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(54) Title: A DIFFERENTIATED MEGAKARYOCYTE LINE PRODUCING NOVEL MEGAKARYOCYTE DIFFERENTIATION FACTORS

(57) Abstract

Differentiated megakaryocytes produced by introducing an activated oncogene into blast-megakaryocytes is disclosed. Also disclosed are novel megakaryocyte differentiation factors and platelets obtained from the differentiated megakaryocytes.

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A DIFFERENTIATED MEGAKARYOCYTE LINE PRODUCING NOVEL MEGAKARYOCYTE DIFFERENTIATION FACTORS

Description

Background of the Invention

Blood platelets are required for the maintenance of normal hemostasis. Platelets initiate blood clot formation and release growth factors that speed the process of wound healing and may serve other functions.

Platelets are the terminal differentiation product of megakaryocytes, which in turn originate from pluripotent stem cells of the bone marrow. The maturation and differentiation processes that begin with pluripotent stem cells and end with blood platelets are complex and incompletely understood. However, it is thought that humoral factors influence different cellular steps in megakaryocyte development.

Thrombocytopenia (i.e., depressed levels of platelets) can result from genetic problems affecting megakaryocytopoiesis in an individual. It can also result

from therapies for cancer (e.g., chemotherapy and radiation) and bone marrow transplantation.

Thrombocytopenia is currently treated mainly with platelet transfusions. However, it is expensive to obtain platelets from human donors. In addition, platelets for transfusions have a relatively short shelf-life and their use can expose the patient to dangerous viruses (e.g., HIV and hepatitis).

A greater understanding of megakaryocytopoiesis, as well as isolation and identification of humoral factors which promote megakaryocytopoiesis are greatly needed.

Summary of the Invention

The present invention relates to Applicants' finding that blast-megakaryocytes, which have been transfected with an activated oncogene, differentiate into mature megakaryocytes. Thus, the invention comprises in one embodiment, a method of producing differentiated megakaryocytes through the introduction of a gene encoding an activated oncogene into blast-megakaryocytes, which subsequently become more differentiated. Preferred blast-megakaryocytes are CMK cells.

In the present method, blast-megakaryocytes are maintained under conditions appropriate for their growth as non-terminally differentiated cells. A gene encoding an activated oncogene is then introduced into these megakaryocytes, thereby producing megakaryocytes containing an activated oncogene. The resulting activated oncogene containing cells are then cultured and a certain number become polyploid and terminally differentiate. The invention also particularly relates to differentiated

megakaryocytes produced by the method and uses therefor. Other aspects of the present invention relate to platelets produced by the method of the subject invention and uses therefor.

A further aspect of the present invention relates to novel differentiation factors produced by the differentiated megakaryocytes of the subject invention and uses therefor. These megakaryocyte differentiation factors (MDFs) are present in the supernatant of ras-transfected megakaryocytes. When megakaryocyte cell lines are cultured in the presence of the MDF, as present in the supernatant, there is a marked increase in proliferation of megakaryocyte cell lines as well as increase in ploidy number. These differentiation factors are heat sensitive and protease sensitive, suggesting that these factors are proteinaceous in nature. In addition, these MDFs do not have any detectable GM-CSF, IL3, IL6 or IL1B activity, making it clear that they are not one of these previously identified lymphokines or cytokine. suggesting that these are novel differentiation factors.

Brief Description of the Drawings

Figure 1 is a graphic representation of the effect of ras-transfected megakaryocyte supernatant on CMK11-5 ploidy.

Figure 2A is a graphic representation of the effect of ras-transfected megakaryocyte supernatant on growth factor-dependent cell line UT7.

Figure 2B is a graphic representation of the effect of ras-transfected megakaryocyte supernatant on growth factor-dependent cell line MO-7.

Figure 3 is a graphic representation of the effect of blocking ras-transfected megakaryocyte supernatant activity with anti-cytokine antibodies.

Figure 4 is a graphic representation of the effect of ras-transfected megakaryocyte supernatant on colony formation by murine bone marrow (MB) cells.

Detailed Description of the Invention

The present invention relates to Applicants' finding that blast-megakaryocytes transfected with an activated oncogene differentiate into mature megakaryocytes. Thus, the invention comprises in one embodiment, a method of producing differentiated megakaryocytes by introducing a gene encoding an activated oncogene into blast-megakaryocytes or other appropriate precursor cell.

Blast-megakaryocytes are morphologically recognizable by their size, which ranges from about 15 to 40 μm, their lobulated nuclei and their ploidy, which ranges from 2N to 8N. In addition they may contain a few cytoplasmic organelles (e.g., mitochondria, golgi apparatus, endoplasmic reticulum). Blast-megakaryocytes, like mature megakaryocytes, also express platelet specific phenotypic markers. Blast-megakaryocytes mature from megakaryocyte progenitor cells (i.e., the burst forming unit - megakaryocyte (BFU-M) and the colony forming unit - megakaryocyte (CFU-M)), proceeding through a phase characterized by mitotic division of progenitor cells followed by a wave of nuclear endoreplication, occurring in a nonproliferating cell.

For purposes of the present method of producing differentiated megakaryocytes and their products, preferred blast-megakaryocytes are CMK cells. (Komatsu, N.

et al., Blood 74:42-48 (1989)). However, examples of other blast-megakaryocyte cell lines useful in the present invention are DAMI (Greenberg, S.M. et al., Blood 72:1968-1977 (1988)) and CHRF (Wilte, D.P. et al., Cancer 58:238 (1986)).

A preferred activated oncogene to introduce into a blast-megakaryocyte is ras, which has been implicated in transformation in vitro and tumorigenesis in vivo. However, other cytoplasmic oncogenes (e.g., avian myb) may also be useful in the subject invention. Cytoplasmic oncogenes, in contrast to nuclear oncogenes, exert their effect in the cell's cytoplasm.

A gene encoding an activated cytoplasmic oncogene such as ras can be introduced into a blast-megakaryocyte using known genetic engineering transfer techniques. For example, the gene can be introduced by incubating megakaryocytes with a calcium phosphate precipitate of DNA, electroporation, direct intracellular micro-injection, infection of cells with modified vectors (e.g., retroviral vectors) or fusion of cells with other cells, sperm or with liposomes, which contain the oncogene. Alternatively, an inactivated form of the gene can be introduced into blast-megakaryocytes and then can be activated in vivo (e.g., chemically or by amplifying the number of copies).

Example 1 sets forth in detail a method of introducing the activated ras oncogene into blast-megakaryocytes. In general, blast-megakaryocytes were maintained under conditions appropriate for their growth as non-terminally differentiated cells. Two plasmids, one containing a gene encoding an activated oncogene and the

other containing a gene encoding a selectable marker were then co-transfected into the megakaryocytes by electroporation. Transfected cultures were maintained for three weeks in selective medium, which was changed every three days. Resistant colonies were picked by the limiting dilution method.

The differentiated megakaryocytes produced by introducing a gene encoding an activated oncogene into blast-megakaryocytes differ from non-oncogene containing blast-megakaryocytes in the following respects: 1) they are larger in size, (e.g., their size ranges from 60 to 120 μm; 2) they have greater ploidy values (e.g., ploidy ranging from 16N-32N); and 3) they contain many cytoplasmic organelles (e.g., mitochondria, golgi apparatus and endoplasmic reticulum).

In addition, some of these differentiated megakaryocytes produce novel differentiation factors, megakaryocyte differentiation factors (MDFs).

In particular, supernatant from H-ras transfected megakaryocytic cell clones, 2B and 2D, demonstrate a significant effect on megakaryocyte differentiation in vitro. One megakaryocytic clone (ras meg A, herein referred to as 2D) producing differentiation factors was deposited (June 27, 1991) under terms of the Budapest Treaty at the American Type Culture Collection (Rockville, Maryland) under ATCC accession number CRL 10817.

Growth factor dependent human megakaryocytic cell lines are used as assay targets in the studies of the MDFs produced by the H-ras transfected megakaryocytes. These cell lines proliferate in response to cytokines, such as GM-CSF, interleukin-3, or erythropoietin.

Initial studies were performed using primary bone marrow megakaryocytes (CMK11-5) as targets cultured with supernatants from the megakaryocytes transfected with H-ras. This resulted in an increase in megakaryocyte ploidy number. These assays were done with crude, unconcentrated unfractionated supernatant, as well as with crude, unconcentrated, unfractionated supernatant supplemented with PMA. (See Figure 1)

As described in Example 2, supernatants from the H-ras transfected megakaryocytes induced a marked increase in tritiated thymidine incorporation in parent (unaltered) CMK and growth factor dependent UT7 and MO7 cells. (See Figures 2A and 2B)

Characterization of this activity revealed that it was heat sensitive. The activity was destroyed by heating to 56°C for 30 minutes. Treatment of this supernatant material with proteinase-K also destroyed MDF activity. These results suggest that MDFs are protenaceous in nature.

To verify whether the MDF activity observed was due to a known cytokine, neutralization experiments using specific antibodies for IL-3, IL-6, GM-CSF and IL-1β were performed. These experiments revealed that the MDF activity was not neutralized by these antibodies singly or in combination. Thus, it is reasonable to suggest that the activity from the transfected megakaryocyte supernatants are potential new growth factors. (See Figure 3)

To examine whether the MDF activity alone or in combination with IL-3, IL-6 and GM-CSF could act synergistically, ploidy assays were performed after 4 days on bone marrow isolated megakaryocytes. These experiments

revealed that marrow megakaryocytes isolated by immunomagnetic beads coated with anti-GpIIb/IIIa antibody were predominantly 2N, with a small proportion containing 4N ploidy. Following treatment with 2B supernatant, an increase in 4N (8.3%) and 8N (2.7%) cells as compared with cells cultured in RPMI alone, were observed (See Table. In combination with IL-6, the 8N population was increased in the presence of 2B or 2D. These results suggest that the factors released by transfected megakaryocyte cells have an effect on megakaryocyte ploidy and may act synergistically with IL-6.

TABLE

The Effects of ras Transfected Megakaryocyte Supernatants on Ploidy of Isolated Megakaryocytes

A.

	%2N	4N	8	
RPMI	84.6	6.3	---	
11-5 sup	76.5	8.9	1.7	†
2B	79.0	8.3	2.7	†
2D	79.0	6.8	1.1	†

B.

+ IL-6	%2N	4N	8N	
RPMI	76.0	8.9	0.9	
11-5 sup	72.9	11.5	4.2	†
2B	74.1	10.8	3.2	†
2D	79.3	9.2	2.7	†

Supernatants from ras transfected megakaryocytes were also assessed for their effects on megakaryocyte colony formation, as described in Example 3. Supernatants from the 2B and 2D clones showed significant increases in the formation of total hematopoietic cell colonies, as well as increases in megakaryocyte colony forming units (CFU-meg) and single megakaryocyte cells. (See Figure 4)

A differentiation factor can be isolated from megakaryocytes which contain an activated oncogene using known methods. For example, supernatants from megakaryocytes which contain an activated oncogene can be purified by anion exchange chromatography or reverse phase high pressure liquid chromatography (HPLC). To stabilize the activity of the differentiation factor, protease inhibitors (e.g., antipain, aprotinin, leupeptin and pepstatin) can be added.

Alternatively, a gene encoding a megakaryocyte differentiation factor can be isolated from a cDNA library made, for example, from the total RNA of a megakaryocyte which contains an activated oncogene, using known techniques. Once isolated, the gene encoding a megakaryocyte differentiation factor can be cloned into an expression vector, which can be used to express the MDF in vitro or in vivo.

In addition to producing differentiation factors, the differentiated megakaryocytes of the subject invention may prove useful for producing platelets, both in vitro and in vivo. A therapeutically effective amount of either platelets produced in vitro from differentiated megakaryocytes or differentiation factors isolated from differentiated megakaryocytes can be administered to a

vertebrate (e.g., a human) with a platelet-associated bleeding disorder (e.g., thrombocytopenia) to treat the bleeding disorder. A therapeutically effective amount is that amount sufficient to significantly reduce or eliminate the symptons or effects of the platelet disorder.

The number of platelets or quantity of differentation factors to be administered to a vertebrate for therapeutic effectiveness can be determined on an individual basis and will be based, at least in part, on considerations of the individual's size, the severity of the symptons being treated, and the results sought. A therapeutically effective amount can be determined by one of ordinary skill in the art using no more than routine experimentation. Administration of platelets or differentiation factors can be by any route appropriate for the condition being treated, but typically platelets will be administered parenterally (e.g., via intravenous (IV) or intra-arterial (IA) injection); and MDFs will be administered subcutaneously or parenterally.

The present invention will now be illustrated by the following Examples, which are not to be seen as limiting in any way.

Example 1 Transfection of CMK cells with activated ras

Cell Culture

CMK 11-5 cells were grown in RPMI 1640 medium containing 5% platelet poor plasma, L-glutamine, penicillin and streptomycin (Mitjavila, M.T. et al., J. Cell Physiol., 734:93 (1986)).

Plasmids

Two plasmids were used for co-transfection experiments: pSV2neo (Mulligan, R.C., et al., Science, 19:1422 (1980)) and pSVET24 (Seeburg, PH. et al., Nature, 312:71 (1984)).

Transfections

Transfections were performed by electroporation, essentially as described in Andreason and Evans Bio Techniques, 6:650 (1988). For cotransfections and selection, 5μg of plasmid DNA pSVET 24 were mixed with 0.5μg of pSV2neo. Twenty-four hours after transfection, the cells were plated onto 100mm dishes, and gentamicin (G418; Gibco Laboratories) was added to 400μg/ml. Cultures were maintained for three weeks with the medium being changed every three days. At the end of this period, resistant colonies were picked by the limiting dilution method. The clones were expanded and analyzed further for their morphology and for the presence of platelet specific markers.

Example 2 Testing supernatants from ras transfected CMK11-5 cells on CMK cells

Spectrophotometric measurement of DNA content of CMK cells:

CMK cells were seeded at 2 x 10^5 cells and fed again after two days. Cells were harvested after 5 days and the nuclei were isolated, stained with propidium iodide, and analyzed on a Becton Dickinson FACS Analyzer (Mountain View, CA) as previously described (Greenberg,

S.M. et al., Blood, 72:1968 (1988)). Freshly prepared lymphocytes were used to mark the position of the 2N cells.

Proliferation assays:

Cell proliferation and viability was assessed by ^{3}[H] thymidine incorporation and by trypan blue exclusion (0.4% trypan blue stain in 0.85% saline, Gibco Laboratories). Supernatants were added in a volume of 50μl in 96-well flat-bottom tissue culture plates (Costar, Cambridge, MA) where CMK11-5 cells were seeded at 2 x 10^{4} cells in the appropriate growth medium containing either 1% platelet poor plasma or 1% fetal calf serum (FCS) in a final volume of 100μl. For ^{3}[H] thymidine incorporation assays, cells were seeded at time zero (50μl volume) and the plates were incubated at 37°C in a humidified atmosphere of 5.5% CO_2 for 48 hours. Cells were pulsed with 0.5 Ci per well of ^{3}[H] thymidine (25 A/mmol, NEN, Boston) and incubated for an additional five hours. Samples were harvested onto glass fiber filters and counted by liquid scintillation spectrometry.

Example 3 Colony Forming Assay

Murine bone marrow cells (BM) were cultured under standard conditions in the presence of Murine IL3 (control), RPMI media with and without platelet poor plasma (PPP) or PMA, or the 2B and 2D clone supernatants, with or without cytokine supplements.

After incubation for approximately 14 days, total colonies formed, CFU-meg colonies and single megakaryocyte cells were tabulated. The results, as shown in Figure 4,

indicate that the 2B and 2D supernatants cause significant increases in total colonies and single megakaryocytes formed, with or without supplemented cytokines.

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 6, line 26 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet ☐ [3]

Name of depositary institution [4]

American Type Culture Collection

Address of depositary institution (including postal code and country) [4]

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit [5]	Accession Number [6]
27 June 1991	CRL10817

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☒

In respect of those designations in which a European Patent is sought, the Applicant hereby informs the European Patent Office under European Rule 28(4) that, until the publication of the mention of the grant of the European Patent or until the date on which the European Application has been refused or is withdrawn or is deemed to be withdrawn, the availability of the biological material deposited with the American Type Culture Collection under Accession No. (Cont'd Appendix A)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

Europe (EP), Australia, Canada and Japan

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

Virginia L Lally
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

APPENDIX A

Box B Continued

shall be effected only by the issue of a sample to an expert nominated by the requester in accordance with European Rule 28(5). In accordance with Regulation 3.25 of the Patents Regulations (Australia Statutory Rules 1991 No. 71), samples of materials deposited in accordance with the Budapest Treaty in relation to this Patent Request are only to be provided before: The patent is granted on the application; or the application has lapsed or been withdrawn or refused; to a person who is: a skilled addressee without an interest in the invention; and nominated by a person who makes a request for the furnishing of those samples.

CLAIMS

1. A megakaryocyte differentiation factor present in the supernatant of a differentiated megakaryocyte cell which contains an activated cytoplasmic oncogene.

2. A megakaryocyte differentiation factor of Claim 1 wherein the activated cytoplasmic oncogene is ras and the megakaryocyte is a CMK cell.

3. A megakaryocyte differentiation factor of Claim 2 which is a heat-sensitive, protease-sensitive factor.

4. A megakaryocyte differentiation factor of Claim 2 having no detectable GM-CSF, IL-3, IL-1β or IL-6 activity.

5. A megakaryocyte differentiation factor of Claim 2 which induces an increase in ploidy number in megakaryocytes cultured in the presence of said factor.

6. A megakaryocyte differentiation factor, present in the supernatant of a differentiated human megakaryocyte cell which contains an activated ras gene, which is heat-sensitive and protease-sensitive and has no detectable GM-CSF, IL-3, IL-1β or IL-6 activity.

7. A method of producing platelets that comprising contacting megakaryocytic blast cells with a megakaryocyte differentiation factor present in the supernatant of a differentiated megakaryocyte cell which contains an activated cytoplasmic oncogene.

8. A method of treating an individual with a platelet-associated bleeding disorder, comprising administering to the individual a therapeutically effective amount of a megakaryocyte differentiation factor present in the supernatant of a differentiated megakaryocyte cell which contains an activated cytoplasmic oncogene.

9. A method of stimulating platelet production in an individual, comprising administering orally or parenterally to the individual a therapeutically effective amount of a megakaryocyte differentiation factor present in the supernatant of a differentiated megakaryocyte cell which contains an activated cytoplasmic oncogene.

10. A therapeutic composition comprising megakaryocyte differentiation factor present in the supernatant of a differentiated megakaryocyte cell which contains an activated cytoplasmic oncogene in a physiological compatible carrier.

11. A method of producing differentiated megakaryocytes, comprising introducing into megakaryocytes an activated cytoplasmic oncogene thereby producing megakaryocytes containing an activated cytoplasmic oncogene and culturing the resulting megakaryocytes under conditions appropriate for growth and differentiation.

12. A method of Claim 11 wherein the activated cytoplasmic oncogene is ras.

13. A megakaryocyte differentiation factor according to any one of the Claims 1 to 6 for use in therapy for example (a) the treatment of an individual with a platelet-associated bleeding disorder, or (b) by oral or parenteral administration for stimulating platelet production in an individual.

14. Use of a megakaryocyte differentiation factor according to any one of Claims 1 to 6 for manufacture of a medicament for (a) the treatment of an individual with a platelet-associated bleeding disorder, or (b) by oral or parenteral administration for stimulating platelet production in an individual.

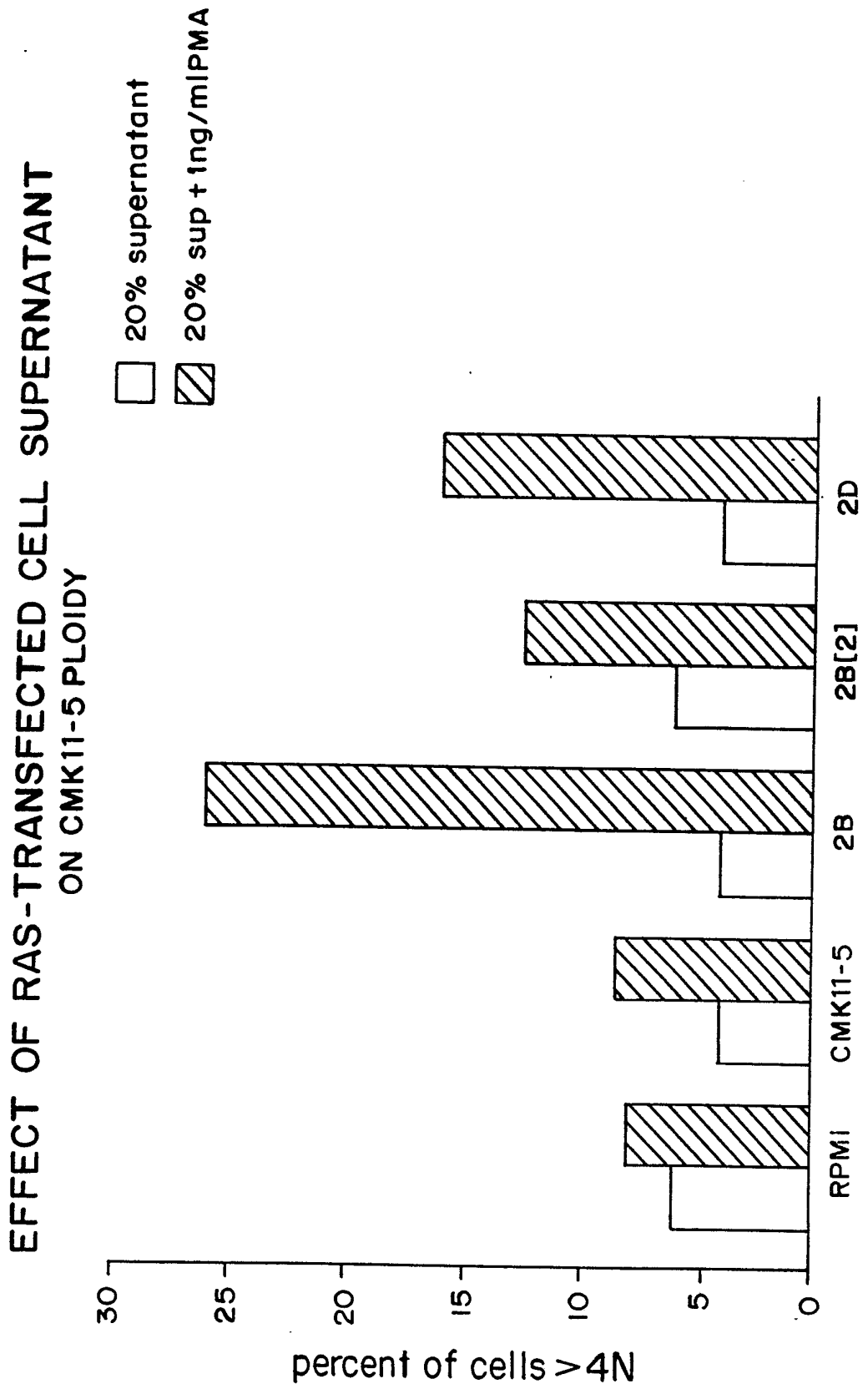

EFFECT OF RAS-TRANSFECTED CELL SUPERNATANT
ON CMK11-5 PLOIDY
20% supernatant
20% sup + 1ng/ml PMA
RPMI
CMK11-5
2B
2B[2]
2D
0
5
10
15
20
25
30
percent of cells >4N

FIG. 1

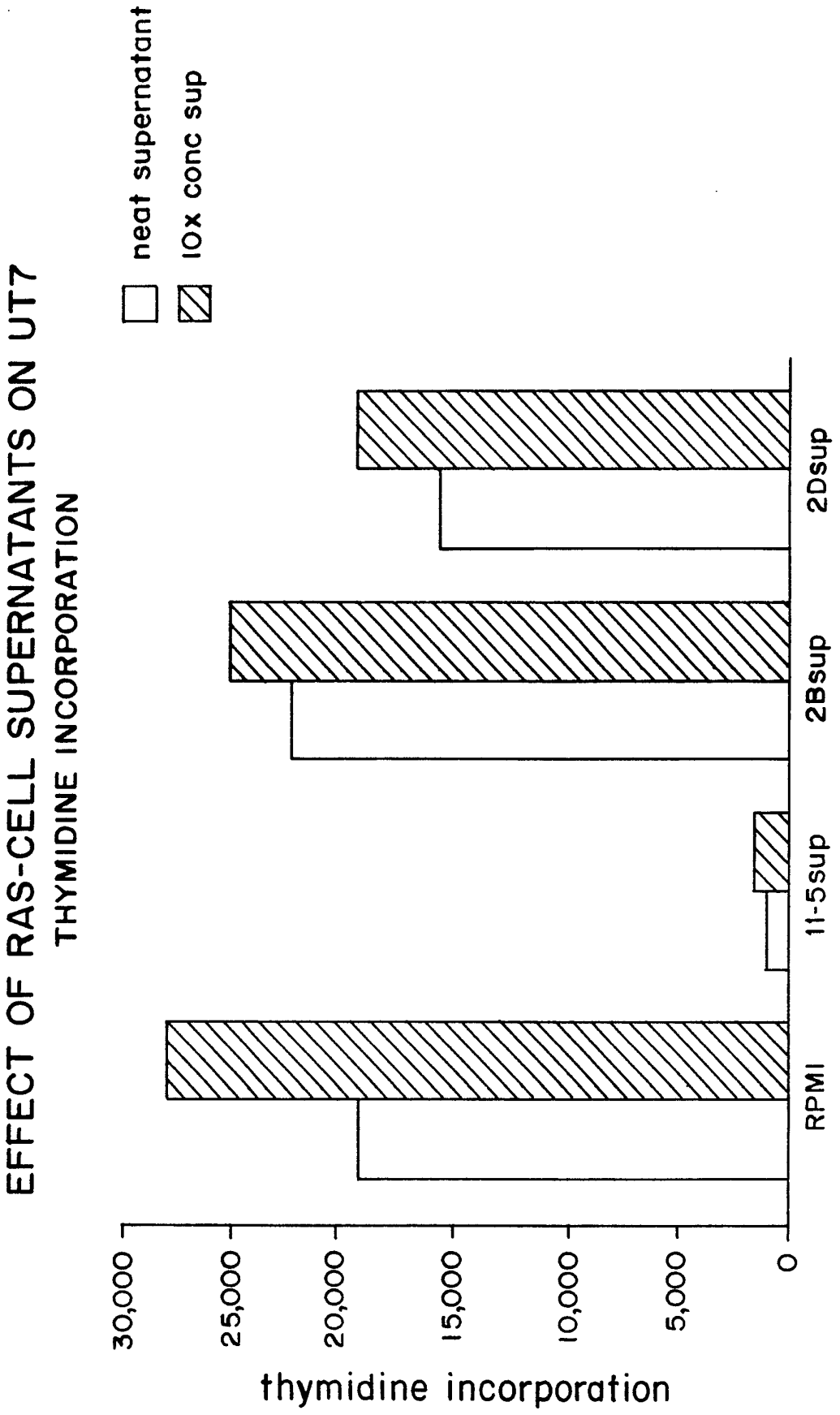

EFFECT OF RAS-CELL SUPERNATANTS ON UT7
THYMIDINE INCORPORATION
neat supernatant
10x conc sup
30,000
25,000
20,000
15,000
10,000
5,000
0
thymidine incorporation
RPMI
11-5sup
2Bsup
2Dsup

FIG. 2A

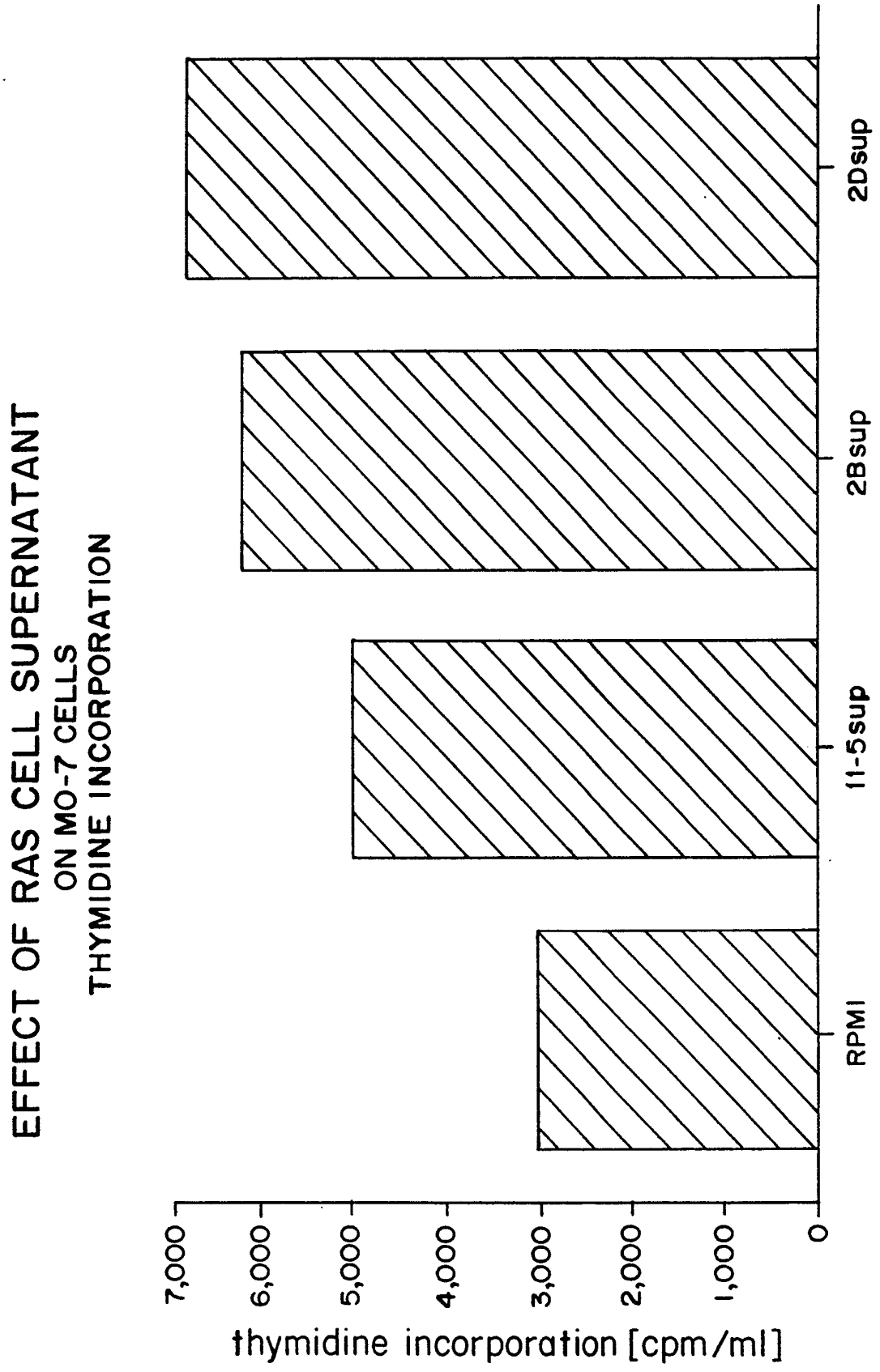

EFFECT OF RAS CELL SUPERNATANT
ON MO-7 CELLS
THYMIDINE INCORPORATION
thymidine incorporation [cpm/ml]
7,000
6,000
5,000
4,000
3,000
2,000
1,000
0
RPMI
11-5sup
2Bsup
2Dsup

FIG. 2B

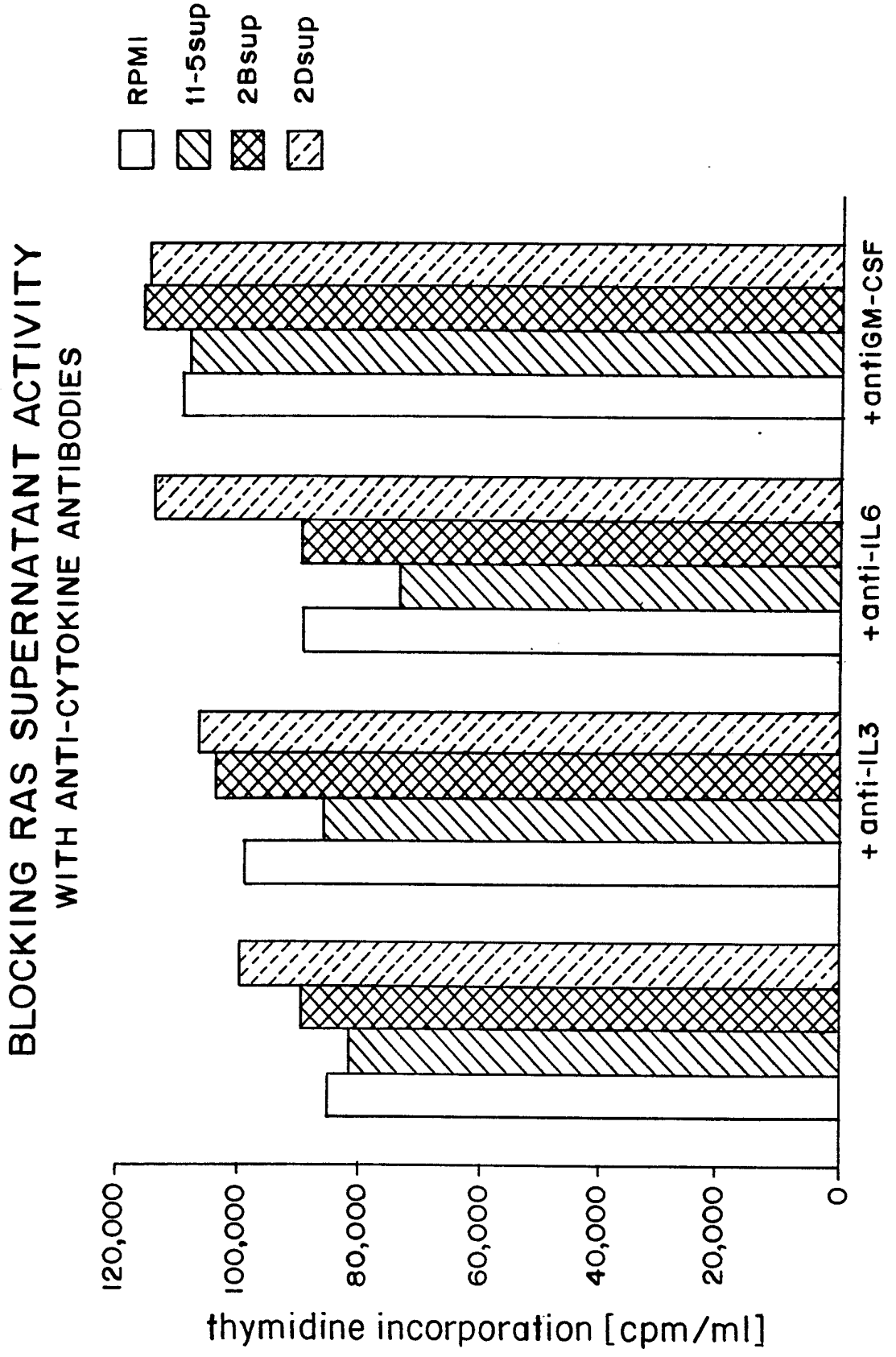

BLOCKING RAS SUPERNATANT ACTIVITY
WITH ANTI-CYTOKINE ANTIBODIES
RPMI
11-5sup
2Bsup
2Dsup
+antiGM-CSF
+anti-IL6
+anti-IL3
120,000
100,000
80,000
60,000
40,000
20,000
0
thymidine incorporation [cpm/ml]

FIG. 3

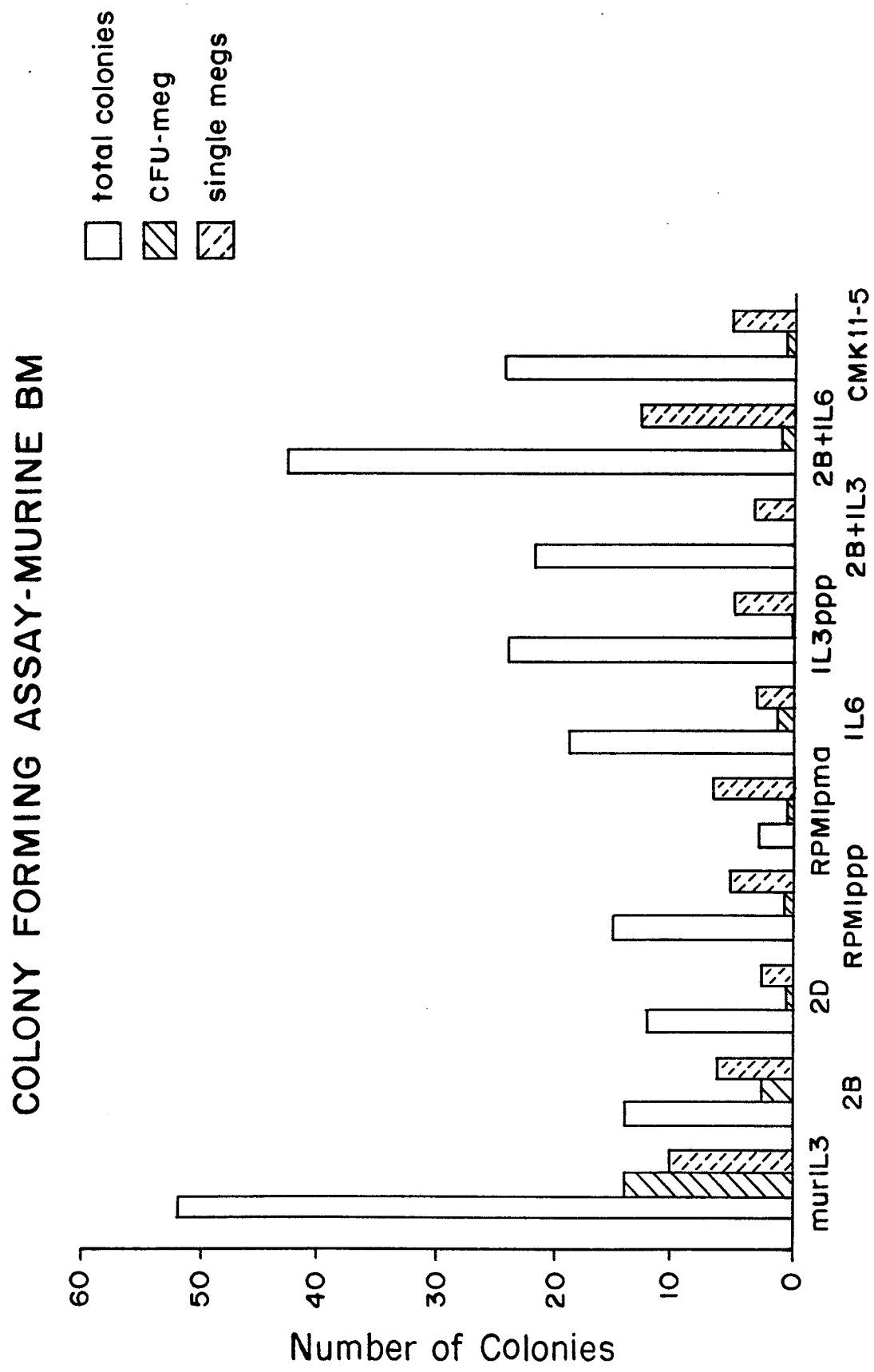

COLONY FORMING ASSAY-MURINE BM
total colonies
CFU-meg
single megs
Number of Colonies
0
10
20
30
40
50
60
murIL3
2B
2D
RPMIppp
RPMIpma
IL6
IL3ppp
2B+IL3
2B+IL6
CMK11-5

FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/05427

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 C12N15/19; A61K37/02; C12N5/10; C12P21/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K ; C12P ; C12N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,A	Section Ch, Week 9151, Derwent Publications Ltd., London, GB; Class B04, AN 91-373424 & JP,A,3 251 189 (MOCHIDA PHARM KK) 8 November 1991 see abstract	1-14
A	CHEMICAL ABSTRACTS, vol. 114, no. 15, 15 April 1991, Columbus, Ohio, US; abstract no. 141233n, FUSE, AKIRA ET AL 'Interleukin 6 , a possible autocrine growth and differentiation factor for the human megakaryocytic cell line, CMK.' page 590 ;column R ; see abstract & BR. J. HAEMATOL. vol. 77, no. 1, 1991, pages 32 - 36	1-14

-/--

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"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.

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
27 OCTOBER 1992	04. 11. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	LE CORNEC N.D.R.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	BLOOD vol. 74, no. 1, 1989, NEW YORK;US pages 42 - 48 KOMATSU , NORIO ET AL 'Growth and differentiation of a human megakaryocytoblastic cell line ,CMK.' cited in the application see the whole document	1-14
A	EP,A,0 260 918 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 23 March 1988 see the whole document	1-14
A	EP,A,0 235 113 (SMITHKLINE BECKMAN CORPORATION) 2 September 1987 see claims	11-12
A	WO,A,9 102 001 (GENETICS INSTITUTE , INC.) 21 February 1991 see the whole document	1-14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/05427

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 8-9 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9205427 SA 62035

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 27/10/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0260918	23-03-88	US-A-	4894440	16-01-90
		AU-B-	598297	21-06-90
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