and G; anti-CD34 antibody QBEND 10: panels H, J and K; anti-CD44 antibody 50 B4: panels L, M and N; anti CD71 antibody OKT9. Panels A, D, H and L; untreated control KG1a cells: panels B, E, J and M; cells treated with neuraminidase before staining: panels C, F, K and N; cells treated with glycoprotease before staining: panel G; cells treated sequentially with neuraminidase and glycoprotease before staining. Data analysed on an Epics profile flow cytometer. Mean fluorescence channel values are indicated in the upper right corner of each panel.

Figure 2

Effects of *P. haemolytica* glycoprotease on O-sialo-glycoproteins

Radio-iodinated KG1a cells were lysed before (-) or after (+) cleavage with glycoprotease. Cell-lysates and cell-free cleavage products (P), were subjected to immunoprecipitation with specific antibodies as indicated. Tracks "S" contain radioactive standards (Amersham).

Figure 3

Effects of *P. haemolytica* glycoprotease on non-O-glycosylated glycoproteins

Radio-iodinated KG1a cells were lysed before (-) or after (+) cleavage with glycoprotease. Cell-lysates and cell-free cleavage products (P), were subjected to immunoprecipitation with monoclonal antibodies.

Figure 4

Effects of *P. haemolytica* glycoprotease on epitopes

detected by CD45 antibodies PBMCs were stained with anti-CD45 (histograms A), anti-CD45 RA (B) anti-CD45 RB (C), anti-CD45 RO (D) or HLA class I antibodies (E) and analyzed by flow cytometry on an Epics Profile. Data were prepared and printed using "Elite Software" (Coulter Electronics). For each antibody, the upper histogram (-) represents untreated cells and lower histogram (+) represents the staining of glycoprotease-cleaved cells.

Figure 5

Effects of neuraminidase and *P. h.* glycoprotease cleavage on CD34 epitopes

KG1 cells were stained with anti-CD34 antibodies and analyzed as in Fig. 4. For each antibody, the lower histogram (i) represents the staining of untreated cells; the middle histogram (ii) represents the staining of VCN-treated cells; the upper histogram (iii) represents the staining of the glycoprotease-treated cells.

Figure 6

Flow cytometry of CD34-positive cell lines after magnetic sorting and treatment with *P. h.* glycoprotease

A mixture of KG1 and HSB2 cells was separated using QBEND 10-coated magnetic beads. Antibody staining and flow cytometry were performed as described in Fig 4. A: Cells which remained unattached after magnetic sorting, stained with ICH3; B: Control KG1 cells stained with ICH3; C: CD34+ cells separated from the mixture, and released from the beads by glycoprotease treatment, stained with ICH3; D: CD34+ cells isolated as in (C) but stained with the class III antibody 115.2; E: Control KG1 cells stained with 115.2.

Figure 7

Flow cytometry of leukemic cells after magnetic sorting and treatment with P. h. glycoprotease

- Top left; light-scattering properties of unfractionated MNC and (right) fluorescence histogram (using QBEND 10) of the 'blast/lymphocytes' cells gated into bitmap A. Middle left; light-scattering properties of magnetically-sorted CD34+ cells after release with glycoprotease and (right) fluorescence histogram using 8G12 of the cells gated into bitmap A. Lower left; light-scattering properties of the magnetically-sorted CD34-cells and (right) fluorescence histogram using 8G12 of the cells gated into bitmap A.

15 Figure 8

SDS-PAGE analysis of radioiodinated CD34 antigen

- Immune complexes were made from ¹²⁵I-lactoperoxidase-labeled KG1 lysates with TUK3 and isolated on Protein A sepharose (track A). Immune complexes were cleaved with glycoprotease prior to analysis (track B). Track C contains the supernatant from the cleavage of immune complexes from track B. Immune complexes were made with TUK3 (track D) or B1.3C5 (track G) from lysates of control KG1 cells, or from glycoprotease treated cells (tracks E and H). Immune complexes made with TUK3 (track F) or B1.3C5 (track K), from the cell-free supernatant of the glycoprotease-treated cells.

30 Figure 9

Flow cytometry of normal bone marrow MNC fractions

- Top: Unfractionated normal bone marrow MNCs were stained with QBEND 10 and fluoresceinated goat anti-MIg. Left: Cells were gated according to light scattering properties into the "blast/lymphocyte bitmap "A", or the granulocyte/macrophage bitmap "B". Right: fluorescence histogram of cells gated into bitmap "A".

Bottom. CD34+ cells post release by the P. h. glycoprotease were stained with TUK3 and fluoresceinated goat anti-MIg. Left: Light scattering properties of released cells. Right: fluorescence histogram of cells
5 gated into bitmap "A".

Figure 10

Photomicrographs of Giemsa-stained cytocentrifuge preparations

10 Top (A): Final CD34+ fraction post enzyme treatment (low magnification).

Middle(B): Final CD34+ fraction post enzyme treatment (high magnification).

Bottom(C): Unseparated MNC population (low
15 magnification).

The growth and differentiation of normal haematopoietic progenitor cells into mature functional leukocyte subsets is accompanied by the acquisition or loss of specific cell-surface glycoproteins (2-1, 2-2).
20 These structures, some of which are involved in the reception and transmission of signals from external sources (2-3, 2-4), play important roles in the fate of cells and their development. The varied arrays of glycoproteins displayed by these cell-types also
25 delineate their associations, interactions and responses to their environmental stimuli. We have discovered that the novel protease from *Pasteurella haemolytica* cleaves the O-glycosylated glycoproteins which include those identified by the antigenic entities selected from the
30 group consisting of CD34, CD43, CD44, and CD45.

As previously demonstrated the protease secreted by *P. haemolytica* cleaves the erythrocyte glycoprotein, glycophorin A (2-10, 2-11), which contains 1 N-linked glycan and 15 O-linked glycans of the mono- or
35 di-sialylated Gal β 1-3GalNAc-R³ type (2-12). Proteins which lacked O-sialo-carbohydrates were not cleaved. The purified enzyme, which has an M_r of about 35,000 on

SDS-PAGE, is a neutral metallo-protease and is non-toxic to cultured cells, including human leukocytes, bovine pulmonary macrophages, cultured bovine endothelial cells and erythrocytes (2-10, 2-11).

5 Until recently, no substrate had been found for the glycoprotease other than the erythrocyte sialoglycoprotein, glycophorin A (2-10, 2-11). None of thirty proteins and glycoproteins tested was cleaved by the enzyme. No hydrolysis was seen for human IgA₁ or IgA₂, so
10 the glycophorin-degrading enzyme is not identical to IgA protease, a microbial neutral metallo-protease (2-15). Similarly, the *P. haemolytica* glycoprotease does not degrade bovine α -1-acid glycoprotein, bovine
15 β -lactalbumin, hen ovalbumin, BSA, glyceraldehyde-3-phosphate dehydrogenase, soybean trypsin inhibitor, bovine carbonic anhydrase, trypsinogen, chymotrypsinogen, insulin A or B chains, or cytochrome c. The removal of sialyl residues from glycophorin A by treatment with
20 neuraminidase destroys the susceptibility of this glycoprotein to hydrolysis by the enzyme. This high degree of specificity for a sialylated O-linked glycoprotein, of a type commonly found on mammalian cell surfaces, further underlines the unique substrate specificity of this enzyme.

25 We have determined and can demonstrate that several O-sialoglycoproteins, found on the surfaces of human cells, can be cleaved by the *P. haemolytica* glycoprotease. These substrates include CD34, CD43, CD44, and CD45 entities, which exhibit distinct patterns of
30 expression in the haematopoietic system. These entities are well-characterized leukocyte antigens with diverse functions.

35 Of these structures, the CD34 antigen is of particular interest. CD34 expression is restricted to only 1-3% of normal bone marrow cells which have been shown by colony-forming assays to include virtually all

unipotent (BFU-E, CFU-G/M, CFU-Meg) and multipotent progenitors (CFU-GEMM) as well as pre-CFU (3-11 - 3-13). While mature lymphoid colony-forming cells do not express the CD34 antigen, putative B lymphoid cell precursors with nuclear terminal deoxynucleotidyl transferase activity do express it (3-12 - 3-14). These studies, together with others using fresh leukemia samples, strongly suggest that mature blood cells of all lineages are derived from the CD34-positive (CD34+) fraction of normal marrow (3-13 - 3-17). This notion is supported by the observation that CD34+ bone marrow cells can reconstitute all lineages of the haematopoietic system in lethally-irradiated baboons (3-18) and rhesus monkeys (3-19). A recent report of the transplant of CD34+ cells in patients with disseminated cancer supports the view that isolated CD34+ marrow cells are also capable of reconstituting haematopoiesis in humans (3-20). Recent single-cell cloning experiments demonstrate that highly purified CD34+ cells, which lack co-expression of any myeloid, T- or B-cell antigens, are capable of generating several types of colonies when grown over irradiated stromal cells *in vitro* (3-21). As will be discussed in more detail there are at least seven CD34 antibodies, recently assessed by the International Workshop (3-22), which immunoprecipitate a monomeric structure of 110 kD from lysates of acute myelogenous leukemia-derived cell lines KG1 and KG1a (3-7, 3-11 - 3-13, 3-15). Similar bands can be isolated from fresh acute leukemias of primitive myeloid, B-lymphoid and T-lymphoid phenotypes (3-11 - 3-13, 3-15 - 3-17). Extensive structural and carbohydrate analyses indicate the presence of both complex-type N-linked and heavily sialylated O-linked glycans. However, partial amino-acid sequence analysis has not revealed the function of the CD34 antigen or any homology with previously-described structures (3-7).

CD34 antibodies recognize a variety of distinct epitopes on this antigen, some of which (MY10, B1.3C5, 12.8, ICH3) as discussed later are differentially dependent on the presence of sialic acid residues (3-7, 3-15, 3-22). We have therefore determined the ability of the *P. haemolytica* glycoprotease to remove the epitopes recognized by all seven CD34 antibodies (3-22), as monitored by fluorescence microscopy, quantitative flow cytometry and biochemical techniques.

The epitopes MY10, B1.3C5, 12.8 and ICH3, are efficiently removed by the *P. h.* glycoprotease as will be later demonstrated. These epitopes have been designated class I. The enzyme also removed the sialic acid-independent epitope detected by QBEND 10 antibody designated as (class II). The epitopes detected by TUK3 and 115.2 antibodies which were not cleaved by the enzyme, were referred to as class III. Class III epitopes are therefore closer to the extracellular membrane surface than the class I and class II epitopes. The enzyme-treated cells exhibited normal quantitative expression and distribution of the CD34 antigen, as assessed by staining with class III CD34 antibodies TUK3 (4-23) and 115.2 (4-5), that detect epitopes on a 75kD cell-bound fragment which remains after treatment with the glycoprotease (4-24). The differential sensitivity of the various CD34 epitopes to cleavage with this novel glycoprotease demonstrate that the enzyme can be used in the recovery of CD34-positive cells, isolated from heterogeneous leukocyte populations by CD34-affinity matrices.

In a preclinical model using immunomagnetic beads, we separated CD34-positive KG1 cells (4-25) with high yield (90-95%) and high purity (94-98%), from sham mixtures containing 50% CD34-negative cells. We also separated CD34-positive blast cells from a patient in megakaryoblastic crisis of chronic myeloid leukemia, with similar high purity and recovery.

We have demonstrated that the *P. h.* glycoprotease releases CD34-positive cells from immunomagnetic beads following their separation from unfractionated normal bone marrow mononuclear cells. In addition, we have
5 demonstrated functional competence of the CD34-positive cells after treatment by the *P. h.* glycoprotease.

The isolation and characterization of the glycoprotease of *P. haemolytica* used in the above generally described techniques, has been achieved by
10 molecular exclusion HPLC techniques. The glycoprotease has also been isolated, free from other *P. haemolytica* proteins by expression in *E. coli* of the recombinant gene which codes for the enzyme (1-11 1-12, 1-13). The enzyme has a M_r of about 35,000 kD. The enzyme is secreted into
15 the medium of *P. haemolytica* cultures, but when it is expressed in transformed *E. coli*, the enzyme is not fully secreted but is trapped in the periplasmic space. Therefore the recombinant gene product can be readily isolated free from most host proteins by osmotic shock
20 treatment.

The isolation, purification, and cloning of the cDNA sequence encoding the glycoprotease is described in detail in the aforementioned U.K. patent application SN 9100825.0 the subject matter of which is hereby
25 incorporated by reference. For ease in understanding the isolation, a brief description is provided as follows. From DNA library of the *P. haemolytica* genome, two identical 3.2 kbp plasmid PBR322 inserts were found that code for the glycoprotease. One of these clones was
30 transformed into *E. coli* CSR603 and gave rise to expression of the glycoprotease. The recombinant enzyme was visible on SDS-PAGE gels as a 35 kD band, and the native product was able to cleave 125 I-glycophorin A. A smaller 2.3 kbp BamHI-HindIII restriction endonuclease
35 fragment, which contained the gene, was constructed from the original insert, cloned into the phage M13 in both orientations, and transformed into *E. coli* TG-1. Single

stranded recombinant DNA templates were sequenced by the dideoxy sequencing technique. The entire DNA insert was sequenced by generating overlapping deletions in M13 mp18 and M13 mp19. All regions of each strand were sequenced at least twice, independently. The sequence shows an open reading frame of 975 nucleotides which encodes 325 amino acids with a predicted mol. wt. of 35.2 kD. These estimates are in agreement with the size of the expressed protein. The calculated isoelectric point for this protein is 4.85. No homology with other known bacterial or eucaryotic proteolytic enzymes can be detected at the DNA or protein level.

The gene encoding the glycoprotease was designated *gcp*. Upstream from the gene is a region which resembles the promoter sequences commonly found in *E. coli*. In particular, two sequences which resemble the TATAAT consensus promoter sequence (1-14, 1-15) can be identified. Further upstream are sequences similar to the consensus RNA polymerase binding site, TTGACA. In addition to these potential promoter sequences, a putative ribosome-binding site can be found preceding the initiation codon of *gcp*. The deduced RNA sequence of this site resembles somewhat that of the *E. coli* consensus sequence AAGGAGGU (1-16). It is likely that some of these features are involved in the expression of the glycoprotease gene. With respect to termination of transcription, a mRNA structure consisting of a 14 bp stem and loop region, very similar to the rho-independent transcriptional termination signals of *E. coli* (1-15) could be identified downstream from *gcp*.

We have recently used the high expression vector pTTQ18 carrying the glycoprotease gene to transform *E. coli* HB101, using induction of the *tac* promoter with isopropyl- β -D-thiogalactoside. The transformed cells expressed high levels of the glycoprotease enzyme, as seen by Coomassie blue staining of SDS-PAGE gels. The gene product was blotted onto polyvinylidene difluoride

(PVDF) membranes and subjected to N-terminal sequencing by the gas-phase automated Edman procedure. The N-terminal sequence of 15 amino-acids corresponded exactly to the longest of two putative open reading frames seen in the DNA sequence, and confirmed the identity of the gene and its product.

The low concentration of glycoprotease was found to be unstable when isolated by HPLC from serum-free culture supernatants. This is in marked contrast to the remarkable stability of the enzyme activity in freeze-dried pH 4 precipitates of culture supernatant, in which activity is maintained for many months at room temperature. The increased protein concentrations of the recombinant gene product expressed in high expression vectors overcome the lability of the enzyme at low protein concentrations. The glycoprotease is only a minor protein component of the culture supernatant of *P. haemolytica* even in bacteria grown in serum-free media. Consequently it has been difficult to isolate a homogenous preparation of the glycoprotease, except by laborious chromatographic methods. The recent identification of the recombinant glycoproteins as a 35 kD ³⁵S-labelled band on SDS-PAGE, the high level of expression of this product, and the recognition that the recombinant product is located within the periplasmic space of *E. coli* HB101 enables us to obtain large amounts of highly purified product.

Prior to 1990, no substrate had been found for the glycoprotease other than the erythrocyte sialoglycoprotein, glycophorin A (1-10). None of thirty proteins and glycoproteins tested previously was cleaved by the enzyme. When glycoproteins from various sources were radiolabelled with ¹²⁵I-iodine and incubated with partially-purified enzyme, no hydrolysis of these substrates could be detected, by SDS-PAGE and autoradiography. No hydrolysis was seen for human immunoglobulin A1 (IgA1) or human immunoglobulin A2

(IgA2), so that the glycophorin-degrading enzyme is not identical to IgA protease, a microbial neutral metallo-protease (1-17). Similar procedure showed that the *P. haemolytica* protease does not degrade [¹²⁵I]-labelled bovine α -1 acid glycoprotein, bovine β -lactalbumin, or hen ovalbumin. The [¹²⁵I]-labelled proteins, bovine serum, albumin, glyceraldehyde-3-phosphate dehydrogenase, soybean trypsin inhibitor, bovine carbonic anhydrase, trypsinogen and chymotrypsinogen were not cleaved by the *P. haemolytica* protease. Other proteins were tested as substrates by incubation with active enzyme fractions, and enzyme action was monitored by SDS-PAGE and Coomassie blue staining of the substrate and products. The enzyme did not hydrolyze insulin chain A, insulin chain B, or cytochrome c. Partially purified enzyme preparations with high activity against glycophorin were inactive in cleavage of dye-casein conjugates (Azocasein) or dye-collagen conjugates (Azocool). Thus the weak casein-degrading activity reported in culture supernatants of *P. haemolytica* (1-6) was not found in the glycoprotease-enriched extracts used here.

The removal of sialyl residues from glycophorin by treatment with neuraminidase destroys the susceptibility of the glycoprotein to hydrolysis by the enzyme. This high degree of specificity for an O-linked sialoglycoprotein, of a type commonly found on mammalian cell surfaces, is a unique property of this enzyme. Our results suggest that a major cleavage of glycophorin A occurs at the Arg₃₁-Asp₃₂ and this has been confirmed by N-terminal analysis of the one product, but there are indications that there are other sites of cleavage. These other sites appear to be in the glycosylated N-terminal region close to the O-linked sialoglycosylated residues, but no requirement for specific amino acyl residues is apparent.

We have demonstrated that several O-sialoglycoproteins, found on human cell surfaces, are substrates

for the *P. haemolytica* glycoprotease (4-22). These new substrates CD34, CD43, CD44 and CD45 are well characterized leukocyte antigens with diverse functions.

We demonstrate in the following examples that the
5 O-glycosylated cell-surface antigens CD34, CD43, CD44, and CD45 are cleaved by a novel glycoprotease from *Pasteurella haemolytica*. On the other hand, glycoproteins containing only N-linked structures were not cleaved. These data, together with previous observations on the
10 cleavage of the major erythrocyte glycoprotein, glycophorin A (2-10, 2-11), demonstrates that the *P. h.* glycoprotease represents a new type of protease whose substrate specificity is highly restricted to glycoproteins rich in O-linked glycans. The novel nature
15 of the *P. h.* glycoprotease has been confirmed by the nucleotide sequence of its gene, which shows no homology with other bacterial or eukaryotic protease genes (2-2 - 2-13).

The enzyme has for the molecules which are
20 substrates thereof, many potential uses. For example, as noted above, the CD34 antigen is cleaved by the *P. h.* glycoprotease. However, not all epitopes detected by various CD34 antibodies, were removed by its action. The epitope detected by the TUK3 antibody, for example, is
25 retained on the cell surface, and must be proximal to the membrane on the extracellular side of the cell compared to either the B1.3C5 or QBEND 10 epitopes as will be demonstrated.

A second example of the enzyme's utility derives
30 from its cleavage of the CD45 antigen and hence is useful in the affinity matrix purification thereof. The cDNA sequences of this family of molecules have been determined and an O-glycosylated, serine/threonine-rich stretch of amino acids (encoded by exons 3-8) is found at
35 the extreme NH₂-terminus (2-33).

The enzyme also has uses in the study of structure-function relationships of some O-glycosylated

cell-surface antigens. For example, the family of CD45 molecules have been implicated in T cell activation phenomena (2-36); the intracytoplasmic domains of CD45 exhibit intrinsic tyrosine phosphatase activity (2-37 - 2-38) and CD45 expression has been shown to be required for antigen-induced, T-lymphocyte proliferation (2-39). Within the T cell compartment, antigen-unprimed or 'virgin' T cells exhibit the CD45 RA+/CD45 RO- phenotype. In contrast, antigen-primed, activated T cells lose expression of the CD45 RA epitope and acquire the CD45 RO structure and maintain this phenotype in their post-activation phase as 'memory' T cells (2-40). The glycoprotease can be used to analyze the role of the O-glycosylated domains in the CD45 isoforms and to assess the role of the glycoprotease-cleavable sequences in the signal transduction pathways which modulate the tyrosine-specific phosphatase activity. Additionally, once the individual ligands for the various CD45 isoforms have been determined, the glycoprotease can be used in locating the ligand-binding domains of the CD45 structures.

The CD43 structure, another substrate molecule for the P. h. glycoprotease, has also been implicated in immune cell function and one antibody to CD43 can activate T cells directly (2-41). Signal transduction via anti-CD43 antibody appears to be independent of the CD3:T cell receptor complex (2-42). Differentially O-glycosylated forms of the same polypeptide are expressed on all nucleated haematopoietic cells, with the exception of resting B cells, in a lineage specific manner (2-22). CD43 is also the major surface protein which is structurally altered, or whose expression is drastically reduced on leukocytes from patients with Wiscott-Aldrich syndrome, an X-chromosome-linked disease (2-43 - 2-44).

The use of the P. h. glycoprotease also cleaves the CD44 antigen. This heavily glycosylated molecule was

recently shown to function as the receptor for hyaluronic acid and may also be a receptor for other components of the extracellular matrix such as chondroitin sulphates (2-23).

5 For susceptible structures, the location of the individual epitopes with respect to the glycoprotease cleavage points, has significant implications for the affinity purification and recovery of the leukocyte subsets which express them. For example, it should be
10 possible to use the P. h. glycoprotease to rapidly obtain pure populations of either CD45 RA+ 'virgin' T cells or CD45 RO+ 'memory' T cells (2-40) for further study in isolation of other 'contaminating' leukocyte subsets.

In accordance with a particular embodiment of this
15 invention, this enzyme in being useful for the purification of leukocyte subsets, derives such use from its removal of the epitopes detected by the appropriate CD34 antibodies. The cleavage of CD34 by the enzyme suggests that CD34-positive cells, isolated from
20 heterogeneous leukocyte populations by CD34-affinity matrices, are released from these matrices by the enzyme. A further advantage of this technique is that the purity of the released CD34-positive cells can be assessed using other CD34 antibodies, such as TUK3, whose epitopes are
25 retained on the cell-surface after cleavage by the glycoprotease. The ability to rapidly purify functionally competent, hematopoietic progenitor cells using this non-toxic enzyme has important implications for autologous and allogeneic 'stem-cell' bone marrow
30 transplantation.

Four monoclonal antibodies MY10 (1-19), B1.3C5 (1-20), 12.8 (1-21) and ICH3 (1-22) raised against KG1 or KG1a cells have been shown to identify an antigen on a small population of bone marrow cells. The procedures
35 for making these antibodies are fully identified in the noted references the subject matter of such references being incorporated by reference. This sub-population is

shown by colony-forming assays to include virtually all unipotent (BFU-E, CFU-G M, CFU-Meg) and multipotent (CFU-GEMM and pre-CFU) progenitors (1-19 - 1-20). MY 10 has also been shown to bind to blast colony-forming cells in
5 cord blood (1-23).

The above five of the seven epitopes identified by the CD34 antibodies (3-11 - 3-15, 3-22 - 3-24) are cleaved by the enzyme. All epitopes which are dependent upon the presence of sialic acid residues, i.e. MY10,
10 B1.3C5, 12.8 and ICH3, are efficiently removed by the P. h. glycoprotease. Thus we have designated these epitopes, class I. The enzyme also cleaves the sialic acid-independent epitope detected by QBEND 10 (class II). The epitopes detected by TUK3 and 115.2 which are not
15 cleaved by either enzyme, are referred to as class III. Class III epitopes are therefore more proximal to the extracellular side of the cell membrane than the class I and class II epitopes.

Neither partial amino acid sequence analysis (3-7)
20 nor the full-length cloning of the cDNA has revealed the function of the CD34 antigen. However, the sensitivity of the CD34 antigen to the P. h. glycoprotease is in keeping with known and anticipated characteristics of the CD34 molecule. The full length human cDNA predicts a type I
25 integral membrane protein of about 40 kD with a maximum of 9 potential N-glycosylation-sites. Since the de-N-glycosylated and desialylated forms are 90 kD and 150 kD respectively, the native molecule must contain a considerable number of O-linked glycans (3-7).
30 Accordingly, over 35% of the amino acids in the 145 amino acid NH₂-terminal domain of this antigen are serine or threonine residues. The presence of high levels of serine and threonine residues indicates the possibility that it may be the carbohydrate moiety, rather than
35 primary amino acid sequence *per se*, which determines the functional capabilities of this part of the CD34 structure. The clusters of O-linked glycans in this

domain, some of which at least may be large and/or complex (3-7), probably ensure that the protein takes on the conformation of an extended 'rod' (3-33).

We have demonstrated in the following examples, that

5 the major product of the P. h. glycoprotease cleavage of CD34 is a cell-bound fragment of about 75 kD. Using TUK3, this fragment was detected in lysates of radiolabeled KG1 cells, cleaved before lysis, as well as in isolated immune complexes after their cleavage with the enzyme.

10 When immune complexes were made from non-cleaved, radiolabeled cells with class I (B1.3C5) or class II (QBEND 10) antibodies, the same 75 kD fragment was recovered in the supernatant rather than associated with the immune complexes. Any minor components of the CD34

15 antigen which may have remained associated with the immune complexes of the class I and II reagents were not identifiable in SDS gels capable of resolution down to about 10 kD. Together, these experiments indicate that the class I and class II CD34 epitopes are probably

20 contained in the distal 25-35 kD region of the NH₂-terminal domain of CD34.

The differential sensitivity of the various CD34 epitopes to cleavage with this novel glycoprotease demonstrates that the enzyme may be of use in the

25 recovery of CD34-positive cells, isolated from heterogeneous leukocyte populations by CD34-affinity matrices. Recent studies in baboons (3-18), rhesus monkeys (3-19), and humans (3-20), together with single cell-cloning experiments (3-21), suggest that

30 affinity-purified populations of CD34-positive bone marrow cells contain the primordial hematopoietic stem cell. 'Positive selection' of hematopoietic stem/progenitor cells represents an alternative strategy to 'negative selection' or purging for the manipulation of

35 bone marrow cells prior to transplantation (3-34), as well as possibly providing potential target cells for genetic manipulation studies *in vitro*. As previously

discussed in more general terms, it is difficult to isolate the most primitive (i.e. CD34-positive) progenitor cells from normal bone marrow populations for effective reconstitution of haematopoietic function in human recipients. Current technologies fail to provide pure populations of CD34-positive progenitor cells in the quantities required on a reliable and cost-effective basis. While fluorescence-activated cell-sorting produces relatively pure populations of CD34-positive cells for *in vitro* experiments, the method is too slow to isolate the 1%-3% CD34-reactive cells, on the scale required for transplantation. 'Panning' techniques require rigorous and laborious procedures to remove naturally adherent cells (macrophages, granulocytes etc.) prior to panning on anti-CD34-coated surfaces in large plastic vessels. CD34-positive cells produced by variations of this technique may routinely be contaminated with more than 30% CD34-negative cells and the overall yields of CD34-positive cells are low. This technique is also relatively expensive in that it requires large quantities of highly purified antibodies. Another approach, which employs the use of avidin-biotin affinity column chromatography, can generate CD34-positive progenitor cells in acceptable yields (25-58%) from tumor patient's bone marrow with purities in the 35-92% range (3-20).

As will be demonstrated, we separated CD34-positive KG1 cells from sham mixtures of KG1 and the primitive T-cell-line HSB2 using anti-CD34-coated immunomagnetic beads. After release from the beads with the *Pasteurella haemolytica* glycoprotease, the yields and purities of the CD34-positive cells were 90-95% and 94-98% respectively. All the magnetic beads were released from the cells in 20-30 minutes at 37°C and as will be demonstrated, the enzyme does not have detrimental effects on cell viability either at 30 minutes or after overnight incubation of the treated cells. The enzyme-treated cells showed normal quantitative expression and distribution of

the CD34 antigen, as assessed by staining with the glycoprotease-resistant epitopes identified by TUK3 (3-24), 115.2 (3-13) or the directly conjugated anti-CD34 antibody 8G12 (3-29).

5 In a second model system, we separated CD34-positive megakaryoblasts from a peripheral blood sample of a patient in blastic transformation of chronic myeloid leukemia. Even without prior removal of adherent cells or any other pre-sort manipulations, we efficiently
10 separated the CD34-positive cells from the rest of the sample and recovered 94% of the blasts from the starting heterogeneous cell preparation. The purity of the CD34-positive cells after release from the magnetic beads was also high at 93%, with a few granulocyte-series cells
15 as the major contaminating cell-type.

 In the cell-line model system cited, we used CD34 antibody-coated beads, whereas in the second system, we used antibody-coated leukemia cells. Coated beads were used in the first experiment because after enzyme
20 treatment to detach the magnetic beads, an aliquot of the released cells was stained with CD7 antibodies to identify contaminating HSB2 cells. Had this experiment been performed with antibody-coated cells, any CD34 antibody remaining on the KG1 cells after glycoprotease
25 treatment would have been detected with the secondary, fluorescein- conjugated anti-immunoglobulin reagent.

 The two model systems show that CD34-positive cells, separated from heterogeneous leukocyte populations by magnetic immuno affinity matrices, can be released from
30 these matrices by the glycoprotease from *P. haemolytica*. This technique is rapid, it requires less antibody than the 'panning' method, and it produces CD34-positive cells of high purity and high yield. The procedure is also flexible and can be used both for small-scale and
35 large-scale isolation of cells.

 We have also demonstrated a simple, rapid and flexible method to isolate primitive hematopoietic

progenitor cells from normal bone marrow. These cells, which express the CD34 antigen, were according to this particular embodiment, positively selected using antibodies (class I or class II) to the CD34 antigen, and immunomagnetic beads. Prior depletion of naturally adherent cells was not required. After magnetic selection of CD34+ cells/magnetic beads, the cells were detached from the beads by incubation with the P.h. glycoprotease. The purity of the released cells was assessed by immunofluorescence techniques using either of the class III CD34 antibodies, TUK3 or 115.2, whose epitopes are not removed by the glycoprotease (4-24). In all experiments, the purity of the enzyme-released cells was high. The yield of CD34+ cells was also high when experiments were performed in media other than IMDM as will be demonstrated in the examples. The cells purified and isolated by this technique exhibited the morphological characteristics of undifferentiated blasts. The enzyme-treated cells also display the same light-scattering properties characteristic of CD34+ cells isolated from normal marrow by other techniques such as fluorescence activated cell sorting (4-30). The purified cells generated normal numbers of hematopoietic colonies and reconstituted hematopoiesis in long-term culture, demonstrating that the functional competence of CD34+ progenitor cells *in vitro*, was unaffected by P. h. glycoprotease treatment.

The technique according to this invention offers several potential improvements over previously described procedures for the purification of primitive hematopoietic progenitor cells. Prior enrichment of the progenitor-cell pool by laborious removal of naturally adherent cells is not required. Thus, the potential loss of primitive progenitor cells (which may be crucial for long-term hematopoietic reconstitution), by non-specific adhesion to plastic (4-34) can be avoided. Negative selection with a panel of monoclonal antibodies to remove

mature leukocyte subsets, prior to positive immunomagnetic selection of CD34+ cells, is also avoided because many of the CD34+ progenitor cells also express these structures.

5 Despite the use of DNase in the culture medium (to reduce cell aggregation during the experimental procedures), some naturally adherent cells such as macrophages and mature granulocytes did bind to the magnetic microspheres. These non-specifically adsorbed
10 cells were not released from the beads by the P.h. glycoprotease thereby confirming the highly specific action of this enzyme, and hence the high purity of the isolated progenitor cells.

 In our experiments we demonstrate better recovery of
15 CD34+ cells from normal marrow after treatment with P.h. glycoprotease by selection of an appropriate medium. In a pre-clinical model system using mixtures of KG1 cells with CD34-negative cells, almost 100% recovery of CD34+ cells can be obtained using RPMI medium. IMDM has been
20 was used for the normal bone marrow separations. When duplicate separations were performed with a chronic myeloid leukemia sample containing 25% CD34+ blasts, the yield of CD34+ cells isolated in the presence of IMDM was only about 20% of that obtained from the parallel
25 experiment performed in the presence of RPMI. The most likely component of IMDM which may be responsible for the differences in enzyme efficiency in the two media is the presence of Na_2SeO_3 in IMDM (4-35) which is absent from RPMI (4-36). Since the *Pasteurella* enzyme is a putative
30 zinc-binding, metallo-protease (4-21), it is possible that the selenite ion may disrupt enzyme activity by displacing the metal ion cofactor. Subsequent isolation of CD34+ cells from normal marrow used RPMI or other similar media which is free of certain metal ions.
35 Experiments 3-5 resulted in consistently improved recovery of purified cells with the P.h. glycoprotease.

In clonogenic culture, the purified CD34+ cells were highly enriched for colony-forming cells, including multi-lineage progenitors. The degree of enrichment is comparable with that seen with CD34+ cells isolated by flow cytometry (4-29).

Using a modified LTBM system long term marrow-repopulating ability was determined. Confluent stromal layers derived from normal LTBMcs were used as feeder layers after irradiation of the layers to eradicate any residual haemopoietic activity, and then inoculated with the CD34+ cells released from the beads by P.h. glycoprotease. The subsequent generation of CFU-GM from these cultures over 7 weeks provided a pattern of generation of CFU-GM from the CD34+ cells was similar to that seen with ACD cells on identically-prepared layers. The results are also similar to those obtained previously using purified CD34+ cells isolated by flow cytometry (4-27). Thus it is apparent that the CD34+ cells isolated after P.h. glycoprotease cleavage from the beads contained precursors of colony-forming cells. Moreover, the ability of these cells to proliferate when co-cultured with stromal layers was not compromised by the action of the enzyme. It would appear that O-sialoglycosylated peptide moieties, lost after enzyme release of CD34+ cells, are resynthesized or are not essential, for early progenitors to initiate long-term haematopoiesis *in vitro*. The cleavage of the CD34 antigen on KG1 cells by P.h. glycoprotease generates a major cell bound fragment of about 75 kD identified by the antibody TUK3 which appears to be the essential functional component of the CD34 antigen, if the antigen plays a role in long-term haematopoiesis.

The invention therefore provides a rapid means of isolating CD34+ cells in high purity and yield, and without cytotoxicity. Since the functional capacity of early progenitors is unaffected by the procedure, this method provides large scale isolation of purified

hematopoietic progenitor cells, for medical treatment *in vivo* or for *in vitro* gene transfer studies.

It is apparent that the method according to this invention for recovering viable cells is not only dependent upon the specific activity of the P. *haemolytica* glycoprotease but as well the binding affinity of the affinity matrice used to isolate the desired cell in solution. Although the preferred embodiments have been directed to the use of particular antibodies, it is appreciated that other entities which have affinity for the particular O-glycosylated proteins may be used. Such entities include receptors, natural ligands antibody fragments, receptor fragments, recombinantly produced protein sequences which have affinities for the particular determinant on the desired cells, synthetic peptides having engineered binding sites, lectins, and cell-adhesion molecules from natural sources. The glycoprotease has a highly restricted specificity for cleaving solely the O-glycosylated protein portions having the determinants.

Before describing in detail the various experiments to demonstrate enzyme specificity, use of the enzyme in purification of O-glycosylated cell surfaces and viability of the treated cells, the following more general discussions of certain embodiments of the invention is provided.

CD34 is highly glycosylated 110 kD molecule in leukaemic cells

CD34 antibodies immunoprecipitate a monomeric structure of 110 kD lysates of acute myelogenous leukaemia-derived cell lines KG1 and KG1a (1-19, 1-20, 1-21, 1-22). Similar bands can be isolated from fresh acute leukaemias of primitive myeloid, B-lymphoid and T-lymphoid phenotypes (1-20, 1-27). Most CD34 antibodies identify denaturation-resistant epitopes in western blots, though with widely different efficiencies (1-35 -

1-36). These antibodies recognize a variety of distinct epitopes on this antigen, some of which (MY10, B1.3C5, 12.8) we have shown to be dependent on the presence of sialic acid residues. Extensive structural and carbohydrate analyses indicate the presence of O-linked glycans (1-34, 1-36). Partial amino-acid sequence analysis has revealed no similarities with previously-described structures (1-36). The CD34 cDNA has recently been cloned using a mammalian expression system, COS-7 cells. It appears that CD34 is a type I integral membrane protein of 40 kD, with 9 potential N-glycosylation sites. Since the de-N-glycosylated and desialylated forms are 90 kD and 150 kD respectively (1-36), the native molecule must contain considerable number of O-linked glycans. Accordingly over 35% of the amino acids in the N-terminal domain of this antigen are serine or threonine residues. The clusters of O-linked glycans in this domain probably ensure that it takes on the conformation of an extended "rod". Thus the NH₂-terminus of the CD34 antigen can be expected to extend a considerable distance out from the cell membrane.

CD34 is a substrate for the P.H. glycoprotease

The progenitor-cell-restricted antigen CD34 on KG1 cells is readily cleaved by the *P. haemolytica* glycoprotease as shown by the loss of reactivity of this antigen with the anti-CD34 monoclonal antibody B1.3C5 (1-18, 1-20) which detects a sialic acid-dependent epitope on this glycoprotein (1-22, 1-36). We have assessed the ability of this enzyme to remove CD34 epitopes recognized by all seven previously described CD34 antibodies designated by the 4th Leukocyte Antigen Workshop (1-35). We have shown by fluorescence microscopy, quantitative flow cytometry and western blotting, that sialic acid-dependent epitopes, recognized by antibodies B1.3C5, MY10 (1-19) and 12.8 (1-21) and ICH3 (1-22), are totally removed, as is the sialic acid-independent epitopes

recognized by QBEND 10 (1-36). In contrast, the sialic acid-independent epitopes recognized by 115.2 (1-21) and TUK3 (1-35) are totally resistant to the action of the glycoprotease.

5 The differential sensitivity of certain CD34 epitopes to cleavage with either neuraminidase and/or glycoprotease establishes the aforementioned 3 classes of epitopes: (Class I) those like MY 10, B1.3C5, 12.8 and ICH3 and which differentially sensitive to neuraminidase
10 and totally removed by the glycoprotease; (Class II) those like QBEND 10 which is removed only by the glycoprotease; (Class III) those like TUK 3 and 115.2 which are not removed by either enzyme. These results have important implications for the purification and
15 recovery of the rare CD34+ cells from normal bone marrow, for stem-cell transplantation and gene transfer studies.

 As described, the progenitor-cell-restricted antigen CD34 is readily cleaved by the *P. haemolytica* glycoprotease which results in the loss of reactivity of
20 this antigen with the anti-CD34 monoclonal antibody B1.3C5 (1-18). We have used this observation to isolate CD34+ cells which have been separated from heterogeneous populations of leukocytes, by immobilized antibodies specific for CD34. The transplantation of highly
25 purified CD34+ haematopoietic stem cells has widespread therapeutic potential, especially in cancer patients.

The P.h. glycoprotease has been used with magnetic immunoselection of CD34+ cells, to give high purities and
30 yields of haematopoietic stem cells from bone marrow cell preparation

Studies on KG1 cells.

 It is difficult to isolate the most primitive (i.e. CD34+) progenitor cells from normal bone marrow
35 populations for effective reconstitution of haematopoietic function in recipients. Current technologies fail to provide pure populations of CD34+

progenitor cells in the quantities required. Previous attempts have used one of three approaches: (a) fluorescence activated cell sorting (FACS); (b) "panning" methods using antibody-coated plastic surfaces; (c) other affinity chromatographic methods using biotinylated CD34 antibodies and avidin affinity columns. In the first approach, while relatively pure population of CD34+ cells can be produced for small scale *in vitro* experiments, the method is too slow to isolate the 1%-4% CD34-reactive cells, on the scale required for transplantation. In the second approach, rigorous and laborious procedures to remove naturally adherent cells (macrophages, granulocytes etc.) are required before bone marrow mononuclear cells can be "panned" by binding to anti-CD34-coated surfaces in large plastic vessels. CD34+ cells produced by variations of this technique may be contaminated with up to 30% CD34-negative cells and the overall yields of CD34+ cells are low. The "panning technique" is also relatively expensive in that it requires large quantities of highly purified antibodies. The third approach, which employs the use of avidin-biotin affinity column chromatograph, has been successful in generating relatively pure CD34+ progenitor cells in acceptable yields from normal bone marrow (1-29, 1-30).

According to this invention, a separation system has been developed using magnetic immunoselection techniques. Cells precoated with anti-CD34 antibodies are attached to magnetic microspheres conjugated with a secondary anti-mouse immunoglobulin. The CD34+ cells which bind to the microspheres are then removed with a magnet. In preliminary experiments, we immunomagnetically separated KG1 cells with high yield (90-95%) and high purity (94-98%) from sham mixtures containing 50% CD34-negative cells. This technique has several advantages in that it is rapid, it requires less antibody than "panning", and it produces CD34+ cells of high purity and high yield (1-

18). The approach is also flexible and can be used both for small-scale and large-scale isolation of cells. A major disadvantage has been that overnight incubation at 37°C has been required to remove the magnetic
5 microspheres from the positively-sorted cells, by the process of capping and antigen turnover.

According to this invention, overnight incubation is avoided by removing the magnetic microspheres by treatment with the *P. haemolytica* glycoprotease. We have
10 shown that a 30 min incubation with the enzyme is sufficient to remove all magnetic microspheres from KG1 cells, as assessed by microscopy. The free beads can then be separated from the treated cells by another round of magnetic selection. The enzyme treatment appeared to
15 have no detrimental effect on cell viability, either at 30 min or after overnight (16 h) incubation of the treated cells. The treated cells showed a normal quantitative expression and distribution of CD34 antigen as assessed with the glycoprotease-resistant epitopes
20 detected by Class III antibodies (1-18). In control experiments, the 16 h capping technique was shown to be less efficient at releasing the microspheres and about 90% of the control cells retained one or two beads. In contrast to the glycoprotease-
25 treated cells, the CD34 antigen on the control cells was only detectable in tight caps on pseudopod-like outgrowths, directly under the remaining magnetic beads. The final yield of cells prepared by the 16 h capping technique was also much lower because some of them were
30 still attached to magnetic beads and so were removed along with the free beads in the second magnetic selection.

Studies on bone marrow cells.

35 Similar experiments have been performed on normal haematopoietic progenitors isolated from bone marrow cells. It is more difficult to isolate CD34+ cells from

bone marrow mononuclear cell preparations than from sham mixtures of cell-lines, since normal progenitors express less CD34 antigen on their surfaces than do KG1 cells. CD34+ antibodies have been titrated against bone marrow

5 cells to optimize the separation of positive from negative cells. The sensitivity of the system has been amplified by the use of secondary rabbit antibody against mouse immunoglobulins, followed by magnetic microspheres coated with protein A (available from Dynal or Advanced

10 Magnetics). "Naturally" adherent cells which bind non-specifically to beads can present another problem in the fractionation of bone marrow cells. This problem can be avoided by gentle rocking of the cells during the bead-absorption phase of the isolation. Furthermore, cells

15 which adhere to the beads unspecifically are not detached by glycoprotease treatment.

Our cell-line experiments show that different anti-CD34 antibodies can affect the rate and efficiency of removal by the enzyme of the magnetic beads. In

20 particular, bead cleavage was more rapid and cell recovery was higher, when the QBEND 10 antibody was used, compared with the MY10 antibody. Since MY10 (group I) detects a sialic acid-dependent epitope, and the glycoprotease works best on fully sialylated

25 glycoproteins (glycophorin, CD44 and CD34), MY10 and the enzyme appear to compete for binding to a common sialate-rich region. In contrast, QBEND 10 (group II) binds to a sialic acid-independent epitope, and the glycoprotease cleaved all the beads from all cells in the experiment.

EXPERIMENTS AND RESULTS

Cleavage of the cell-surface O-sialoglycoprotein

CD34, CD43, CD44 and CD45

Materials and Methods

P. haemolytica glycoprotease preparation

P. haemolytica A1 (biotype A, serotype 1) was originally obtained from E. L. Biberstein, Univ, of California, Davis. The microorganism is available from ATCC under deposit No. _____. Stock organisms were stored as lyophilized cultures after freeze-drying in distilled water containing 5% (w/v) dextran, $M_r=70,000$: 7% (w/v) sucrose and 1% (w/v) monosodium glutamate. A 4.5 h brain-heart infusion broth culture of *P. haemolytica* was used to inoculate RPMI medium 1640 (Gibco Laboratories, Grand Island, N. Y.) containing 7% heat-inactivated FCS. After 3-4 h incubation at 37°C on a shaker, the culture was centrifuged (10,000 x g) and the supernatant filtered through a 0.2 mm Millipore filter. The filtrate was dialyzed against distilled water for 48 h at 4°C. The dialyzed culture supernatant was processed as described in detail elsewhere and the final preparation assayed for its ability to cleave human glycophorin A (2-10, 2-11). Although the gene encoding the glycoprotease has been cloned and expressed in *E. coli*, the recombinant form is less active than the partially purified culture supernatant form described above (2-14). Furthermore, when the partially purified culture supernatant-derived glycoprotease is purified to homogeneity by HPLC, the enzyme is less stable (2-13). Therefore, for the purposes of this study, batches of partially purified, supernatant-derived enzyme (2-10, 2-11) were titrated against 10^6 KG1a cells for 30 min at 37°C. After washing in ice-cold media supplemented with 0.02% sodium azide, cells were assessed for the loss of expression of the CD34 epitope detected by B1.3C5 (2-16) using fluorescence microscopy and flow cytometry. For subsequent studies,

twice as much enzyme was used as was required to cleave all the B1.3C5 epitopes.

Cells and antibodies

The human cell-line, KG1a, a primitive subline of the acute myeloblastic leukemia-derived cell-line KG1 (2-17, 2-18) was obtained from the American Tissue Culture Collection (Rockwell, Maryland) ATCC # CCL 246, CCL 246.1 and CRL 8031 and described in U.S. patent 4,678,751. The cells were grown in RPMI 1640 medium supplemented with L-glutamine (300 mg/ml), penicillin (100 U/ml), streptomycin (100 U/ml), and 10% heat-inactivated FCS. For some experiments, fresh peripheral blood mononuclear cells were prepared by density gradient sedimentation on Ficoll-Hypaque.

Antibodies to the following CD antigens (2-2) were used: to CD7; WT-1 (IgG₂) (2-6) and Leu-9 (IgG₂) (Becton Dickinson, Mountain View CA); to CD18/11^{a,b,c}; 60.3 (IgG₂) (2-19); to CD34; B1.3C5 (IgG₁) (2-16) QBEND 10 (IgG₁) (2-20) and TUK3 (IgG₃) (2-21); to CD43; rabbit anti-leukosialin (2-22); to CD44/hyaluronic acid receptor (2-23), 50 B4 (IgG₂) (2-24); to CD45; T29/33 (IgG₂) (2-25), Hybritech, La Jolla, CA); to CD71/transferrin receptor; OKT9 (IgG₁) (2-26). Antibodies to 'restricted' epitopes of the CD45 antigen, i.e. CD45 RA; 2H4 (IgG₁) (2-27), CD45 RB; MT3 (2-28), and CD45 RO UCHL1 (IgG₂) (2-29) were also used. The non-CD antibodies used were W6/32 (IgG₂) to HLA class 1 antigens (2-30), and 8A3, (IgG₂) which detects an activation antigen expressed on KG1a cells (2-31). All of the above antibodies are described in the noted publications, some of which are commercially available such as MY10 and 8G12.FITC from Becton Dickinson, QBEND-10 from GenTrak and AMAC, TUK3 from DAKO, ICH3 from Caltag, B1.3C5 from Sera Lab and 11.1.6 from Oncogene Science. The remaining antibodies are available from sources noted in the above publications and/or may be prepared in accordance with

the techniques noted in these publications. For purposes of preparing the antibodies, the subject matter of these publications is hereby incorporated by reference. For fluorescence-microscopy and flow-cytometry, fluorescein-conjugated, F(ab')₂ fragments of affinity-purified goat antibodies to pooled mouse immunoglobulins, cross-adsorbed with normal human immunoglobulins were obtained from Western Blotting Enterprises, Oakville, Ontario.

Radiolabeling, immunoprecipitation, and biochemical analysis

Cells were surface-labeled by the ¹²⁵I/lactoperoxidase technique (2-5, 2-6) (Na¹²⁵I from Amersham, Oakville, Ontario). After labelling, cells were washed in ice-cold PBS, divided into 2 x 50 µl aliquots. 10 µl of P. h. glycoprotease were added to one aliquot and both were incubated at 37°C for 20 min. The cells were pelleted and the supernatant from the enzyme-treated cells was carefully decanted. After a further wash in ice-cold PBS supplemented with 2mM EDTA, the cells were subjected to detergent lysis in 1% NP40. Processing of lysates and immunoprecipitations with specific antibodies and protein A-Sepharose was performed as described in detail elsewhere (2-5, 2-6). The supernatant from the enzyme-cleaved cells was brought to 1% NP40 by addition of a 1/10th vol of 10% NP40, and thereafter processed identically to the cell-lysates. For immunoprecipitations performed with monoclonal antibodies which did not bind to protein A, 5 µg affinity-purified rabbit antibodies to pooled mouse immunoglobulins, prepared as above were used to precoat protein A-Sepharose (Pharmacia, Piscataway, New Jersey) prior to addition to the immune complexes (2-6).

Immunofluorescence analysis.

For immunofluorescence analysis, 5x10⁵ cells suspended in 50 µl of PBS were incubated in the presence

or absence of P. h. glycoprotease as described above before addition of specific monoclonal antibodies. In some experiments, the KG1a cells were treated with 20 μ l *Vibrio cholerae* neuraminidase (BDH Chemicals, Poole, England) prior to glycoprotease treatment. The binding of monoclonal antibodies was detected with fluorescein-conjugated F(ab')₂ fragments of affinity purified goat anti-mouse immunoglobulin. Samples were analyzed by either epi-illumination fluorescence microscopy or flow cytometry (Epics Profile, Coulter Electronics Burlington, Ontario).

RESULTS

Flow Cytometric analysis of the cleavage of the cell-surface O-sialoglycoproteins CD34 and CD44 by P. h. glycoprotease.

Several of the CD34-specific antibodies, including B1.3C5 (2-16), depend upon the presence of sialic acid residues on the CD34 antigen, for their binding, whereas others, such as QBEND 10 (2-20) and TUK3 (2-21), do not (2-20, 2-32, 2-33). As shown in Fig. 1A, the binding of B1.3C5, was, as expected, greatly reduced in KG1a cells pre-treated with neuraminidase (Fig. 1B). Pre-treatment of the KG1a cells with P. h. glycoprotease totally abrogated the binding of B1.3C5 (Fig. 1C). These experiments were repeated with the sialic acid-independent anti-CD34 antibody QBEND 10 (2-20, 2-32, 2-33) and, although neuraminidase treatment predictably had little effect (Fig. 1E versus 1D), binding of QBEND 10 was markedly decreased after P. h. glycoprotease cleavage (Fig. 1F). To see if cleavage of the CD34 molecule was affected by prior removal of sialic acid residues, KG1a cells were treated with neuraminidase, washed and subjected to cleavage by P. h. glycoprotease. The result of a representative experiment is shown in Fig. 1G, and although the staining of QBEND 10 was greatly reduced, the efficiency of enzyme cleavage

of the desialylated CD34 molecule was reduced, compared to its cleavage of the fully sialylated form.

Neuraminidase had little or no effect (Fig. 1J) on the binding of the CD44 antibody, 50 B4 to KG1a cells (Fig. 1H). However, the P. h. glycoprotease removed most of the CD44 epitopes detected by 50 B4 (Fig. 1K).

As assessed by fluorescence microscopy and flow cytometry, the binding of antibodies to transferrin receptors/CD71 (Fig. 1L) was not detectably altered by prior treatment of the cells with neuraminidase (Fig. 1M) or the P. h. glycoprotease (Fig. 1N) under conditions where CD34 and CD44 were cleaved. Similarly, neither neuraminidase nor the glycoprotease affected the binding of antibodies to CD7, CD18/11^{a,b,c}, CD45, HLA class I and 8A3 antigens. Interestingly, the binding of the anti-CD34 antibody TUK3 (2-22), which detects a sialic acid-independent epitope (2-32, 2-33) was similarly unaffected by the action of the P. h. glycoprotease. Together, these data indicate that the O-sialoglycosylated structures, CD34 and CD44, were both cleaved by the P. h. glycoprotease. Nevertheless, the lack of evidence for cleavage of the other antigens does not prove that these structures are not substrates for the enzyme. These apparently negative results may reflect the fact that the individual epitopes detected by the various antibodies are associated with the remaining (cell-bound) fragments of their respective antigens. To address this issue, SDS-PAGE analysis of immune complexes made from the glycoprotease-treated cells was performed alongside those made from control (untreated) cells.

SDS-PAGE analysis of the cleavage of the O-sialoglycoproteins CD34, CD43, CD44, and CD45 by P. h. glycoprotease.

Immunoprecipitates made with specific antibodies and cell lysates from untreated control cells, P. h. glycoprotease-treated cells, and enzyme-cleaved,

cell-free, soluble fragments showed that the CD34, CD43, CD44 and CD45 antigens were all cleaved by the P. h. glycoprotease (Fig. 2). As shown in track D, immunoprecipitations performed with the CD34 antibody B1.3C5 produced the expected band at $M_r=110,000$ (track D). No evidence for this band was detected in lysates of treated cells, indicating that virtually all of the CD34 antigen had been cleaved under the conditions employed (track E). Discrete bands were not detected in immunoprecipitates made with B1.3C5 and the cell-free products (track F), indicating either that very small fragments (8 kD or less) were generated by the action of the glycoprotease, or that the epitope detected by this antibody was destroyed by the enzyme.

Immunoprecipitations performed with the anti-CD44 antibody, 50 B4, generated a heavily labeled band at $M_r=85,000$, (track K) as described previously for this reagent (2-24). Other than a small residual amount of uncleaved 85 kD material associated with the lysate from glycoprotease-treated cells, no additional bands were detected (track L). In contrast, two heavily labeled bands were resolved at $M_r=50,000$ and $40,000$ in immunoprecipitations performed on the cell-free products (track M). These data clearly demonstrate that the epitope detected by the 50 B4 antibody is associated with the glycoprotease-generated soluble fragments and that there may be two susceptible sites of cleavage by P. h. glycoprotease in the CD44 molecule.

Immunoprecipitations performed with polyclonal anti-CD43 antibodies on the untreated cell lysate generated the expected bands at $M_r=100-110,000$ (track G). Discrete bands of comparable intensity of radiolabel were not detectable in either the glycoprotease-treated cell-lysate (track H) or the cell-free products (track J). Weakly labeled bands were however detectable in track H at about $M_r=90,000$, and in track J at $M_r=43,000$ and $45,000$. Although the identity of these bands remains

unclear, similar bands were also detected in the corresponding immunoprecipitations with other antibodies, e. g. CD45, track P. These results indicate that almost all of the CD43 molecules were cleaved by the glycoprotease and that the fragments generated were either too small to be resolved or that the epitopes recognised by the antibodies were all destroyed by the action of the enzyme. Even though the polyclonal antibody used here (2-22) would be expected to contain antibodies to intracellular epitopes of the CD43 structure, these remaining cell-bound fragments would not be detectable if all the radioactive label from the surface iodination was associated with glycoprotease-sensitive NH_2 -terminal domains of the CD43 molecule.

Immunoprecipitates made with the anti-CD45 'framework' antibody T29/33 and the untreated cell-lysate, contained a series of 3 or 4 bands in the $M_r=180,000$ to $220,000$ range (track N), in keeping with known characteristics for this family of CD45 isoforms (2-25, 2-34). In contrast, the treated cells yielded a major band at $M_r=175,000$ together with a minor one at about $M_r=180,000$ (track O). No material was immunoprecipitated from the enzyme-generated cell-free products, confirming the flow cytometry analysis which indicated that the epitope detected by the T29/33 antibody was retained on the major cell-bound fragment (track P). These results strongly suggest that the P. h. glycoprotease cleaves the CD45 structures only at their most distal, NH_2 -terminal domains. This interpretation is consistent with the known structural characteristics of the isoforms of the CD45 family, the individual members of which contain variable numbers of O-glycosylated, serine/ threonine-rich sequences in their NH_2 -terminal domains (reviewed in 34).

The CD7 molecule (track A), which we have shown to contain only a small amount of O-linked carbohydrate

(2-6), exhibited little or no diminution in apparent molecular weight after cleavage by P. h. glycoprotease (track B). No product of cleavage was detectable in immunoprecipitates made from the cell-free products (track C), consistent with the flow cytometric evidence, cited above, that the two CD7 antibodies used in this study, WT-1 and Leu-9, detect the cell-bound fragments.

P. h. glycoprotease does not cleave glycoproteins which lack O-linked glycans.

A series of immunoprecipitations were performed on enzyme-treated and control cell-lysates, with antibodies to cell-surface glycoproteins which are known to lack O-sialoglycan moieties. Neither CD18/11^{a,b,c}, CD71, HLA class 1 nor 8A3 antigens were detectably cleaved by the glycoprotease (Fig. 3, tracks A-M).

P. h. glycoprotease cleaves CD45 isoforms.

The observation that all CD45 isoforms expressed on KG1a cells were apparently cleaved by the glycoprotease suggested that epitopes detected by individual CD45 R (isoform-'restricted') antibodies may be susceptible to its action. Since KG1a cells expressed only very low levels of the CD45 RO epitope detectable with UCHL1, fresh PBMCs were utilised for this experiment. As shown in figure 4 A (-), and Table 1, the epitope detected by control antibody T29/33 (CD45 framework) was not removed from the cell-surface by the P. h. glycoprotease (+). In contrast, the fluorescence intensity of untreated PBMCs stained with the anti-CD45 RA antibody (Fig 4B) was very heterogeneous, containing a population of unstained cells as well brightly stained and relatively weakly stained cells. P. h. glycoprotease-treated PBMCs showed a much less heterogeneous pattern of staining with the CD45 RA antibody. Although 8% of the cells were very weakly stained (mean fluorescence intensity of 1.5), strongly stained cells were no longer detectable by flow cytometry

(see Table 1) or by fluorescence microscopy. When similar experiments were performed with the CD45 RB antibody MT3 (Fig 4C), virtually all the leukocytes were stained in both the non-treated (-) as well as the glycoprotease-treated (+) cells. However, the mean fluorescence intensity of the glycoprotease-treated cells (+) was much lower than for the untreated sample (Table 1). Similar to the staining with CD45 RA, the CD45 RO antibody UCHL1 (Fig 4D) showed a very similar distribution of negative, variably weakly stained, and brightly stained cells. After glycoprotease treatment (+), only relatively small numbers of stained cells were detectable (Table 1). It is not clear why there is incomplete cleavage of the CD45 R epitopes (a reproducible phenomenon over a range of glycoprotease concentrations), in contrast to the complete cleavage of CD34 and CD43. It is likely that, in addition to the differences in primary sequences of the CD45 R isoforms, there is also heterogeneity in the glycosylation of the O-linked glycans for each isoform. It may be significant that the CD45 RA isoform, which contains the highest number of potentially O-glycosylated serine/threonine rich domains, is the form which is most sensitive to the action of the glycoprotease. In contrast, enzyme treatment had no effect on the staining of PBMCs by the antibody W6/32, which detects the glycoprotease-resistant HLA class I antigen (Fig 4E).

Differential Sensitivity of CD34 epitopes to cleavage by *P. haemolytica*

MATERIALS AND METHODS

Cells and antibodies

The acute myeloid leukemia-derived cell-lines KG1 and KG1a (3-25, 3-26) and the T-cell acute lymphoblastic leukemia-derived cell-line HSB-2 were obtained from the American Tissue Culture Collection (Rockwell, Maryland), and maintained in RPMI 1640 with 10% FCS. Peripheral blood mononuclear cells (MNC) from a patient in blastic

transformation of chronic myelogenous leukemia, were prepared from venous blood by Ficoll-Hypaque density gradient centrifugation. Anti-CD34 antibodies MY10 (3-11) B1.3C5 (3-12), 12.8, 115.2 (3-13), ICH3 (3-15), QBEND 10 (3-23) and TUK3 (3-24) were obtained as previously described.

IMMUNOMAGNETIC CELL SEPARATION

A. Cell-lines

Magnetic microspheres (Dynal) conjugated with sheep anti-mouse IgG₁ antibodies were obtained from P&S Biochemicals (Gaithersburg, MD 20877). Thirty μ l of bead suspension (equivalent to about 5 mg of sheep anti-mouse IgG₁) were further coated for 30 min at room temperature with 5 mg anti-CD34 antibodies MY10 or QBEND 10. The beads were washed twice in a magnetic particle concentrator (MPC, Dynal) with RPMI supplemented with 10% FCS, to remove unbound antibodies. A standard test mixture of 5×10^6 KG1 cells (CD34+) with 5×10^6 HSB2 cells (CD34-) was prepared in 100ml RPMI/FCS and mixed with the magnetic bead suspension in a total volume of 200 μ l. The approximate bead:KG1 cell ratio in the mixture was 7.5:1. After 30 min at room temperature with occasional gentle agitation, the cell/bead suspension was brought to 5 ml by addition of RPMI/FCS and placed in the MPC for 2-3 min. Free cells remaining in the suspension were carefully decanted and placed in a separate tube. The tube containing the bead-coated cells was removed from the MPC and the beads were gently resuspended in another 5 ml of media. The cell/bead suspension was placed in the MPC again for 2-3 min. The second aliquot of bead-free supernatant was pooled with the first, and the free cells were counted. The test-tube containing the bead-coated cells was removed from the MPC and the cell/bead mixture washed to the bottom of the tube by addition of 200 ml media. *P. haemolytica* glycoprotease was added to the suspension and the capped tube was placed in a water bath

at 37°C. After 30 min, the magnetic beads/cell suspension was brought to 5 ml with media and the tube placed into the MPC again. The cells remaining in the supernatant were carefully decanted and placed in a separated tube. As before, the magnetic beads were resuspended in 5 ml of media and subjected to another round of separation. The detached cells were pooled and carefully enumerated. Aliquots of the cells from the first and second sort were stained for immunofluorescence analysis with anti-CD34 antibodies ICH3, or 115.2, or the anti-CD7 antibody WT1 (3-27).

B. Fresh leukemic samples

Peripheral blood samples from consenting adult leukemia patients, obtained under Institutional Review Board-approved protocols, were collected into heparin and a mononuclear fraction obtained by separation on Ficoll-Hypaque. The numbers of CD34-positive cells in the unfractionated mononuclear cell suspension was assessed by morphology as well as by fluorescence microscopy and flow cytometry using QBEND 10 or TUK3. For magnetic separation, 5×10^6 cells were suspended in 250 μ l RPMI 1640 supplemented with 10% FCS. The cells were coated with QBEND 10 for 30 min at 4°C and washed twice with the same media. The washed cells were added to a 30 ml suspension of washed magnetic beads and incubated at 4°C for 30 min with occasional gentle agitation. Separation, recovery and enumeration of the purified CD34-positive cells was performed as described above.

Susceptibility of CD34 epitopes to VCN and *P. haemolytica* glycoprotease.

As shown in Figure 5, the epitopes detected by the monoclonal CD34 antibodies B1.3C5 and 12.8 (lower histograms respectively) were removed by prior treatment of the KG1 cells with VCN (middle histograms). The epitopes detected by MY10 and ICH3 were also susceptible

to VCN treatment but to a lesser extent. In contrast, the epitopes detected by the QBEND 10, TUK3 and 115.2 were not cleaved by VCN. The neuraminidase-sensitive epitopes detected by MY10, B1.3C5, 12.8 and ICH3 were all cleaved by the *P. haemolytica* glycoprotease (upper histograms). Similarly, the VCN-resistant epitope identified by QBEND 10 was also sensitive to the action of the glycoprotease. However, the VCN-resistant epitopes identified by TUK3 and 115.2 were not cleaved by this glycoprotease. These data, which are summarized in Table 2, indicate that the epitopes detected by the CD34 antibodies can be separated into three classes, based upon their sensitivity to neuraminidase and *P. haemolytica* glycoprotease.

Immunomagnetic sorting of CD34-positive cell-lines

CD34-positive KG1 cells were mixed (1:1) with the undifferentiated T-lymphoblastoid cell-line HSB2 and incubated with anti CD34 antibody-coated magnetic microspheres as described above. After removing the bead-coated cells on the MPC, the remaining cells were decanted and stained with the anti-CD34 antibody ICH3 (class I, see Table 2). As shown in Figure 3 (histogram A) and Table 3, the suspension of free cells ('negative sorted') contained only 2.5% CD34-positive cells. A control population of KG1 cells stained with the same antibody is shown for comparison in histogram B. After removal of the magnetic beads with the *P. haemolytica* glycoprotease, the bead-attached cells ('positive sort') were also stained with the class I antibody, ICH3 (histogram C). As can be seen from the quantitative analysis shown for these cells in Table 3, the mean fluorescence of the glycoprotease-treated cells was considerably lower than the control KG1 cells when stained with the same antibody (histogram B). The partially biphasic nature of histogram C is probably due to incomplete cleavage of the ICH3 epitopes on a few cells.

When cells from the 'positive sort' were stained with the class III antibody 115.2, 98.6% of the cells were stained with a mean fluorescence intensity of 41.8 (histogram E). For comparison, the staining of pure KG1 cells with the 115.2 antibody is shown in histogram D. The mean fluorescence of this population (45.7) is almost identical to the cells stained with the same antibody from the 'positive sort' (Table 3). The few unstained cells in the 'positive sort' (histogram E) were determined by fluorescence microscopy to be residual HSB2 cells, due to their very characteristic size and shape. To confirm this, an aliquot of cells from the 'positive sort' were stained with the anti-CD7 antibody, WT1, which binds to HSB2 cells, and less than 2% of the cells were stained (data not shown). The recovery of CD34+ cells after enzyme treatment and removal of detached beads was in excess of 90% in two separate experiments.

Immunomagnetic sorting of CD34-positive leukemic blasts

A mononuclear cell (MNC) suspension was prepared from the peripheral blood of a patient in blast crisis of chronic myelogenous leukemia. Approximately 25% of the cells exhibited the morphologic characteristics and the composite immuno-phenotype of megakaryoblasts (B/T-cell lineage-negative, CD34 and GPIIb/IIIa (CD41)-positive). After staining with the class II anti-CD34 antibody QBEND 10, the peripheral MNC fraction contained two major populations on flow cytometric analysis (Fig 7, top left). 40% of the cells had the light-scattering properties characteristic of CD34-positive bone marrow progenitor cells (3-28) and were gated into the 'blast/lymphocyte' bitmap A. Virtually all the other cells were separated into the 'granulocyte' bitmap B, in keeping with the clinical status of this patient (see differential blood counts, Table 4). On fluorescence analysis, about 62% of the cells in bitmap A (representing 24% of the total MNC) were stained (ie

fluorescence channel 0.5 or greater), with a mean fluorescence of 3.1 (top right); the majority of the unstained cells were lymphocyte (data not shown). Less than 2% of the granulocytes (represented in bitmap B) were weakly stained. After magnetic sorting and release of the sorted cells with the *P. haemolytica* glycoprotease, the released cells were counted. Approximately 94% of the CD34+ cells were recovered. An aliquot of recovered cells was stained with a directly fluorescein-conjugated, CD34 antibody 8G12 (3-29), which recognizes a class III epitope. As shown in Fig 7 (middle left), virtually all of the released cells were gated into the 'blast/lymphocyte' bitmap A. 93% of these cells were stained (mean fluorescence 3.2) by the 8G12 antibody (middle right). The cells remaining after the removal of the CD34-positive fraction with the magnet, were also stained with the CD34 antibody 8G12. Only 22% of the cells in this CD34-depleted population were gated into the 'blast/lymphocyte' bitmap A (lower left). 40% of these cells (representing 7% of the CD34-depleted population) were stained by the class III CD34 antibody (lower right). However, the staining of these positives was extremely weak (mean fluorescence 1.2), and was not effectively detectable by fluorescence microscopy.

The unsorted MNCs, the CD34-positive/ glycoprotease released, and the CD34-depleted fractions were examined after Giemsa staining and the full differential counts are shown in Table 4. The blast cells remaining in the 'CD34-negative sort' probably correspond to the 7% of the cells which express very low levels of the CD34 antigen (Fig 7, lower right). These cells are probably blast cells at a more advanced stage of differentiation, in which the CD34 antigen is no longer highly expressed (3-22).

Identification of a major glycoprotease-generated CD34 fragment.

Immune complexes were made with lysates of ^{125}I /lactoperoxidase-labeled KG1 cells using either class I (B1.3C5) or class III (TUK3) anti-CD34 antibodies. Both TUK3 (Fig 8 track D) and B1.3C5 (track G) identified the same 110 kD band in lysates of control untreated KG1 cells. The class III anti-CD34 antibody TUK3 identified a major cleavage product of the CD34 antigen of about 75 kD in lysates of the glycoprotease-treated cells (track E). In contrast, B1.3C5 did not immunoprecipitate this fragment from the glycoprotease-treated cell-lysates (track H), which confirms that the epitope detected by this antibody is removed by the enzyme. The supernatant from the enzyme-treated, radiolabeled cells was also used for immunoprecipitations with TUK3 (track F) and B1.3C5 (track K). In neither case were we able to identify any soluble products of the cleaved CD34 molecule even in SDS-polyacrylamide gels capable of resolving down to about 10 kD. In an attempt to do so, immune complexes were made from uncleaved lysates with TUK3, which represents the most efficient antibody in immunoprecipitation assays (DRS unpublished observations). The washed immune complexes were divided into two aliquots and one of them cleaved by the glycoprotease. Both the cleaved immune complexes and the soluble products of this cleavage were analysed by SDS-PAGE. Control untreated immune complexes made with TUK3 contained the expected band at 110 kD (fig 8 track A) while the cleaved immune complexes (track B) contained a similar band of about 75 kD to that identified in lysates of cleaved cells (track D). Other fragments of the CD34 molecule were not detectable in the supernatant (track C). When this experiment was repeated using either class I (B1.3C5) or class II (QBEND 10) antibodies, the 75 kD bands were resolved in the supernatants from the

cleaved immune complexes but the fragments remaining with the immune complexes were not detectable.

**RETENTION OF PROGENITOR CELL FUNCTION IN CD34+ CELLS
PURIFIED USING A NOVEL O-SIALOGLYCOPROTEASE
MATERIALS AND METHODS**

Cells

Bone marrow cells were obtained under Institutional Review Board-approved protocols from normal healthy donors and bone marrow transplant donors at the time of marrow harvest. In experiments 1 and 2 (see Results) marrow cells were collected into 10% fetal bovine serum (FBS) in Iscove's Modified Dulbecco's Medium (IMDM, Gibco) but for experiments 3-5, RPM1 1640 (Gibco) was used throughout instead of IMDM. DNase I (Sigma) was added to all medium at a final concentration of 70 U/ml to reduce cell aggregation. The light density mononuclear cell (MNC) fraction was isolated on a Percoll gradient (density 1.077 g/cm³, Pharmacia).

Antibodies

Either of the two IgG₁ CD34 monoclonal antibodies, MY10 (4-3) or QBEND 10 (4-15, 4-16) were used for isolation of CD34+ cells. The antibodies were titrated against unfractionated normal marrow MNCs and used at half saturating concentrations as assessed by flow cytometry. The respective epitopes recognized by MY10 and QBEND 10, are both cleaved by P.h. glycoprotease. After release of magnetic beads from CD34+ cells by P.h. glycoprotease treatment, the purity of the cells was assessed using TUK3 (IgG₃) (4-23), or 115.2 (IgG₁) (4-5), anti-CD34 antibodies whose epitopes are not removed by P. h. glycoprotease treatment (4-22, 4-24).

Immunomagnetic selection of CD34+ cells

CD34+ cells were positively selected from the rest of the MNC fraction using magnetic beads coated with

sheep anti-mouse IgG₁, (Dynabeads M-450, Dynal, Great Neck, NY). The beads were washed three times in 5% FBS in phosphate buffered saline (PBS) prior to use. Marrow MNC were resuspended in 5% FBS in PBS at $1.0-1.5 \times 10^7$ cells/ml incubated with either MY10 or QBEND 10 for 30 min at 4°C and washed twice. One magnetic bead was used per 2 MNC, and the mixture of beads and cells was gently rotated for 30 min at 4°C in a 15 ml round-bottomed polycarbonate test tube. After the addition of 5 ml of PBS/FBS to the beads/cells, the tube was placed in a magnetic particle concentrator (Dynal) for 1.5 min to separate the bead-coated cells from the rest of the MNCs. The cells remaining in suspension were carefully removed and the remaining (bead-coated) cells were gently washed three more times, by resuspension in 5 ml FBS/RPMI and separation in the magnetic particle concentrator. The bead-coated cells were counted. Aggregates of beads and cells, which consisted of large granular cells and/or non-viable cells, were not scored as CD34+ cells. The magnetic beads were cleaved from the CD34+ cells using P.h. glycoprotease. Bead-coated cells were incubated with P. h. glycoprotease at a concentration of 2×10^6 /ml in 10% FBS/RPMI at 37°C. After 30 min, the mixture of beads and detached cells was brought to 5 ml with FBS/RPMI and the tube placed in the MPC magnetic particle concentration to separate the free beads from the detached CD34+ cells.

Morphology

Preparations of the unseparated MNCs, the CD34-depleted fraction, and the CD34+ cells obtained after enzyme cleavage, were cytocentrifuged onto glass slides and stained with May-Grunwald-Giemsa. Slides were examined by light microscopy.

Colony forming assays

Modification of an established colony-forming assay (4-26) was used to determine the frequency of mixed

lineage progenitors (CFU-GEMM) as well as early erythroid (BFU-E), granulocytic (CFU-G) and monocyte/macrophage (CFU-M) progenitor cells. MNC and CD34-depleted fractions were plated at 10^5 /ml and CD34+ cells at 2.5×10^3 /ml in 1.3 % methyl-cellulose (Terry Fox Laboratories, Vancouver), 10% phytohemagglutinin-stimulated leucocyte conditioned medium (PHA-LCM), 30% normal human plasma and 2 units recombinant human erythropoietin (Ortho Pharmaceuticals, Ontario). Cultures were incubated at 37°C in 5% CO₂ in air and scored in duplicate on day 14. CFU-GEMM were identified by granulocytic and erythroid cells, with or without the presence of megakaryocytic cells, in single colonies. BFU-E consisting of three or more clusters were identified by their orange/red coloration and CFU-G and CFU-M resolved by their characteristic colony morphology.

Long term bone marrow cultures (LTBMC)

The ability of CD34+ cells (released by P. h. glycoprotease from magnetic beads) to proliferate in the presence of irradiated normal allogeneic LTBMC adherent layers was examined in a known manner as previously described (4-27). Normal LTBMC were established with nucleated marrow cells which had been depleted of red cells by sedimentation in 0.1% methyl-cellulose. Marrow cells (2×10^7) were inoculated into 10 ml of medium comprising 10% FBS, 10% horse serum, 5×10^{-7} M hydrocortisone (Sigma) in IMDM (350 mOsm/kg). The cultures were maintained at 33°C and half the medium changed weekly (4-28, 4-29). Between weeks 3 and 6, the confluent stromas were irradiated with 15 Gy and used as feeder layers for CD34+ cells, inoculating a dose of 5×10^4 cells for each experiment. Thereafter the nonadherent layer CFU-GM were assayed weekly for at least 7 weeks. As a control, an adherent cell-depleted (ACD) population of cells was obtained from the same marrow MNC fraction, as described previously (4-27), and 5×10^6 ACD cells

inoculated onto an irradiated normal LTBM layer similar to the one used as a feeder layer for the CD34+ cell fraction. The cultures that had been inoculated with ACD cells were maintained under identical conditions to those with CD34+ cells.

RESULTS

Expression of CD34 antigen on marrow MNCs

The number of CD34+ cells in the unfractionated bone marrow MNCs was assessed by flow cytometry and fluorescence microscopy. Additionally, the total blast count in the unfractionated marrow MNCs was assessed by light microscopy of Giemsa-stained cytocentrifuge preparations. For the flow cytometry analysis, CD34+ cells which exhibited the light-scattering properties characteristic of bone marrow progenitor cells (4-30) were gated into the 'blast/lymphocyte' bitmap A (Figure 9, top left). In a representative experiment (experiment 5), 3.4% of the cells in bitmap A, were stained with the class II anti-CD34 antibody, QBEND 10 (Fig. 1, top right). Since about 35% of the total MNCs scattered in this bitmap, the overall % of CD34+ cells in the unfractionated MNCs in this sample was approximately 1.1% (see Table 5). This figure was in good agreement with estimates of CD34+ cells by fluorescence microscopy and by total blast count of cytopsin preparations (see Table 6). For all experiments reported herein, the mean numbers of CD34+ cells detected by flow cytometry was $1.5\% \pm 0.48$ (range 0.43-3.1) (Table 5).

Release of CD34 cells from magnetic beads by P.h. glycoprotease

After separation of the CD34+/bead-coated cells from the other mononuclear cells with the magnetic particle concentrator, the bead-coated fraction was incubated with the P. h. glycoprotease. After 30 minutes at 37°C, free magnetic beads, together with any cells from which beads

had not been detached, were separated from the released cells. The released cells were carefully removed with a pasteur pipette and counted. In the first series of experiments, typified by those numbered 1 and 2 in Table 5, the recovery of CD34+ cells after P.h. glycoprotease treatment of cells bound to immuno-magnetic beads was only around 20%. Microscopic examination showed that many of the cells in these experiments remained bound to the beads after enzyme treatment. This observation contrasted with previous experiments using mixtures of cell-lines or fresh leukemia samples, in which the post-enzyme recovery of CD34+ cells ranged from 80-95% (DRS et al. Exp. Hematol., in press). Since all previous CD34+ cell-sorting using the *Pasteurella* glycoprotease had been performed in RPMI, this media was substituted for IMDM in experiments numbered 3-5 (Table 5). This modification resulted in a significant increase in recovery of CD34+ cells (range 60-85%). The bead-coated cells remaining after P.h. glycoprotease treatment in the latter series of experiments consisted of very large clusters of beads and cells, single large phagocytic cells, or dead cells.

The viability of CD34+ cells released by P.h. glycoprotease was at least 95% in all experiments.

Purity of CD34+ cells released by P.h. glycoprotease

The purity of the CD34+ cells released from immunomagnetic beads by P.h. glycoprotease was assessed by fluorescence microscopy and flow cytometry. Released cells were stained with either TUK3 or 115.2, class III CD34 antibodies which detect epitopes which are not removed by the glycoprotease. Flow cytometry of the stained cells (from experiment 5) demonstrated that virtually all of the CD34+ cells released by the enzyme had low to medium forward and low right angle light scatter properties. These cells were located in the "blast/lymphocyte" bitmap A (Figure 9, lower left), typical for cells expressing the CD34 antigen. Between 63

and 95% (mean 81%) of the enzyme-released cells were CD34⁺ as determined by flow cytometry in experiments 1, 4, and 5 (Table 5). When the starting marrow sample was too small to yield sufficient enzyme-released cells for analysis by flow cytometry, purity was assessed by fluorescence microscopy. By this technique, the purity of CD34⁺ cells in the enzyme-released fraction was assessed to be in excess of 90% in both experiments 2 and 3.

Morphology of CD34⁺ cells

The proportion of blasts in Giemsa-stained cytospin preparations of the released cells ranged from 68-95% (mean 87%). For each individual experiment, the proportion of blast cells counted was very similar to the percentage of CD34⁺ cells determined using immunofluorescence techniques (Table 5). The cells from the the CD34⁺/enzyme-released fraction from experiment 5 are shown in Figures 10A and 10B. In contrast, unfractionated marrow MNCs from the same experiment exhibits a range of cell-types typical of those from normal bone marrow (Figure 10C). The morphologic characteristics of the unfractionated marrow MNCs, the the CD34-depleted fractions and the CD34⁺/enzyme-released fractions were determined for each individual experiment. As shown in Table 6, the composition of the unfractionated MNCs was quite variable with respect to the percentages of the different cell-types in each experiment. In contrast, the percentage of blasts in the CD34⁺/enzyme-released fraction was consistently high, and was 93% or better in experiments 2, 3, and 5.

Colony-forming assays

Colony-forming assays were performed on unfractionated MNCs, the CD34-depleted fraction, the CD34⁺/enzyme-released fraction and the 'residual' CD34⁺/enzyme-treated cells which remained bound to the beads. As shown in Table 7, in comparison with the

unfractionated marrow, the CD34+ fraction in each experiment was highly enriched for hematopoietic progenitor cells when grown in clonogenic culture. Mean enrichments of 81 fold (range 15-220) for CFU-GEMM, 45 fold (range 21-78) for CFU-G, 13 fold (range 5-31) for CFU-M and 26 fold (range 4-66) for BFU-E were obtained for the CD34+ cells compared with the MNC fraction from experiments 1 to 5. For the later experiments (3-5) in which CD34+ cells were more efficiently released from the beads in the presence of RPMI rather than IMDM (see above), the degree of enrichment of the multi-lineage progenitors (CFU-GEMM) in this fraction was particularly striking.

Less than 1% of the total CFU-G, 4% of the CFU-M, and 0% of BFU-E were grown from the 'residual' cell fraction which remained bound to the beads after glycoprotease treatment performed in RPMI (experiments 3-5). In contrast, the equivalent fraction generated in the presence of IMDM in earlier experiments (1 and 2) contained a mean of 12% CFU-G and 4% BFU-E. This fraction additionally contained a mean of 20% of the total CFU-M colonies

Multi-lineage colonies did not grow from either the CD34-depleted fraction or the 'residual' fraction containing the cells which failed to be detached by the P. h. glycoprotease. These data indicate that the depletion of CD34+ cells in all experiments was highly efficient. Furthermore, the recovery of CFU-GEMM in the CD34+ fraction using the *Pasteurella* glycoprotease was also very efficient, particularly when the experiments were performed in RPMI.

P. h. glycoprotease-treated CD34+ cells in LTBM

The generation of hematopoietic progenitors, from the enzyme-released CD34+ cells that were inoculated onto normal irradiated LTBM stromal feeder layers, was assessed in each experiment. The CFU-GM generated weekly

were comparable in numbers and duration to those generated from ACD marrow cells isolated from the same bone marrow, with evidence of sustained hematopoiesis for at least 7 weeks (see Table 8).

The following is a listing of the aforementioned Tables.

TABLE 1. Effects of *P. haemolytica* glycoprotease on epitopes detected by CD45 antibodies; flow cytometric data of histograms shown in Figure 1.

ANTIBODY	% POSITIVE CELLS (LOG MEAN FLUORESCENCE)			
	UNTREATED		GLYCOPROTEASE TREATED	
	Window 1 ¹	Window 2 ²	Window 1	Window 2
CD45 ³	99% (29)	97% (30)	99% (35)	98% (36)
CD45 RA	58% (8)	30% (24)	8% (1.5)	<1% (0)
CD45 RB	98% (47)	97% (48)	94% (10.5)	68% (15)
CD45 RO	48% (10)	30% (24)	33% (3)	5% (12)
W6/32	97.5% (50)	97% (51)	97.7% (58)	97.5% (61)

Footnotes to Table 1.

1; Window 1 was set to measure the mean fluorescence of cells having a fluorescence intensity of 1.5 (arbitrary fluorescence units) or more.

2; Window 2 was set to measure the mean fluorescence of cells having a fluorescence intensity of 7.5 or more. The relative positions of windows 1 and 2 are shown in Figure 4.

3; % positive cells for CD45 'framework' antibody excludes unstained contaminating red blood cells. See histograms 'A' in Fig 4.

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TABLE 2 Effects of neuraminidase or *P. haemolytica* glycoprotease on epitopes detected by CD34 antibodies; flow cytometric data of histograms shown in Figure 5.

ANTIBODY	MEAN FLUORESCENCE (LOG SCALE)		
	UNTREATED	NEURAMINIDASE	GLYCOPROTEASE
CLASS I			
MY10	29.4	21.3	1.8
B1.3C5	13.3	2.2	1.6
12.8	25.4	1.5	2.2
ICH3	39.6	16.6	1.7
CLASS II			
QBEND 10	34.5	36.1	2.6
CLASS III			
TUK3	96.8	93.3	104.9
115.2	39.1	47.7	43.5

TABLE 3 Purification of CD34-positive KG1 cells from sham mixtures using immunoaffinity magnetic separation and P. h. glycoprotease; flow cytometric data from histograms A-E in Figure 6

CELLS	% CD34-POSITIVE CELLS* (LOG MEAN FLUORESCENCE)	
	ICH3 (CLASS I)	115.2 (CLASS III)
NEGATIVE SORT (CD34-DEPLETED)	2.5% (16.5) HISTOGRAM A	Not Done
CONTROL KG1	92.5% (24.6) HISTOGRAM B	99.8% (45.7) HISTOGRAM D
POSITIVE SORT: CD34+VE CELLS AFTER GLYCOPROTEASE TREATMENT	24.1% (7.9) HISTOGRAM C	98.6% (41.8) HISTOGRAM E

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TABLE 4. Differential blood counts on unsorted mononuclear cells, CD34-positive/glycoprotease-treated cells and CD34-depleted cells.

FRACTION	% CELLS IN FRACTION				
	BLASTS	LYMPHS	GRANS	MONOS	ERYTHBLAST
UNSORTED	40	5	46	3	6
CD34+VE SORT	92	1	5	1	1
CD34-VE SORT	8	6	83	2	1

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Table 5. Recovery and purity of CD34+ marrow mononuclear cells.

Expt.# (medium)	% CD34+		% BLASTS	% RECOVERY
	Pre-sort MNC	Post-enzyme	% blasts post-enzyme	post-enzyme/ pre-sort
1. (IMDM)	3.1	85	86	21
2. (IMDM)	2.0	90*	94	19
3. (RPMI)	0.4	90*	93	60
4. (RPMI)	1.0	63	68	85
5. (RPMI)	1.1	95	95	74

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Table 6 Morphological Characteristics of Purified Cells.

% CELLS IN EACH FRACTION

EXPT #	FRACTION	Blasts	Lymphs	Grans	Monos	E'blasts
1. (IMDM)	MNC	2	19	25	13	41
	CD34-	4	18	29	6	43
	CD34+	86	5	5	0	4
2. (IMDM)	MNC	1	35	41	8	15
	CD34-	2	32	44	8	15
	CD34+	94	3	3	0	0
3. (RPMI)	MNC	1	40	35	16	8
	CD34-	4	41	41	7	7
	CD34+	93	4	0	2	0
4. (RPMI)	MNC	1	9	54	30	6
	CD34-	2	6	57	29	6
	CD34+	68	6	10	8	8
5. (RPMI)	MNC	2	8	60	23	7
	CD34-	4	7	65	18	6
	CD34+	95	1	3	1	0

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Table 7 Colony-Forming Assays of Marrow Mononuclear Cell Fractions.

COLONIES (per 10 ⁵ cells)					
EXPT #	FRACTION	CFU-GEMM	CFU-GM	CFU-M	BFU-E
1. (IMDM)	MNC	2	46	58	17
	CD34+ cells	30	1710	1790	380
	CD34- cells	0	8	20	9
	residual cells*	0	220	355	5
2. (IMDM)	MNC	1	35	61	19
	CD34 + cells	20	1120	1240	430
	CD34- cells	0	7	40	8
	residual cells	0	155	395	30
3. (RPMI)	MNC	2	36	72	15
	CD34+ cells	60	740	480	60
	CD34- cells	0	4	29	5
	residual cells	0	0	20	0
4. (RPMI)	MNC	2	23	34	28
	CD34+ cells	180	1800	240	480
	CD34- cells	0	5	9	5
	residual cells	0	15	25	0
5. (RPMI)	MNC	1	87	62	10
	CD34+ cells	220	4700	280	660
	CD34- cells	0	7	19	0
	residual cells	0	2	3	0

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Table 8. Generation of Hematopoietic Progenitors from Enzyme-released CD34+ cells on Irradiated LTBM Stromas.

CFU-GM PER FLASK

Expt #	Cells	weeks in culture							
		0	1	2	3	4	5	6	7
1. (IMDM)	CD34+	1750	927	126	106	102	50	21	--
	ACD	3200	444	332	137	46	4	9	--
2. (IMDM)	CD34+	1180	57	64	64	22	142	--	20
	ACD	--	--	--	--	--	--	--	--
3. (RPMI)	CD34+	610	7	109	120	72	55	48	22
	ACD	2400	88	126	40	39	22	37	3
4. (RPMI)	CD34+	1020	312	33	131	82	59	15	9
	ACD	1500	72	36	108	84	--	36	26
5. (RPMI)	CD34+	2350	447	154	340	67	68	32	63
	ACD	5050	290	328	217	69	47	125	89

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FOOTNOTES TO TABLES

Table 3

% CD34+ cells refer to the percentage of cells stained with a fluorescence channel number of 4 or greater (4 decade log scale).

Table 5

Insufficient yield for analysis by flow cytometry. Purity estimated using fluorescence microscopy.

Table 6

Lymphs, lymphocytes; Grans, all granulocytic cells excluding blasts; Monos, monocytes; E'blasts, erythroblasts.

Table 7

Values shown represent mean for each assay performed in duplicate.

Residual cells refers to the single cells or aggregates of cells with single or multiple beads still attached after glycoprotease treatment.

Table 8

At week '0', 5×10^4 CD34+ cells (post enzyme) and 5×10^6 ACD cells were inoculated onto LTBM stromal layers. CFU-GM were assayed from the non-adherent layer of each culture. For experiment number 2, insufficient MNCs were available for the

ACD control culture. Results represent total number of CFU-GM present in the culture at each given time point.

REFERENCES

- 1-1.
5 Newsome, I.E., Cross, F. 1932, J. Am. Vet. Assoc. 80:711.
- 1-1A.
10 Civin, C.I., Strauss, L.C., Fackler, M.J., Trischman, T.M., Wiley, J.M., Loken, M.R., 1990, Prog. Clin. Biol. Res. 333-387.
- 1-2.
15 Martin, S.W., Meek, A.H., Davis, D.G., Thomson, R.G., Johnson, J.A., Lopez, A., Stephens, L., Curtis, R.A., Prescott, J.F., Rosendal, S., Savan, M., Zubaidy, A.J., Bolton, M.R., 1980, Can. J. Comp. Med. 44:1.
- 1-3.
20 Yates, W.D.G. 1982, Can. J. Comp. Med. 46:225.
- 1-4.
Biberstein, E.L. 1978, Methods Microbiol. 10:253.
- 25 1-5.
Frank, G.H., Smith, P.C., 1983, Am. J. Vet. Res. 44:981.
- 1-6.
30 Otulakowski, G.L., Shewen, P.E. Udoh, A.E., Mellors, A., Wilkie, B.N. 1983, Infect. Immun. 42:54.
- 1-7.
Shewen, P.E., Wilkie, B.N. 1982, Infect. Immun. 35:91.
- 35 1-8.
Gonzalez-Rayos, C., Lo, R.Y.C., Shewen, P.E., Beveridge, T.J. 1986, Infect. Immun. 53:505.
- 1-9.
40 Frank, G.H., Tabatabai, L. 1981, Infect. Immun. 32:1119.
- 1-10.
Udoh, A.E. 1986, Ph.D. Thesis, Univ. of Guelph
- 45 1-11.
Abdullah, K.M., Udoh, A.E., Mellors, A. Lo, R.Y.C., Shewen, P.E. 1988, Proc. Canad. Fed. Biol. Socs. (abstract) and ms. submitted.
- 50 1-12.
Abdullah, K.M., Lo, R.Y.C., Mellors, A. 1990, Biochem. Soc. Trans. 18:901.
- 1-13.
55 Abdullah, K.M., Lo, S.S., Lo, R.Y.C., Mellors, A. 1990,

Proc. Canad. Fed. Biol. Socs. (abstract) and ms. in preparation.

- 1-14.
5 Hawley, D.K., McClure, W.R. 1983, Nucleic Acid Res. 11:2237.
- 1-15.
10 Rosenberg, M., Court, D. 1979, Annu. Rev. Genet. 13:319.
- 1-16.
Shine, J., Dalgarno, L. 1974, PNAS 71:1342.
- 1-17.
15 Plaut, A.G. 1983, Annu. Rev. Microbiol. 37:602.
- 1-18.
Sutherland, D.R., Abdullah, K.M., Mellors, A., Davidson, J., Baker, M.A. 1990 Blood (in press).
20
- 1-19.
Civin, C., Strauss, L.C., Brovall, C., Fackler, M.J., Schwartz, J.F., Shaper, J.H. 1984, J. Immunol. 133:157.
- 1-20.
25 Katz, F.E., Tindle, R.W., Sutherland, D.R., Greaves, M.F. 1985, Leuk. Res. 9, 191.
- 1-21.
30 Andrews, R.G., Singer, J.W., Bernstein, I.D. 1986, Blood 67:842.
- 1-22.
Watt, S.M., Karhi, K., Gutter, K., Furley, A.J.W., Katz, F.E., Healy, L.E., Atlass, L.J., Bradley, N.J., Sutherland, D.R., Levinsky, R. and Greaves, M.F. 1987 Leukaemia 5:417.
35
- 1-23.
40 Strauss, L.C., Rowley, S.D., La Russa, V.F., Sharkis, S.J., Stuart, R.K., Civin C.I. 1986, Exp. Hematol. 14:878.
- 1-24.
45 Katz, F.E., Tindle, R.W., Sutherland, D.R., Greaves, M.F. 1986, Leucocyte Typing II, Oxford Univ. Press, pp. 305.
- 1-25.
50 Ryan, D. Kossover, S.A., Mitchell, S., Frantz, C., Hennessey, L., Cohen, H.J. 1986 Blood 68:417.
- 1-26.
Ryan, A., Chapple, C.W., Kossover, S.A., Sandberg, A.A., Cohen, H.J. 1987 Blood 70:814.
55

- 1-27.
Schuh, A., Horsfall, W., Sutherland D.R. Mills, G.B.,
Dube, I., Baker, M.A., Keating, A. 1990, Leukaemia (in
press).
- 5 1-28.
Fackler, M.J., Civin, C.I., Sutherland, D.R., Baker,
M.A., May, W.S., 1990, J. Biol. Chem. 265, 11056.
- 10 1-29.
Berenson, R.J., Andrews, R.G., Bensinger, W.I., Kalamasz,
E., Knitter, G., Buckner, C.D., Bernstein, I.D. 1988, J.
Clin. Invest. 81:951.
- 15 1-30.
Berenson, R.J., Andrews, R.G., Bensinger, W.I., Kalamasz,
D., Knitter, G., Bernstein, I.D., Buckner, C.D. 1988,
Exp. Hematol. 16:252a.
- 20 1-31.
Andrews, R.G., Singer, J.W., Bernstein, I.D. 1990. J.
Exp. Med. 172, 355.
- 1-32.
25 Beschomer. W.E., Civin, C.I., Strauss, L.C. 1985, Am. J.
Path. 119:1.
- 1-33.
Sutherland, D.R., Karhi, K., Watt, S.M., Gatter, K.,
30 Levinsky, R., Keating, A. Baker, M.A., Greaves, M.F.
1986, Blood 68:187a.
- 1-34.
Fina, L, Molgaard, H., Robertson, D., Bradley, N.,
35 Monahan, P., Sutherland, D.R., Baker, M.A., Greaves, M.F.
1990, Blood 75, 2417.
- 1-35.
Civin, C.I., Trischmann, T.M., Fackler, M.J., Bernstein,
40 I.D., Buhning, H.J., Campos, L, Greaves, M.F., Damoun,
M., Katz, D.R., Landsdorp, P.M., Look, A.T., Seed, B.,
Sutherland, D.R., Tindle, R.W., Uchanska-Zeigler, B.
1989, Leucocyte Typing IV Oxford Univ. Press, pp. 818.
- 45 1-36.
Sutherland, D.R., Watt, S.M., Dowden, G., Karhi, K.,
Baker, M.A., Greaves, M.F., Smart, J.E., 1988, Leukaemia
2:793.
- 50 2-1.
Foon, K. A., and R. F. Todd, III. 1986. Immunological
classification of leukemia and lymphoma. Blood 68: 1.
- 2-2.
55 Knapp, W., B. Dorken, P. Rieber, R. E. Schmidt, H. Stein,

- A. E. G. Kr. von dem Bourne, eds. 1989. Leukocyte typing IV. Oxford University Press, Oxford.
- 2-3.
- 5 Metcalfe, D. 1990. The molecular control of cell division, differentiation commitment and maturation in haematopoietic cells. *Nature* 339: 27.
- 2-4.
- 10 Moore, M. A. S. 1991. Clinical implications of positive and negative hematopoietic stem cell regulators. *Blood* 78: 1.
- 2-5.
- 15 Schneider, C., D. R. Sutherland, R. A. Newman, and M. F. Greaves. 1982. Structural features of the cell surface receptor for transferrin that is recognized by the monoclonal antibody OKT9. *J. Biol. Chem.* 257: 8516.
- 20 2-6.
- Sutherland, D. R., C. Rudd, and M. F. Greaves. 1984. Isolation and biochemical characterization of a human T cell associated glycoprotein. *J. Immunol.* 133: 327.
- 25 2-7.
- Newsome, I. E., and F. Cross. 1932. Some bipolar organisms found in pneumonia in sheep. *J. Am. Vet. Assoc.* 80: 711.
- 30 2-8.
- Martin, S. W., A. H. Meek, D. G. Davis, R. G. Thomson, J. A. Johnson, A. Lopez, L. Stephens, R. A. Curtis, J. F. Prescott, S. Rosendal, M. Savan, A. J. Zubaidy and M. R. Bolton. Factors associated with mortality in feedlot cattle: the Bruce county beef cattle project. 1980. *Can. J. Comp. Med.* 44: 1.
- 35 2-9.
- Yates, W. D. G. A review of infectious bovine rhinotracheitis. 1982. *Can. J. Comp. Med.* 46: 225.
- 40 2-10.
- Otulakowski, G. L., P. E. Shewen, A. E. Udoh, A. Mellors and B. N. Wilkie. 1983. Proteolysis of sialoglycoprotein by *Pasteurella haemolytica* cytotoxic culture supernatant. *Infect. Immun.* 42: 54.
- 45 2-11.
- Abdullah, K. M., R. Y. C. Lo and A. Mellors. Distribution of glycoprotease activity and the glycoprotease gene among serotypes of *Pasteurella haemolytica*. 1990. *Biochem. Soc. Trans.* 18: 901.
- 50 2-12.
- Tomita, M., H. Furthmayr, and V. T. Marchesi. 1978. Primary structure of human erythrocyte glycophorin A.
- 55

Isolation and characterization of peptides and complete amino acid sequence. Biochemistry 17: 4756.

- 2-13.
5 Abdullah, K. M., E. A. Udoh, P. E. Shewen and A. Mellors. A neutral glycoprotease from *Pasteurella haemolytica* A1 specifically cleaves O-sialoglycoproteins. Infect. Immun. In Press, 1991.
- 10 2-14.
Abdullah, K. M., R. Y. C. Lo, and A. Mellors. 1991. Cloning, nucleic acid sequence and expression of *Pasteurella haemolytica* strain A1 glycoprotease gene. J. Bacteriol. 173: 5597.
- 15 2-15.
Plaut, A. G. 1983. The IgA₁ proteases of pathogenic bacteria. Annu. Rev. Microbiol. 37: 602.
- 20 2-16.
Katz, F., R. W. Tindle, D. R. Sutherland and M. F. Greaves. 1985. Identification of a membrane glycoprotein associated with hemopoietic progenitor cells. Leuk. Res. 9: 191.
- 25 2-17.
Koeffler, H. P., R. Billing, J. Lysis, R. Sparkles and D. W. Golde. 1980. An undifferentiated variant derived from human acute myelogenous leukemia cell line (KG1). Blood 56: 265.
- 30 2-18.
Furley, A. J., B. R. Reeves, S. Mizutani, L. J. Altass, S. M. Watt, M. C. Jacob, P. Van der Elsen, C. Terhorst, and M. F. Greaves. 1986. Divergent molecular phenotypes of KG1 and KG1a myeloid cell lines. Blood 68: 1101.
- 35 2-19.
Beatty P. G., J. A. Leadbetter, P. J. Martin, T. H. Price and J. A. Manson. 1983. Definition of a common leukocyte cell-surface antigen (Lp95-150) associated with diverse cell-mediated immune functions. J. Immunol. 131: 1:2913.
- 40 2-20.
Sutherland, D. R., S. M. Watt, G. Dowden, K. Karhi, M. A. Baker, M. F. Greaves and J. S. Smart. 1988. Structural and partial amino acid sequence analysis of the human haemopoietic progenitor cell antigen CD34. Leukemia 2: 793.
- 50 2-21.
Uchanska-Ziegler, B., S. Petrasch, J. Michel and A. Ziegler. 1989. Characterization of the CD34-specific monoclonal antibody TUK3. Tissue Antigens 33: 230 (abstract).
- 55

- 2-22.
Carlsson, S. R., and M. Fukuda. 1986. Isolation and
characterization of leukosialin, a major
sialoglycoprotein on human leukocytes. J. Biol. Chem.
5 261: 12779.
- 2-23.
Aruffo, A., I. Stamenkovic, M. Melnick, C. B. Underhill
and B. Seed. 1990. CD44 is the principal cell surface
10 receptor for hyaluronate. Cell 61: 1303.
- 2-24.
Letarte, M., S. Iturbe and E. J. Quackenbush. 1985. A
glycoprotein of molecular weight 85,000 on human cells of
15 B-lineage: detection with a family of monoclonal
antibodies. Mol. Immunol. 22: 113.
- 2-25.
Omary, M. B., I. S. Trowbridge, H. A. Battifora. 1980.
20 Human homologue of murine T200 glycoprotein. J. Exp. Med.
152: 842.
- 2-26.
Sutherland, D. R., D. Delia, C. Schneider, R. Newman, J.
25 Kemshead and M. F. Greaves. 1981. Ubiquitous, cell
surface glycoprotein on tumor cells is proliferation
associated receptor for transferrin. Proc. Natl. Acad.
Sci. USA. 78: 4515.
- 30 2-27.
Morimoto, C., N. L. Letvin, J. A. Distaso, W. R. Aldrich,
and S. F. Schlossman. 1985. The isolation and
characterization of the human suppressor inducer T cell
subset. J. Immunol. 134: 1508.
35
- 2-28.
Poppema, S., L. Visser, R. Lai, A. Shaw, and J. Slupsky.
1989. Antibody MT3 is reactive with a 190 kDa band of the
leukocyte common antigen in plasmacytomas. In; Leukocyte
40 typing IV. Knapp, W., B. Dorken, P. Rieber, R. E.
Schmidt, H. Stein, A. E. G. Kr. von dem Bourne, eds.
Oxford University Press, Oxford.
- 2-29.
45 Smith, S. H., M. H. Brown, D. Rowe, R. E. Callard, and P.
C. L. Beverley. 1986. Functional subsets of human
helper-inducer cells defined by a new monoclonal
antibody, UCHL1. Immunology 58: 63.
- 50 2-30.
Barnstable, C. J., W. F. Bodmer, G. Brown, G. Galfre, C.
Milstein, A. F. Williams and A. Ziegler. 1978. Production
of monoclonal antibodies to group A erythrocytes, HLA and
other human cell surface antigens-new tools for genetic
55 analysis. Cell 14: 9.

- 2-31.
Sutherland, D. R., E. L. Yeo, A. Ryan, G. B. Mills, D. Bailey and M. A. Baker. 1991. Identification of a cell-surface antigen associated with activated T lymphoblasts and activated platelets. *Blood* 77: 84.
- 2-32.
Civin, C., T. Trischman, M. Fackler, I. Bernstein, H. Buhning, L. Campos, M. F. Greaves, M. Kamoun, D. Katz, P. Lansdorp, T. Look, B. Seed, D. R. Sutherland, R. Tindle and B. Uchanska-Zeigler. 1989. Summary of CD34 cluster workshop section. In: *Leukocyte Typing IV* (W. Knapp et al, eds). Oxford University Press: p818.
- 2-33.
Sutherland, D. R., J. C. W. Marsh, J. Davidson, M. A. Baker, A. Keating, and A. Mellors. Differential sensitivity of CD34 epitopes to cleavage by *Pasteurella haemolytica* glycoprotease: implications for purification of CD34-positive cells. *Exp. Hematol.*, in press.
- 2-34.
Thomas, M.L. 1989. The leukocyte common antigen family. *Annu. Rev. Immunol.* 7: 339.
- 2-35.
Streuli, M., L. R. Hall, Y. Saga, S. F. Schlossman, and H. Saito. 1987. Differential usage of three exons generates at least five different mRNAs encoding human leukocyte common antigens. *J. Exp. Med.* 166: 1567.
- 2-36.
Leadbetter, J. A., N. K. Tonks, E. H. Fischer, and E. A. Clark. 1988. CD45 regulates signal transduction and lymphocyte activation by specific association with receptor molecules on T or B cells. *Proc. Natl. Acad. Sci. USA.* 85: 8628.
- 2-37.
Charbonneau, H., N. K. Tonks, K. A. Walsh, and E. H. Fischer. 1988. The leukocyte common antigen (CD45): A putative receptor-linked protein tyrosine phosphatase. *Proc. Natl. Acad. Sci. USA.* 85: 7182.
- 2-38.
Ostergaard, H. L., D. A. Shackleford, T. R. Hurley, P. Johnson, R. Hyman, B. M. Sefton, and I. S. Trowbridge. 1989. Expression of CD45 alters phosphorylation of the lck-encoded tyrosine protein kinase in murine lymphoma cell lines. *Proc. Natl. Acad. Sci. USA.* 86: 8959.
- 2-39.
Pingel, J. T. and M. Thomas. 1989. Evidence that the leukocyte-common antigen is required for antigen-induced T lymphocyte proliferation. *Cell* 58: 1055.

- 2-40.
Sanders, M. E., M. W. Makgoba, S. O. Sharrow, D. Stephany, T. A. Springer, H. A. Young, and S. Shaw. 1988. Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3, CD2, and LFA-1) and three other molecules (UCHL1, CDw29, and Pgp-1) and have enhanced IFN-g production. *J. Immunol.* 140: 1401.
- 2-41.
10 Mentzer S. J., E. Remold-O'Donnell, M. A. V. Crimmins, B. E. Bierer, F. S. Rosen, and S. J. Burakoff. 1987. Sialophorin, a surface sialoglycoprotein defective in Wiscott-Aldrich syndrome, is involved in human T lymphocyte proliferation. *J. Exp. Med.* 165: 1383.
- 15 2-42.
Silverman, L. V., R. C. K. Wong, E. Remold-O'Donnell, D. Vercelli, J. Sancho, C. Terhorst, F. Rosen, R. Geha, and T. Chatila. 1989. Mechanism of mononuclear cell activation by an anti-CD43 (sialophorin) agonistic antibody. *J. Immunol.* 142: 4194.
- 20 2-43.
Remold-O'Donnell, E., D. M. Kenney, R. Parkman, L. Cairns, B. Savage, and F. S. Rosen. 1984. Characterization of a human lymphocyte surface sialoglycoprotein that is defective in Wiscott-Aldrich syndrome. *J. Exp. Med.* 159: 1705.
- 25 2-44.
Greer L. W., E. Higgins, D. R. Sutherland, A. Novodrodsky, I. Brockhausen, M. Peacock, L. A. Rubin, M. A. Baker, J. W. Dennis and K. A. Siminovitch. 1989. Altered expression of leucocyte sialoglycoprotein in Wiskott-Aldrich syndrome is associated with a specific defect in O-glycosylation. *Biochem Cell Biol* 67: 503.
- 30 35 2-45.
Greaves M. F., J. Brown, H. V. Molgaard, N. K. Spurr, D. Robertson, D. Delia, and D. R. Sutherland. (1991). Molecular features of CD34: a hemopoietic progenitor cell-associated molecule. *Leukaemia*: In press.
- 2-46.
45 Berenson, R. J., R. G. Andrews, W. I. Bensinger, D. Kalamasz, G. Knitter, C. D. Buckner, I. D. Bernstein. 1988. Antigen CD34-positive marrow cells engraft lethally irradiated baboons. *J. Clin. Invest.* 81: 951.
- 50 2-47.
Berenson R. J., W. I. Bensinger, R. S. Hill, R. G. Andrews, J. Garcia-Lopez, D. F. Kalamaz, B. J. Still, G. Spitzer, D. Buckner, I. D. Bernstein, E. D. Thomas. 1991. Engraftment after infusion of CD34+ marrow cells in patients with breast cancer or neuroblastoma. *Blood* 77: 1717.
- 55

- 2-48.
Leonard, W. J., J. Depper, T. Uchiyama, K. A. Smith, T. A. Waldman and W. C. Greene. 1983. Blockade of interleukin-2 receptor by anti-Tac antibody: inhibitors of human lymphocyte activation. *J. Immunol.* 131: 690.
- 2-49.
Newman R. A., D. R. Sutherland, T. W. LeBien, J. H. Kersey and M. F. Greaves. 1982. Biochemical characterisation of a leukaemia-associated antigen (p24) defined by the monoclonal antibody BA-2. *Biochim Biophys Acta.* 701: 318.
- 2-50.
Piruccello, S. J. and T. W. Le Bien. 1986. The human B cell-associated antigen CD24 is a single chain sialoglycoprotein. *J. Immunol.* 136: 3779.
- 2-51.
Dittman, W. A. and P. W. Majerus. 1990. Structure and function of thrombomodulin: a natural coagulant. *Blood* 75: 329.
- 2-52.
Russell, D.W., W. J. Schneider, T. Yamamoto, K. Lusky, M. S. Brown and J. L. Goldstein. 1984. Domain map of the LDL receptor: Sequence homology with the epidermal growth factor precursor. *Cell* 37: 577.
- 3-1.
Newsome IE, and Cross F (1932) Some bipolar organisms found in pneumonia in sheep. *J Am Vet Assoc* 80:711
- 3-2.
Martin SW, Meek AH, Davis DG, Thomson RG, Johnson JA, Lopez A, Stephens, L, Curtis RA, Prescott JF, Rosendal S, Savan M, Zubaidy AJ and Bolton MR (1980) Factors associated with mortality in feedlot cattle: the Bruce county beef cattle project. *Can J Comp Med* 44:1
- 3-3.
Yates WDG (1982) A review of infectious bovine rhinotracheitis. *Can J Comp Med* 46:225
- 3-4.
Otulakowski GL, Shewen PE, Udoh AE, Mellors A and Wilkie BN (1983) Proteolysis of sialoglycoprotein by *Pasteurella haemolytica* cytotoxic culture supernatant. *Infect Immun* 42:54
- 3-5.
Abdullah KM, Lo RYC and Mellors A (1990) Distribution of glycoprotease activity and the glycoprotease gene among serotypes of *Pasteurella haemolytica*. *Biochem Soc Trans* 18:901

- 3-6.
Abdullah KM, Lo RYC and Mellors A (1991) Cloning, nucleic acid sequence and expression of *Pasteurella haemolytica* A1 glycoprotease gene. J Bacteriol 173: 5597.
- 5 3-7.
Sutherland DR, Watt S.M, Dowden G, Karhi K, Baker MA, Greaves MF and Smart JS (1988) Structural and partial amino acid sequence analysis of the human haemopoietic progenitor cell antigen CD34. Leukemia 2:793
- 10 3-8.
Carlsson SR, and Fukuda M (1986) Isolation and characterization of leukosialin, a major sialoglycoprotein on human leukocytes. J Biol Chem 261:12779
- 15 3-9.
Stamenkovitch I, Amiot M, Pesando JM, and Seed B (1989) A lymphocyte molecule implicated in lymph node homing is a member of the cartilage link protein family. Cell 56:1057
- 20 3-10.
Thomas ML (1989) The leukocyte common antigen family. Annu Rev Immunol. 7:339
- 25 3-11.
Civin C, Strauss LC, Brovall C, Fackler MJ, Schwartz JF, and Shaper JH (1984) Antigenic analysis of hematopoiesis III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG1a cells. J Immunol 133:157
- 30 3-12.
Katz F, Tindle RW, Sutherland DR, and Greaves MF (1985) Identification of a membrane glycoprotein associated with hemopoietic progenitor cells. Leuk Res 9:191
- 35 3-13.
Andrews RG, Singer JW, and Bernstein ID (1986) Monoclonal antibody 12.8 recognizes a 115-kd molecule present on both unipotent and multipotent colony-forming cells and their precursors. Blood 67:842
- 40 3-14.
Ryan D, Kossover S, Mitchell, S, Frantz, C, Hennessey, L, Cohen, HJ (1986) Subpopulations of common acute lymphoblastic leukemia antigen-positive lymphoid cells in normal bone marrow identified by hematopoietic differentiation antigens. Blood 68:417
- 45 3-15.
Watt SM, Karhi K, Gatter K, Furley AJW, Katz FE, Healy LE, Altass LJ, Bradley NJ, Sutherland DR, Levinsky R, and Greaves MF (1987) Distribution and epitope analysis of
- 50

the cell membrane glycoprotein (HPCA-1) associated with Human haemopoietic progenitor cells. Leukaemia 1:417

3-16.

- 5 Schuh A, Sutherland DR, Horsfall W, Mills GB, Dub I, Baker MA and Keating A (1990) Chronic myelogenous leukaemia arising in a progenitor common to T cells and myeloid cells. Leukaemia 4:631

10 3-17.

Fackler, MJ, Civin CI, Sutherland DR, Baker MA, and May WS (1990) Activated Protein Kinase C Directly Phosphorylates the CD34 Antigen on Hematopoietic Cells. J Biol Chem 265:11056

15

3-18.

- Berenson RJ, Andrews RG, Bensinger WI, Kalamasz D, Knitter G, Buckner CD, and Bernstein ID (1988) Antigen CD34-positive marrow cells engraft lethally irradiated baboons. J Clin Invest 81:951

20

3-19.

- Wagermaker G, van Gils FCJM, Bart-Baumeister JAK, Weilenger JJ, Levinsky RJ (1990) Sustained engraftment of allogeneic CD34 positive hemopoietic stem cells in rhesus monkeys [abstr]. Exp Hematol 18:704

25

3-20.

- Berenson RJ, Bensinger WI, Hill RS, Andrews RG, Garcia-Lopez J, Kalamaz DF, Still BJ, Spitzer G, Buckner D, Bernstein ID, Thomas ED (1991) Engraftment After Infusion of CD34+ Marrow Cells in Patients with Breast Cancer or Neuroblastoma. Blood 77:1717

30

3-21.

- Andrews RG, Singer JW, Bernstein ID (1990) Human hematopoietic precursors in long-term culture: single CD34+ cells that lack detectable T cell, B cell, and myeloid cell antigens produce multiple colony-forming cells when cultured with marrow stromal cells. J Exp Med 172:355

40

3-22.

- Civin C, Trischman T, Fackler MJ, Bernstein I, Buhning H, Campos L, Greaves MF, Kamoun M, Katz D, Lansdorp P, Look T, Seed B, Sutherland DR, Tindle R. and Uchanska-Ziegler B (1989) Summary of CD34 cluster workshop section. In: Leucocyte Typing IV W. Knapp et al, (eds). Oxford Univ Press, p818

50

3-23.

- Fina L, Molgaard HV, Robertson D, Bradley NJ, Monaghan P, Delia D, Sutherland DR, Baker MA and Greaves MF (1990) Expression of the CD34 gene in vascular endothelial cells. Blood 75:2417

55

- 3-24.
Uchanska-Ziegler B, Petrasch S, Michel J and Ziegler A
(1989) Characterization of the CD34-specific monoclonal
antibody TUK3 [abstr]. Tissue Antigens 33:230
- 5 3-25.
Koeffler HP, Billings R, Lysis J, Sparkles R and Golde DW
(1980) An undifferentiated variant derived from human
acute myelogenous leukemia cell line (KG1). Blood 56:265
- 10 3-26.
Furley AJ, Reeves BR, Mizutani S, Altass LJ, Watt SM,
Jacob MC, Van der Elsen P, Terhorst C, and Greaves MF
(1986) Divergent molecular phenotypes of KG1 and KG1a
15 myeloid cell lines. Blood 68:1101
- 3-27.
Sutherland DR, Rudd C and Greaves MF (1984) Isolation and
biochemical characterization of a human T cell associated
20 glycoprotein. J Immunol 133:327
- 3-28.
Civin CI, Banquerigo ML, Strauss LC and Loken MR (1987)
Anigenic analysis of hematopoiesis. VI. Flow cytometric
25 characterization of My10-positive progenitor cells in
normal human bone marrow. Exp Hematol 15:10
- 3-29.
Lansdorp PM, Sutherland HJ, and Eaves CJ (1990) Selective
30 expression of CD45 isoforms on functional subpopulations
of CD34+ hemopoietic cells from human bone marrow. J Exp
Med 172:363
- 3-30.
Sutherland DR, Abdullah KM, Baker MA, and Mellors A
35 (1991) Cleavage of the O-glycosylated cell-surface
glycoproteins CD34, CD43, CD44, and CD45 by a novel
glycoprotease from *Pasteurella haemolytica* [abstr].
Glycoconjugate Journal 8;208
- 40 3-31.
Brown J, Greaves MF, and Molgaard HV (1991) The gene
encoding the stem cell antigen CD34 is conserved in mouse
and expressed in haemopoietic progenitor cell lines,
45 brain, and embryonic fibroblasts. Int Immunol 3:175
- 3-32.
Greaves MF, Brown J, Molgaard HV, Spurr NK, Robertson D,
Delia D, and Sutherland DR (1991) Molecular features of
50 CD34: a hemopoietic progenitor cell-associated molecule.
Leukaemia:In press
- 3-33.
Jentoft N (1990) Why are proteins O-glycosylated? Trends
55 in Biochem Sci 15:291

- 3-34.
Civin CI, Strauss LC, Fackler MJ, Trischmann TM, Wiley JM
(1990) Positive stem cell selection - basic science. *Prog Clin Biol Res* 333:387
- 5 4-1.
Foon K. A. and R. F. Todd, III. 1986. Immunological
classification of leukemia and lymphoma. *Blood* 68: 1.
- 4-2.
10 Knapp W., B. Dorken, P. Rieber, R. E. Schmidt, H. Stein,
A. E. G. Kr. von dem Bourne, eds. 1989. *Leukocyte typing*
IV. Oxford University Press, Oxford.
- 4-3.
15 Civin C., L. C. Strauss, C. Brovall, M. J. Fackler, J. F.
Schwartz, and J. H. Shaper. 1984. Antigenic analysis of
hematopoiesis III. A hematopoietic progenitor cell
surface antigen defined by a monoclonal antibody raised
against KG1a cells. *J. Immunol.* 133: 157.
- 20 4-4.
Katz F., R. W. Tindle, D. R. Sutherland, and M. F.
Greaves. 1985. Identification of a membrane glycoprotein
associated with hemopoietic progenitor cells. *Leuk. Res.*
25 9: 191.
- 4-5.
Andrews R. G., J. W. Singer, and I. D. Bernstein. 1986.
Monoclonal antibody 12.8 recognizes a 115-kd molecule
30 present on both unipotent and multipotent colony-forming
cells and their precursors. *Blood* 67: 842.
- 4-6.
35 Ryan D., S. Kossover, S. Mitchell, C. Frantz, L.
Hennessey, H. J. Cohen. 1986. Subpopulations of common
acute lymphoblastic leukemia antigen-positive lymphoid
cells in normal bone marrow identified by hematopoietic
differentiation antigens. *Blood* 68: 417.
- 40 4-7.
Schuh A, D. R. Sutherland, W. Horsfall, G. B. Mills, I.
Dub, M. A. Baker and A. Keating. 1990. Chronic
myelogenous leukaemia arising in a progenitor common to T
cells and myeloid cells. *Leukaemia* 4: 631.
- 45 4-8.
Fackler M. J., C. I. Civin, D. R. Sutherland, M. A.
Baker, and W. S. May. 1990. Activated Protein Kinase C
Directly Phosphorylates the CD34 Antigen on Hematopoietic
50 Cells. *J. Biol. Chem.* 265: 11056.
- 4-9.
Andrews R. G., J. W. Singer, I. D. Bernstein. 1990. Human
hematopoietic precursors in long-term culture: single
55 CD34+ cells that lack detectable T cell, B cell, and
myeloid cell antigens produce multiple colony-forming

cells when cultured with marrow stromal cells. J. Exp. Med. 172: 355.

4-10.

- 5 Berenson R. J., R. G. Andrews, W. I. Bensinger, D. Kalamasz G. Knitter, C. D. Buckner, and I. D. Bernstein. 1988. Antigen CD34-positive marrow cells engraft lethally irradiated baboons. J. Clin. Invest. 81: 95.

10

4-11.

- Wagermaker G., F. C. J. M. van Gils, J. A. K. Bart-Baumeister, J. J. Weilenger, R. J. Levinsky. 1990. Sustained engraftment of allogeneic CD34 positive hemopoietic stem cells in rhesus monkeys. Exp. Hematol. 18: 704 (abstr).

4-12.

- 20 Berenson R. J., W. I. Bensinger, R. S. Hill, R. G. Andrews, J. Garcia-Lopez, D. F. Kalamaz, B. J. Still, G. Spitzer, D. Buckner, I. D. Bernstein, E. D. Thomas. 1991. Engraftment after infusion of CD34+ marrow cells in patients with breast cancer or neuroblastoma. Blood 77: 1717.

25

4-13.

- Civin C. I., L. C. Strauss, M. J. Fackler, T. M. Trischmann, J. M. Wiley. 1990. Positive stem cell selection -basic science. In: Gross, S., A. P. Gee and D. Worthington-White eds. Progress in Clinical and Biological Research: Bone Marrow Purging and Processing, Alan R. Liss, Inc. 333: 387.

4-14.

- 35 Civin C., T. Trischman, M. Fackler, I. Bernstein, H. Buhning, L. Campos, M. F. Greaves, M. Kamoun, D. Katz, P. Lansdorp, T. Look, B. Seed, D. R. Sutherland, R. Tindle and B. Uchanska-Zeigler. 1989. Summary of CD34 cluster workshop section. In: Leukocyte Typing IV (W. Knapp et al, eds). Oxford University Press: p818.

40

4-15.

- 45 Watt S. M., K. Karhi, K. Gatter, A. J. W. Furley, F. E. Katz, L. E. Healy, L. J. Altass, N. J. Bradley, D. R. Sutherland, R. Levinsky, and M. F. Greaves. 1987. Distribution and epitope analysis of the cell membrane glycoprotein (HPCA-1) associated with Human haemopoietic progenitor cells. Leukaemia 1: 417.

50

4-16.

- Sutherland D. R., S. M. Watt, G. Dowden, K. Karhi, M. A. Baker, M. F. Greaves and J. S. Smart. 1988. Structural and partial amino acid sequence analysis of the human haemopoietic progenitor cell antigen CD34. Leukemia 2: 793.

55

- 4-17.
Brown J., M. F. Greaves and H. V. Molgaard. 1991 The gene encoding the stem cell antigen CD34 is conserved in mouse and expressed in haemopoietic progenitor cell lines,
5 brain, and embryonic fibroblasts. *Int Immunol* 3: 175.
- 4-18.
Greaves M. F., J. Brown, H. V. Molgaard, N. K. Spurr, D. Robertson, D. Delia and D. R. Sutherland. 1991. Molecular
10 features of CD34: a hemopoietic progenitor cell-associated molecule. *Leukaemia*: In press.
- 4-19.
Otulakowski G. L., P. E. Shewen, A. E. Udoh, A. Mellors
15 and B. N. Wilkie. 1983. Proteolysis of sialoglycoprotein by *Pasteurella haemolytica* cytotoxic culture supernatant. *Infect. Immun.* 42: 54.
- 4-20.
20 Abdullah K. M., R. Y. C. Lo and A. Mellors. Distribution of glycoprotease activity and the glycoprotease gene among serotypes of *Pasteurella haemolytica*. 1990. *Biochem. Soc. Trans.* 18: 901.
- 4-21.
25 Abdullah K. M., R. Y. C. Lo, and A. Mellors. 1991. Cloning, nucleic acid sequence and expression of *Pasteurella haemolytica* strain A1 glycoprotease gene. *J. Bacteriol.* 173: 5597.
30
- 4-22.
Sutherland D. R., K. M. Abdullah, M. A. Baker, and A. Mellors. 1991. Cleavage of the O-glycosylated
cell-surface glycoproteins CD34, CD43, CD44, and CD45 by
35 a novel glycoprotease from *Pasteurella haemolytica*. *Glycoconjugate Journal* 8: 208 (abstract).
- 4-23.
40 Uchanska-Ziegler B., S. Petrasch, J. Michel and A. Ziegler. 1989. Characterization of the CD34-specific monoclonal antibody TUK3. *Tissue Antigens* 33: 230 (abstract).
- 4-24.
45 Sutherland D. R., K. M. Abdullah, A. Mellors, J. Davidson, and M. A. Baker. 1990. Cleavage of the lymphohematopoietic progenitor cell antigen CD34 by a novel glycoprotease from *P. Haemolytica*. *Blood* 76: 122 (abstract).
50
- 4-25.
Koeffler H. P., R. Billing, J. Lysis, R. Sparkles and D. W. Golde. 1980. An undifferentiated variant derived from human acute myelogenous leukemia cell line (KG1). *Blood*
55 56: 265.

- 4-26.
Fauser A. A., and H. A. Messner. 1978.
Granuloerythropoietic colonies in human bone marrow,
peripheral blood and cord blood. Blood 52: 1243.
- 5
- 4-27.
Marsh J. C. W., J. Chang, N. G. Testa, J. M. Hows, and T.
M. Dexter. 1991. In vitro assessment of marrow "stem
cell" and stromal cell function in aplastic anemia. Br.
10 J. Hematol. 78: 258.
- 4-28.
Coutinho L. H., Testa NG, Dexter TM: The
myelosuppressive effect of recombinant interferon in
15 short-term and long-term marrow cultures. Br. J.
Hematol. 63:517, 1986.
- 4-29.
Marsh J. C. W., J. Chang, N. G. Testa, J. M. Hows, T. M.
20 Dexter. 1990. The hematopoietic defect in aplastic anemia
assessed by long-term marrow culture. Blood 76:1748.
- 4-30.
Civin C. I., M. L. Banquerigo, L. C. Strauss, and M. R.
25 Loken. 1987. Anigenic analysis of hematopoiesis. VI. Flow
cytometric characterization of My10-positive progenitor
cells in normal human bone marrow. Exp. Hematol. 15: 10.
- 4-31.
30 Gorin N. C., R. P. Gale and J. O. Armitage for the
Advisory Committee of the International Transplant
Registry (ABMTR). 1989. Autologous bone marrow
transplants: different indications in Europe and North
America. Lancet 2: 312.
- 35
- 4-32.
Keating A. 1991. Is marrow purging necessary or
clinically useful? Canadian Bone Marrow Transplantation
Symposium (Cooper, B. and G. Fulop, Eds) Bone Marrow
40 Transplantation 7 (Suppl. 1): 61.
- 4-33.
Gross S. 1990. Perspectives in marrow purging. In: Gross,
S., A. Gee and D. Worthington-White eds. Progress in
45 Clinical and Biological Research: Bone Marrow Purging and
Processing, Alan R. Liss, Inc. 333: 29.
- 4-34.
Gordon M. Y., G. P. Riley and M. F. Greaves. 1987.
50 Plastic adherent progenitor cells and human bone marrow.
Exp. Hematol. 15: 772.
- 4-35.
Iscoe N. N. and F. Melchers. 1978. Complete replacement
55 of serum by albumin transferin and soy bean lipid in

cultures of lipopolysaccharide-reactive B lymphocytes. J. Exp. Med. 147: 923.

4-36.

- 5 Moore G. E., R. E. Gerner and H. A. Franklin. 1967.
Culture of normal human leukocytes. JAMA 199: 519.

4-37.

- Andrews R. G., J. W. Singer and I. D. Bernstein. 1989.
10 Precursors of colony-forming cells in humans can be
distinguished from colony-forming cells by expression of
the CD33 and CD34 antigens and light scatter properties.
J. Exp. Med. 169: 1721.

15

CLAIMS:

1. A method for recovering viable cells having surface protein determinants having portions rich in O-glycosylated carbohydrates where such proteins having said determinants are substrates sensitive to a P. haemolytica derived neutral metallo-glycoprotease, said glycoprotease having highly restricted specificity for cleaving solely from said cell surfaces said O-glycosylated protein portions having said determinants while retaining cell viability, said process comprising:
- i) contacting said cells in solution with affinity matrices which bind specifically to one or more of said determinants which are part of said glycoprotease sensitive substrates and allowing said affinity matrices to bind said determinants on said cells,
 - ii) separating said cells to which said affinity matrices are bound from any remaining matter in said solution, said affinity matrices having sufficient binding affinity for said determinants to remain bound to said determinants during said separation,
 - iii) contacting said glycoprotease with said separated cells in sufficient concentration and duration to cleave solely said protein substrate portions having said determinants and said affinity matrices and thereby release said cells from said affinity matrices, said released cells retaining viability due to said restricted specificity of said glycoprotease cleaving solely O-glycosylated protein portions having said determinants, and,
 - iv) recovering said released cells.

2. A method of claim 1 wherein said cells are haematopoietic progenitor cells.

35

3. A method of claim 2 wherein said progenitor cells have antigen glycoproteins selected from the group

identified by entities consisting of CD34, CD43, CD44 and CD45.

4. A method of claim 1 wherein said affinity
5 matrices are selected from the group consisting of
antibodies, receptors, antibody fragments, receptor
fragments, (list to be completed by inventor).

5. A method of claim 4 wherein said determinants
10 are epitopes and said affinity matrices are antibodies.

6. A method of claim 5, wherein said progenitor
cells have antigenic glycoproteins selected from the
group identified by entities consisting of CD34, CD43,
15 CD44 and CD45, said antibodies being specific to a
selected one of said antigenic glycoprotein entities.

7. A method of claim 6 wherein said antibody is
specific to CD34.
20

8. A method of claim 7 wherein said antibodies are
selected from the group consisting of entities MY10,
B1.3C5, 12.8, ICH3 and QBEND 10.

9. A method of claim 1 wherein said affinity
25 matrices are immobilized on a solid support.

10. A method of claim 9 wherein said solid support
is of magnetic material, bound by said affinity matrices
30 being separated from remaining matter in said solution by
magnetically capturing said solid support and washing
said captured solid support to remove any remaining
matter.

11. A method of claim 10, wherein magnetic capture
35 of said solid support is removed after washing of said
support and before treatment with said glycoprotease.

12. A method of claim 11 wherein said solid support is a plurality of magnetizable beads which are adapted to immobilize said affinity matrices.

5 13. A method of claim 12 wherein said affinity matrices are first antibodies, said plurality of beads being coated with second antibodies for binding said first antibodies.

10 14. Modified viable haematopoietic progenitor cells having removed therefrom by a glycoprotease, O-glycosylated portions of an antigenic glycoprotein epitope selected from the group of epitopes identified by entities CD34, CD43, CD44 and CD45, said modified cells
15 being viable for long term haematopoiesis.

15 15. Modified cells of claim 14 wherein said cells can reconstitute long term haematopoiesis when grown on irradiated human bone marrow *in vitro* and when
20 transplanted.

16. Modified cells of claim 14 wherein said cells have long term haematopoiesis when grown in clonogenic culture.

25

17. Modified cells of claim 14, 15 or 16 wherein said cells are identified by said CD34 entity.

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CD34/B1.3C5

UNTREATED

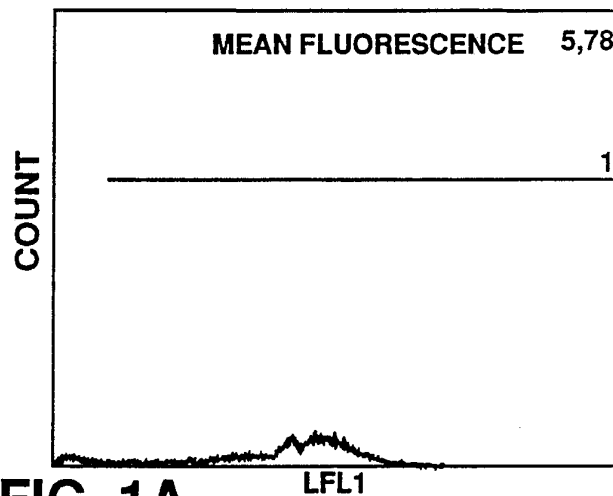


FIG. 1A.

+VCN

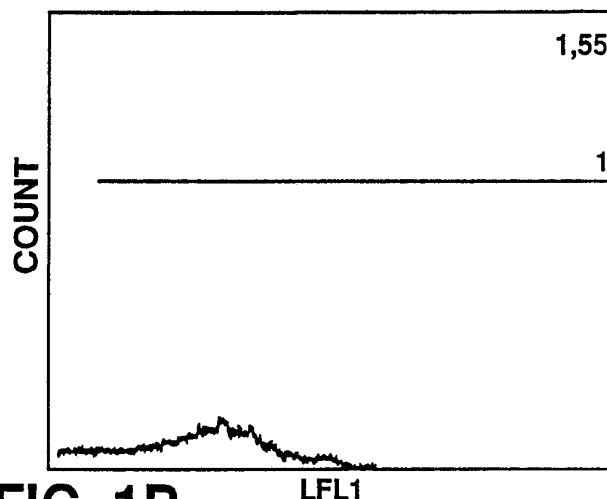


FIG. 1B.

+GLYCOPROTEASE

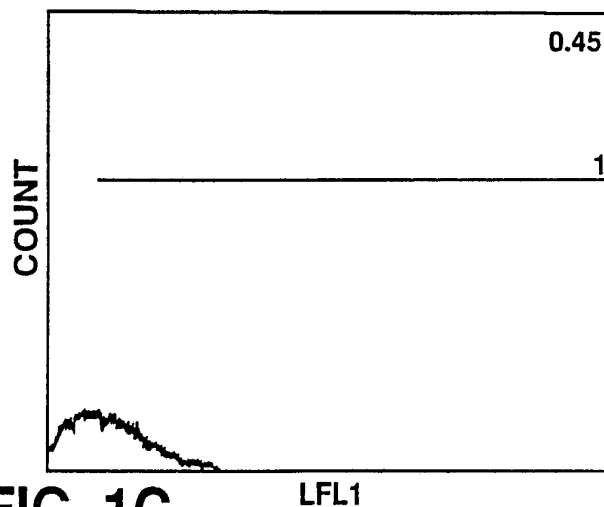


FIG. 1C.

SUBSTITUTE SHEET

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CD34/QBEND10

UNTREATED

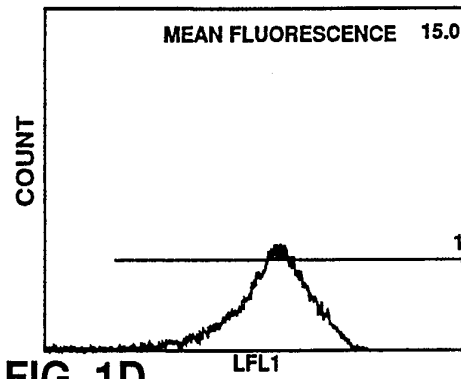


FIG. 1D.

+VCN

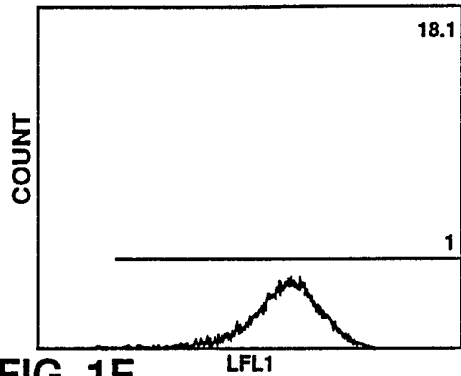


FIG. 1E.

+GLYCOPROTEASE

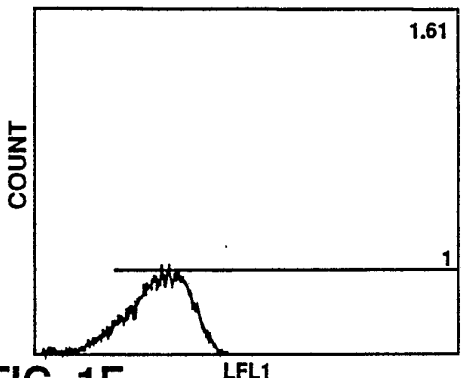


FIG. 1F.

VCN/
GLYCOPROTEASE

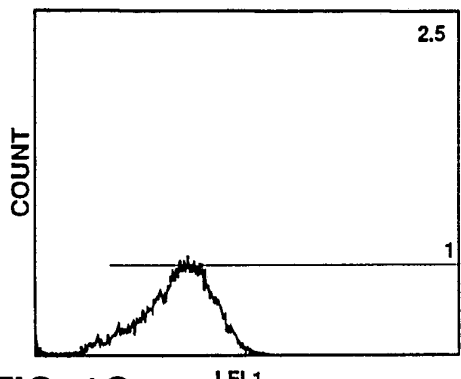
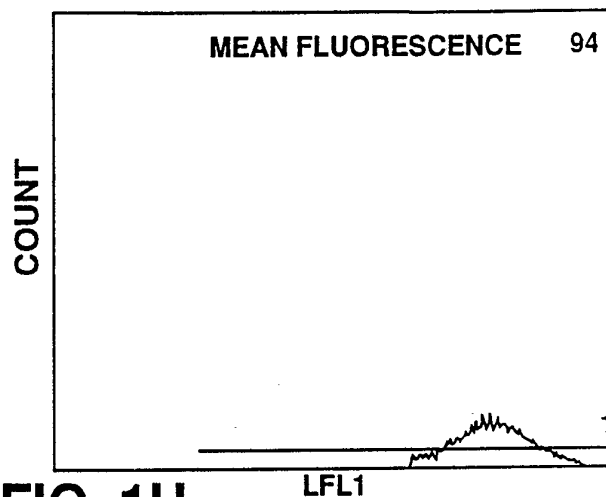
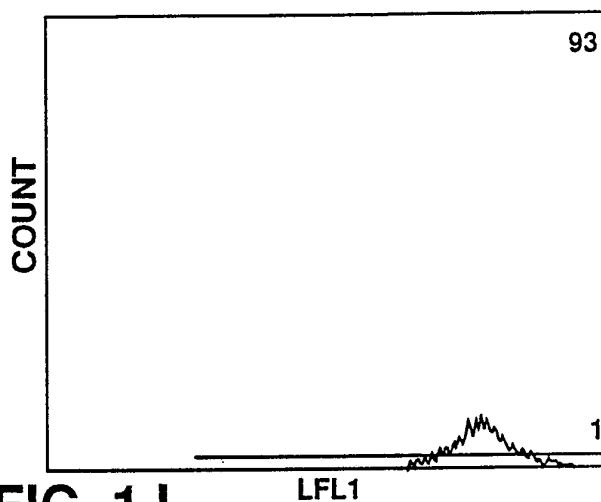
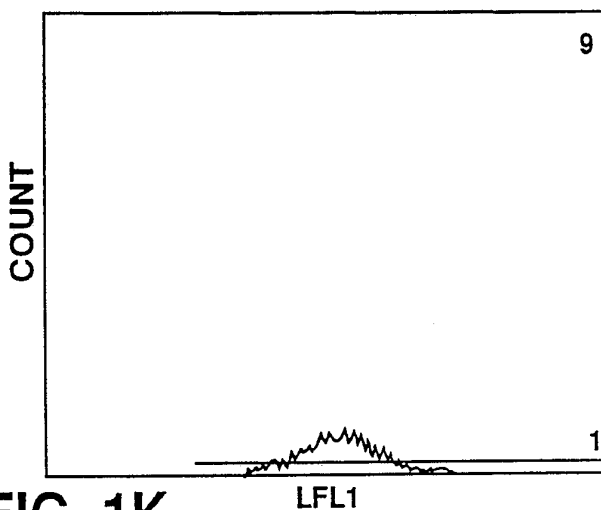


FIG. 1G.

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CD44/50B4**UNTREATED****FIG. 1H.****+VCN****FIG. 1J.****+GLYCOPROTEASE****FIG. 1K.****SUBSTITUTE SHEET**

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CD71/OKT9

UNTREATED

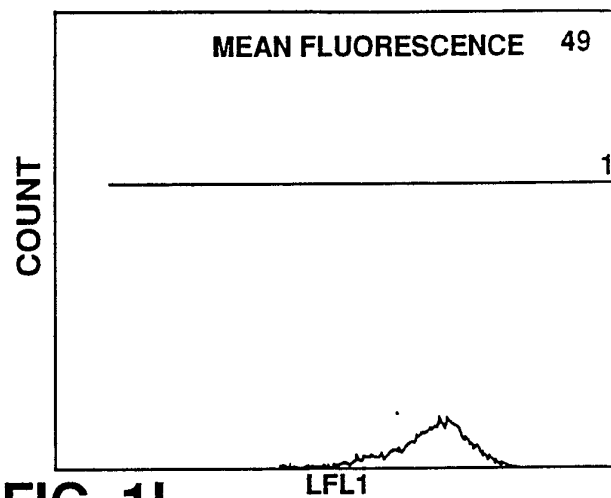


FIG. 1L.

+VCN

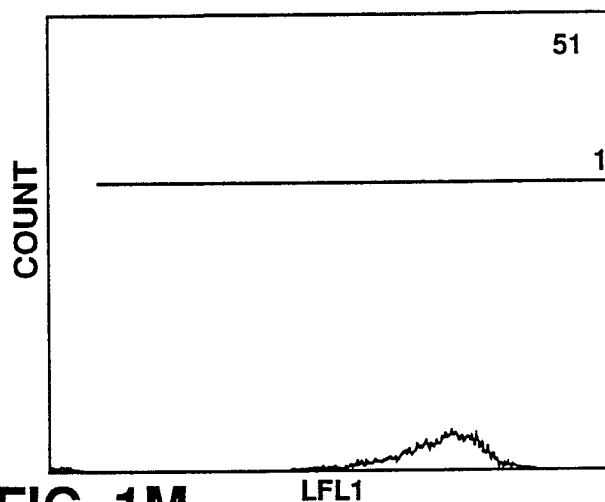


FIG. 1M.

+GLYCOPROTEASE

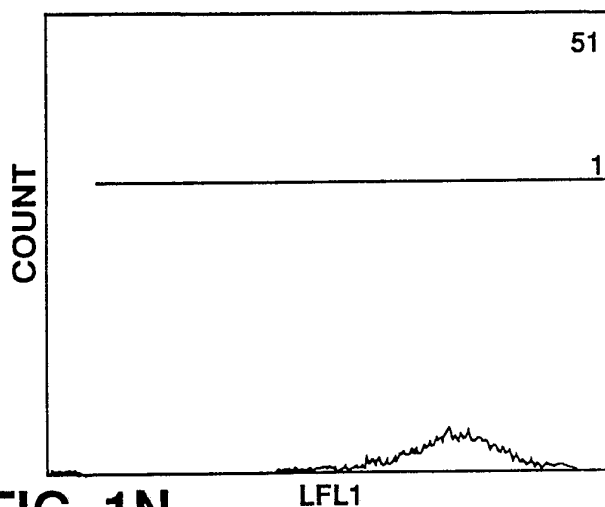


FIG. 1N.

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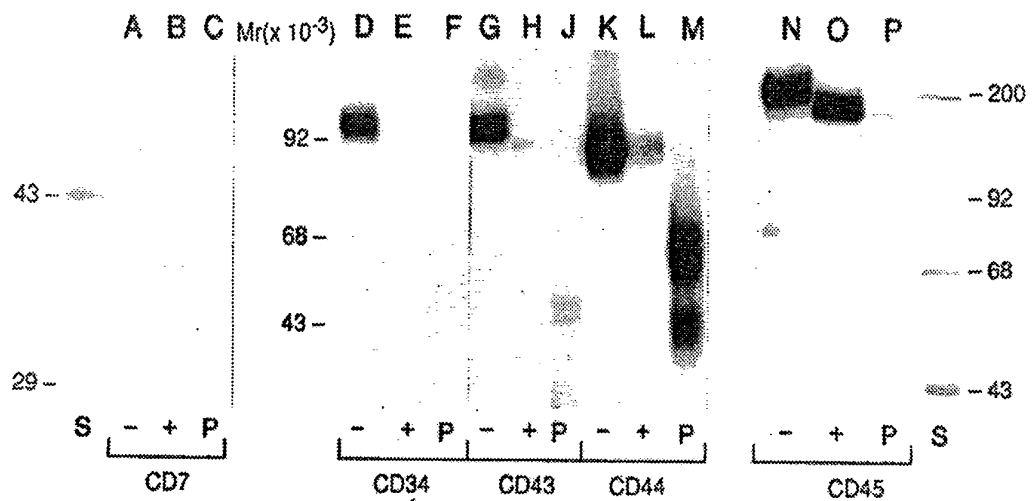


FIG.2.

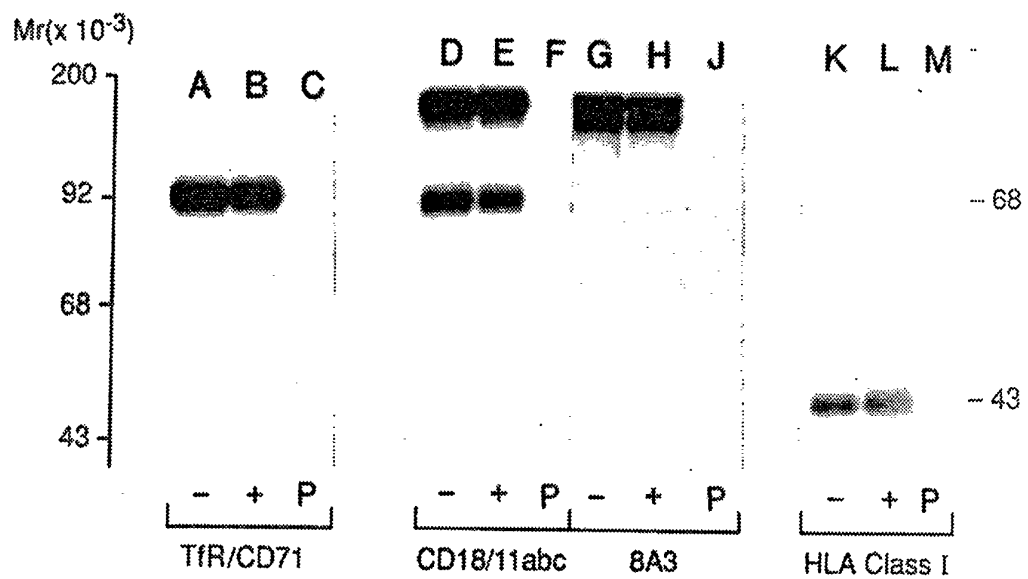
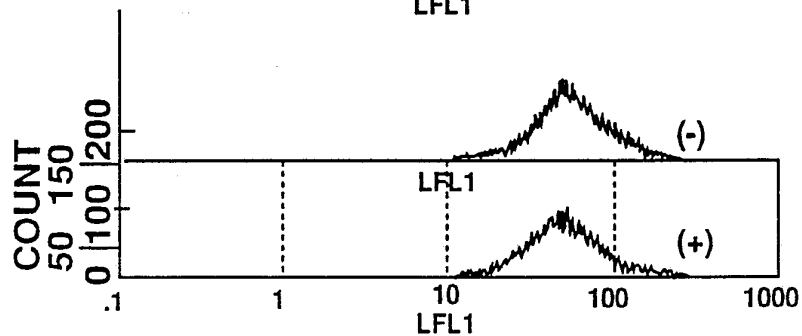
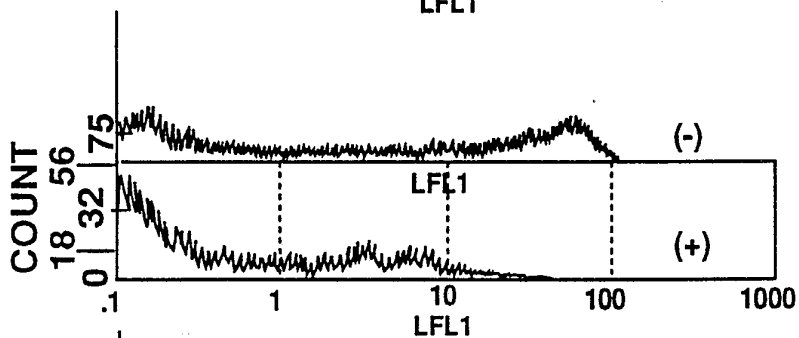
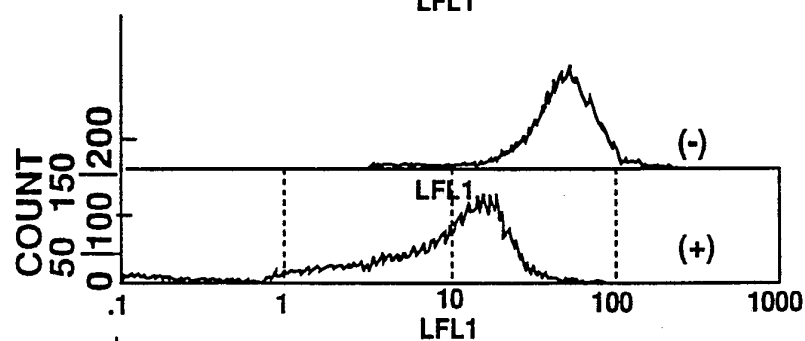
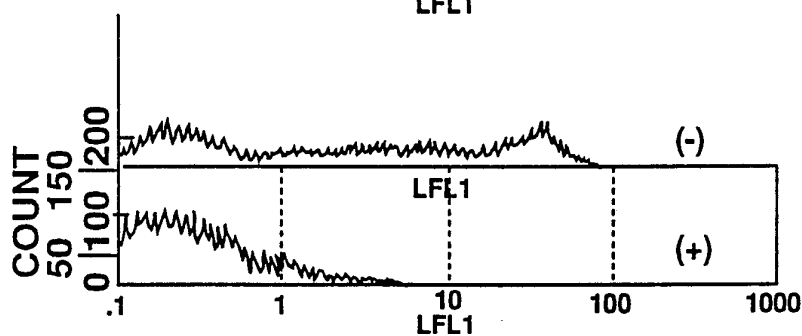
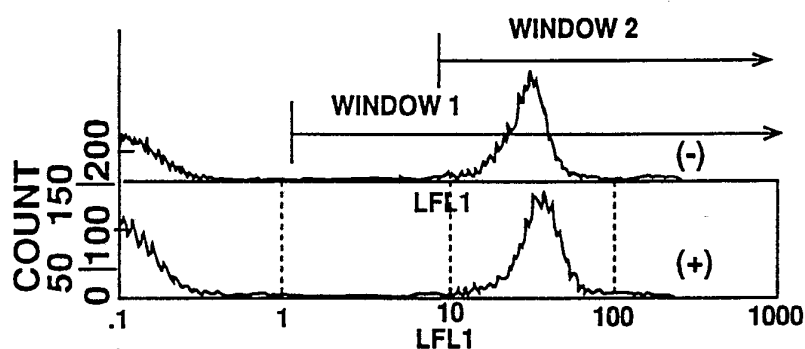


FIG.3.

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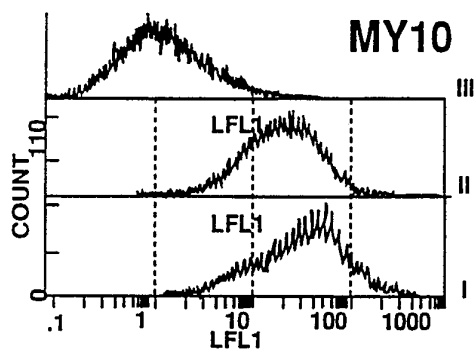


FIG. 5A.

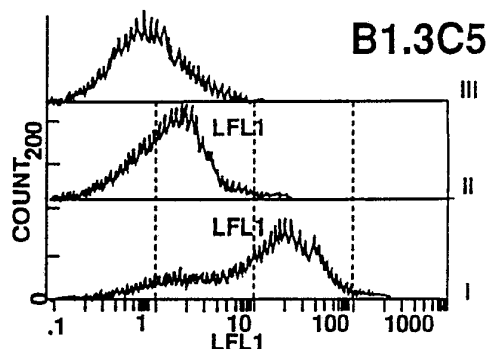


FIG. 5B.

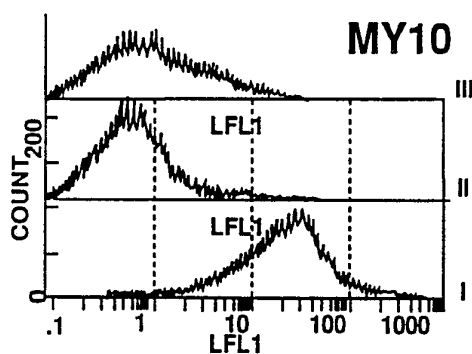


FIG. 5C.

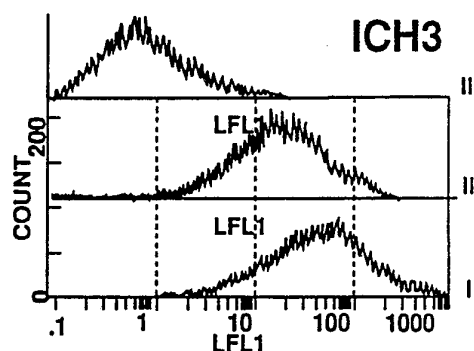


FIG. 5D.

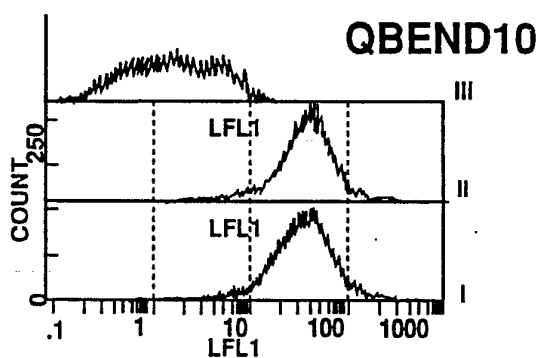


FIG. 5E.

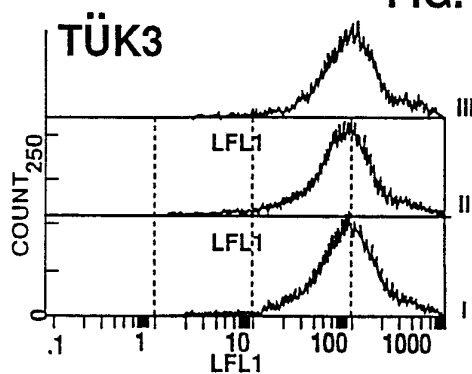


FIG. 5F

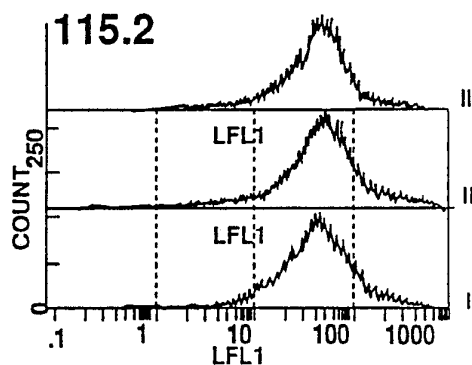


FIG. 5G.

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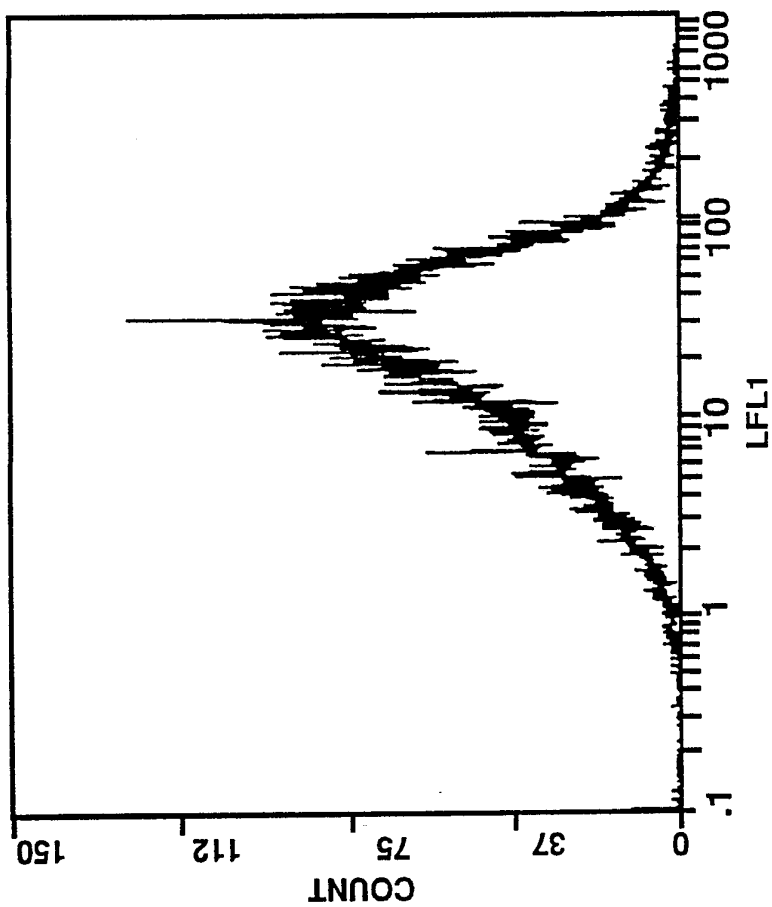


FIG. 6B.

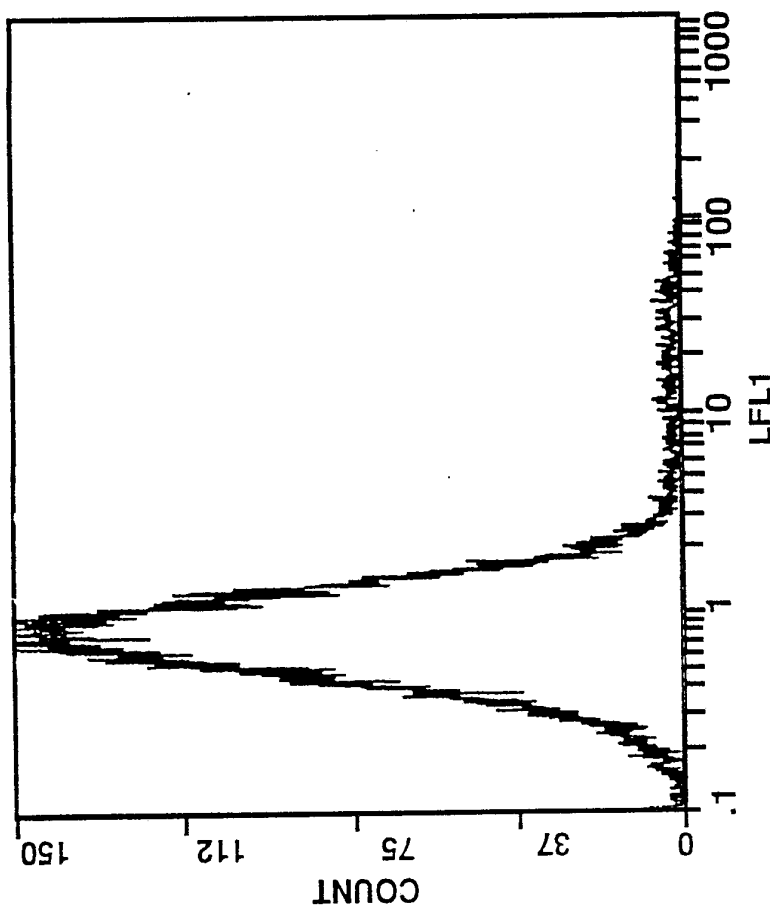


FIG. 6A.

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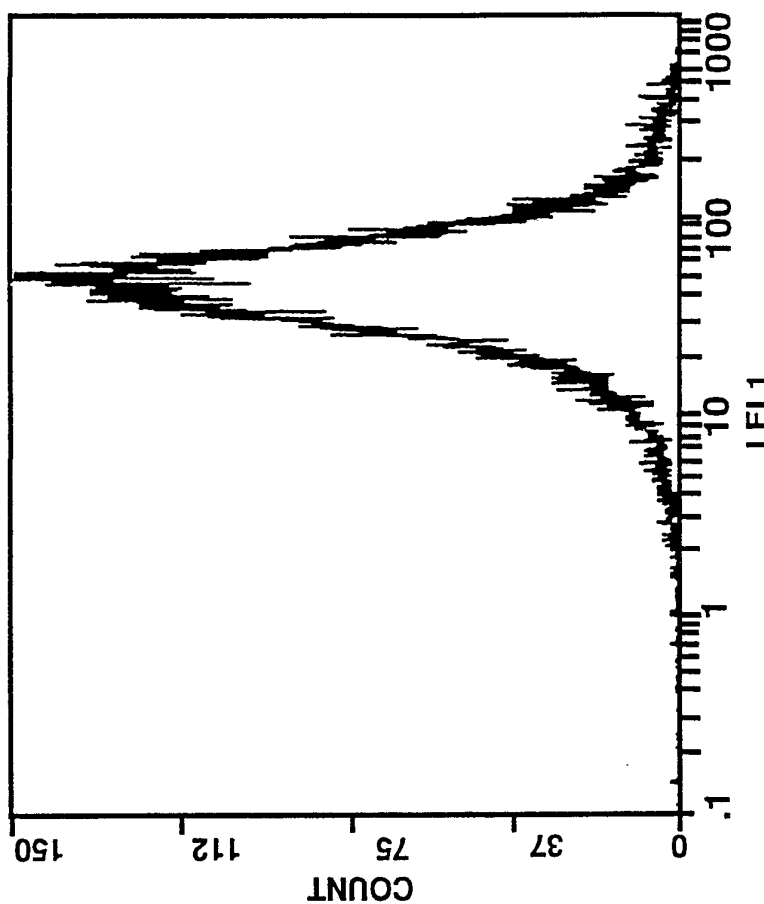


FIG. 6D.

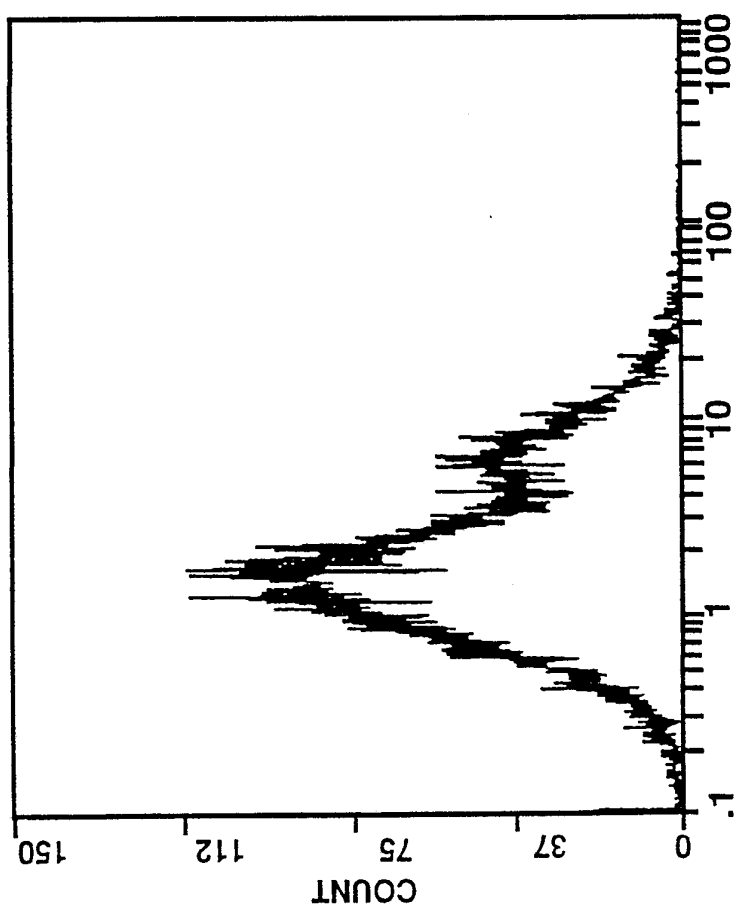


FIG. 6C.

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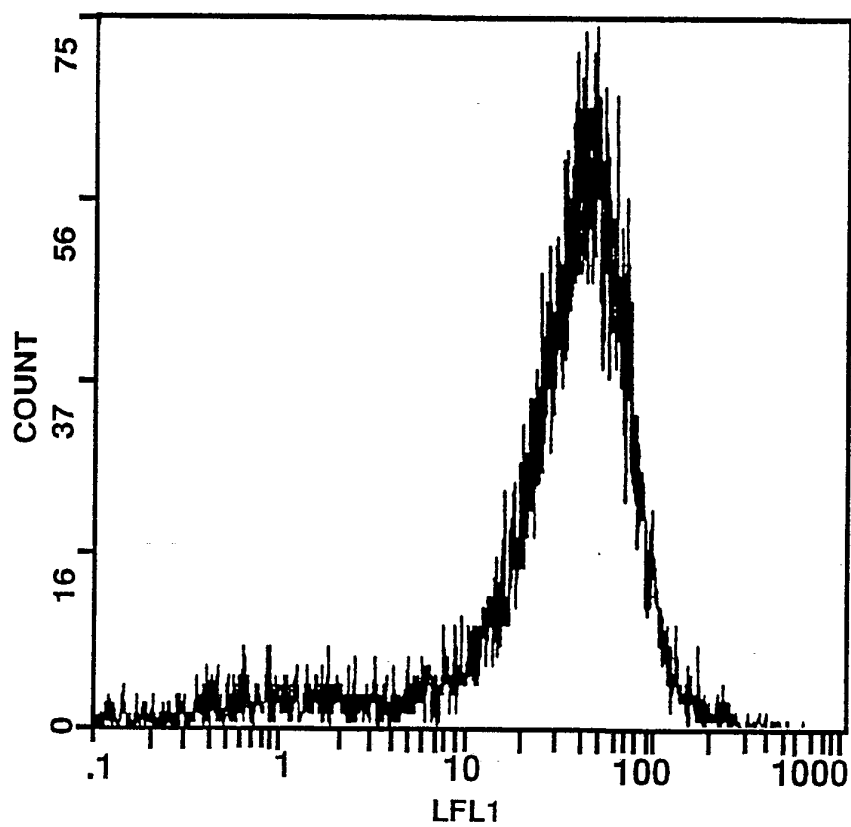


FIG.6E.

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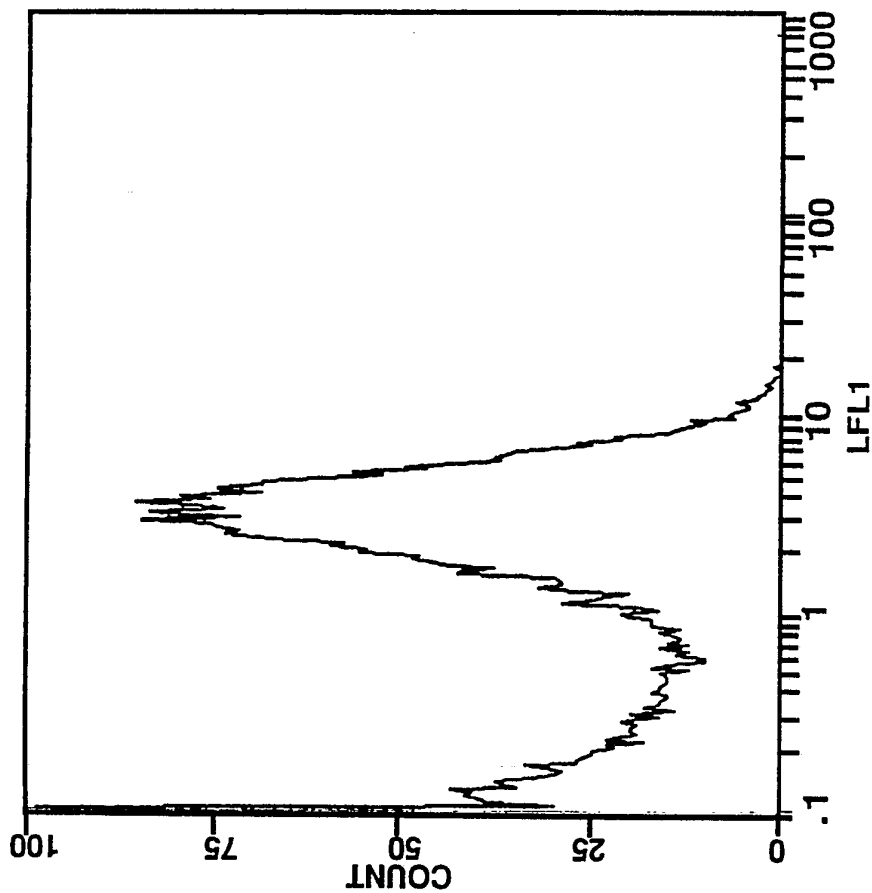


FIG. 7B.

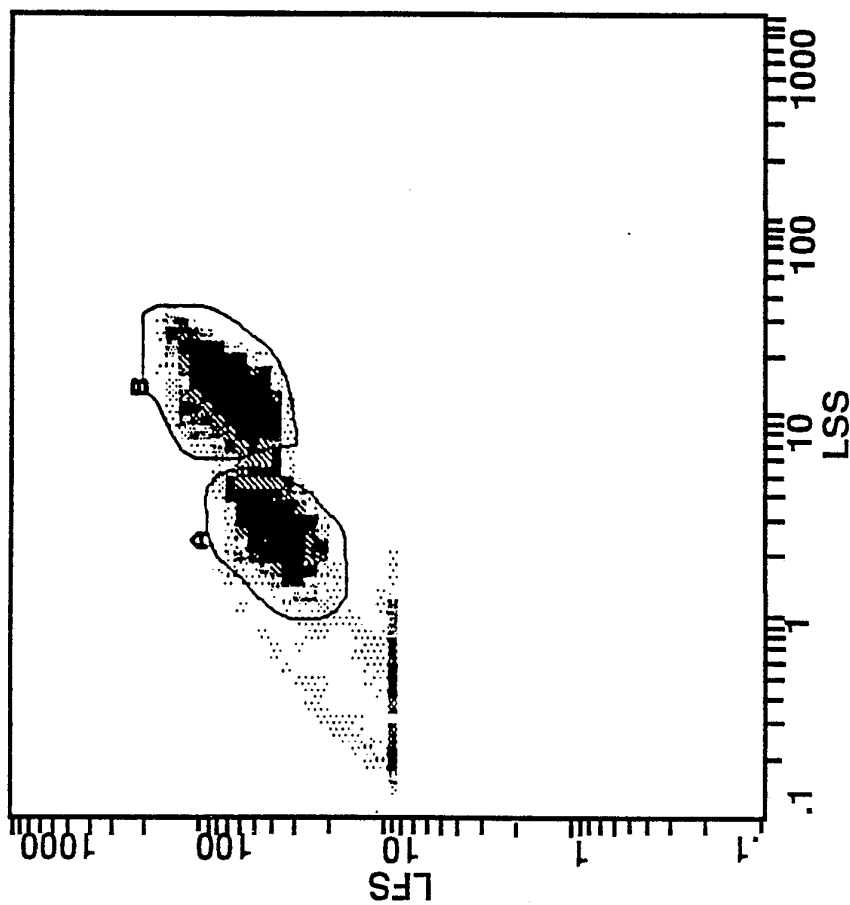


FIG. 7A.

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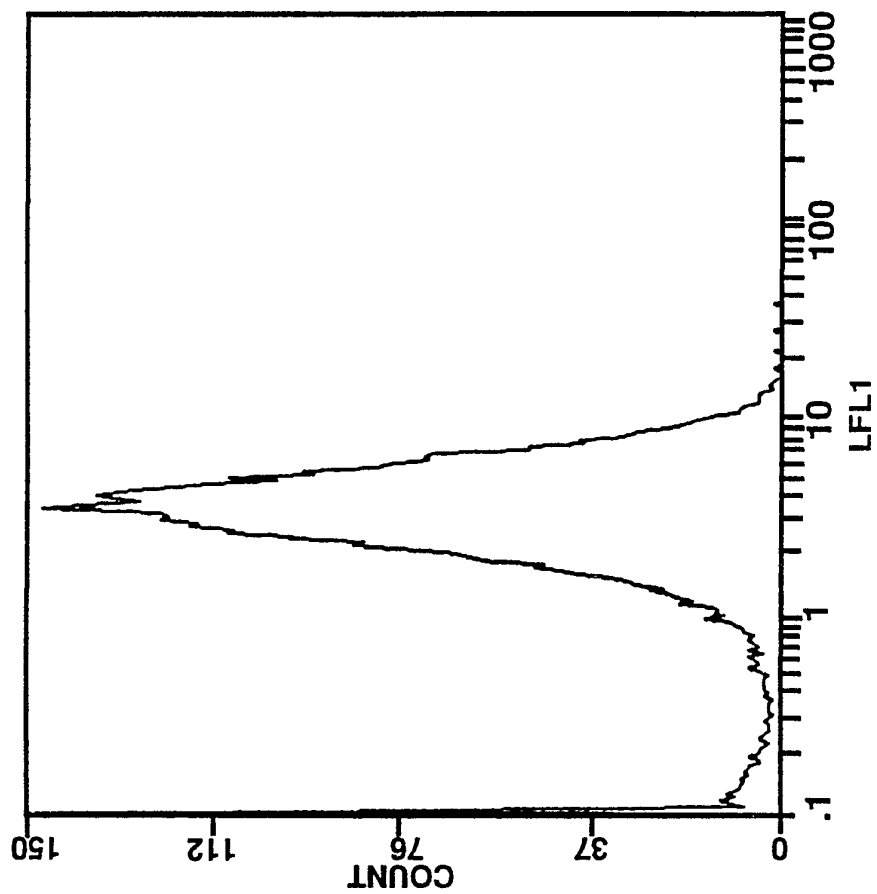


FIG. 7D.

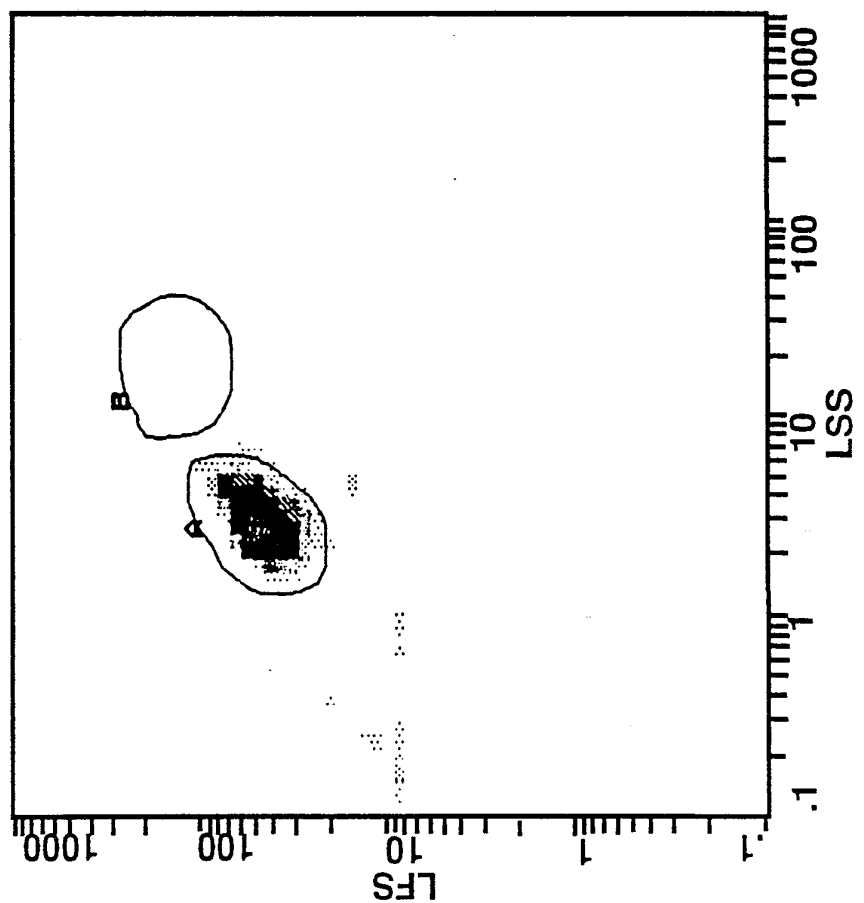


FIG. 7C.

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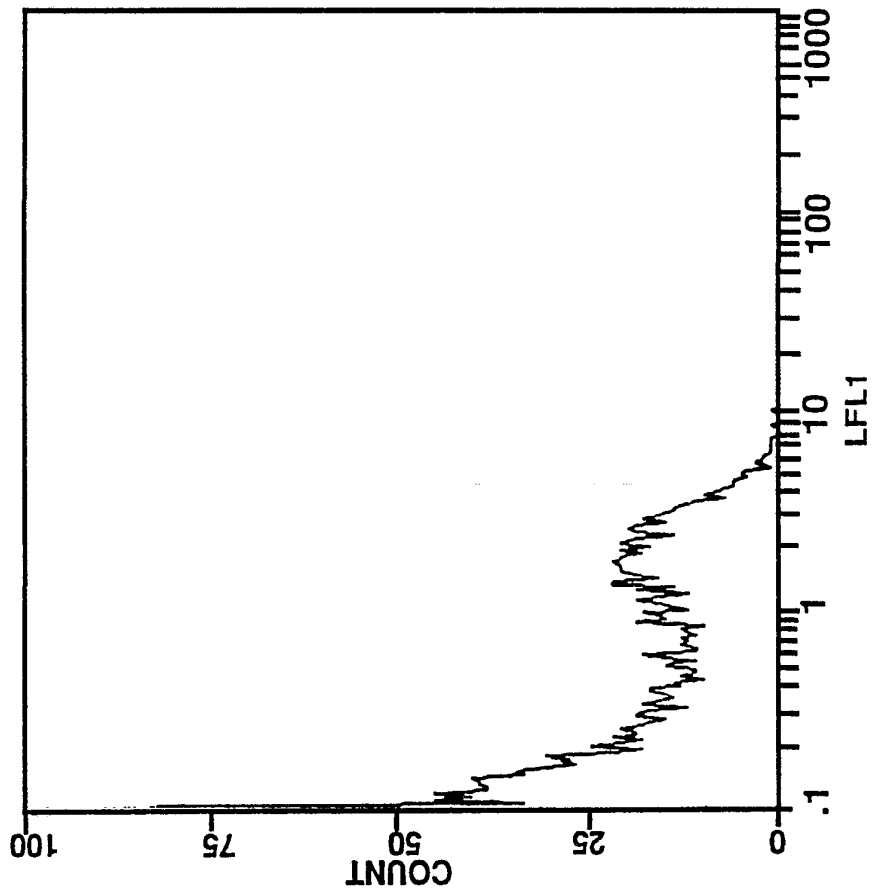


FIG. 7F.

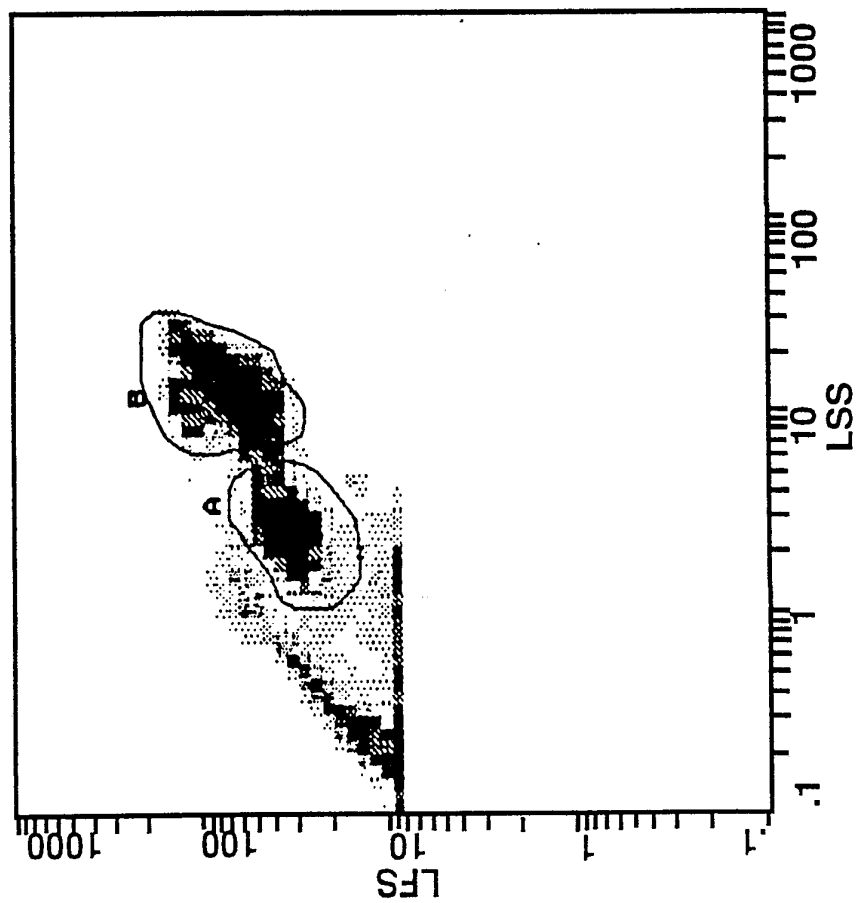


FIG. 7E.

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Mr($\times 10^{-3}$) A B C D E F G H K

92 —   

68 —  

45 —

29 —

FIG. 8.

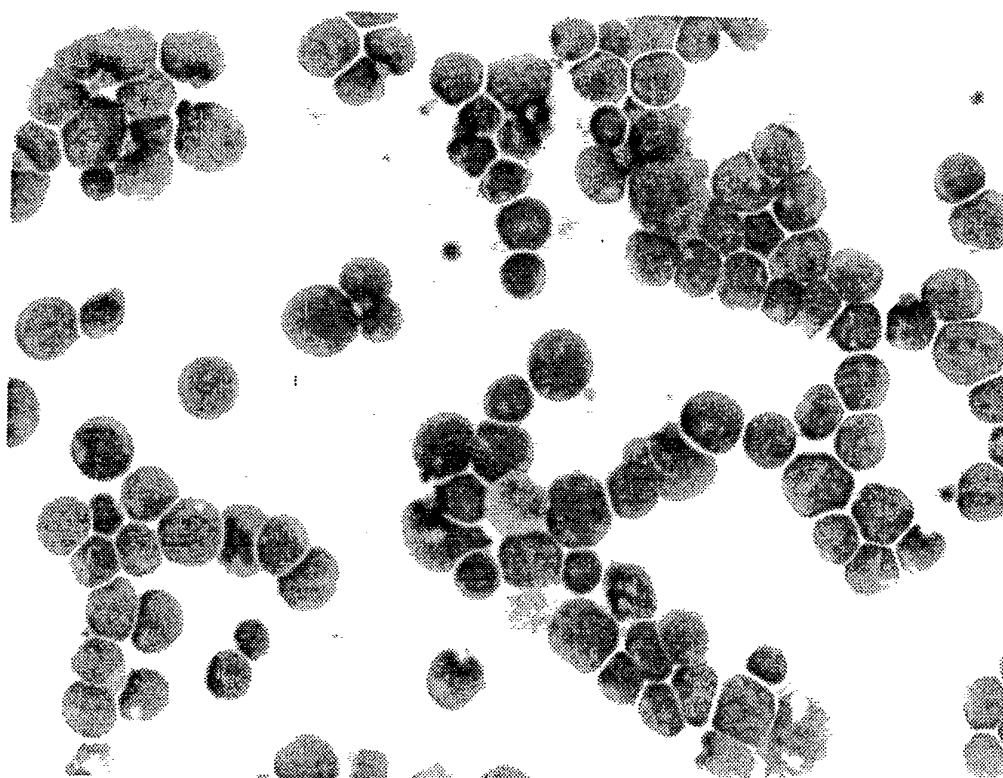
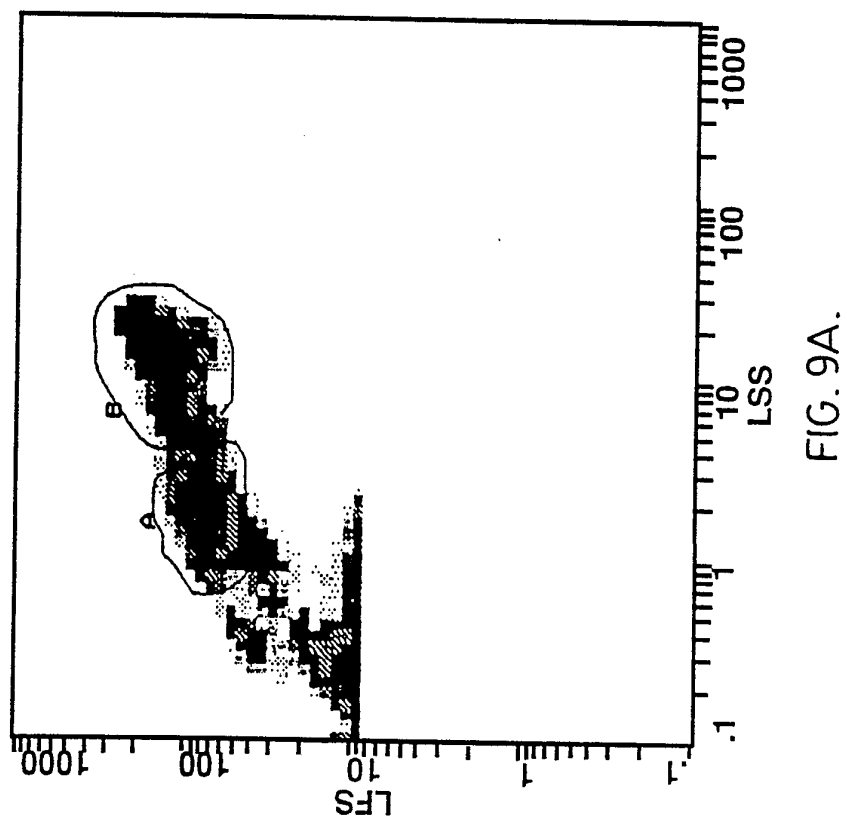
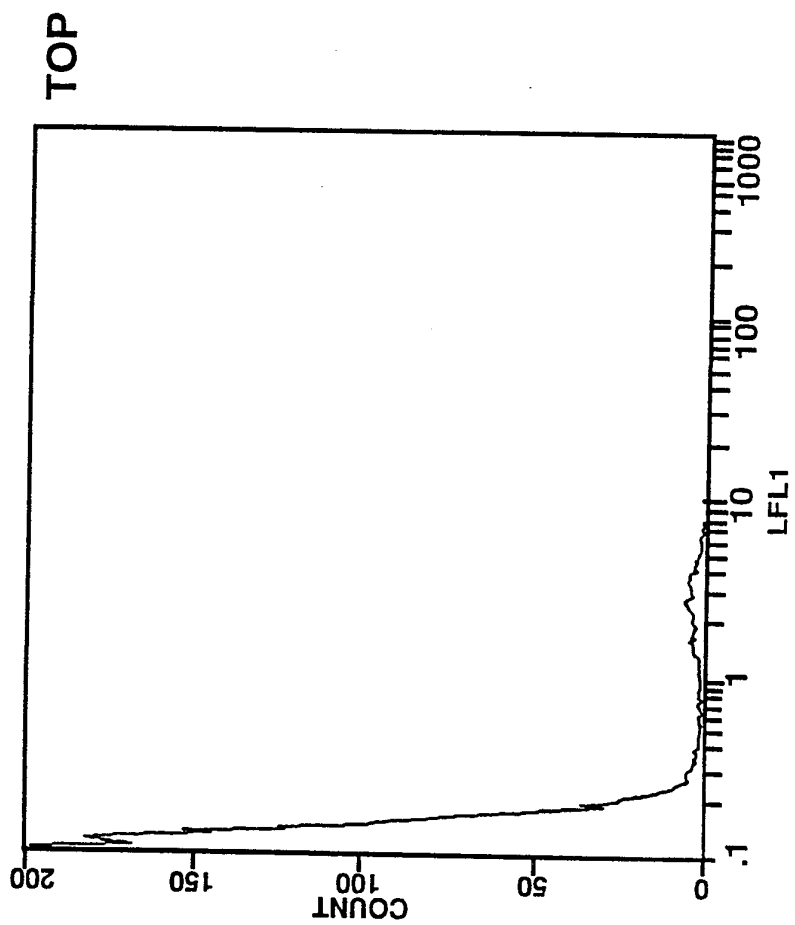


FIG. 10A.

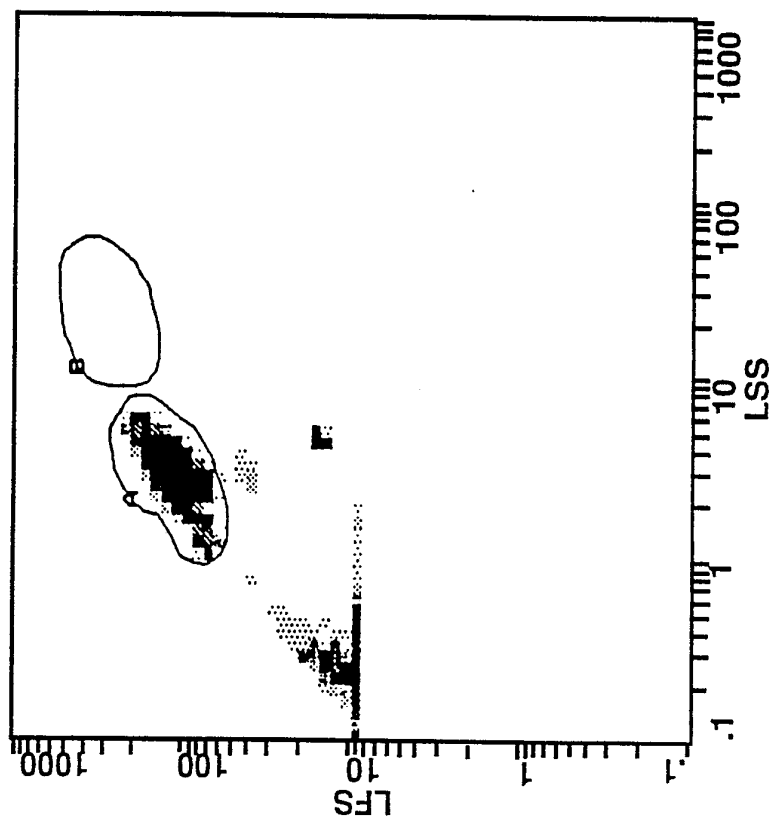
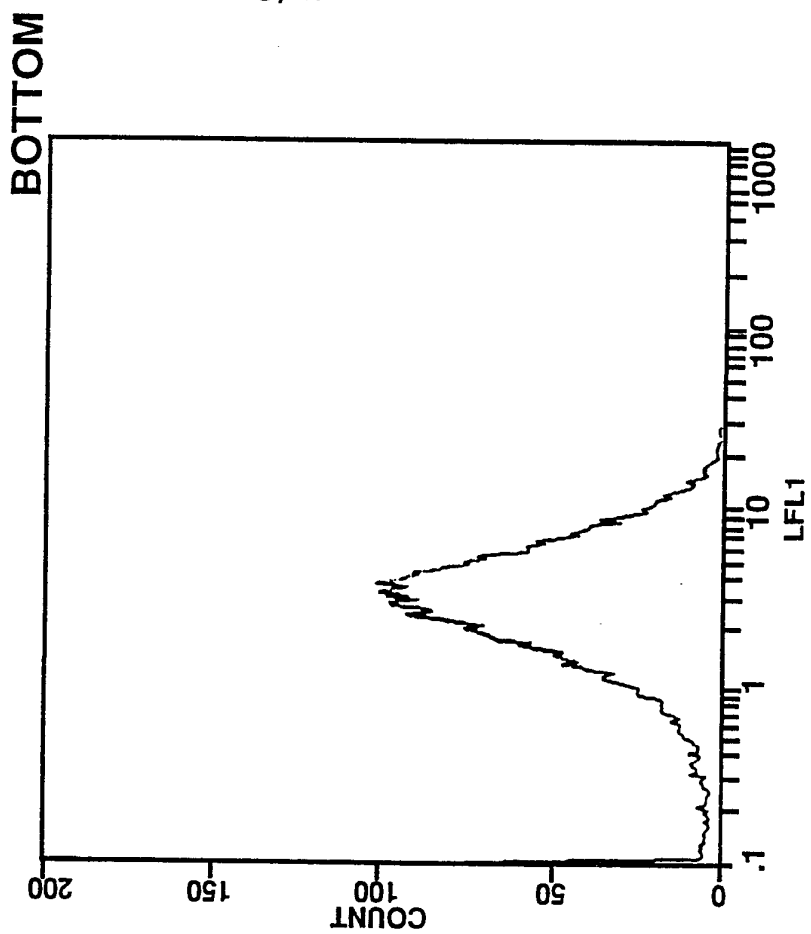
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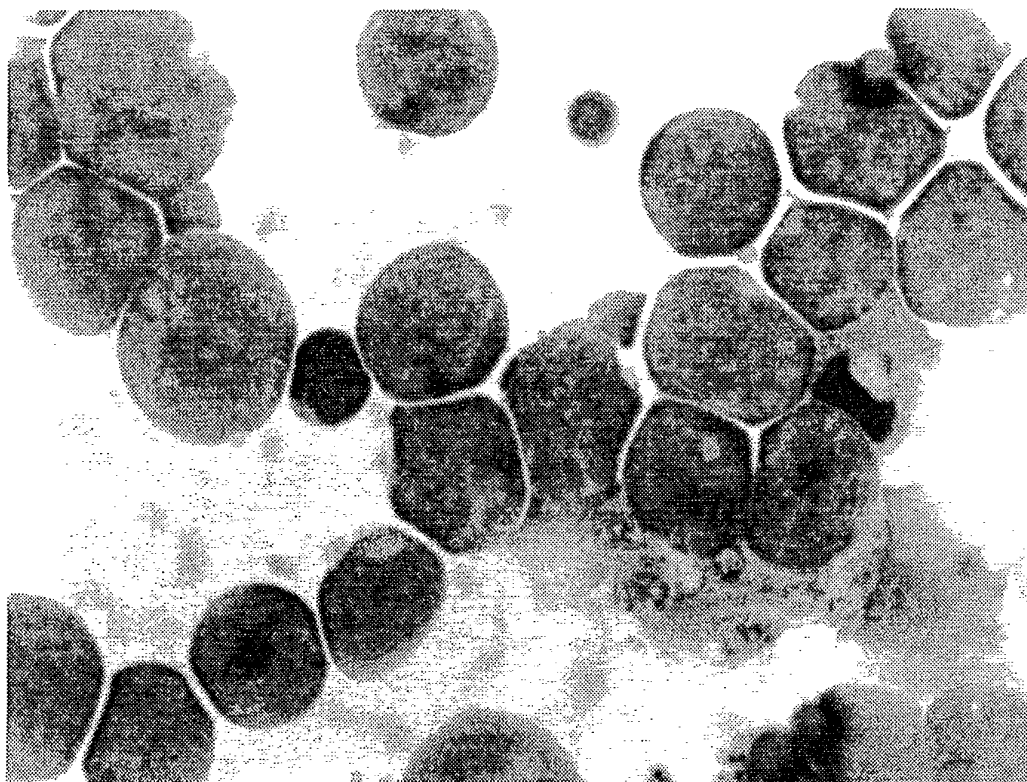


FIG.10B.

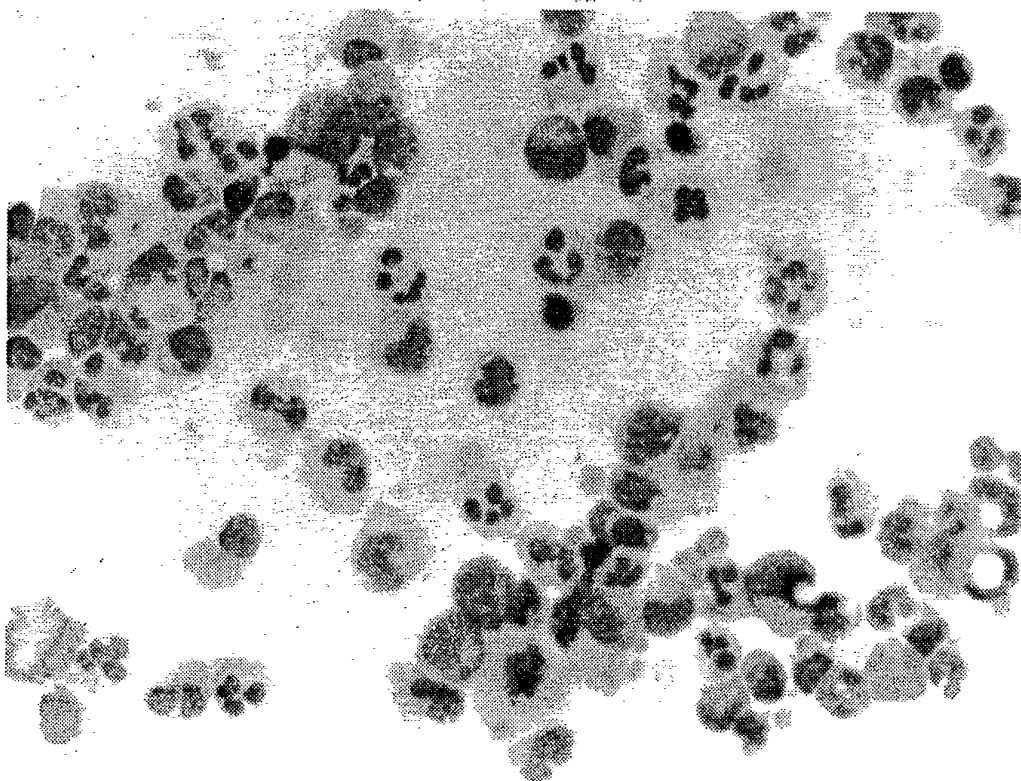


FIG.10C.

INTERNATIONAL SEARCH REPORT

PCT/CA 91/00454

International Application No

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C12Q1/24; C12N5/08; //A61K35/28		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	A61K ; C12Q ; G01N ; C12N C07K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claims No. ¹³
X,P	EP,A,0 395 355 (THE JOHNS HOPKINS UNIVERSITY) 31 October 1990 see page 3, line 40 - line 50 see page 4, line 34 - line 51 see examples 1-14 see claims 1-10	14-17
Y,P	---	1-13
Y	US,A,4 906 571 (ALAN MELLORS) 6 March 1990 see the whole document	1-13
X	---	14-17
X	US,A,4 714 680 (CURT I. CIVIN) 22 December 1987 see the whole document	14-17
	---	14-17
	EP,A,0 341 966 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 15 November 1989 see the whole document	14-17

¹⁰ Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
25 FEBRUARY 1992	11. 03 92	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	FERNANDEZ Y BRA F.	

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. CA 9100454
SA 54571**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 25/02/92

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0395355	31-10-90	CA-A- 2014965 JP-A- 3061480	25-10-90 18-03-91
US-A-4906571	06-03-90	None	
US-A-4714680	22-12-87	US-A- 4965204	23-10-90
EP-A-0341966	15-11-89	JP-A- 2042978	13-02-90