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(54) Title: NEW ASSAYS FOR PREIMPLANTATION FACTOR AND PREIMPLANTATION FACTOR PEPTIDES

(57) **Abrégé/Abstract:**

The present invention relates to assay methods used for detecting the presence of PIF, and to PIF peptides identified using this assay. In particular, the present invention relates to flow cytometry assays for detecting PIF. It is based, at least in part, on the observation that flow cytometry using fluorescently labeled antilymphocyte and anti-platelet antibodies demonstrated an increase in rosette formation in the presence of PIF. It is further based on the observation that flow cytometry demonstrated that monoclonal antibody binding to CD2 decreased in the presence of PIF. The present invention further relates to PIF peptides which, when added to Jurkat cell cultures, have been observed to either (i) decrease binding of anti-CD2 antibody to Jurkat cells; (ii) increase expression of CD2 in Jurkat cells; or (iii) decrease Jurkat cell viability. In additional embodiments, the present invention provides for ELISA assays which detect PIF by determining the effect of a test sample on the binding of anti-CD2 antibody to a CD2 substrate.



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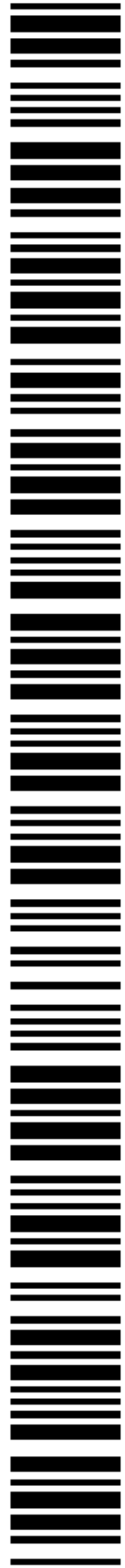
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NEW ASSAYS FOR PREIMPLANTATION FACTOR AND PREIMPLANTATION FACTOR PEPTIDES

1. INTRODUCTION

The present invention relates to preimplantation factor ("PIF") a very early marker of fertilization and embryo viability, to new methods for detecting PIF activity and to PIF peptides.

2. BACKGROUND OF THE INVENTION

Infertility is a major health care concern affecting millions of couples worldwide. Contributing to this problem, early demise of the human conceptus is a common event. Approximately 73 % of natural single conceptions are lost before reaching week 6 of gestation (Boklage CE. Survival probability of human conceptions from fertilization to term. *Int J Fertil* 1990; 35:75). This is mostly due to early embryonic demise prior to implantation or soon after implantation occurs. Data relating to the low fertility rate observed in older women and its improvement by oocyte donation from young women indicate that oocyte quality is an important factor in achieving a successful pregnancy (Navot D, Bergh PA, Williams MA et al. Poor oocyte quality rather than implantation failure as a cause of age-related decline female fertility. *Lancet* 1991;337:1375).

In vitro fertilization ("IVF") is a technology which has been developed to address the problem of infertility. However, maintaining embryo viability is even more problematic under the artificial conditions used for culturing embryos *in vitro* for implantation. *In vitro*, the embryo development rate is lower than *in vivo* and only 25-65 % of embryos typically develop to the blastocyst stage (Gardner DK, Lane M, KOuridakis K, Schoolcraft WB. Complex physiologically based serum-free culture media increase mammalian embryo development. In: Gomel V, Leung PCK, eds. *In vitro* fertilization and assisted reproduction. Procc 10th World Congress, 1997:187). The state of the art is not yet able to identifying embryos likely to implant and survive. Human chorionic gonadotrophin ("hCG"), the currently used marker for fertilization *in vivo* and early embryo implantation, can only be detected several days after implantation. As a result of the lack of a suitable marker for embryo viability,

nowadays many embryos incapable of implanting are being transferred, thus lowering the chance for achieving successful pregnancy.

To address the possibility that embryos may not be viable, a greater number of embryos are simultaneously transferred into a potential mother. The transfer of a high number of embryos may lead to multiple pregnancies, which are inherently risky, while transfer of a small number of embryos carries the risk that none would implant, losing a whole IVF cycle. Clearly, there is a need to improve embryo selection and define accurate markers to determine embryo viability. In addition, using non-invasive methods by testing culture media for products specific to viable, implantation-competent embryos would allow selection of those most likely to result in successful pregnancies, without causing embryo damage.

Another factor involved in determining whether a pregnancy is successful or not is the interaction between the conceptus and the mother's immune system. Shortly after fertilization a systemic maternal recognition of pregnancy should occur. The mother's immune system modulation triggered by specific early embryo signals could be the key of this process. Once the oocyte is fertilized, the zygote up to hatching blastocyst is surrounded by the zona pellucida, a hard semi permeable membrane. Therefore the embryo-maternal communication must occur simultaneously while the embryo is developing in the oviduct and uterine cavity through compounds that are secreted by the embryo.

It has been shown that pregnant sera and viable embryo conditioned culture media can produce an increase in rosette formation by platelets and T lymphocytes in the presence of CD2 antibody. As disclosed in United States Patent No. 5,646,003 by Barnea et al., issued July 8, 1997, and in United States Patent No. 5,981,198 by Barnea et al., granted November 9, 1999, the presence of Preimplantation Factor ("PIF") can be detected by mixing lymphocytes, platelets, heat inactivated serum from a pregnant subject, guinea pig complement, and T11 (anti-CD2) monoclonal antibody (Dakko, Denmark), where rosette formation between platelets and lymphocytes is increased by PIF in pregnant subjects. PIF has been found to be (i) secreted by viable early human and mouse embryos from the two-cell stage onward; detectable in the peripheral circulation 3-4 days after embryo transfer following IVF; (iii) associated with 73% take home babies vs 3 % in early negative PIF results; (iv) detectable 5-6 days after intrauterine insemination; (v) absent in non-pregnant serum, or non-viable embryos; and (vi) present in various pregnant

mammals in addition to humans, including mice, horses, cows and pigs. In addition, PIF has been observed to disappear from the circulation two weeks before hCG secretion declines in cases of spontaneous abortion.

The monoclonal antibody used in the above-mentioned PIF assay is directed toward the lymphocyte associated antigen referred to as CD2. CD2 is present on about 80-90% of human peripheral blood lymphocytes, greater than 95% of thymocytes, all T lymphocytes that form erythrocyte rosettes and a subset of NK cells. Various roles for CD2 in T cell activation have been proposed, including function as an adhesion molecule which reduces the amount of antigen required for T cell activation and as a costimulatory molecule or direct promoter of T cell activation. Moreover, CD2 has been implicated in the induction of anergy, the modulation of cytokine production and the regulation of positive selection of T-cells.

The natural ligand for CD2 is the structurally related IgSF CAMs CD58 (LFA-3), a cell-surface adhesive ligand with broad tissue distribution. In addition, CD2 can interact with CD48, CD59 and CD15 (Lewis x)-associated carbohydrate structure. CD2 binds CD58 with very low affinity and an extremely fast dissociation constant. The lateral redistribution of CD2 and its ligand CD58 also affect cellular adhesion strength. Regulation of CD2 adhesiveness affects the ability of CD2 to enhance antigen responsiveness. CD2-cell lines incapable of avidity regulation exhibit a marked deficiency in an antigen-specific response. Strength of adhesion resulting from increased CD2 avidity contributes directly to T-cell responsiveness independently of CD2-mediated signal transduction.

3. SUMMARY OF THE INVENTION

The present invention relates to assay methods used for detecting the presence of PIF, and to PIF peptides identified using this assay. In particular, the present invention relates to flow cytometry assays for detecting PIF. It is based, at least in part, on the observation that flow cytometry using fluorescently labeled anti-lymphocyte and anti-platelet antibodies demonstrated an increase in rosette formation in the presence of PIF. It is further based on the observation that flow cytometry demonstrated that monoclonal antibody binding to CD2 decreased in the presence of PIF.

The present invention further relates to PIF peptides which, when added to Jurkat cell cultures, have been observed to either (i) decrease binding of anti-

CD2 antibody to Jurkat cells; (ii) increase expression of CD2 in Jurkat cells; or (iii) decrease Jurkat cell viability. In additional embodiments, the present invention provides for ELISA assays which detect PIF by determining the effect of a test sample on the binding of anti-CD2 antibody to a CD2 substrate.

4. BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1A-B. PIF purification from mouse embryo culture conditioned medium (MECCM); (A) shows a high performance liquid chromatography ("HPLC") profile of MECCM-3kDA ultra-filtrate previously purified by MabCD2 affinity chromatography; (B) shows the profile following additional HPLC purification of a PIF-active fraction from (A).

FIGURE 2A-C. Western blot analysis of different PIF peptides purified from MECCM; MabCD2 was used as a primary antibody and anti-sense mouse horseradish peroxidase (HRP)-biotin streptavidin complex was used as secondary antibody. Specific PIF bands were identified by the ECL detection reagents (Amersham Pharmacia Biotech).

FIGURE 3A-C. (A) shows flow cytometric determination of lymphocyte-platelet rosette formation (L-P) in the presence of fresh culture medium (CM) and mouse embryo culture conditioned medium (MECCM) and MabCD2. Fluorescent labeled specific antibodies to L (MabCD45-PE) and to P (MabCD42a-FITC) were used to detect the L-P complex. MECCM gave 30-40 percent higher formation of L-P compared to culture medium (CM). (B) shows FC of MECCM effect on MabCD2 binding to Jurkat cells (JC). JC were incubated with samples and further with MabCD2 Cy5. Antibody binding to CD2 decreased by PIF present in MECCM. Arrows indicate PIF activity.

FIGURE 4A-C. Mass spectrum from PIF peptides purified from MECCM. Molecular weight (MW) of PIF-active fractions from MECCM purified by ultrafiltration, diafiltration, HPLC, MabCD2-affinity chromatography and by additional HPLC was determined by mass spectroscopy. MW of PIF peptides were A) 610-995 Da; B) 963-1848 Da; and C) 1807-1846 Da.

FIGURE 5A-F. Flow cytometric analysis of PIF negative effects on MabCD2 binding (A), fluorescence (B) and viability (C) in Jurkat cells, and of PIF positive effects on MabCD2 binding (D), fluorescence (E) and viability (F). PIF in positive samples competes with CD2 (arrows indicate PIF activity).

FIGURE 6. Effect of Synthetic PIF peptides on CD2 expression on Jurkat cells.

5. DETAILED DESCRIPTION OF THE INVENTION

In a first set of embodiments, the present invention provides for a method for determining the presence of preimplantation factor in a sample, comprising the step of detecting whether the sample contains a component which inhibits the binding of an anti-CD2 antibody to CD2 antigen; wherein the ability to inhibit the binding of anti-CD2 antibody to CD2 has a positive correlation with the presence of preimplantation factor.

Such a method may, for example, be employed in a flow cytometry method or in an enzyme-linked immunosorbent assay method, using techniques otherwise known in the art. A non-limiting example of a flow cytometry method for detecting anti-CD2 antibody binding to CD2 is presented in Section 7, below.

An anti-CD2 antibody, as that term is used herein, may be a monoclonal or polyclonal antibody which specifically binds to CD2. Such a monoclonal antibody is sold by Pharmigen (see below).

CD2 antigen may be in the form of purified CD2 antigen or may be carried by a cell. In non-limiting embodiments of the invention, the cell is a Jurkat cell. Other CD2-expressing cell lines are known in the art.

The sample may be a serum sample (for example, serum from a subject to be tested for fertilization/implantation/persistence of embryo), may be a sample of culture fluid (for example, to determine the viability of embryos prior to transfer for IVF), or may be a solution to be tested for the presence of a PIF peptide (for example, during the purification of PIF acting agents; see Section 6, below).

The subject may be a human subject (for example, a human suspected of being pregnant) or a non-human subject (for example an agricultural animal or a zoo animal).

In a second set of embodiments the present invention provides for a method for determining the presence of preimplantation factor in a sample, comprising the step of detecting, by flow cytometry, whether the sample contains a component which increases the formation of rosettes between lymphocytes, platelets, and anti-CD2 antibodies, where an increase in rosette formation has a positive correlation with the presence of preimplantation factor. Such an assay may be

performed, for example, using fluorescently labeled antibodies directed toward lymphocytes and platelets, where preferably different labels are used for anti-platelet and anti-lymphocyte antibodies.

The increase is relative to a known negative control.

The present invention also provides for the following isolated peptides:

(1) An isolated peptide having a sequence selected from the group consisting of: Met-Val-Arg-Ile-Lys-Pro-Gly-Ser-Ala; Met -Val-Arg-Ile-Lys-Pro-Gly-Ser-Ala-Asn-Lys-Pro-Ser; Met -Val-Arg-Ile-Lys-Pro-Gly-Ser-Ala-Asn-Lys-Phe-Ser-Asp; and Met -Val-Arg-Ile-Lys-Pro-Gly-Ser-Ala-Asn-Lys-Pro-Ser-Asp-Asp, or an isolated peptide comprising said peptide which binds to anti-CD2 antibody and which is not a circumsporooite protein;

(2) An isolated peptide having a sequence Ser-Gly-Ile-Val-Ile-Tyr-Gln-Tyr-Met-Asp-Asp-Arg-Tyr-Val-Gly-Ser-Asp-Leu, or an isolated peptide comprising said peptide which binds to anti-CD2 antibody and which is not an HIV protein;

(3) An isolated peptide having a sequence Val-Ile-Ile-Ile-Ala-Gln-Tyr-Met -Asp or an isolated peptide comprising said peptide which binds to anti-CD2 antibody; and

(4) An isolated peptide having a sequence selected from the group consisting of Ser-Gln-Ala-Val-Gln-Glu-His-Ala-Ser-Thr and Ser-Gln-Ala-Val-Gln-Glu-His-Ala-Ser-Thr-Asn-Xaa-Gly, where Xaa can be any amino acid, or an isolated peptide comprising said peptide which binds to anti-CD2 antibody and which is not a silencing mediator for human retinoid and thyroid hormone.

6. EXAMPLE: IDENTIFICATION OF PIF PEPTIDES

PIF was isolated from a large volume of MECCM using ultra filtration, lyophilization, high performance chromatography (HPLC), affinity chromatography and western blot. Two-cell-to blastocyst stage mouse embryos were cultured for several days in Ham's F-10 medium with penicillin, streptomycin, MgSO₄, NaHCO₃, KHCO₃, and Ca lactate supplemented with 0.1% BSA. MECCM collected was stored at -80°C until used.

One liter of MECCM was purified by ultra filtration through an Amicon membrane (3 kDa cut-off; YM- 3 kDa, Amicon. Millipore Co, USA). Concentrated MECCM was further diafiltered using 300 ml of pure water. In addition, fresh culture media (CM, without embryos) was processed in the same way. Only

MECCM-3kDa ultra filtrate and diafiltrated demonstrated PIF activity and then they were pooled and concentrated by lyophilization.

It was observed that PIF is able to bind to anti-CD2 monoclonal antibody ("MabCD2"). Therefore, PIF-active fractions were purified first by affinity chromatography performed with agarose-hydrazide-MabCD2 activated gels. An antibody affinity matrix was prepared as follows. 1.5 mg of MabCD2 (clone RPA-2.10, Pharmigen, Becton Dickinson) was buffer exchanged with the coupling buffer pH 5.5 using the Econo-Pac 10DG desalting column provided and further oxidized with sodium periodate and coupled to 2 ml of agarose-hydrazide activated gel following the manufacturer's indications (Affi-gel Hydrazide immunoaffinity kit, BioRad Laboratories, CA, USA). Then, MECCM-3kDa ultra filtrate-diafiltrate lyophilized powder was further purified using the MabCD2-affinity chromatography column (10 x 20 mm). 2 g of MECCM-3kDa powder were dissolved in 10 ml of pure water, pH neutralized, filter-out through a 0.22 µm syringe sterile filter (Corning Inc., NY, USA) and passed 5 times through the affinity chromatography column at gravity flow. The column was washed-out with 5 volume bed of 100 mM phosphate saline buffer, pH 7.2, followed by washing with 5 volume bed of 0.5 M NaCl.

The bound PIF was eluted with 3 ml of 0.1 M acetic acid. PIF-eluted fractions were pooled, assayed for PIF activity and concentrated by lyophilization.

A total of 300 mg of MECCM-3 kDa ultra filtrate further purified by affinity chromatography were run in three batches by HPLC on a Cliepus C18 preparative column (Higgins Analytical, Inc., USA). Preparative HPLC running parameters were: flow, 15 ml/min. Buffers: A= 0.1 % trifluoroacetic acid (TFA); B= 0.1 % TFA in 99.9% acetonitrile (CH₃CN). Gradient: 0%B, during 5 min plus 0-60%B for 30 min and 0-100%B for 3 min.

Fractions from HPLC were further concentrated by evaporation. HPLC concentrated fractions were pH neutralized and re-assayed for PIF-activity. Several fractions showed high PIF-activity (see Fig 1A). These fractions were purified by additional HPLC on a Vydac C8 analytical column (4.6x250 mm; Hesperia, CA, USA). Additional HPLC running parameters: flow, 1 ml/min. Buffers: A= 0.1 % trifluoroacetic acid (TFA); B= 0.1 % TFA in 99.9% acetonitrile (CH₃CN). Gradient: 0%B, during 5 min plus 0-60%B for 30 min and 0-100%B for 3 min. Several eluted fractions showed PIF activity (see Fig 1B) and were further sequenced for amino acid composition and their molecular weight (MW) was determined by mass-spectrometry.

PIF active fractions purified from MECCM gave positive signals in Western blots ("WB"). Solutions from CM ultra filtrate- lyophilize fraction was used as negative control in WB. The WB conditions were as follows. For gels, SDS-PAGE pre-casting gels (BioRad) were used, having a 16.5% agarose resolving gel and a 4% agarose stacking gel. The gels were run in 100 mM Tris, 100mM Tricine, 0.1 % SDS, pH 8.3 (Tris-tricine running buffer). Samples consisting of 30 microliters of PIF–MECCM purified fractions plus 10 microliters of Tricine sample buffer [200 mM Tris (hydroxymethyl) aminomethane (Tris-HCl) pH 6.8, 2 % sodium duodecyl sulphate (SDS), 40% glycerol, 0.04% Coomassie blue brilliant (CBB-G250)] (BioRad) were incubated at 95°C during 5 min. After cooling, the samples were loaded into the wells of the SDS-polyacrylamide gels (PAGE). To determine the molecular weight of low molecular weight (MW) polypeptides, 10 microliters of a 1:20 water dilution of SDS-PAGE standards (BioRad) were loaded into a well of each gel. For electrophoresis, samples and standards were run at 175 v during 5min plus 60 v for 1 h.

The resulting gels were then electro-blotted using, as transfer buffer, 100 mM CAPS [3-(cyclohexylamino)-1-propanesulfonic acid) buffer, pH 11. Electro- blotting was performed at 80 mA during 1h onto a 0.22 µm nitrocellulose membrane (BioRad).

Then, nitrocellulose membranes were blocked with 5 % blocking solutions (Amersham, Pharmacia, Biotech, NJ, USA) at room temperature during 18 h., and then were washed-out 4 times during 20 min with PBS-T [phosphate saline buffer -0.05% polyoxyethylenesorbitan monolaurate (Tween 20)]. For the primary antibody incubation, blocked membranes were incubated with 2 µg/ml MabCD2 (Pharmigen) – PBS-T solutions at room temperature during 2h, and then washed as above. For the secondary antibody incubation, the membranes were incubated with anti mouse IgG-horse radish peroxidase conjugate (1:1000 in PBS-T) solution at room temperature during 1h. PIF bands were then visualized using the ECL-chemiluminescent system (Amersham). Figure 2 shows a typical WB of PIF peptides purified from MECCM.

A flow cytometric methodology (FC) for measuring PIF was developed to improve the efficiency and reproducibility of methods set forth in United States Patent Nos. 5,646,003 and 5,981,198. In particular, rosette formation was evaluated by FC with pregnant and non-pregnant human and porcine serum, MECCM, CM and isolated PIF-fractions using MabCD2 or MabCD2-Cy5 (Cy-

chrome conjugated antibody), MabCD45-PE (phycoerythrin conjugated antibody) and MabCD41a-FITC (fluorescein isothiocyanate conjugated antibody), all antibodies were from Pharmingen. The ratio of labeled P-L complex was higher by 30-40 % with MECCM versus CM (Fig 3A). Further, it was found that pre-incubation of MECCM or pregnant sera with immobilized MabCD2 prevented the P-L formation in the assay. The addition of a MabCD58 (lymphocyte function-associate antigen-3 or LFA-3) antibody to L-P did not prevent totally the rosette formation by effect of PIF-active samples in the assay.

A FC-PIF quantitative assay using Jurkat cells (JC) and MabCD2-Cy5 was developed (Fig 3B). The use of an immortalized leukemia cell line avoids the need for fresh donor blood to assess the PIF activity by the bioassay. The JC-FC assay was validated with human serum samples (see Table I) and was used to assess PIF activity of fractions during PIF purification.

MW of purified PIF-active fractions was determined by mass spectral analysis on a Voyager-RP Biospectrometry MALDI-TOF Workstation from Perseptive Biosystems (Cambridge, MA, USA). Samples were mixed with a matrix consisting in a 1:2 mixture of acetonitrile:water containing 1% trifluoroacetic acid. Spectra were averages of approximately 200 scans. PIF- peptides from MECCM have MW between 610 - 1845 Da (Fig 4).

Further, it was assessed that pre-incubation of PIF-active fractions with MabCD2 abolished the PIF-activity. These data indicated that PIF could be a portion of CD2 or homologue peptides. However, after the sequencing of purified PIF peptides it was demonstrated that these peptides are not a portion of CD2 and their amino acid sequences are unique.

Using the JC-FC assay it was demonstrated that MECCM-PIF peptides have three different effects on CD2 expressed by T cells. These effects are related to: decreasing MabCD2 binding to the JC; up-regulating CD2 expression by JC; or decreasing JC viability.

Purified PIF active fractions from mouse embryos were sequenced by Edman degradation on an Applied Biosystems Pulsed Liquid Sequencer (model 477A). Released amino acids were derivatized with phenylisothiocyanate to give the PTH-amino acids which were detected by reverse phase-HPLC on a HPLC system in line with the sequencer. Several of the PIF fractions yielded unique sequences. Several peptides gave sequences whose N-terminal nine and ten residues were

identical indicating that the peptides were various truncated forms of common molecules (see Table II). PIF peptides were identified as a least three unique families of embryo-derived and pregnancy-related small peptides. The amino acid sequence of a family of three PIF peptides matches 100% with a region of Circumsporozoite protein (malaria parasite: *Plasmodium falciparum*). This family of PIF peptides up regulates the CD2 expression by JC. A PIF peptide (14 amino acids) that shares only the five first amino acid residues with the former described PIF-peptide's family and another PIF peptide (18 amino acids) that matches in 11 amino acids to the sequence of HIV-1 RNA directed DNA polymerase (reverse transcriptase, EC 2.7.7.49) also up regulate the CD2 expression by JC. In addition, another family of two PIF peptides (9 and 13 amino acids) matches in 10 amino acids with the sequence of the human receptor-interacting factor, a silencing mediator for retinoid and thyroid hormone receptor (SMRT) (Chen and Evans, 1995). The shorter member of this PIF-peptide family shows a competitive effect for the binding of MabCD2 to JC and the longer PIF-peptide decrease the viability of JC. It is worth to notice that transcriptional silencing mediated by nuclear receptors is important in development, differentiation and oncogenesis.

PIF peptides were synthesized by solid-phase peptide synthesis (SPPS) on an Applied Biosystems Peptide Synthesizer employing Fmoc (9-fluorenylmethoxycarbonyl) chemistry in which the amino nitrogen of each amino acid is blocked with Fmoc. Coupling was performed by activation of the carboxyl groups of the N-protected amino acids using 3 mol/ml of 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate/1-hydroxybenzotriazole on the presence of diisopropylethylamine. Activated amino acids were sequentially added to the nascent peptide. Upon completion of the synthesis, final purification was carried out by reversed-phase HPLC and identity was verified by MALDI-TOF mass spectrometry and amino acid analysis. PIF synthetic peptides demonstrated to have similar effect on CD2 phenomenon in Jurkat cells (Fig. 6), and were also immunodetected by the Mab CD2.

7. FLOW CYTOMETRY ASSAY FOR PIF

7.1. MATERIALS

Materials included Jurkat leukemia cells (JC); cloning medium; Falcon tubes for flow cytometry measurements; Mab CD2-Cy5 (Cy-chrome conjugated

antibody, clone RPA-2.10, Pharmigen, Becton Dickinson); the biological sample (which could be a human serum to be assayed for PIF activity, or could be a solution of a putative or synthetic PIF peptide); PBS- 2 % BSA (100 mM phosphate saline buffer -2% bovine serum albumin; a negative control); trypan-blue dye; a CO₂-incubator for cell culture; and a flow cytometer.

7.2. METHOD

To prepare the JC suspension:

- . Check the viability of the JC culture using Trypan blue dye exclusion staining. Cell viability should be between 80-90%.
- . Wash twice the JC with 10 ml of PBS-2 %BSA.
- . Prepare a JC suspension in cloning medium or PBS-2 %BSA containing 5,000,000 cells/ml.
- . Dispense 50 ml of JC suspension into falcon tubes (250,000 cells/tube).

For the sample incubation:

- . Add 200 ul samples, serum from early pregnancy controls (3 positive controls) or PBS-2% BSA (negative control). Mix gently.
- . Incubate at room temperature for 20-30 min.
- . Add 200 ul of MabCD2-Cy5 diluted 1:200 in PBS-2%BSA. Mix gently.
- . Incubate at room temperature for 20-30 min.

For flow cytometric determination:

- . Measure the fluorescence of each tube (488 nm laser excitation wavelength). Compare the fluorescence of alive and total cells and total dead cells (see Fig 5) with controls.
- . Calculate PIF activity as follows:

Fluorescence of total cells / % dead cells x Fluorescence of alive cells

Interpretation of the results

PIF negative activity should be in the range of : 130-340

Positive PIF samples are out side of the negative reference range

CLINICAL VALIDATION OF THE FLOW
CYTOMETRIC PIF ASSAY IN SERUM
FROM 37 PATIENTS

Samples	Sensitivity (%)	Specificity (%)	Positive predictive Value (%)	Negative predictive Value (%)
Early pregnancy	91	92	92	92
Mid pregnancy	66	92	86	73
Late pregnancy	100	92	83	92
All pregnant samples	86	92	88	86

TABLE I.

Table II. Amino acid sequence of PIF peptides derived from pre-implantation mouse embryos

PEPTIDE CODE/ AMINO ACID NUMBER	SEQUENCE	T CELL RELATED CD2 ACTIVITY	SEQUENCE MATCHING (REFERENCE)
MECCM-4 (9 AA)	[H]-m-v-r-i-k-p-g-s-a-[OH]	Increase of CD2 expression	Match 100% in 9 AA with circumsporozoite protein (malaria parasite-Plasmodium falciparum)
MECCM-6 (13 AA)	[H]-m-v-r-i-k-p-g-s-a-n-k-f-s-[OH]	Increase of CD2 expression	Match 100% in 9 AA with circumsporozoite protein (malaria parasite-Plasmodium falciparum)
MECCM-3 (15 AA)	[H]-m-v-r-i-k-p-g-s-a-n-k-p-s-d-d-[OH]	Increase of CD2 expression	Match 100% in 9 AA with circumsporozoite protein (malaria parasite-Plasmodium falciparum)
MECCM-5 (14 AA)	[H]-m-v-r-i-k-y-g-s-y-n-n-k-p-s-d-[OH]	Increase of CD2 expression	Match 100% in 5 AA with circumsporozoite protein (malaria parasite-Plasmodium falciparum)
MECCM-7 (18 AA)	[H]-s-g-i-v-i-y-q-y-m-d-d-r-y-v-g-s-d-l-[OH]	Increase of CD2 expression	Match 100% in 11 AA with HIV- RNA-directed DNA polymerase (EC 2.7.7.49)
P14-6 (9 AA)	[H]-v-i-i-i-a-q-y-m-d-[OH]	Competition with MabCD2 for binding	No match
P13-5 (10 AA)	[H]-s-q-a-v-q-e-h-a-s-t-[OH]	Decrease of cell viability	Match 100% in 10 AA with a silencing mediator for human retinoid and thyroid hormone receptor (SMRT)
P4-3 (13 AA)	[H]-s-q-a-v-q-e-h-a-s-t-n-ta-g-[OH]	Decrease of cell viability	Match 100% in 10 AA with a silencing mediator for human retinoid and thyroid hormone receptor (SMRT)

SEQUENCE LISTING

<110> BioIncept, LLC

<120> NEW ASSAYS FOR PREIMPLANTATION FACTOR
AND PREIMPLANTATION FACTOR PEPTIDES

<130> PAT 58426W-1

<140> 2,490,538

<141> 2002-06-28

<150> PCT/US02/20599

<151> 2002-06-28

<150> US60/302,607

<151> 2001-02-07

<160> 8

<170> FastSEQ for Windows Version 4.0

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<211> 9

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<213> Mus musculus

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1

5

<210> 2

<211> 13

<212> PRT

<213> Mus musculus

<400> 2

Met Val Arg Ile Lys Pro Gly Ser Ala Asn Lys Pro Ser

1

5

10

<210> 3

<211> 14

<212> PRT

<213> Mus musculus

<400> 3

Met Val Arg Ile Lys Pro Gly Ser Ala Asn Lys Phe Ser Asp

1

5

10

<210> 4

<211> 15

<212> PRT

<213> Mus musculus

2

<400> 4

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1				5					10					15

<210> 5

<211> 18

<212> PRT

<213> Mus musculus

<400> 5

Ser	Gly	Ile	Val	Ile	Tyr	Gln	Tyr	Met	Asp	Asp	Arg	Tyr	Val	Gly	Ser
1				5					10					15	

Asp Leu

<210> 6

<211> 9

<212> PRT

<213> Mus musculus

<400> 6

Val	Ile	Ile	Ile	Ala	Gln	Tyr	Met	Asp
1				5				

<210> 7

<211> 10

<212> PRT

<213> Mus musculus

<400> 7

Ser	Gln	Ala	Val	Gln	Glu	His	Ala	Ser	Thr
1				5					10

<210> 8

<211> 13

<212> PRT

<213> Mus musculus

<220>

<221> VARIANT

<222> 12

<223> Xaa = any amino acid

<400> 8

Ser	Gln	Ala	Val	Gln	Glu	His	Ala	Ser	Thr	Asn	Xaa	Gly
1				5					10			

CLAIMS:

1. An isolated peptide comprising the sequence Met-Val-Arg-Ile-Lys-Pro-Gly-Ser-Ala (SEQ ID NO. 1), which binds to anti-CD2 antibody.
2. An isolated peptide which binds to anti-CD2 antibody, said isolated peptide comprising the sequence selected from the group of Met-Val-Arg-Ile-Lys-Pro-Gly-Ser-Ala-Asn-Lys-Pro-Ser (SEQ ID No. 2) and Met-Val-Arg-Ile-Lys-Pro-Gly-Ser-Ala-Asn-Lys-Pro-Ser-Asp-Asp (SEQ ID No. 4).
3. The isolated peptide of claim 1, wherein said isolated peptide comprises Met-Val-Arg-Ile-Lys-Pro-Gly-Ser-Ala-Asn-Lys-Pro-Ser (SEQ ID No. 2).
4. The isolated peptide of claim 1, wherein said isolated peptide comprises Met-Val-Arg-Ile-Lys-Pro-Gly-Ser-Ala-Asn-Lys-Pro-Ser-Asp-Asp (SEQ ID No. 4).
5. An isolated peptide which binds to anti-CD2 antibody, said isolated peptide comprising the sequence Ser-Gly-Ile-Val-Ile-Tyr-Gln-Tyr-Met-Asp-Asp-Arg-Tyr-Val-Gly-Ser-Asp-Leu (SEQ ID No: 5).
6. An isolated peptide which binds to anti-CD2 antibody, said isolated peptide comprising the sequence Val-Ile-Ile-Ile-Ala-Gln-Tyr-Met-Asp (SEQ ID No. 6).
7. An isolated peptide which binds to anti-CD2 antibody, said isolated peptide consisting of the sequence selected from the group of Ser-Gln-Ala-Val-Gln- Glu-His-Ala-Ser-Thr (SEQ ID No. 7) and Ser-Gln-Ala-Val-Gln-Glu-His-Ala-Ser-Thr-Asn-Xaa-Gly (SEQ ID No. 8), wherein Xaa is methionine.
8. The isolated peptide of claim 7, wherein said isolated peptide consists of Ser-Gln-Ala-Val-Gln-Glu-His-Ala-Ser-Thr (SEQ ID No. 7).

9. The isolated peptide of claim 7, wherein said isolated peptide consists of Ser-Gln-Ala-Val-Gln-Glu-His-Ala-Ser-Thr-Asn-Xaa-Gly (SEQ ID No. 8), wherein Xaa is methionine.
10. An isolated peptide which binds to anti-CD-2 antibody, said isolated peptide comprising the sequence Met-Val-Arg-Ile-Lys-Tyr-Gly-Ser-Tyr-Asn-Lys-Pro-Ser-Asp (SEQ ID No. 3).

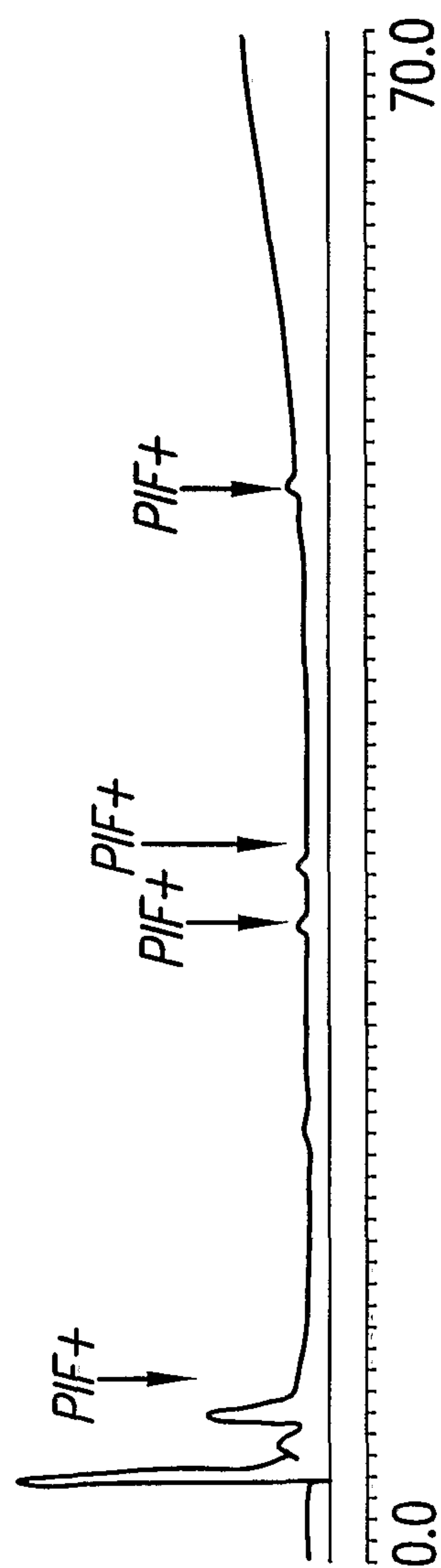


FIG. 1A

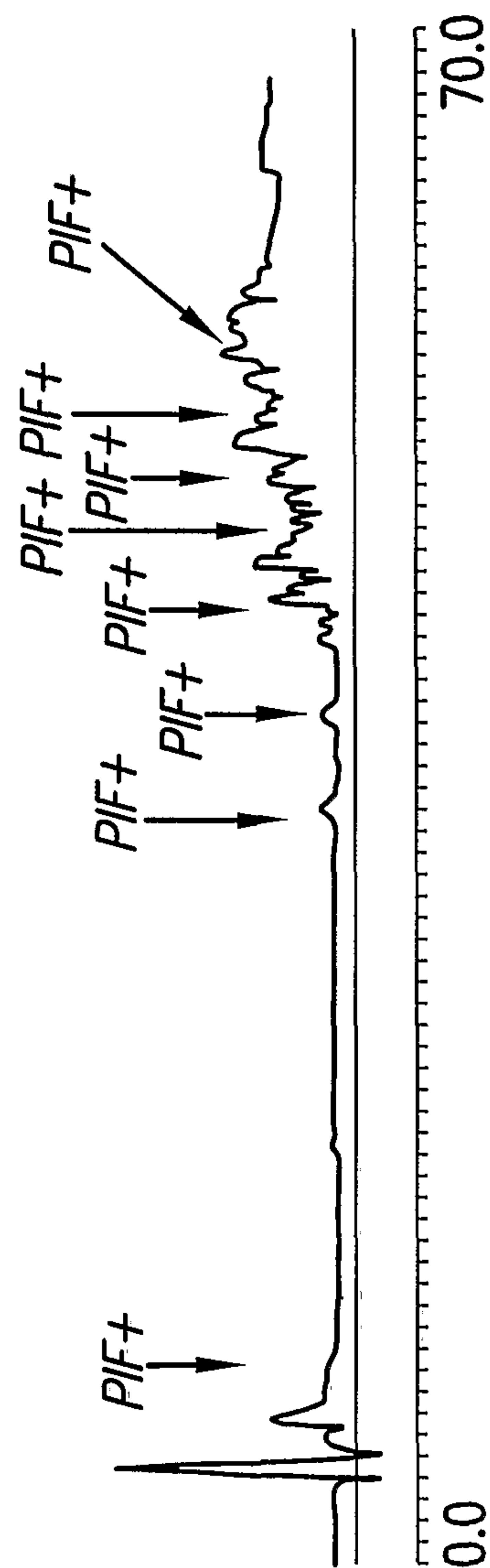


FIG. 1B



FIG. 2A

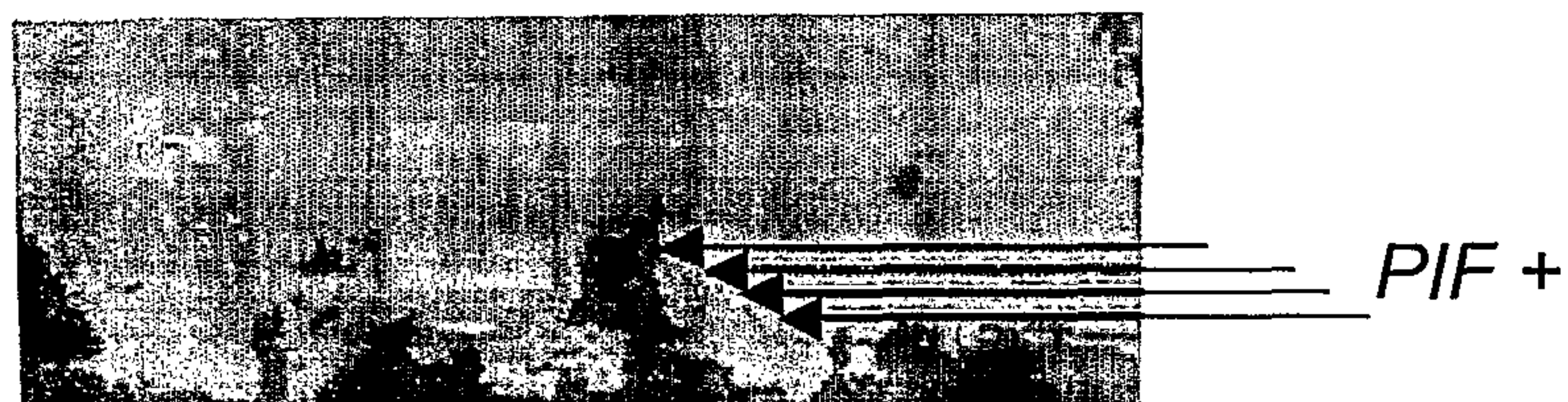


FIG. 2B

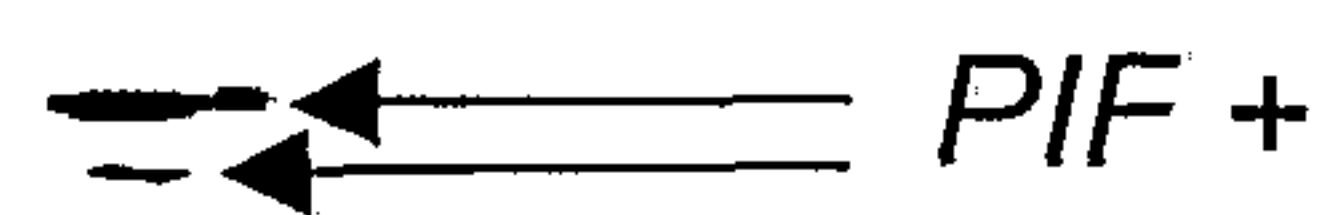


FIG. 2C

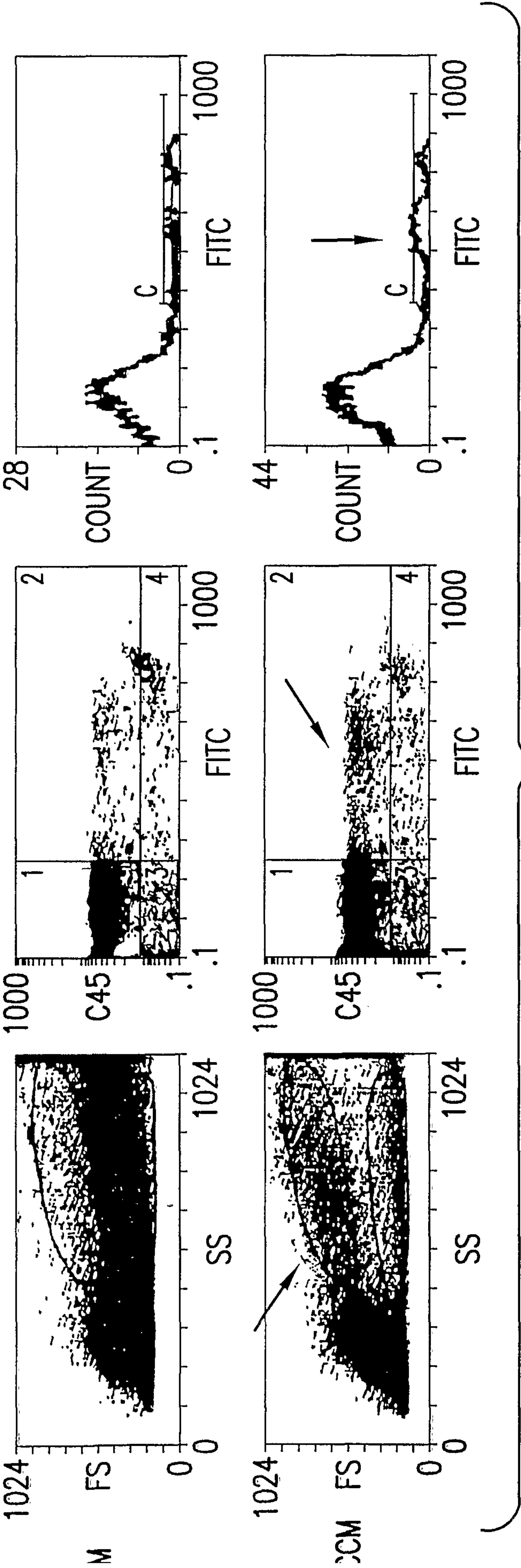
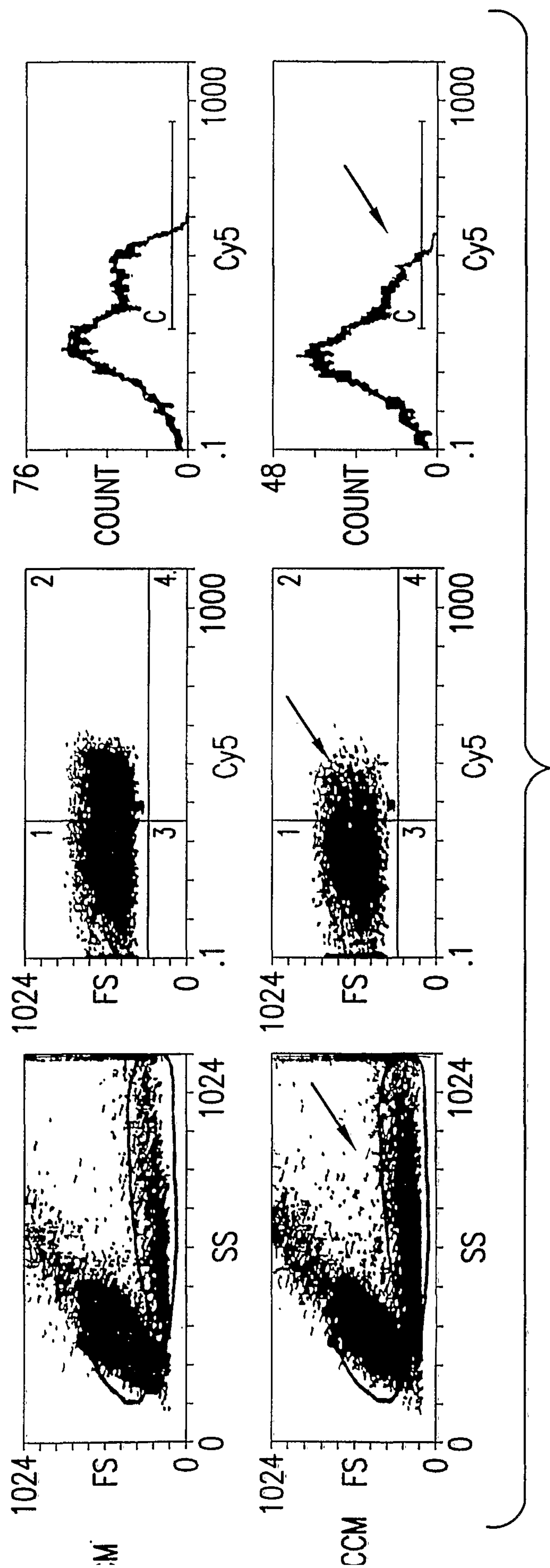
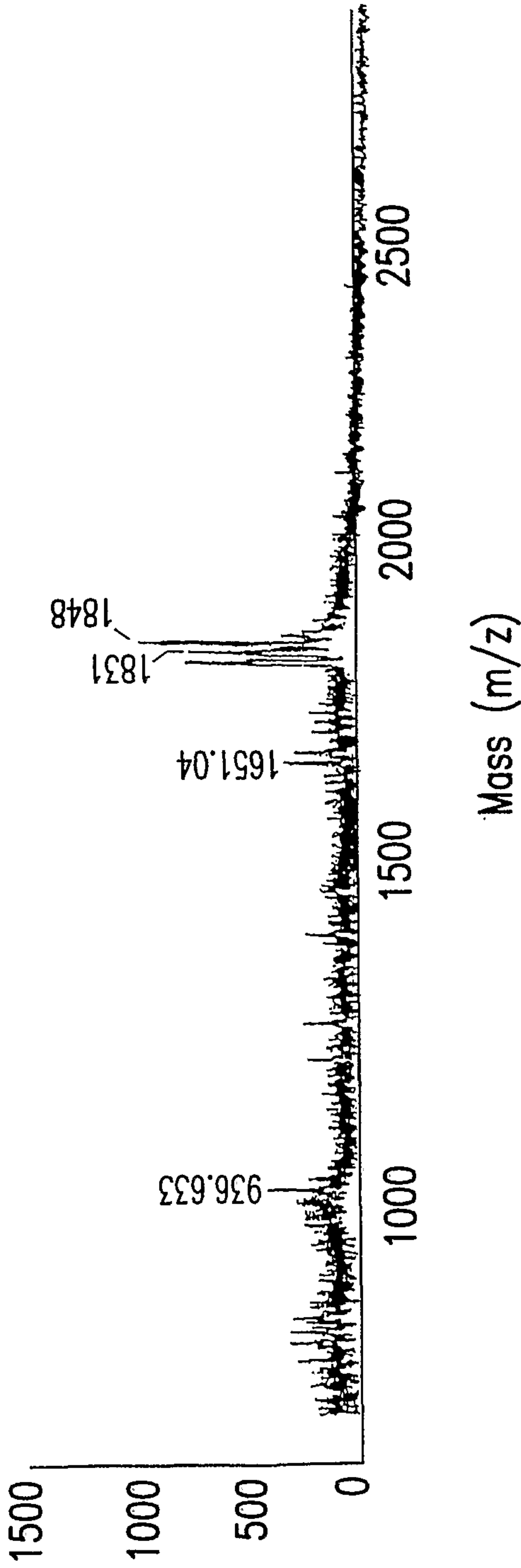
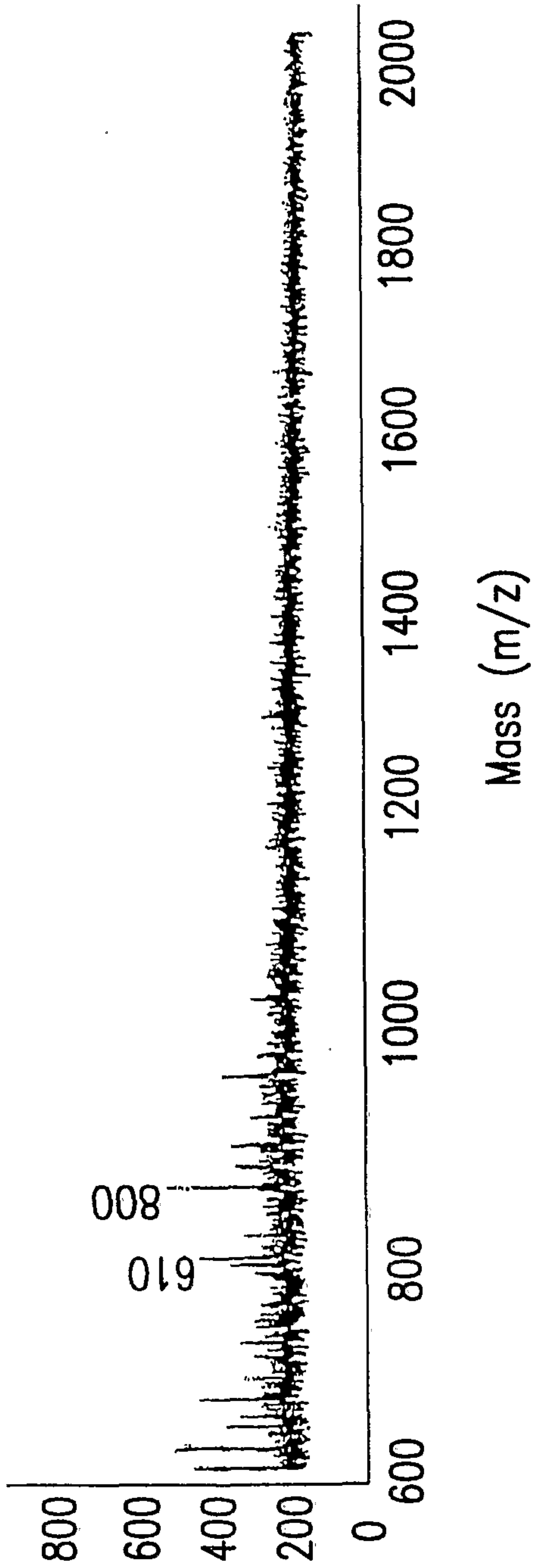


FIG. 3A





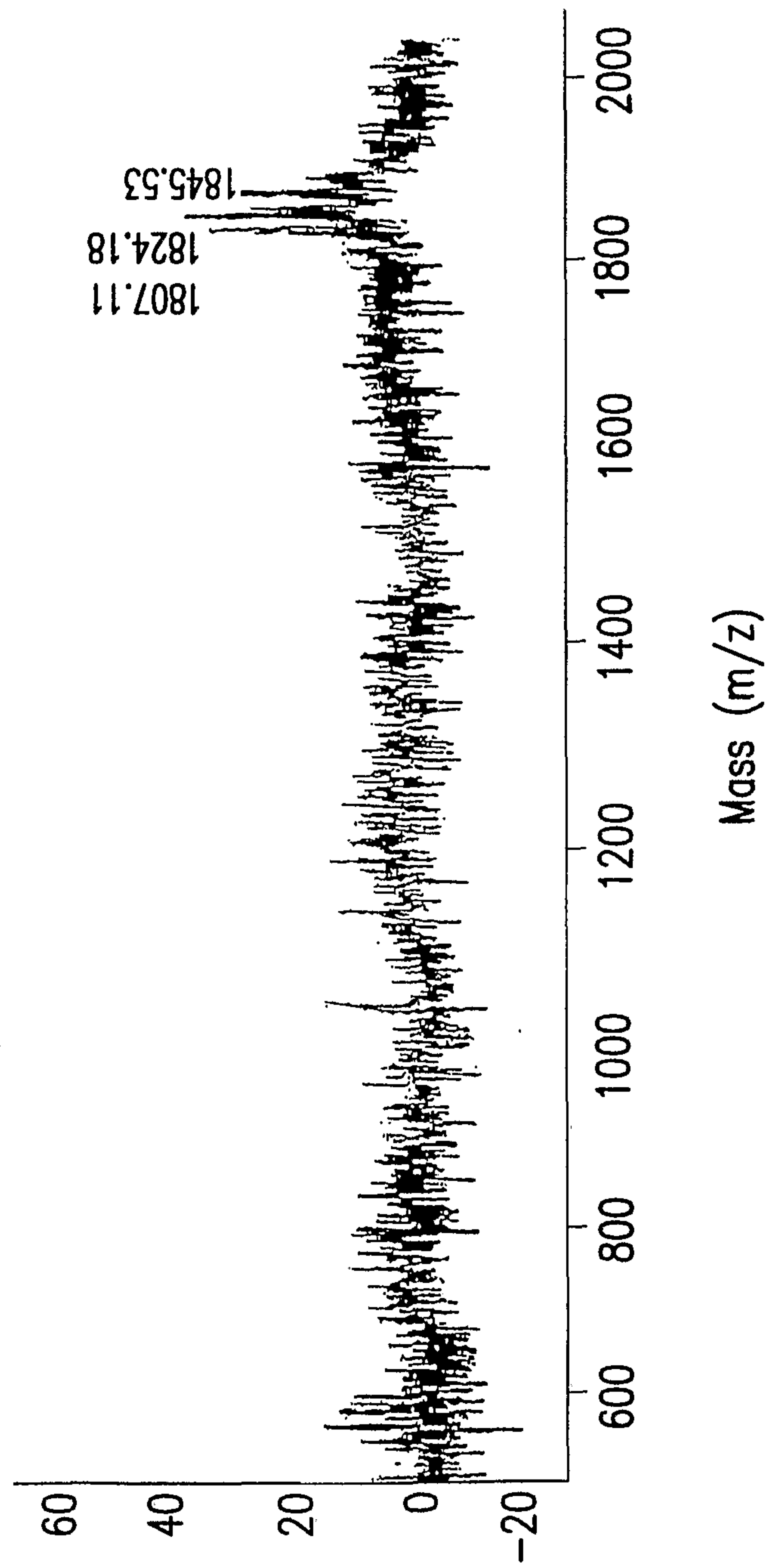


FIG. 4C

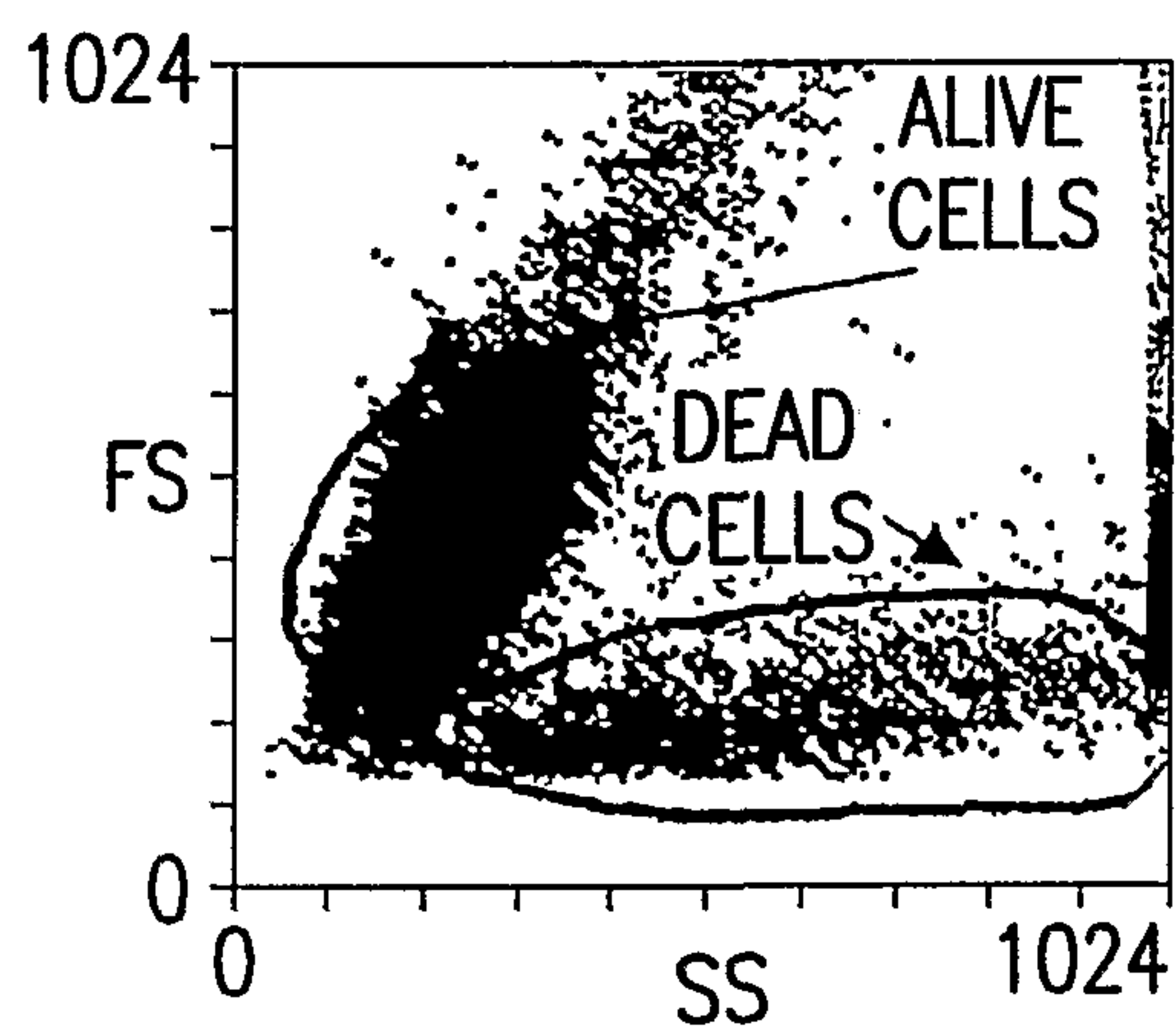


FIG. 5A

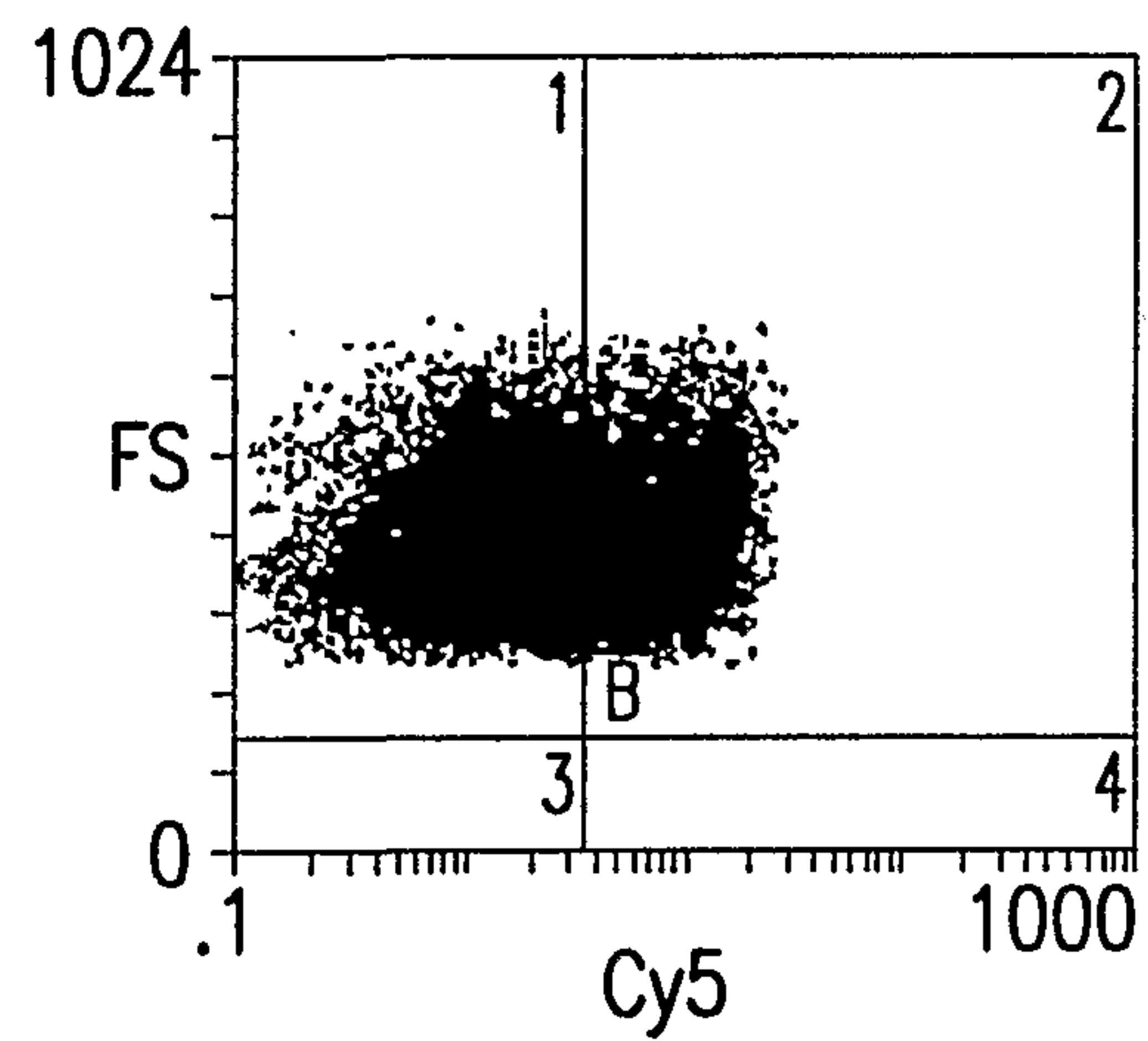


FIG. 5B

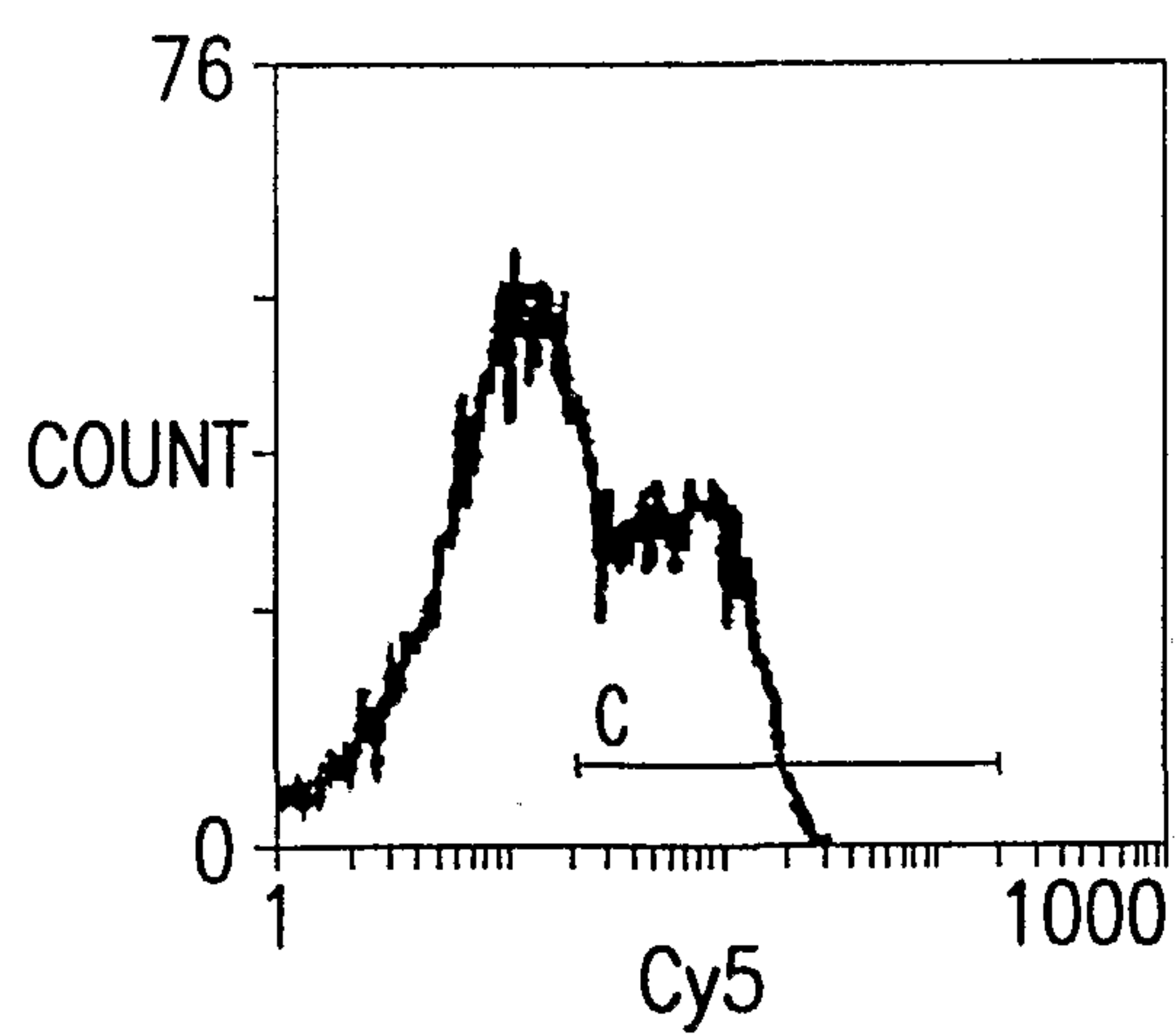


FIG. 5C

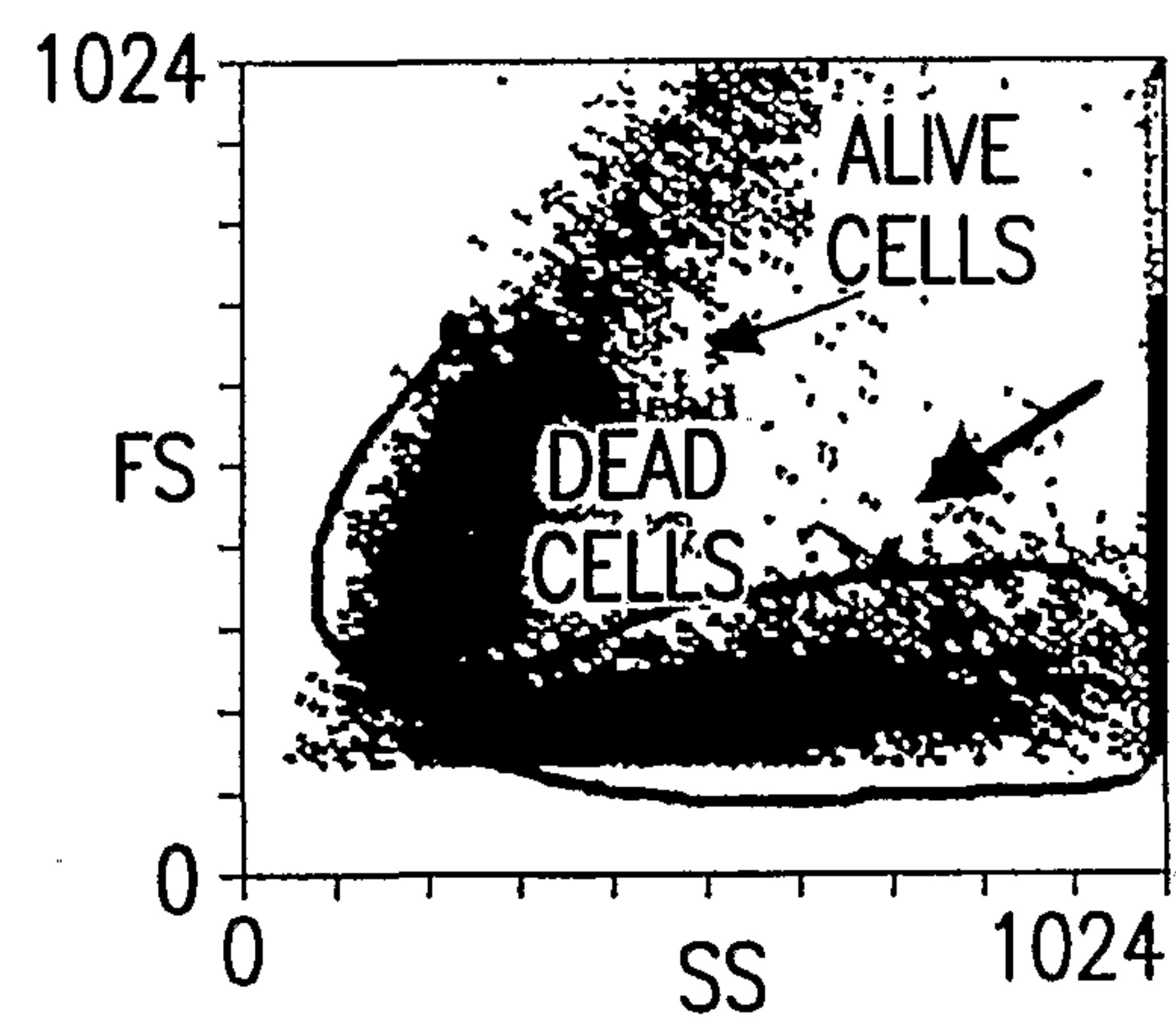


FIG. 5D

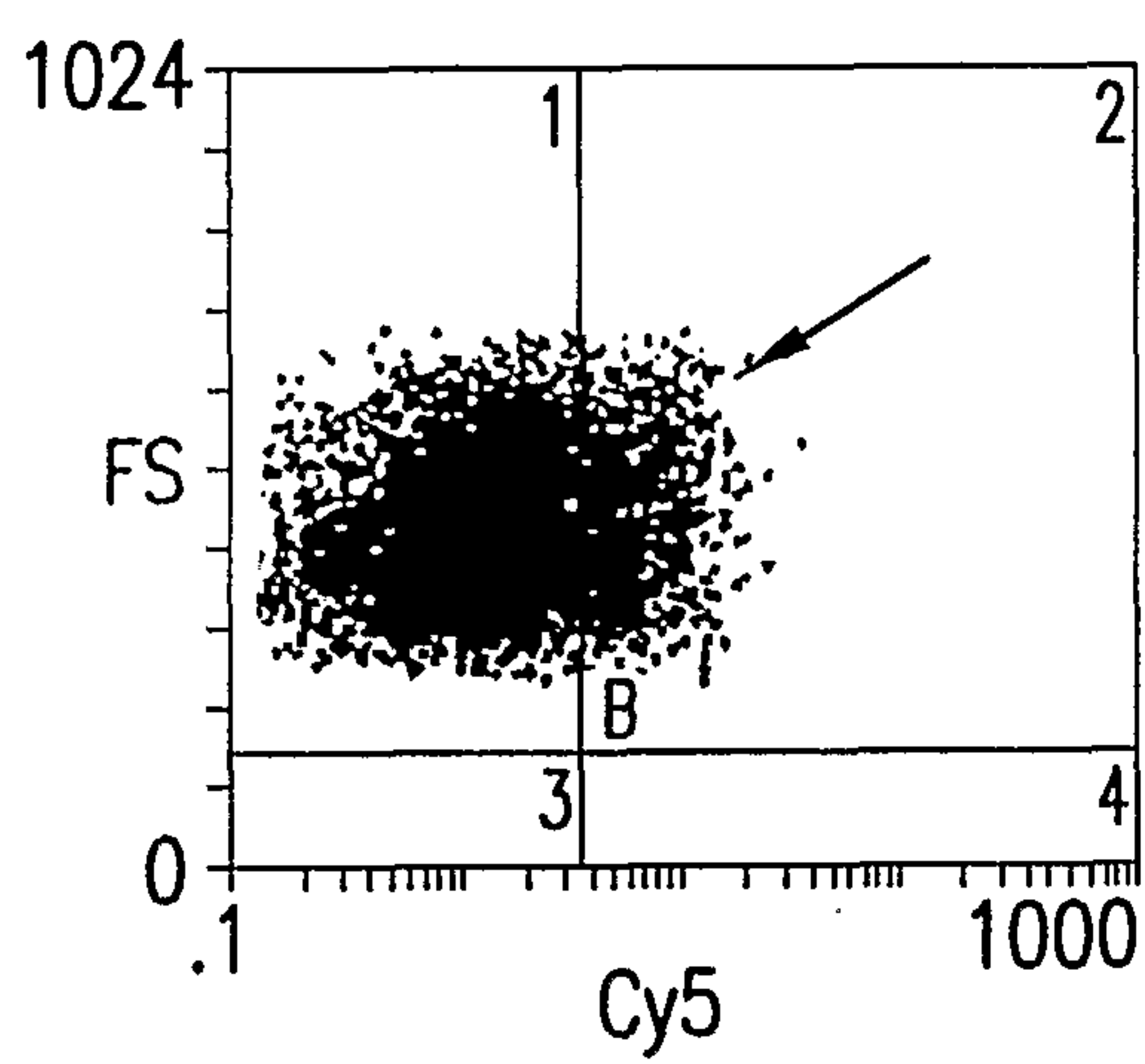


FIG.5E

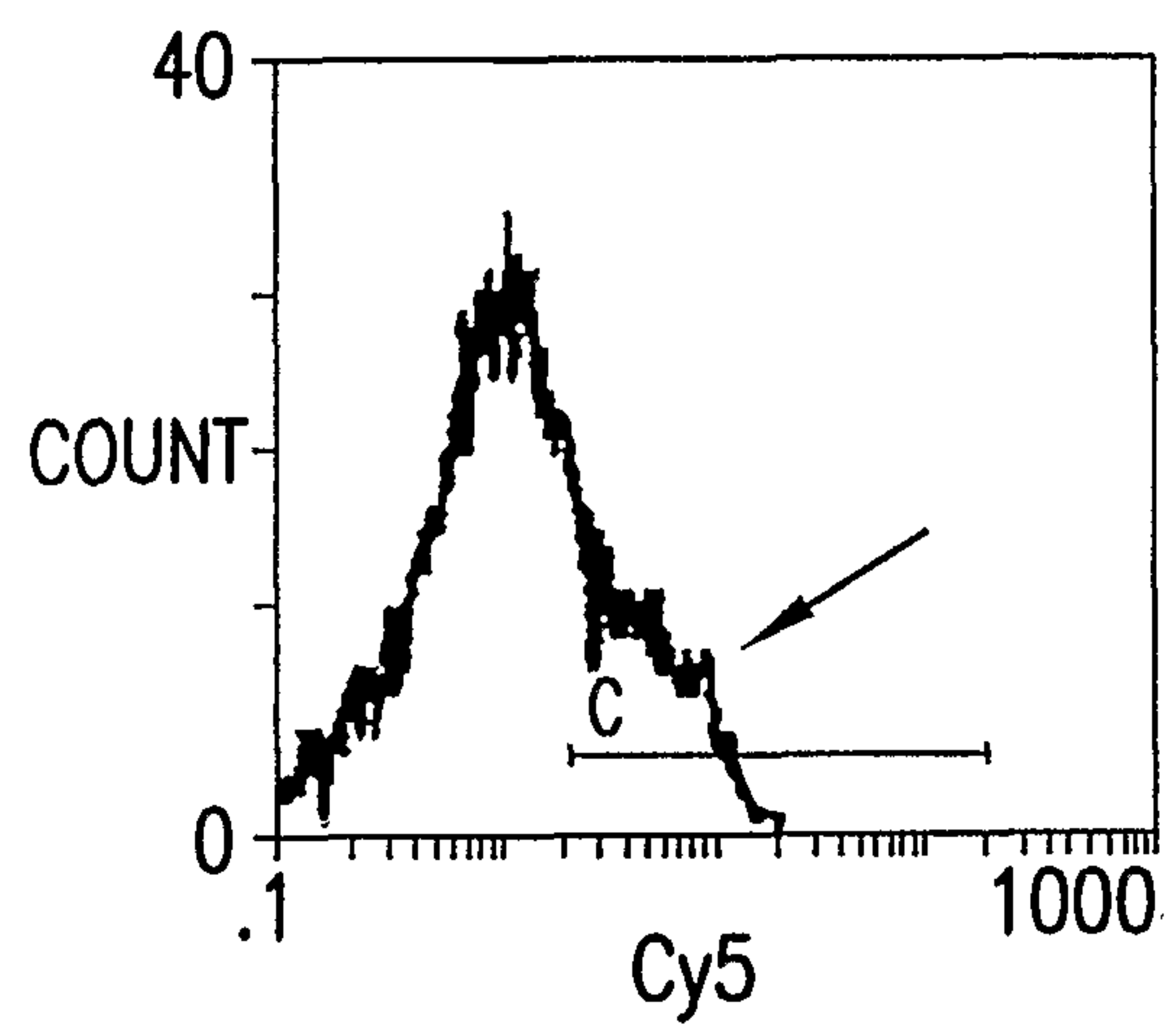


FIG.5F

EFFECT OF SYNTHETIC NOVEL PIF
PEPTIDES ON CD2 EXPRESSION ON JURKAT
CELLS

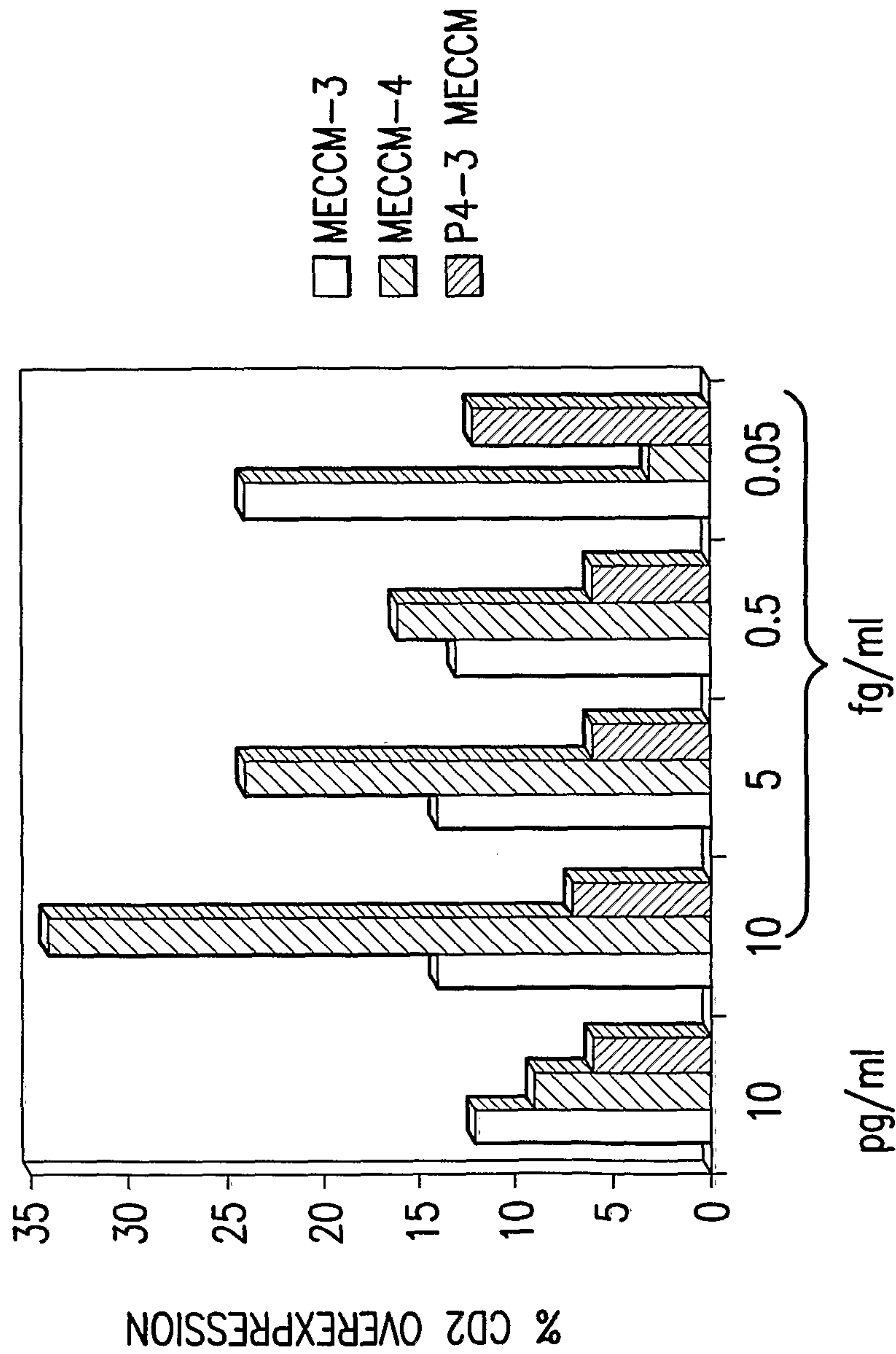


FIG.6