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COMMONWEALTH OF AUSTRALIA

Patents Act 1952

APPLICATION FOR A STANDARD PATENT

I/WE YEDA RESEARCH AND DEVELOPMENT COMPANY LIMITED, a company organized and existing under the laws of the State of Israel of P.O. Box 95, Rehovot, Israel,

hereby apply for the grant of a Standard Patent for an invention entitled

CYTOTOXIC PROTEIN

which is described in the accompanying complete specification.

This application is made under the provision of Part XVI of the Patents Act 1952 and is based on an application for a patent or similar protection made

in Israel on 20 December 1984 (No. 73883)

in on (No.

My/Our address for service is: F.B. RICE & CO.,
28A Montague Street,
Balmain, NSW 2041

Dated this 17th day of December 1985.

YEDA RESEARCH AND DEVELOPMENT COMPANY LIMITED

APPLICATION ACCEPTED AND AMENDMENTS ALLOWED 21.6.90

By:

Patent Attorney

To : The Commissioner of Patents
COMMONWEALTH OF AUSTRALIA

F.B. RICE & CO.,
Patent Attorneys,
Sydney

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DECLARATION IN SUPPORT

In support of the (Convention) Application made by: Yeda Research & Development Company Limited

for a patent for an invention entitled: "A Cytotoxic Protein"

I (We) David Schlachet

of and care of the applicant company do solemnly and sincerely declare as follows:

~~a) I am (We are) the applicant(s) for the patent~~

~~or~~

b) I am (We are) authorised by the applicant(s) for the patent to make this declaration on its behalf.

Delete the following if not a Convention Application.

The basic application(s) as defined by section 141 (142) of the Act was (were) made

on December 20 1984 in Israel (73,883)

~~or~~ ~~in~~

~~or~~ ~~in~~

by Yeda Research & Development Company Limited

The basic application(s) referred to in this paragraph is (are) the first application(s) made in a Convention country in respect of the invention the subject of the application.

~~a) I am (We are) the actual inventor(s) of the invention~~

~~or~~

b) David Wallach resides at 14, Hadar Street, Rehovot, Israel

is (are) the actual inventor(s) of the invention and the facts upon which the applicant company is (are) entitled to make the application are as follows:

The applicant is a person who would if a patent were granted upon application made by the actual inventor be entitled to have the patent assigned to it.

Declared at Rehovot this 23 day of January 19 90

YEDA Research & Development Co. Ltd.

Signed ..... Status General Manager

Declarant's Name David Schlachet

F. B. RICE & CO PATENT ATTORNEYS

This form is suitable for any type of Patent Application. No legalisation required

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(57) Claim

1. A hybridoma cell line expressing a monoclonal antibody which specifically recognizes and binds human TNF-alpha said hybridoma being formed by fusion of spleen cells from a mouse previously immunized with human TNF-alpha and murine myeloma cells.

7. A process for the purification of human TNF-alpha which comprises:

a. providing a preparation containing human TNF-alpha;
b. absorbing the TNF-alpha from said preparation onto controlled pore glass beads;
c. desorbing the TNF-alpha in a state of enhanced purity from said controlled pore glass beads;
d. contacting the desorbed TNF-alpha with an immunoadsorbent, said immunoadsorbent comprising a monoclonal antibody against the TNF-alpha produced by a hybridoma according to claim 1; and
e. eluting TNF-alpha from the immunoadsorbent.

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19. A pharmaceutical composition for selectively treating virus-infected and tumor target cells in humans, which comprises a therapeutically effective amount of at least purified homogeneous human TNF-alpha as claimed in claim 16 or a salt thereof, together with a pharmaceutically acceptable excipient or in combination with interferons and/or a metabolic blocker in amount sufficient to sensitize said cells to human TNF-alpha but not to sensitize normal cells.

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COMPLETE SPECIFICATION
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This document contains the amendments made under Section 49 and is correct for printing.

Name of Applicant : YEDA RESEARCH AND DEVELOPMENT COMPANY LIMITED

Address of Applicant : P.O. Box 95, Rehovot, Israel

Actual Inventor : David Wallach of 14, Hadar Street, Rehovot, Israel,

Address for Service : F.B. RICE & CO.,
Patent Attorneys,
28A Montague Street,
BALMAIN. 2041.

Complete Specification for the invention entitled:
CYTOTOXIC PROTEIN

The following statement is a full description of this invention including the best method of performing it known to us :-

The present invention relates to monoclonal antibodies which specifically recognize and bind human tumor necrosis factor - alpha (herein TNF-α), to hybridoma cell lines expressing said monoclonal antibodies, and to a process for the purification of TNF-α using said monoclonal antibodies.

Background of the invention

Proteins which exert a toxic effect on cells (cytotoxins - CTXs) were found to be secreted, in response to stimulation, by mononuclear cells of various kinds. T-cells, of probably both the helper and the supressor subsets, can respond to antigens recognized by them as well as to mitogenic lectins, by secreting such cytotoxic proteins (Granger, G.A. and Kolb, W.P., J. Immunol. 101, 111-120 (1968): Ruddle, N.H. and Waksman, B.H., J. Exp. Med. 128, 1267-1279 (1968): Eardley, D.D., Shen, F.W., Gershon, R.K. and Ruddle, N.H., J. Immunol. 124, 1199-1202 (1980)). Monocytes and macrophages produce cytotoxic proteins in response to certain bacterial toxins (reviewed by Ruff, M.R. and Gifford, G.E. in Lymphokines, E. Pick and M. Landy editors, Academic Press, Inc., New York, 235-272 (1981)). Natural killer (NK) cells secrete cytotoxic proteins (natural killer cytotoxic factor - NKCF) upon incubation with appropriate target cells (Wright, S.C. and Bonavida, B., J. Immunol. 129, 433-439 (1982)) while cells of certain continuous B lymphocyte lines were found to produce spontaneously cytotoxic proteins (Rosenau, W.,

Stites, D. and Jemtrud, S., Cell. Immunol. 43, 233-244 (1979)).

There is some ambiguity in the terminology of the CTXs. Proteins produced in lymphocyte cultures, by induction with antigens or with mitogenic lectins, are usually referred to as lymphotoxins (LTs), while the term tumor necrosis factor (TNF) is used for cytotoxic proteins produced by cultures of monocytes or of macrophages. In accordance with the proposed new nomenclature, the TNF induced in mononuclear cells is now named TNF-α, while the LT is named TNF-β (see Wallach, D. Interferon 7, Academic Press Inc. (London) Ltd. (1986) pp. 90-124).

So far, only a single lymphotoxin produced spontaneously by cells of B lymphoblastoid line has been characterized in some detail. It was purified to homogeneity and its molecular weight was estimated to be about 20,000 daltons (Aggarwal, B.B., Moffat, B. and Harkins, R.N., J. Biol. Chem. 259, 686-691 (1984)).

According to the present invention, a cytotoxic protein referred to as cytotoxin (CT) was produced by induction of human peripheral blood mononuclear cells (PBMC) with Sendai virus, phytohemagglutinin-P (PHA) or concanavalin A (Con A), separated from the other proteins secreted by the mononuclear cells and purified to homogeneity by immunoaffinity purification with monoclonal antibodies raised against said cytotoxin. A purified cytotoxin was obtained with a molecular weight of about

17,000±500 daltons, as determined by SDS/PAGE analysis.

After filing the present patent application, it was verified that the 17,000 dalton cytotoxin that we have isolated according to the process of this invention is identical to the 17,000 dalton monocyte-derived human cytotoxin described by Pennica, D. et al., (1984) Nature (London) 312, 724-729, and designated "tumor-necrosis factor". We have already shown this identity in our publication: Hahn,T. et al, Proc. Natl. Acad. Sci. USA, 82, 3814-3818 (June 1985), as well as in later publications (Aderka, D. et al, J. Immunol. 136, 2938-2942 (1986); Israel, S. et al, Immunol. Letters 12, 217-224 (1986)). Since the cytotoxin of the present invention is identical to TNF-α, this nomenclature shall be used herein without deviating from the scope of the original disclosure.

The study of the functions of the cytotoxins and their application has for a long time been hampered by difficulties in purifying these proteins. In trying to purify cytotoxins by chromatographic procedures, we have repeatedly encountered significant losses of cytotoxic activity resulting, most likely, from instability of these proteins in a partially purified state. However, in spite of this extensive decrease in biological activity, preparations of CTXs which have been subjected to partial purification could induce, when injected into mice, significant titers of antibodies to CTXs. We therefore tried now to approach the isolation of TNF-α

indirectly by first raising monoclonal antibodies (Mabs) against the TNF-α, using mice which had been immunized with partially purified TNF-α preparation, and then applying these Mabs for immunoaffinity purification of the TNF-α. In other words, to isolate the cells which make antibodies to this protein as an antecedent step to the purification of the protein itself.

Description of the Invention

This invention provides hybridoma cell lines expressing monoclonal antibodies which specifically recognize and bind human TNF-α, said hybridomas being formed by fusion of spleen cells from a mouse previously immunized with human TNF-α and murine myeloma cells. Preferably, impure preparations of TNF-α are used for immunization of the mouse, although pure TNF-α preparations are also used according to the invention. The hybridoma cell line designated by us as Cell Line CT-1, producing the preferred monoclonal antibody against TNF-α according to the invention, was deposited with the National Culture Collection of Microorganisms of Institute Pasteur, Paris, France, under Deposition No. I-472, on July 16, 1985 (Collection Nationale de Cultures de Microorganismes - C.N.C.M.). as hereinafter defined.

The present invention also provides monoclonal antibodies, which specifically recognize and bind human TNF-α or neutralize its cytotoxicity. The invention further provides a process for the purification of TNF-α which comprises:

a. providing a preparation containing TNF-α;

b. absorbing the TNF-α from said preparation onto controlled pore glass beads;

c. desorbing the TNF-α in a state of enhanced purity from said controlled pore glass beads by means of a desorption buffer including 0.5M tetramethyl ammonium chloride; and

d. contacting the desorbed TNF-α with an immunoadsorbent, said immunoadsorbent comprising a monoclonal antibody against TNF-α according to the invention and eluting the TNF-α immunoadsorbent under mild dissociating conditions, by means of about 0.2M NH_4OH.

Purified homogeneous TNF-α is further provided whenever purified by th. immunoaffinity process, using the monoclonal antibodies of this invention.

In order to achieve these results, TNF-α was induced in human PBMC by Con A and phorbol 12-myristate 13-acetate (PMA) and purified with monoclonal antibodies raised against preparations of cytotoxins enriched with TNF-α by chromatographic procedures, following the scheme set out in Fig. 1.

The detection of monoclonal antibodies in the sera of immunized mice was performed by the techniques set out in Fig. 2.

The screening of hybridomas derived from splenocytes of the immunized mice for the production of TNF-α-binding antibodies

was done by the procedures set out in Fig. 3. The critical step was the screening of a large number of hybridomas for detecting a few hybridomas producing the desired antibodies against TNF-α. A solid-phase assay for the detection of antibodies which bind TNF-α was thus established and used for screening the multiple hybridomas derived from splenocytes of mice immunized with partially purified, phytohemagglutinin (PHA) induced preparations containing this protein. Among multiple hybridoma cultures which were screened by that technique a few were found to produce antibodies that bound TNF-α. The selectivity of their binding activity is demonstrated in Fig. 4, which compares the binding of TNF-α by one of the monoclonal antibodies (CT-1; in A) to the binding of IFN-gamma by a monoclonal antibody against the latter lymphokine (in B) and to the lack of the binding of either, to a third, unrelated, monoclonal antibody (against dinitrophenyl-DNP, in Fig. 4, C).

SDS/PAGE analysis of the proteins bound to CT-1 showed that of the many proteins present in crude preparations of cytotoxins CTX, only a single minor protein, with an apparent molecular weight of about 17,000 daltons is recognized by the antibody. Fig. 5 shows in lane C the TNF-α purified on an immunoadsorbent column constructed with the monoclonal antibody CT-1, as detected by Coomassie blue staining, following electrophoresis on an acrylamide gel in the presence of SDS.

Fig. 6 shows how the molecular weight of the TNF-α is estimated

by comparison to the mobility, on acrylamide gel, of the standard proteins shown in Fig, 5D.

Examples

1. Induction of TNF-α: Human peripheral blood mononuclear cells (PBMC) were isolated on a Ficoll-Hypaque cushion (Pharmacia, Upsala, Sweden) from the "buffy coats" of freshly donated blood and depleted of platelets by differential centrifugation. The cells were suspended at a concentration of 10^7 cells/ml and incubated at 37°C in minimal essential EM alpha-medium (Gibco, Grand Island, N.Y.). TNF-α was induced in these cells by one of the following techniques:

(A) TNF-α preparations used for the immunization of mice were induced by stimulating PBMC with phytohemagglutinin-P (PHA). Prior to that stimulation, the cells were first incubated for 12 h in the presence of a crude preparation of lymphokines (0.2 µg/ml). This treatment did not result in the production of TNF-α but greatly increased the responsiveness of the cells to subsequent stimulation. PHA (5 µg/ml) (Difco, Detroit) was then added and the PBMC were further incubated for 24 h. The medium was then collected, centrifuged at 2500 rpm for 15 min to remove cell debris, and processed for concentrating and enriching the TNF-α as described below.

(B) Preparations of lymphokines used for purifying TNF-α on

immunoadsorbents were advantageously induced with Con A as it was found difficult to fully eliminate traces of PHA in the purification procedure. The cells were first treated for 12 h with 0.25 μg/ml Con A. At this concentration, Con A did not induce significant secretion of TNF-α but it increased the responsiveness of the cells to subsequent stimulation by a higher concentration of Con A. Phorbol 12-myristate 13-acetate (PMA) was then added to a concentration of 5 ng/ml and 3 h later Con A was added to concentration of 10 μg/ml. The cells were incubated for 24 h and then, following replacement with fresh medium containing 5 μg/ml Con A, for a further period of 24 h. The media were combined and centrifuged, α-methyl-D-mannopyranoside (Sigma, St. Louis, Mo.) was added to a final concentration of 50 mM and the media were then further processed for purification on the immunadsorbent as described below.

(C) Alternatively, the TNF-α can effectively be induced in human peripheral blood mononuclear cells, in monocytes isolated from the mononuclear cell population or in cultured cells such as U937 whose properties resemble those of monocytes, by applying to these cells Sendai virus (SV)(500 HA/ml) and incubating the cells for a period of about 12 hours to allow the production of TNF-α. The cell media are then centrifuged and processed for purification of the TNF-α as described below.

2. Quantitation of TNF-α: TNF-α was quantitated by determining

its cytotoxic effect by a bioassay with SV-80 cells (Wallach, D. J. Immunol. 132, 2464-2469 (1984)). Samples to be tested were applied in serial dilutions simultaneously with the application of cyclohemixide (CHI 50µg/ml) into microwells containing confluent cultures of the SV-80 cells. The extent of cell killing, determined by measuring the uptake of neutral red by the cells, was quantitated 12 hours later, by using a MicroELISA Autoreader (Dynatech, Alexandria, VA). One TNF-α unit was defined as the concentration at which 50% of the cells were killed.

3. Enrichment for TNF-α: Crude preparations of TNF-α were first concentrated by adsorption to controlled-pore glass beads (CPG) (PG-350,200, Sigma, St. Louis, Mo.) followed by desorption in 0.5 M tetramethylammonium chloride (TMAC) and then further concentrated by ultrafiltration on an Amicon PM-10 membrane (Amicon, Denvers, Ma). TNF-α preparations applied for immunization of mice were then further purified by one of the two following procedures:

(A) The CPG-concentrated TNF-α preparations from the first step were fractionated by electrophoresis in 7.5% polyacrylamide gels under non-denaturing conditions (Walker, S.M. and Lucas, Z.J., J. Immunol. 113, 813-823 (1974); Lewis, J.E., Carmack, C.E., Yamamoto, R. and Granger, G.A., J. Immunol. Meth. 14, 163-176 (1977)). Fractions eluted from slices of the gels which exhibit cytotoxic activity were pooled, concentrated by ultrafiltration

on an Amicon PM-10 membrane and injected into mice.

(B) CPG-concentrated TNF-α preparations were equilibrated with 1 M NaCl 30% (vol/vol) ethylene glycol 0.1 mM EDTA and 10mM sodium phosphate buffer, pH 7.4, and subjected twice, sequentially, to fractionation on an Ultrogel AcA 44 column. Following each ACA 44 fractionation, fractions exhibiting cytotoxic activity were pooled and concentrated on a PM-10 membrane. The cytotoxic proteins recovered from the second run on the Ultrogel column were applied to further purification by preparative isoelectric focusing in an LKB 8100-1 column containing 1% Ampholine (pH 3.5-10) in a sucrose gradient. Fractions exhibiting maximal cytotoxic activity, peaking at about pH 6.4 were pooled, concentrated, equilibrated with PBS and then injected into mice.

Immunization with TNF-α and Cell Fusion

Four-month-old female CB6 mice were repeatedly injected with samples of 10 µg each of TNF-α preparations at 1 to 3 week intervals - five times with preparations enriched by procedure A, as described above, and another two times with preparations enriched by procedure B. In the first immunization, the proteins were emulsified in complete Freund adjuvant and injected into the foot pads of the mice (0.5 ml/mouse). The second injection was given 3 weeks later, and the rest of the injections which were given at 1 to 2 week intervals, were all given subcutaneously using alumina gel as adjuvant (0.3 µg/0.25

ml/mouse). Immuni- zation was then discontinued for a month and the mouse showing the highest titer of serum antibodies against TNF-α was injected twice, intraperitoneally, at a 1-day interval, with 10 µg of a TNF-α preparation enriched by procedure B. A day after the second immunization, the mouse was sacrificed and its splenocytes were fused with NSO myeloma cells. The fused cells were distributed into multiple wells of microtiter plates and hybridomas were selected by growth in the presence of hypoxanthine/aminopterin/thymidine (HAT) - containing tissue culture medium. Hybridomas found to produce antibodies against TNF-α were cloned in soft agar. For growing these cells in the ascitic fluid of mice, they were innoculated intraperitoneally at 10^7 cells per mouse 2-4 weeks following intraperitoneal injection of 0.5 ml pristane (2,6,10,14-tetramethyl pentadecane).

Quantitation of Antibodies against TNF-α in Mouse Sera and in Hybridoma Growth Media

The level of antibodies against TNF-α in sera of mice was determined by measuring their neutralizing and binding activities.

TNF-α Neutralizing Activity

Samples of TNF-α (1/ U in 50 µl of Dulbecco's modified Eagle's medium containing 2% fetal calf serum (DME/2% FCS) were incubated for 4 h at 37°C with samples of mouse sera (50 µl), serially diluted in DME/2% FCS. They were then further

incubated for 12-16 h at 4°C and then assayed for TNF-α activity at eight 2-fold dilutions.

TNF-α Binding Activity

Samples of crude concentrated TNF-α (30µl, 10^4 U/ml) were incubated for 4 h at 37°C in conical-bottom microtiter plates with samples of mouse sera, serially diluted in DME/2% FCS. Normal mouse serum (20 µl of a 1:40 dilution in PBS - phosphate buffered saline) was added, followed by 60 µl of goat antiserum against mouse $F(ab')_2$ immunoglobulins. The plates were further incubated for 30 min at 37°C and then overnight at 4°C and were then spun at 1200 g for 5 min at 4°C. The immuno- precipitates were rinsed twice with cold PBS and once with unbuffered saline, solubilized by adding 50 µl of 7% mM NH_4OH and then assayed for TNF-α activity at eight 2-fold dilutions.

After five immunizations with preparations of human cytokines which had been enriched for TNF-α, mice yielded sera containing antibodies that bound TNF-α. The titer of this TNF-α-binding activity increased about 25-fold after two further immunizations. On the other hand, TNF-α-neutralizing activity was not detected aftr five immunizations but only following two further ones, and its extent was then significantly lower than that of the binding of TNF-α.

Splenocytes of a mouse that had developed a high titer of serum antibodies to human TNF-α were fused with myeloma cells, and the

hybridoma cells produced were screened for the production of antibodies against TNF-α. Since binding of TNF-α proved to be a more sensitive indicator of the presence of antibodies against these proteins than neutralization activity, we chose to screen the hybridoma cultures on the basis of binding activity.

Solid Phase Assay for Detecting TNF-α Binding Monoclonal Antibodies

A solid-phase modification for measuring TNF-α binding activity was used to screen the hybridoma growth media for the presence of TNF-α binding antibodies (Fig. 3). Polyvinyl chloride microtiter plates (Dynatech, Alexandria, Va) were incubated, with affinity-purified goat antibody against mouse $F(ab')_2$ immuno- globulin fragments (80 µg/ml in PBS, 80 µl/well), then with samples of the hybridoma growth media (50 µl/well) and finally with samples of a crude concentrated TNF-α preparation (10^4 µ/ml, 50 µl/well). Each of the three incubation periods lasted 12-18 h (at 4°C) and following each, the plates were rinsed 3 times with PBS and once with unbuffered saline. The bound TNF-α was dissociated by applying NH_4OH (75 mM containing 0.1% FCS 20 µl/well). A hundred µl of 0.04 M Na-Hepes pH 7.4 in DMEM-10% FCS were added and the eluted cytoxic activity was quantitated on CHI-sensitized SV-80 cells, at four serial 2-fold dilutions.

Among 1300 hybridoma cultures, 3 were found to produce antibodies that bound TNF-α. One of these hybridomas produced

IgG1 and the two others produced IgM.

As hereinafter defined, the most potent monoclonal antibody designated CT-1 is produced by the hybridoma cell line deposited with the Institute Pasteur under the accession number CNCMI-472.

CT-1 monoclonal antibody is of the IgM isotype, it binds TNF-alpha, it does not bind IFN-gamma, it has low affinity to TNF and releases bound TNF at pH 3 or 11.

To determine the specificity of the monoclonal antibodies (mAbs), the most potent of the three (CT-1, an IgM) was tested also for its ability to bind IFN-gamma - a protein present in significant amounts in the crude preparations of TNF-α (Fig. 4A). For comparison, a mAb against IFN-gamma (166-5, an IgG) and a mAb against a third, unrelated antigen (U13-6, a monoclonal IgM against 0-2,4-dinitrophenyl) were also examined for binding of TNF-α and of IFN-gamma (Fig. 4B and C). Selectivity in binding was clearly evident: The antibody against TNF-α bound cytotoxin effectively but did not bind IFN-gamma (Fig. 4A), whereas the antibody against IFN-gamma bound IFN-gamma but not TNF-α (Fig. 4B). The antibody against dinitrophenyl bound neither IFN-gamma nor TNF-α (Fig. 4C).

This selectivity was confirmed by NaDodSO$_4$/PAGE analysis of the proteins that bound to the CT-1 column (Fig. 5). Of the many proteins present in a crude preparation of TNF-α (Fig. 5, lane A), only a single minor protein whose apparent Mr was 17,500 was found to adsorb to the immunoadsorbent constructed from the CT-1 antibody (Fig. 5, lane C).

In the same way as monoclonal antibody CT-1, other monoclonal antibodies were produced. For example, monoclonal antibodies anti TNF-1 and anti-TNF36 were developed using splenocytes of Balb/c mice which were immunized with TNF and screened for, as with CT-1. As shown in Table 1 below, their affinity to TNF was much higher than that of CT-1. Anti TNF-1 and anti-TNF36 are IgG1 while CT-1 is IgM.

Table 1: Comparison of the monoclonal antibodies CT-1, anti TNF-1 and anti TNF-36 for affinity to TNF-α

Eluting buffer	CT-1	anti TNF-1	anti TNF-36
	(% counts eluted at given step)		
50 mM Citric acid, 1 M NaCl	>98%	7.8%	6.7%
5 M Urea	-	26.7%	56.3%
6 M Urea	-	20.2%	73.0%
8 M Urea	-	71.0%	70.1%

The three antibodies were covalently bound to hydrazidagarose (1mg antibody per ml of resin). Samples of 0.1 ml of resin were packed into micro columns. ^{125}I-TNF (5.10^5 cpm 50μCi/μg) was applied and then eluted by sequentially washing the columns with samples of 1.5 ml of the various elution buffers. Given are the percentages of residual bound radioactivity eluted at each step.

Purification of TNF-α on Immunoadsorbents

Monoclonal antibodies were purified from ascitic fluids by precipitation with ammonium sulphate (50%). Those of the IgM isotype were further purified by dialysing against water followed by solubilization of the precipitating IgM in PBS. 10 mg of each

of the purified immunoglobulins were coupled to 1 g Trisacryl GF2000 (LKB) which was derivatized with aminocaproic acid and activated with N-hydroxysuccinimide. Uncoupled antibody was removed by washing the resin with 50 mM Na-citrate pH 2.8 and then with 0.15 NH_4OH.

For purification of TNF-α on the immunoadsorbent, samples of 0.5 ml of the resin were mixed for 2 h at 4°C with 3 ml of TNF-α preparation in the presence of 0.5 M TMAC. The resins were then packed in small columns, and unbound protein was washed with 0.5M TMAC solution. The columns were then further washed with 0.5% Nonidet P-40 in 0.5M TMAC, then with a solution of 1 M NaCl containing also 10 mM sodium phosphate buffer pH 7.4 and then with unbuffered saline. The bound TNF-α was eluted by applying 0.2 M NH_4OH and neutralized with 1 M acetic acid within 10 min of elution. All steps of the immunoaffinity purification procedure were carried out at 4°C.

Analysis of the Purified TNF-α by SDS Gel Electrophoresis

Fig. 5 shows the pattern of proteins in a crude preparation of cytotoxins as analyzed on SDS-polyacrylamide gel (15%). NH_4OH-eluted fraction from an immunoadsorbent constructed with the anti-dinitrophenyl (DNP) monoclonal antibody U13-6 on which the crude TNF-α has been applied is shown in lane B. TNF-α purified from the crude preparation on the CT-1 immunoadsorbent column is shown in lane C, and molecular weight standards (phosphorylase 94K, bovine serum albumin 67K, ovalbumin 43K,

carbonic anhydrase 30K, soybean trypsin inhibitor 20.1K and lysozome 14.4K daltons) is shown in lane D. Lane C shows that the purified TNF-α constitutes a single polypeptide species. As shown in Fig. 6, the molecular weight of the purified protein, as estimated by comparison to the mobility on the acrylamide gel of other proteins with known molecular weights, is about 17.5Kd.

The monoclonal antibodies of this invention are, therefore, useful in the purification of TNF-α, a cytotoxic protein useful for treating virus-infected and tumor target cells.

Since some of the monoclonal antibodies have also a high affinity to TNF-α and neutralize its cytotoxicity, they may be used themselves as pharmaceuticals for neutralizing the deleterious effects of TNF-α when produced by the organism in conditions that cause destruction of normal tissues. One of such severe conditions is septic shock, where high levels of TNF-α in the blood can be rapidly neutralized by anti-TNF-α antibodies in extracorporeal devices for treatment of blood, or by injection of the antibodies to patients.

As mentioned above, TNF-α has a major causative role in the elicitation of septic shock. It is formed in the body in bacterial infections and then greatly potentiates those effects of the bacterial lipopolysaccharides (LPS) which can lead to shock.

To examine whether the monoclonal antibodies against TNF-α which

we have developed can protect *in vivo* against this effect of TNF-α, we have established the following experimental set-up:

Mice were injected with increasing doses of bacterial LPS (from *E. coli* 0127:38) and of homogeneously purified recombinant TNF-α (rTNF) to establish the maximal sublethal dose of the two. They were then injected with combinations of TNF-α and LPS, at various sublethal doses, to determine that combination which is lethal. The effect of our antibody CT-1 was then examined by injecting samples of the purified antibody IP, at various amounts (in 0.5 ml phosphate buffered saline) simultaneously with the IP injection of the combination of TNF-α and LPS. All treatments were done in duplicates using Balb/c mice.

As shown in Table 2 below, injecting mice with the combination of 100 μg LPS and 5.10^6 units of TNF-α but not with either of the two alone resulted, within 24 hr, in death of both mice. The monoclonal CT-1 antibody could not protect the mice when injected at a dose of 50 μg but fully protected the mice from the lethal effect of the TNF-α + LPS combination when injected at doses if 250 μg and above.

Table 2: Protective effect of monoclonal antibody CT-1 against the lethal effect of TNF-α in mice under conditions mimicking elicitation of septic shock

LPS (μg)	rTNF-α (units)	CT-1* (μg)	mice remaining alive in 24 hr	mice remaining alive in 3 days
-	-	-	2/2	2/2
100	-	-	2/2	2/2
-	5.10^6	-	2/2	2/2
-	-	500	2/2	2/2
100	5.10^6	-	0/2	0/2
100	5.10^6	50	0/2	0/2
100	5.10^6	250	2/2	2/2
100	5.10^6	500	2/2	2/2

* Purified by ammonium sulphate precipitation and fractionation on DEAE-cellulose.

These experiments confirmed that CT-1 can neutralize those activities of human TNF which take part in the development of septic shock and establish an experimental set-up for a routine testing of the effectivity of differing batches of the antibody.

This and other indications or methods can be developed with the monoclonal antibodies of this invention.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:-

1. A hybridoma cell line expressing a monoclonal antibody which specifically recognizes and binds human TNF-alpha said hybridoma being formed by fusion of spleen cells from a mouse previously immunized with human TNF-alpha and murine myeloma cells.

2. A hybridoma cell line according to claim 1 wherein impure preparations of human TNF-alpha are used for immunization of the mouse.

3. The hybridoma cell line of claim 1 having the identifying characteristics of CNCM I-472, as hereinbefore defined.

4. A monoclonal antibody produced by a hybridoma according to claim 1, which specifically recognizes and binds human TNF-alpha.

5. A monoclonal antibody according to claim 4 produced by a hybridoma cell line according to claim 1.

6. A monoclonal antibody of the type IgM, said antibody being produced by the hybridoma cell line according to claim 3.

7. A process for the purification of human TNF-alpha which comprises:

a. providing a preparation containing human TNF-alpha;

b. absorbing the TNF-alpha from said preparation onto controlled pore glass beads;

c. desorbing the TNF-alpha in a state of enhanced purity from said controlled pore glass beads;

d. contacting the desorbed TNF-alpha with an immunoadsorbent, said immunoadsorbent comprising a monoclonal antibody against the TNF-alpha produced by a hybridoma according to claim 1; and

e. eluting TNF-alpha from the immunoadsorbent.

8. The process of claim 7 wherein the TNF-alpha is desorbed in step (c) by means of a desorption buffer including 0.5 M tetramethyl ammonium cloride.

9. The process of claim 7 or 8 wherein the TNF-alpha is eluted from the immunoadsorbent by means of about 0.2 M NH_4OH.

10. The process of any of claims 7 to 9 wherein the monoclonal antibody used is the monoclonal antibody according to claim 6.

11. The process of any of claims 7 to 10 where the preparation of step (a) is made from stimulated peripheral blood mononuclear cells.

12. The process of claim 11 when the cells are stimulated with phytohemagglutinin after incubation with lymphokines.

13. The process of claim 11 where the cells are stimulated by concanavalin-A that does not itself induce significant secretion of TNF-alpha.

14. The process of claim 11 in which the cells are stimulated with Sendai virus.

15. A process for the preparation of a purified human TNF-alpha which comprises contacting a preparation containing a TNF-alpha with an immunoadsorbent comprising a monoclonal antibody as defined in claim 6 and eluting the TNF-alpha from said immunoadsorbent.

16. Purified homogenous human TNF-alpha whenever obtained according to the process claimed in any of claims 7 to 15.

~~17. A solid phase immunoassay by which multiple hybridoma~~ cultures can be screened for the production of antibodies which can bind human TNF-alpha, which comprises:

a. Coating protein-binding support means with affinity purified antibody against mouse immunoglobulins;

b. Incubating the tested hybridoma growth media in the coated support means followed by washing;

c. Incubating samples of TNF-alpha in the support means followed by washing;

d. Dissociating the human TNF-alpha which has bound to the support means and determining its amount in a ~~bioassay.~~

17. A process for isolation of human TNF-alpha which comprises:

a. Developing monoclonal antibodies against human TNF-alpha from a hybridoma claimed in claim 1 following immunization with impure preparations of human TNF-alpha fusion of spleen cells of said mice with murine myeloma cells to form hybridomas and cultivating said hybridomas to obtain the desired monoclonal antibodies;

b. Constructing an immunoadsorbent from these antibodies and using the same for purifying human TNF-alpha from crude preparations thereof.

18. A process according to claim 17, characterized in that the monoclonal antibody is the antibody according to claim 6.

19. A pharmaceutical composition for selectively treating virus-infected and tumor target cells in humans, which comprises a therapeutically effective amount of at least purified homogeneous human TNF-alpha as claimed in claim 16 or a salt thereof, together with a pharmaceutically acceptable excipient or in combination with interferons and/or a metabolic blocker in amount sufficient to sensitize said cells to human TNF-alpha but not to sensitize normal cells.

20. The composition of claim 19 where the blocker is cycloheximide.

21. The composition of claim 19 where the blocker is mitomycin C.

22. The composition of claim 19 where the blocker is actinomycin D.

DATED this 7th day of June 1990

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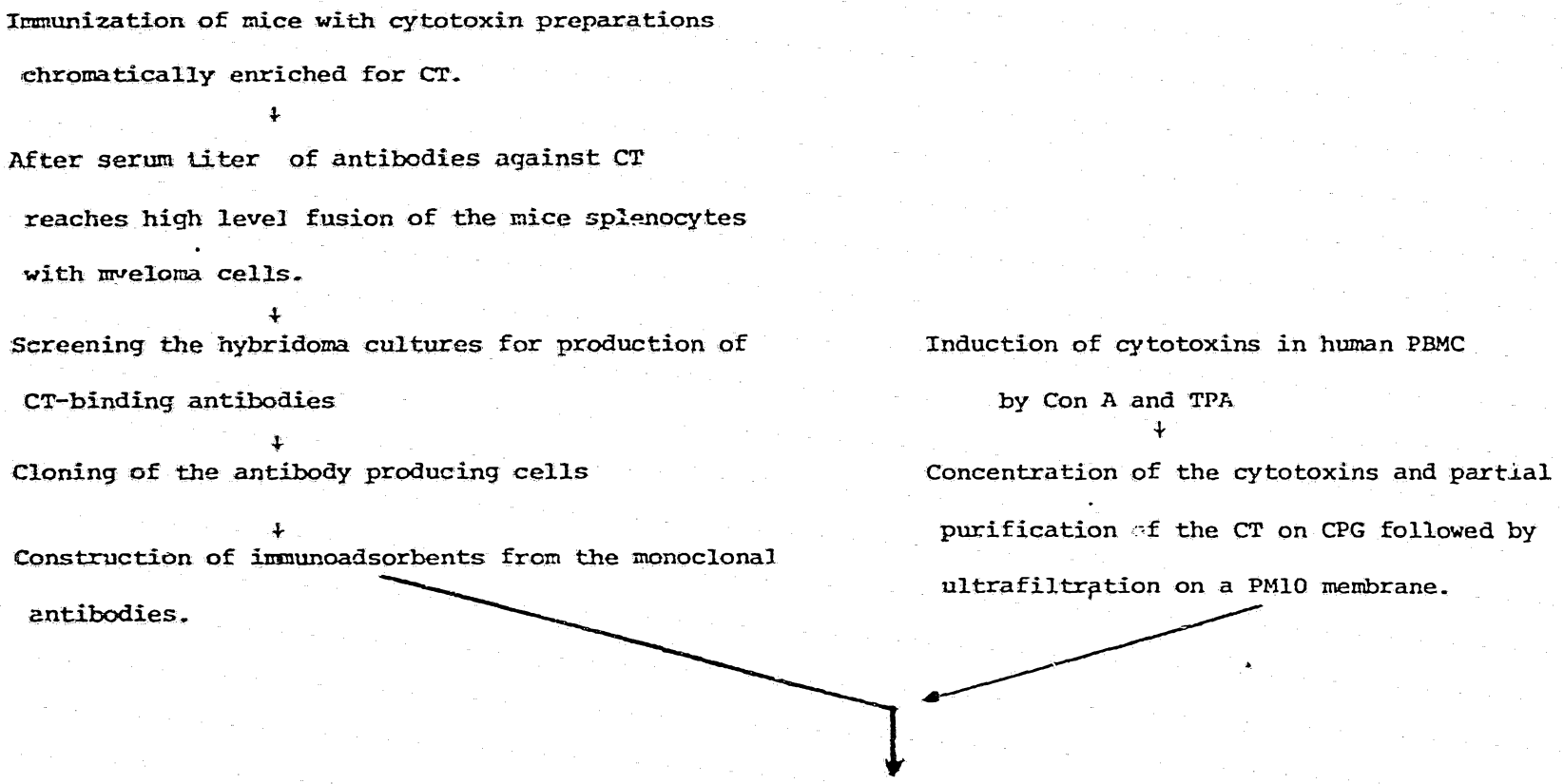

Fig. 1

Alternative Approaches for Detection of Antibodies against CT in Sera of Immunized Animals

a Neutralization assay:	Incubation of CT preparation with antiserum.	→ Determination of CT activity (in the presence of antiserum)		
b Binding assay:	Incubation of CT preparation with antiserum	Precipitation of serum immunoglobulin+bound CT with goat antiserum against mouse immuno-globulins.	→ Washing of immuno precipitate followed by its solubilization at 0.075M ammonia	→ Determination of T activity in solubilized immunoprecipitate

Fig. 2

The Solid Phase Assay for Detecting, CT Binding, Monoclonal Antibodies

Adsorbtion of hybridoma produced immunoglobulin to PVC microwells which had been precoated with affinity purified antibodies against mouse immunoglobulins. → Incubation of CT preparations in microwells. → Rinsing of microwells followed by dissociation of bound antigens at 0.075M ammonia → Determination of CT activity in the proteins eluted from microwells

Fig. 3

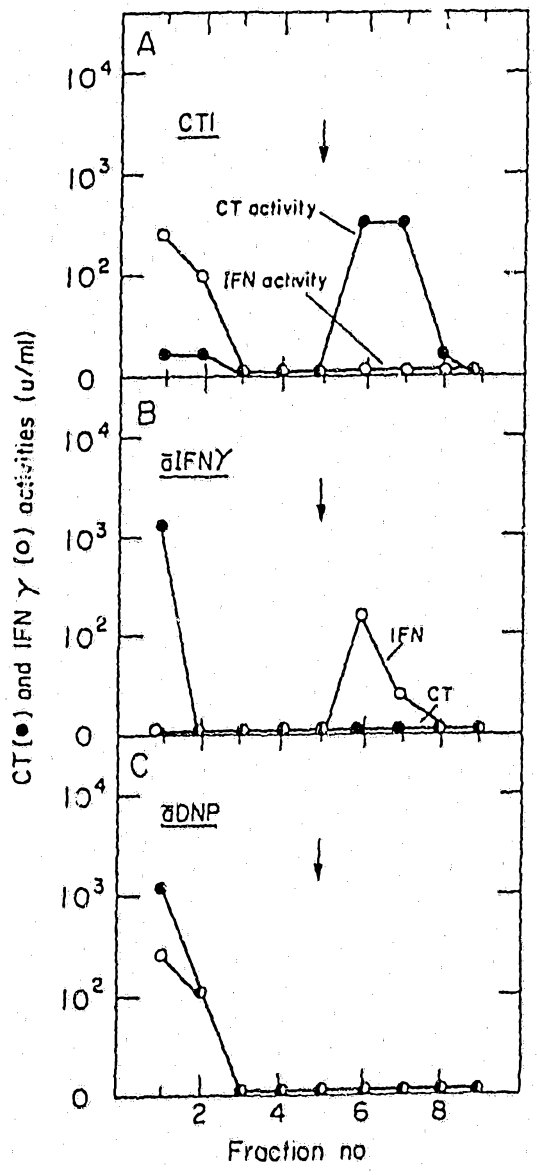
A
CTI
CT activity
IFN activity
B
αIFNγ
IFN
CT
C
αDNP
CT (●) and IFN γ (○) activities (u/ml)
Fraction no

FIG 4

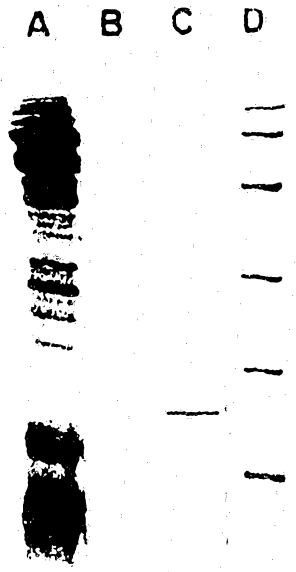
A
B
C
D

FIG 5

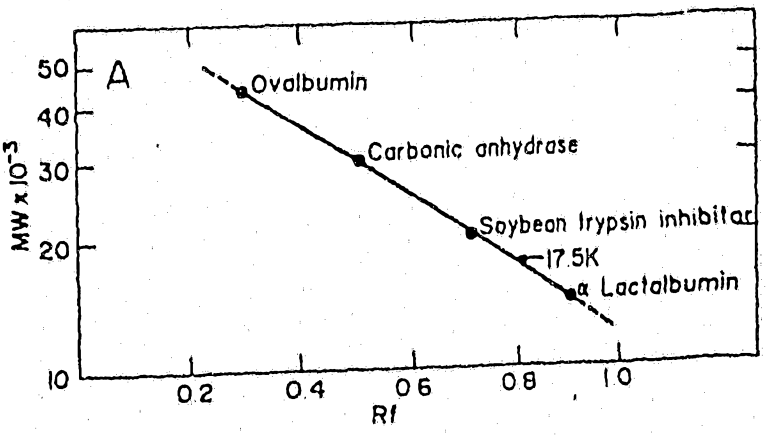
A
Ovalbumin
Carbonic anhydrase
Soybean trypsin inhibitor
17.5K
α Lactalbumin
MW x 10⁻³
50
40
30
20
10
0.2
0.4
0.6
0.8
1.0
Rf

FIG 6

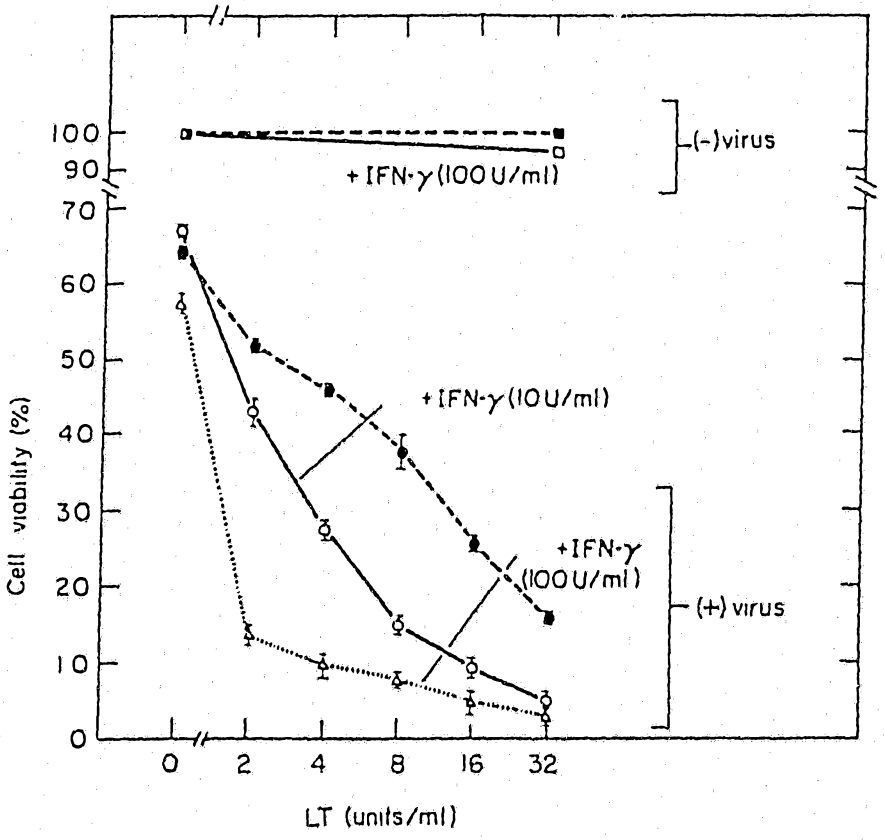
100
90
70
60
50
40
30
20
10
0
Cell viability (%)
0
2
4
8
16
32
LT (units/ml)
(-)virus
+IFN-γ(100U/ml)
+IFN-γ(10U/ml)
+IFN-γ
(100U/ml)
(+) virus

FIG. 7.