THE PRODUCTION AND CHARACTERIZATION OF A PUTATIVE ANTI-IDIOTYPIC ANTIBODY TO TUMOR NECROSIS FACTOR-α

by

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(Abstract)

Tumor necrosis factor-α (TNFα) is primarily a macrophage-derived cytokine. TNFα, in vitro, kills or inhibits growth of approximately one third of surveyed transformed cell lines including the L929 and WEHI 164 murine fibrosarcoma cell lines. Very little is known about the mechanisms of TNFα action. However, recently, it has been theorized that TNFα has no activity of its own and that the receptor for TNFα on the cell surface, when properly triggered, activates the cellular mechanisms which may result in the cell's death.

The objective of this study was to produce an anti-idiotypic antibody to TNFα to be used as a tool to study the mechanisms of TNFα action. A hybridoma that secretes an anti-idiotypic antibody to TNFα (Ab2β) has been produced and isolated following standard procedures. This antibody was found to be of isotype IgG2a as determined by an indirect ELISA test. The Ab2β exhibited TNFα target cell-killing capabilities in vitro. The TNFα-resistant cell lines, SP2/O and NS-1 were resistant to Ab2β and TNFα sensitive cells, L929 and WEHI 164, were sensitive to Ab2β. The cell killing activity of both TNFα and Ab2β could be neutralized by a monoclonal anti-TNFα antibody. Both TNFα and Ab2β acted in parallel having an effect on the killing of Brucella abortus strain RB51 by peritoneal macrophages, whereas neither TNFα nor Ab2β had an effect on the killing of strain 2308 by macrophages. These results, again indicate that TNFα and Ab2β have parallel bactericidal effects and that Ab2β is capable of mimicking TNFα activity. The Ab2β was further characterized by gel electrophoresis and Western blot and was found to have two subunits of 25 and 50 kDa molecular weights similar to IgG.

This anti-idiotypic antibody to TNFα may help in understanding the mechanisms of the cytotoxic activity of TNFα.

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Table of Contents

		Page
	Abstract	ii
	Acknowledgements	iii
	List of Figures	vi
	List of Abbreviations	vii
I.	Introduction and Objectives	1
II.	Literature Review	2
	A. Tumor Necrosis Factorα	2
	1. Structure	3
	2. Actions	4
	3. Pharmacokinetics	14
	4. Clinical Trials	15
	5. Production and Metabolism of Arachidonic Acid	16
	6. Genetics	19
	7. Receptors	20
	8. Intracellular Events	21
	B. Anti-idiotypic Antibodies	24
	1. Jerne's Theory	24
	2. Antigenic Mimicry	26
	C. Cytokines and Resistance to Pathogens	29
1.	Effects of Cytokines on Resistance to Pathogens	29
	2. Effects of TNFα on Brucella abortus	30

II. Materials and Methods..... 32
A. Immunization of Mice..... 32
B. Cytotoxicity Assay..... 32
C. Production of Hybridomas..... 34
1. Cell Fusion..... 34
2. Cloning by Limiting Dilution..... 37
D. Antibody Purification..... 37
E. Determination of Protein Content..... 38
F. Cytotoxicity Neutralization Assay..... 39
G. Isotype and Subisotype determination by ELISA 39
H. Gel Electrophoresis and Western Blotting..... 40
I. Isolation of Peritoneal Macrophages..... 42
J. Bactericidal Assay..... 43
K. Statistical Analysis..... 45
III. Results..... 46
A. Titer Activity..... 46
B. Hybridoma Isolation..... 48
C. Isotype and Subisotype Determination..... 50
D. Further charaterization of Ab2β..... 50
E. Neutralization of cytotoxic activity..... 54
F. Molecular weight determination..... 60
G. Bactericidal assay..... 64
IV. Discussion..... 69
V. Literature Cited..... 76

List of Figures

Figure		Page
1.	Cytotoxicity Assay: antiserum to Anti-TNFα.....	47
2.	Cytotoxicity Assay: polyclonal supernatant.....	49
3a.	Cytotoxicity Assay: 1C and Ab2β on L929.....	51
3b.	Cytotoxicity Assay: CVC.7 on L929.....	52
4a.	Cytotoxicity Assay: rHuTNFα on WEHI 164.....	53
4b.	Cytotoxicity Assay: 1C and Ab2β on WEHI 164.....	55
5a.	Cytotoxicity Assay: rHuTNFα on SP2/O and NS-1....	56
5b.	Cytotoxicity Assay: 1C on SP2/O.....	57
5c.	Cytotoxicity Assay: 1C on NS-1.....	58
6a.	Neutralization Assay: rHuTNFα.....	59
6b.	Neutralization Assay: Ab2β.....	61
6c.	Neutralization Assay: rHuTNFα or Ab2β using CVC.7.	62
7a.	Gel Electrophoresis: Ab2β, TNFα, and dog IgG.....	63
7b.	Western Blot: Ab2β and dog IgG.....	65
8a.	Bactericidal Assay: log (# strain RB51) vs. time..	66
8b.	Bactericidal Assay: log (# strain 2308) vs. Time..	67

List of Abbreviations

ACTH = adrenocorticothyroid hormone

BCG = Bacillus Calmette-Guerin

cfu = colony formong unit

DLF = dose limiting factor

DNA = deoxyribonucleic acid

DMSO = dimethylsulfoxide

ELAM = endothelial leucocyte-adhesion molecule-1

GH = growth hormone

GM-CSF = granulocyte/macrophage-colony stimulating factor

HAT = hypoxanthine-aminopterin-thymidine

HT = hypoxanthine-thymidine

ICAM-1 = intercellular adhesion molecule-1

IFNβ = interferon-β

IFNγ = interferon-γ

IgG = immunoglobulin G

IL-1 = interleukin-1

IL-2 = interleukin-2

IU = international unit

kDa = kilodalton

LPL = lipoprotein lipase

LPS = lipopolysaccharide

mAb = monoclonal antibody

M-CSF = macrophage-colony stimulating factor

MEM = minmum essential media

MHC = major histocompatability complex

MLI = mistletoe lectin I

Mn-SOD = manganese superoxide dismutase

MTD = maximum tolerated dose

NK-kB = nuclear factor-kB

pAb = polyclonal antibody

PAF = platelet activating factor

PBS = phosphate buffered saline

PDGF = platelet derived growth factor

PEG = polyethylene glycol

PGE_2 = prostaglandin E_2

PGG_2 = prostaglandin G_2

PGI_2 = prostaglandin I_2

PKC = protein kinase C

PLA_2 = phospholipase A_2

rHuTNFα = recombinant human tumor necrosis factor-α

RPM = revolutions per minute

SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TBS = Tris buffered saline

TNFβ = tumor necrosis factor-β

VLDL = very low density lipids

VSV = vesicular stomatitis virus

I. INTRODUCTION AND OBJECTIVES

Tumor necrosis factor-α (TNFα) is a cytokine of the immune system. Recent evidence indicates that it plays an important role in regulating a number of biological processes including cell proliferation, hemorrhagic necrosis of transplanted tumors, cytotoxicity, inflammatory, immunoregulatory, and antiviral responses[9]. TNFα, formerly known as cachectin, is also known to cause endotoxic shock and cachexia[30]. However, the mechanism by which TNFα elicits these biological effects still remains obscure. Since anti-idiotypic antibodies retain some of the structural integrity of the original antigen, it was hypothesized that use of an anti-idiotypic antibody to TNFα may shed some light on the mechanisms of these responses. The overall objective was to produce and characterize an anti-idiotypic antibody to TNFα and to determine if the anti-idiotypic antibody would mimic some of the biological activities of TNFα. The specific aims of this research were:

1. to produce and characterize polyclonal antibodies against monoclonal antibodies specific for TNFα (Ab1).
2. to produce and characterize a monoclonal antibody (Ab2) reactive with the Ab1 to TNFα.
3. to determine if Ab2 produced against Ab1 will mimic the cytotoxic effects of TNFα in cell lines susceptible and resistant to TNFα.

4. to determine if Ab2 produced against Ab1 will mimic the effects of TNFα on macrophages infected with Brucella abortus, in vitro.

II. LITERATURE REVIEW

A. Tumor Necrosis Factor-α

TNFα and TNFβ, formerly known as cachectin and lymphotoxin respectively, are functionally similar cytokines of the immune system[60]. TNFβ is primarily produced by T lymphocytes, whereas TNFα is primarily produced by macrophages. To a lesser extent, TNFα is also produced by lymphocytes when exposed to phorbol esters, NK cells, monocytes, B cells, large granular lymphocytes, and mast cells[9,66]. Macrophages can be stimulated in vitro to produce TNFα using lipopolysaccharide (LPS) isolated from E. coli. Interferon-γ (IFNγ), Brucella abortus, Corynebacterium parvum, and bacillus Calmette-Guerin (BCG) can also be used to stimulate TNFα production but not as efficiently as purified lipopolysaccharide (LPS). Mistletoe lectin I (MLI) from the mistletoe, Viscum album, stimulates peripheral blood monocytes to produce TNFα[45]. Stimulated macrophages can be activated to release TNFα by direct contact with some tumor cells but not by nontransformed cells[25]. Once the macrophages are stimulated by LPS to release TNFα, it is estimated to be 1-2% of the

total secretory products[9]. Production of TNFα can be inhibited by carrageenan, in vitro, at the mRNA level. Pentoxyfylline can inhibit TNFα synthesis at the mRNA level, in vitro[61].

1. Structure

TNFα is a polypeptide with a monomer size of 17 kDa. It consists mainly of anti-parallel β-pleated sheets in a jelly roll topology[20]. It has one intrachain disulfide bond between cysteines 69 and 101. TNFα can exist in solution as a dimer, trimer, or pentamer[9,65]. However, most recent studies show that the trimeric form is the most biologically active[9,21,65,69]. TNFα is relatively hydrophobic but is irreversibly denatured by boiling for 5 minutes[9]. Temperatures up to 50°C for any length of time are tolerated by TNFα without significant loss of biological activity[9]. Addition of 6M urea denatures TNFα and its activity can be restored upon dialysis. Interspecies structural homologies of mature TNFα molecules are extensive. Thus, murine and human TNFα are 156 amino acids and 157 amino acids in length, respectively and there is 79% homology between the two polypeptides. A highly conserved area is found between amino acids 115 and 130. Also, there is an invariant c-terminal leucine. TNFα is synthesized in murine and human species as a prohormone having an additional 79 and

76 amino acids, respectively. The prohormones of these two species are 86% conserved. The prohormone is a biologically inactive precursor to the active form of TNFα which is then cleaved to produce the active form of TNFα. A distinct structural difference that is observed between these two polypeptides is the mouse TNFα glycosylation at an N-linked site which has not been detected in human TNFα. The function of the side-chain is unknown. Because of the structural similarities, species preference between human and mouse TNFα is not considered significant[34]. Human and mouse TNFα can be used interchangeably.

2. Actions

In 1975, Carswell et.al. discovered a necrosis factor in the serum of BCG-infected mice treated with endotoxin[13]. It was also noted that cancer patients who showed hemorrhagic necrosis of their tumors had streptococcal infection[60]. The factor found to be responsible for this was termed tumor necrosis factor-α and was soon discovered to be identical to cachectin[10]. As mentioned before, the name TNFα is not very descriptive of its capabilities. Since this polypeptide has pleiotropic properties, it plays a role in regulating a number of different body functions including hematopoiesis, its antiviral properties, its function in immunoregulation, its

antitumor properties, its role in cachexia and in lipolysis, its ability to induce skeletal changes, its effects on the vasculature, and its role in septic shock[23,76].

In hematopoiesis, TNFα causes an early release of immature neutrophils from bone marrow resulting in a "left shift" in a complete blood count[36]. It stimulates the endothelial cells to release neutrophil chemotactic factors and enhances adhesion of neutrophils, as well as other cells, to the vascular endothelium by upregulating the production of intercellular adhesion molecule-1 (ICAM-1). It increases the phagocytic capabilities of neutrophils, the ability of neutrophils to degranulate, increases neutrophil microbicidal activity, and the ability to increase superoxide production in neutrophils[9,36]. TNFα is also known to reorganize the vascular endothelium, such that the cells flatten, overlap, rearrange actin filaments, and lose stainable fibronectin[9]. In humans, mice, and horses, treatment with TNFα leads to an initial neutropenia and lymphophilia, followed by an abrupt reversal to neutrophilia and lymphopenia[36]. The lymphopenia may be due to the up-regulation of leukocyte adhesion molecules, ICAM-1, and endothelial leucocyte-adhesion molecule-1 (ELAM-1)[30].

Detectable levels of TNFα have been found in serum from patients with leishmaniasis and cerebral malaria[23]. TNFα is known to enhance eosinophilic and monocytic toxicity to schistosomula <u>in vitro</u>[9]. These studies support the hypothesis

that TNFα has a role in chronic parasitic infections. Significant levels of TNFα have also been found in patients with Yersinia arthritis, sarcoidosis, hairy cell leukemia, chronic lymphocytic leukemia, some autoimmune disorders, and AIDS[66]. It has been shown that patients with non-Hodgkin's lymphomas treated with granulocyte/monocyte-colony stimulating factor (GM-CSF) show enhanced serum levels of TNFα in less than 24 hours[25]. Patients with bacterial meningitis have detectable levels of TNFα in their cerebrospinal fluid. It can cross the blood-brain barrier in small amounts and may play a role in multiple sclerosis[9]. Elevated serum levels of TNFα were observed in 65% of patients with renal allografts. Only 20% of these showed detectable blood levels before transplantation[49].

It is postulated that TNFα has some antiviral properties since TNFα is known to prevent replication of vesicular stomatitis virus (VSV) in the glioblastoma cell line U87MG, myeloma cell line RPMI-8226, C127 mouse epithelial cells, and RAT-1 fibroblasts. These effects may have occurred because of IFNγ induction instead of TNFα activity since monoclonal antibodies to TNFα did not suppress the antiviral activity[60].

Natural killer cell activity is augmented by TNFα, as is the cytotoxicity of the macrophage[36]. Both activities are further enhanced by co-incubation with interleukin-2 (IL-2).

Some genes are induced by the presence of TNFα including

genes coding for antioxidants like manganese superoxide dismutase (Mn-SOD) and ferritin heavy chain[78]. Some heat shock protein genes including genes for HSP68 and HSP70 may be induced by TNFα and may be involved in the safe disposal of TNFα via lysosomal degradation[38]. TNFα also induces the genes for the major histocompatibility complex (MHC) antigens I and II, and the class II associated invariant chain[58,66]. IFNγ acts synergistically with TNFα to increase mRNA levels for MHC class I heavy- and light-chain genes[31].

TNFα stimulates macrophages to produce platelet activating factor (PAF), prostaglandin E_2 (PGE_2), interleukin-1 (IL-1), GM-CSF, thromboxane, and TNFα itself[9,23,36]. TNFα can induce fibroblasts to produce a number of factors including GM-CSF, PGE_2, IL-1, IL-6, IFNβ, HLA-antigen, glycosaminoglycans, and collagenase[8,9,36]. Synovial cells and dermal fibroblasts produce PGE_2 and collagenase when treated with TNFα. Endothelial cells can be stimulated by TNFα to produce IL-1, platelet derived growth factor (PDGF), PAF, and prostacyclin but production of thrombomodulin is depressed[36]. TNFα enhances IL-2-dependent production of IFNγ by lymphocytes.

TNFα also affects the endocrine system by inducing the release of adrenocorticotropic hormone (ACTH), growth hormone (GH), and thyrotropin from the anterior pituitary. The increased release of GH and thyrotropin caused by TNFα is

suppressed by indomethacin without affecting the release of ACTH[30]. Also, the levels of the stress hormones epinephrine, norepinephrine, and glucagon increase following treatment with TNFα. The general effects of indomethacin on TNFα activity will be discussed later.

TNFα, interferons, and interleukins are classified as cytokines. They function as a network, working together as a team of regulators. TNFα, IL-1, and IL-6 have many similar functions including cytotoxic effects on some malignant cell lines, stimulatory effects on the growth of fibroblasts, activation and adherence of neutrophils, and pyrogenic activation[60]. TNFα is an endogenous pyrogen. It directly affects hypothalamic neurons by triggering PGE_2 production[17]. Production of PGE_2 causes an increase in body temperature due to a direct affect on the neurons and through induction of IL-1 release[17]. Both TNFα and IL-1 can induce the expression of the other. Together they induce IL-6 at the gene level. However IL-6 has not been found to induce either TNFα or IL-1. Production of IL-6, IL-1, and TNFα by macrophages can be induced by cycloheximide, a protein synthesis inhibitor, which may suggest that regulation of these cytokines is under the control of repressor proteins[2]. If the production of the repressor proteins is inhibited, then the cytokine production can occur. Pretreatment of cervical and ovarian carcinoma cell lines, <u>in vitro</u>, with IFNγ and then exposure to TNFα in

the presence of protein synthesis inhibitors, emetine or actinomycin-D, increases TNFα cytotoxic activity[46]. This also supports the repressor protein theory.

The most interesting property of TNFα is its antitumor activity. TNFα, in vitro, kills or inhibits growth of approximately one third of surveyed transformed cell lines including the L929 murine fibrosarcoma cell line and the U937 human histiocytic lymphoma cell line[9]. It can stimulates growth in other cell lines such as the FS-4 fibroblasts. The cell-killing activity can be inhibited by anaerobic conditions[9] suggesting that TNFα needs aerobic conditions to function effectively.

Studies, in vivo, testing the direct effect of TNFα on tumors and biological systems started with mice. Using mice with methylcholanthrene-induced fibrosarcomas, the effects of TNFα on tumor-bearing and nontumor-bearing mice were examined. It was found that the tumor-bearing mice were more susceptible to the deleterious effects of the administered TNFα than the nontumor-bearing mice[3]. Over 50% of the tumor-bearing mice died within 48 hours of exposure to TNFα. Whereas none of the nontumor-bearing mice died when similar doses of TNFα were administered i.v[3]. The administration of TNFα did have some therapeutic effects on tumor-bearing mice[26]. It was noted that weakly immunogenic sarcomas seem to be more susceptible to TNFα action[3]. More established tumors are also more

susceptible most likely because of the tenuous blood supply to the center of the tumor. TNFα causes the production of coagulants resulting in ischemic neccrosis at the center of these more established tumors. For any sort of tumor reduction, the best route for TNFα seems to be intravenous administration even though tumor necrosis is noted at the site of injection with intratumoral administration[14]. Intravenous injection of TNFα into Balb/c mice with methylcholanthrene-induced fibrosarcomas resulted in the destruction of the center of the tumor mass within 24 to 48 hours[14].

Although TNFα can kill certain tumor cells in vitro, the toxic side-effects in vivo were too severe to use TNFα as a therapeutic agent against tumors. The secondary effects caused by TNFα are similar to the ones observed in shock syndrome. The symptoms are hypotension, tachypnea, metabolic acidosis, hemoconcentration, adrenal necrosis, pulmonary congestion, hyperglycemia reversing to hypoglycemia, cecal necrosis, and multiple end-organ damage. Infusion of a large intravenous dose of recombinant human TNFα (rHuTNFα) causes severe gastrointestinal inflammation, cecal hemmorrhage, and blockage of gastric emptying within 4 hours in laboratory animals[29]. This may have occurred because of a stimulated production of platelet activating factor (PAF), a mediator of bowel necrosis in endotoxemia.

A study in horses showed that TNFα is possibly a mediator

of endotoxemia. Thus, experimentally induced endotoxemia in horses revealed increased levels of serum TNFα that was undetectable before[52]. Symptoms noted in these horses were mild colic, depression, tachycardia, increased temperature, and leukopenia reversing to leukocytosis. Also noted was that cyclo-oxygenase inhibition alleviated clinical signs without affecting TNFα activity[52]. The observed clinical findings made scientists look deeper into what effects TNFα was having the on biological activity.

TNFα can cause cachexia and lipolysis. In adipose tissue, lipoprotein lipase (LPL) transcription is suppressed by TNFα causing hypertriglyceridemia despite an anorexic state[9]. While this is occurring, very low density lipids (VLDL) accumulate in the circulation, leading to a reduction in adipose tissue lipid accumulation[30,36]. Other effects of TNFα on adipocytes include the decreased uptake of acetate, the incorporation of glucose into fatty acids, suppression of acetyl CoA carboxylase and fatty acid synthetase, and activation of the release of glycerol from fat cells and "dedifferentiation" induction[9,23].

Major organs of the body are adversely affected in laboratory animals when exposed to TNFα. For example, administration causes hemorrhaging of the adrenal gland. Adrenalectomized mice are extremely sensitive to the lethal effects of TNFα[9]. Kidneys undergo acute tubular necrosis.

Grossly visible punctate hemorrhage occurs in the lungs. In muscle, TNFα lowers the transmembrane potential, increases the transport of glucose, depletes cell gylcogen, and increases cellular efflux of lactate. TNFα causes hepatomegaly, bile duct proliferation, infiltration of the liver by monocytes, depression of cytochrome P450-dependent drug metabolism, and a decrease in albumin production in the liver. Ten percent of an injected dose of TNFα ends up in the gastro-intestinal tract leading to cecal bowel necrosis most likely mediated by the PAF induced by TNFα[9]. TNFα also causes resorption of proteoglycan in cartilage and of bone. Osteoclast-like cells are stimulated to make macrophage-colony stimulating factor (M-CSF) and PGE_2[9].

Antibodies against TNFα have been successfully used to block its deleterious effects <u>in vivo</u>[67]. Thus, a monoclonal antibody (mAb) to TNFα, TN3-19.12, protected mice from LPS-induced shock. In this experiment, mice were injected with irrelevent IgG, saline, or mAb to TNFα and then exposed to LPS which caused the production of TNFα. The first two groups showed detectable levels of TNFα peaking at 90 minutes. The mice injected with the anti-TNFα monoclonal antibody showed no detectable TNFα in circulation. Only the mice injected with the anti-TNFα were protected against the TNFα effects produced by LPS-stimulated macrophages. The anti-TNFα antibody bound to the circulating TNFα inhibited the TNFα from binding to

target cells, inhibiting the deleterious effects of TNFα[67]. The half-life of this mAb is 7 days after intraperitoneal injection into Balb/cByJ mice making it a long enough lasting treatment to continue protecting until TNFα production decreases. Studies using rats injected with the mAb to TNFα showed similar levels of protection against direct administration of TNFα[75].

Some cells are resistant to the effects of TNFα. The difference between sensitive and resistant cell lines has not yet been elucidated. However, it has been hypothesized that resistance may be associated with production of TNFα by the resistant cells[66]. Other studies show that the presence of LPS causes certain cell lines to internalize their TNFα receptors before the TNFα can be produced[18]. Once the TNFα is produced, no receptors are present on the cell surface for the TNFα to bind, avoiding the cell-killing ability of TNFα. It is an indirect resistance since the TNFα cannot attach itself physically to the cell to have an effect. Also, pretreatment with TNFα is known to induce MnSOD which may protect the cell against oxidative stress[38]. Another natural difference is the presence of gap junctions between resistant cells. Sensitive cells lack the gap junctions. However, if gap junctions are induced in the sensitive cells, resistance is acquired[30]. Cell lines resistant to either TNFα or hyperthermia alone become sensitive to a combined treatment of the two for reasons

unknown[70]. Furthermore, cGMP levels may be a key to TNFα-induced cell death. Thus, high levels of cGMP are found in TNFα-sensitive cell lines. This TNFα sensitivity can be induced in normally resistant cell lines by increasing levels of cGMP[29].

3. Pharmacokinetics

The pharmacokinetics of TNFα are still under investigation and current results are contradictory. There are differing viewpoints. In rabbits, the half-life of TNFα is 6 to 7 minutes[9,11,66]. The half-life of TNFα in human has been shown to range from 14 to 18 minutes[23], 20 minutes[66], or as long as 32 minutes[3]. Once in the body, radiolabeled TNFα was traced to the skin (30%), liver (21%), gastrointestinal tract (9%), and kidneys (8%)[11,66]. It has been shown that the half-life of TNFα is not dose-dependent. At 25 $\mu g/m^2$, the half-life was found to be 15.9±3.6 minutes while at 100 $\mu g/m^2$, it was 17±2 minutes[60]. Others have shown that it is dose-dependent. Thus, doses of 40 $\mu g/m^2$ to 160 $\mu g/m^2$ indicated a half-life of 11 to 17 minutes while doses of 200 $\mu g/m^2$ to 280 $\mu g/m^2$ demonstrated a half-life of 54 to 71 minutes[51]. These variations in results are most likely due to differences in the techniques used to measure levels of TNFα or to the differences in the routes of administration.

4. Clinical Trials

TNFα has been tested in phase I clinical trials in human cancer patients. Studies dealt with patients with advanced malignant disease that had been treated unsuccessfully by other methods. Clinically, the most common side-effects were chills/fever, headache, nausea, vomiting, malaise, myalgia, dry mouth, fatigue, confusion, and hypotension. Indomethacin and fluid therapy were given to reduce these severe side-effects. The only factor that demonstrated a dose-dependent response was hypotension[51,72]. Biochemical effects included slight increases in hepatic and renal enzymes, and a drop in leukocyte and platelet levels, all of which returned to normal after TNFα treatment ended. In one study, 50% reduction in tumor size was noted in two patients with gastric carcinoma and one with hepatoma. TNFα injected intratumorally caused necrosis at the injection site[72]. However overall, few studies showed a siginificant reduction in tumor size. Phase II clinical trials of colorectal carcinoma patients were not successful. Increasing doses, given every other day, were not tolerated by these patients. The side-effects were again the same as before with hypotension being the dose limiting factor (DLF). Still no significant antitumor effect was noted at the dose levels used[51].

These studies indicated that the maximum tolerated dose

(MTD) couldn't be increased without controlling the diverse side-effects and therefore the use of TNFα in combination with other cytokines was tested. In vitro, using the L-M cell line, recombinant TNFα increased its cytotoxicity synergistically with mitomycin C, adriamycin, cytosine arabinoside, daunomycin cisplatin, vincristin, or 5-fluorouracil[56]. A phase I clinical study using a combined TNFα and IFNγ treatment in patients with gastrointestinal cancer revealed that the side-effects were about the same as with TNFα alone. Again, no significant tumor reduction occurred, but some tumor stabilization was noted. However, this combined treatment considerably slowed the growth of a pancreatic tumor and a cholangio carcinoma[1]. The use of heparin, an anticoagulant, in combination with TNFα was tried as a new approach. TNFα alone is known to increase the production of procoagulants and decrease production of thrombomodulin, promoting local coagulation and stopping TNFα circulation at those sites. Heparin augmented TNFα antitumor activity more than the TNFα\IFNγ combination by allowing the TNFα to circulate[71].

5. Production and Metabolism of Arachidonic Acid

TNFα causes peripheral mononuclear cells to secrete arachidonic acid metabolites, murine macrophages to secrete

PGE_2, and human endothelial cells to secrete PGI_2[36]. Increases in arachidonic acid metabolites PGE_2, PGI_2, and thromboxane in peripheral blood causes vasodilation, vasoconstriction, and chemotaxis of inflammatory cells. As mentioned earlier, indomethacin was used to offset severe side-effects. Indomethacin inhibts cyclo-oxygenase, the enzyme that stimulates the production of prostaglandin G_2 (PGG_2) from arachidonic acid. Pretreatment with indomethacin before administering TNFα alleviates many of these problems. It prevents associated hypothermia, fever, drastic changes in blood glucose, acidosis. diarrhea, and cyanosis[33,60]. Ibuprofen, also a cyclo-oxygenase inhibitor, has these same protective properties but to a lesser extent[33]. Indomethacin could not block peripheral blood alterations induced by TNFα[36]. The question is whether or not cyclo-oxygenase inhibitors reduce the cytotoxic effects of TNFα. TNFα causes a 10-fold increase in blood PGE_2 within one hour of TNFα injection. PGE_2 levels do not rise with the addition of indomethacin[33,36,68]. Nevertheless, it has been found that cyclo-oxygenase inhibitors do not affect serum TNFα activity[52]. It seems that the cytotoxic effects of TNFα are largely independent of prostaglandin production suggesting that the metabolism of arachidonic acid is not essential to TNFα action[38]. Therefore, indomethacin can be used in clinical studies of TNFα to offset side-effects without affecting TNFα cytotoxicity[33].

Nevertheless, only a few reports have shown that cyclo-oxygenase inhibitors reduce TNFα cytotoxicity[38,48].

The administration of TNFα induces phospholipase A_2 (PLA_2), an enzyme that stimulates the accumulation of cGMP and the production of arachidonic acid from plasma membrane phospholipids[9]. Glucocorticoids, such as dexamethasone, inhibit PLA_2 activity and reduce TNF-mediated cytotoxicity. When dexamethasone was administered before, not after, stimulation with LPS, TNFα production was found to be inhibited at the transcriptional level[9,66]. The dexamethasone must be administered before transcription is initiated or else it has no suppressive effect[36]. Also, the mRNA that is produced isn't effectively translated[9]. Normally the L929 and U937 cell lines are sensitive to TNFα cytotoxic action. However resistant cell lines of U937 and L929 were produced and these do not release arachidonic acid upon addition of TNFα[38]. This suppression occurs at both transcriptional and post-transcriptional levels. Quinacrine, another PLA_2 inhibitor, inhibits the cytotoxic effects of TNFα[38]. In addition, melitin, a PLA_2 activator, enhances TNFα cytotoxicity. This suggests that the production, but not metabolism, of arachidonic acid is important to TNFα activity.

6. Genetics

The gene for TNFα lies in the major histocompatibility region on chromosome 6 in humans and chromosome 17 in mice. The gene is located 1100 bases to the 5' side of the TNFβ gene, possibly the result of a duplication event many hundred million years ago. This current theory justifies the structural and functional similarities between TNFα and TNFβ[60]. TNFα and TNFβ are 30% homologous in structure. The TNFα gene contains three introns with one interrupting the sequence that codes for the active protein[36]. The gene contains the nuclear binding factors, nuclear factor-kB (NF-kB), AP-1, and Sp1 binding sites upstream from the promoter[2]. Their roles in regulation are unknown. Regulation of TNFα may be under the control of repressor proteins, as discussed before since cycloheximide, a protein synthesis inhibitor, superinduces TNFα transcription[36]. TNFα is made de novo when production is stimulated in macrophages. Transcription is accelerated 3-fold, levels of mRNA rise by a factor of 50 to 100, and protein secretion rises by a factor of 10,000[9]. The post-transcriptional control is less understood. There is a region containing only the nucleic acids, uracil and adenine, in the 3'-untranslated segment of both mouse and human mRNA, but the justification for this region is unknown.

7. Receptors

Two TNFα receptors have been detected. One is a 55 kDa myeloid cell type receptor[41,42,62,73]. The other is a 75 kDa epithelial cell type receptor[30]. The extracellular domain of the 55 kDa receptor is 182 amino acids long, cysteine-rich and shows sequence homology with nerve growth factor, the B lymphocyte activation molecule Bp50 (CDw40), and the T2 antigen of the Shope fibroma virus[2,41,62,76]. There is a 28% homology between the extracellular domains of the two receptors. No homology has been found between the intracellular domains[73]. Both receptors have N-linked carbohydrates[42]. O-linked glycosylation has only been found on the 75 kDa epithelial receptor[73]. TNFα receptors are found on macrophages, lymphocytes, polymorphonuclear cells, fibroblasts, endothelial cells, synovial cells, muscle cells, adipocytes, myeloblasts, and tumor cells[66]. However, there is no correlation between the number of receptors on the cell and its susceptibility to TNFα. Concanavalin A, a mitogen that stimulates T cells, increases the number of receptors but blocks signal transduction for TNFα-induced cytotoxicity. IFNγ also causes an increase in the number of TNFα receptors expressed on the cell surface[60]. A biological response can be elicited by the occupancy of as few as 5% of the receptors by TNFα[23]. The number of 75 kDa receptors required to aggregate

through cross-linkage to produce a response is less than the number of 55 kDa receptors[73]. The half-life of these receptors is 2 hours in the absence of TNFα and 30 minutes in the presence of TNFα. The receptors are constantly synthesized, expressed, internalized, and broken down in the lysosome. No recycling of the receptors has been detected. The receptors can be down-regulated by treatment of cells with phorbol esters, activators of protein kinase C (PKC), and A23187, a calcium ionophore[60]. The TNFα receptor is thought to be associated with a human cell surface component (Fas antigen) in sensitive cell lines. Co-downregulation occurs on sensitive cells when treated with anti-Fas or TNFα. A significant amount of Fas antigen was not found on TNFα resistant cells[80].

8. Intracellular Events

Once TNFα binds to the cell surface receptors, the intracellular mechanisms that are triggered are virtually unknown. Apoptosis and necrosis were both noted in sensitive cell lines. During necrosis, certain intracellular events occur. Irregular clumping of nuclear chromatin is accompanied by swelling of cytoplasmic compartments. Densities appear in the matrix of the mitochondria, holes form in the cellular membrane, and lastly, organelles disintegrate. However,

apoptosis is more commonly noted. Within 2 to 3 hours, mitochondrial damage occurs, followed closely by nuclear membrane damage, and compaction of nuclear chromatin into dense masses. At 4 hours, DNA fragmentation occurs. It is not known, however, if this fragmentation or the elimination of mitochondrial respiration is the cause or the result of TNFα-induced cell death. Half the cells are dead within 8 hours caused by severe nuclear membrane damage and blebbing. Within 16 hours, 90% of the cells are found dead[38]. TNFα causes cell arrest during the G_2 phase of the cell cycle, not affecting the rate of the cycle[60].

Oxygen radicals may play a role in TNFα cytotoxicity. It has been noted that TNFα causes free radical production in sensitive cell lines but not in resistant ones[38]. Malonyldialdehyde (MDA) levels are also increased in sensitive cell lines[38,48]. MDA levels are used to measure lipid peroxidation caused by free radicals. Hydroxyl radical production increased 1.8X within 18 hours of TNFα treatment of L-M and KYM cells, both sensitive cell lines, but not after treatment of the resistant human embryonic lung fibroblasts[79]. Free radical scavengers such as dimethylsulfoxide (DMSO) and nordihydroguaiaratic acid (NDGA) were shown to decrease TNFα cytotoxicity in one study[77]. However, other studies have shown differently. Thus, free-radical scavengers such as vitamin E, thiourea, mercaptoethanol, methimazole, DMSO, or superoxide

dismutase (SOD) did not inhibit TNFα action[26,48]. Administration of SOD reduced the toxic effects of TNFα without decreasing the therapeutic effect on tumor-bearing mice[26]. Pretreatment with bismuth subnitrate increases the survival rates of both tumor-bearing and nontumor-bearing mice treated with TNFα. It induces metallothionein which reduces oxygen free radical metabolites early in the production pathway[35].

As stated before, the intracellular events that occur upon the exposure of TNFα are not clearly understood. However, some cellular actions have been noted. TNFα causes translocation of PKC from the cytosol to the cell membrane in TNFα-sensitive cell lines including the Jurkat, K562, and U937, but not in TNFα-resistant cell lines[47,64]. There is also some evidence that G-proteins may play a role in TNFα action, however, that role is controversial[19,38]. It is beleived that Ca^{2+} is not essential for lysis by TNFα even though Ca^{2+} levels do increase slightly in some TNFα sensitive cell lines[38]. However, Ca^{2+} is necessary for DNA fragmentation which does occur in cells exposed to TNFα. Yet, it still is not known whether the DNA fragmentation observed in TNFα-sensitive cells is caused by the TNFα or is only a result of cell death. Promethazine, a calmodulin inhibitor, does not inhibit TNFα cell-killing[38]. Sodium azide, a mitochondrial respiration inhibitor, decreased cell susceptiblity to TNFα, suggesting a

possible ATP requirement[77]. It has also been noted that once the TNFα/receptor complex is internalized, the TNFα must enter a lysosome to be processed. Inhibitors of lysosomal enzymes have been found to reduce TNFα cytotoxic activity in vitro[39,77]. Some believe that the ability of a cell to process TNFα in the lysosome may determine if a cytotoxic response occurs[38].

B. Anti-idiotypic Antibodies

Since anti-idiotypic antibodies (Ab2β) will contain the "internal image" of the antigen, it was hypothesized that the use of Ab2β may kill TNFα sensitive tumor cells lines without the severe side-effects.

1. Jerne's Theory

The groundwork for the theory of an anti-idiotypic network was developed by Neils Jerne in 1974[67]. Jerne theorized that a foreign antigen (i.e. antigen X) stimulates the production of a specific antibody (Ab1) population which carries many idiotopes determining the antibody's idiotype. The idiotype of an antibody refers to the differences in the hypervariable regions located at the ends of the antibody "arms" and is often represented by the antigen combining site or paratope. Most idiotopes require the participation of both

V_H and V_L chains. The increase of Ab1 induced by the antigen in turn stimulates the production of anti-idiotypic antibodies (Ab2) against the idiotopes of Ab1. This network, theoretically, can go on for a number of generations. Idealistically, Ab2 will contain the "internal image" of the antigen which induced the production of Ab1 (antigen X). The internal image is the determinant on an antibody that resembles or mimics the epitope of an antigen. However, most Ab2's do not contain the "internal image" of the original antigen. Antibodies can be produced to idiotopes which do not share the paratope, the portion of the antibody which binds to the epitope of the antigen. These are placed in two categories, Ab2α and Ab2γ. Ab2γ are antibodies that bind close to but not on the binding site of Ab1. Ab2α binds to Ab1 even farther away from the binding site of Ab1 than Ab2γ. The set of antibodies containing the internal image is called Ab2β. There are certain ideal properties of an internal image anti-idiotype. First, it will contain epitopes that should bind to a substantial portion of the total anti-X population. It will recognize anti-X antibodies from various species. It will compete with the original antigen for binding to the anti-X binding site on Ab1, and lastly, it will display functions similar to antigen X.

No anti-idiotypic antibody to TNFα has been produced until now. Other antibodies that may contain part or possibly

all of the TNFα binding site have been produced. However, they were produced using soluble TNFα receptors and not monoclonal anti-TNFα antibodies. These antibodies produced using the receptors are discussed in the next section.

2. Antigenic Mimicry

Anti-idiotypes bearing the internal image of the antigen should display antigenic mimicry. Certain Ab2 can trigger an anti-tumor immune response. Anti-idiotypic antibodies were administered in patients with colorectal carcinoma that contained the image of the tumor antigen. Consequently, a stronger antibody response to the tumor antigen/Ab2 was elicited[27]. In other studies, an Ab2 to prolactin caused α-lactalbumen synthesis[81] and secretion and the Ab2 to insulin caused the uptake of α-amino-isobutyric acid by the cells, mimicking insulin activity[56]. There are two current theories explaining how the Ab2 can act as the original hormone. The Ab2 may stimulate the hormone receptor by interacting with it through its antigen binding site, therefore presenting itself to the receptors as an internal image, a structure identical to that of the active site of the hormone. Secondly, the activities of Ab2 may resemble the abilities of the original hormone by cross-linking the receptors[21]. A combination of both mechanisms could also occur. An Ab2 to IFNγ was produced

that could compete with IFNγ for binding to the IFNγ receptors on bovine kidney MDBK cells. This Ab2 also exhibited IFNγ-like anti-viral activity[57]. In these cases, the Ab2 could elicit responses similar to those of the original molecule[24]. Ab2 has also been used as cell surface probes for B-adrenergic, acetylcholine, thyrotropin, reovirus, and formyl peptide chemotaxis receptors[57]. Anti-idiotypes can also be used as vaccines. This has been attempted in a number of systems including Herpes simplex, hepatitis B, poliovirus type II, rabies virus, Listeria monocytogenes, Schistosoma mansoni, Trypanosoma rhodesiense, and Streptococcus pneumoniae. To date, however, most of these vaccines have met with limited success[28]. However, anti-idiotypes by their nature are often free of the potentially hazardous effects of the original antigens[24].

A recent theory that has come to the foreground in TNFα research is that TNFα may have no activity of its own. It's the TNFα receptor on the surface of the cell, when properly triggered, that activates the cellular mechanisms which may result in the cell's death[60]. In one study, TNFα was fixed to the surfaces of a monolayer of macrophages with para-formaldehyde. The fixed TNFα was still just as efficient at cell-killing as free TNFα. It also seemed to retain its FS-4 growth-stimulatory activity[60]. In the same study, similar results were obtained with TNFα fixed to WEHI 164 clone 13

cells. TNFα exists naturally on the surface of macrophages in the absence of secreted TNFα and is functional in cell-killing[43]. Engelmann, et. al.[21], collected more evidence that supports this theory. Two soluble TNFα receptors were isolated from human urine and polyclonal antibodies (pAb) against one of these TNFα binding proteins displayed TNF-like activity[21,65]. TNFα sensitive cell lines were also sensitive to the pAb. In addition, the pAb failed to kill HeLa and SV80 cells in the absence of protein synthesis inhibitors as does TNFα. This TNFα-like activity of pAb correlates with their ability to cross-link the TNFα receptors[21]. If monovalent rabbit antibody fragments attached to the receptors were cross-linked using goat antibody to rabbit immunoglobulin, extensive cell death occurred[21]. Monovalent Fab fragments do not have activity until they are cross-linked with a second antibody. This suggests that the cross-linking of TNFα receptors is an important part of the activation process[73]. Also, since antibodies that bind to TNFα receptors can trigger this response, it may be that the actual binding of TNFα to a susceptible cell is the most important event of the cytotoxic activity associated with TNFα.

C. Cytokines and Resistance to Pathogens

1. Effects of Cytokines on Resistance to Pathogens

TNFα is capable of stimulating and activating macrophages. By doing so, the macrophages become more effective in killing intracellular pathogens. Examples of such pathogens include Listeria monocytogenes, Mycobacterium avium, Mycobacterium bovis, Mycobacterium lepraemurium, and the protozoan parasites, Leishmania major and Leishmania donovani. Recombinant human TNFα enhanced mouse resistance to L. monocytogenes infection in a dose dependent manner. IL-1α and TNFα work syngergistically to enhance mouse resistance to L. monocytogenes[59]. No clinical side-effects were noted at the doses of TNFα used. Decreased growth of M. avium in human macrophages was noted in the presence of TNFα, while IFNγ had no effect[16]. TNFα works syngergistically with IL-2 to increase intracellular killing of M. avium. At first it was thought that IFNγ had the same bactericidal effect against M. avium, however, IFNγ was only stimulating the macrophages to produce TNFα since anti-TNFα antibodies stopped any "IFNγ activity"[6]. Resistance to M. bovis BCG has been shown to be TNFα-mediated[15]. Anti-TNFα injections made the disease worse[15]. In another system, TNFα worked synergistically with IFNγ, both at very low doses, to kill L. major in stimulated mouse

peritoneal macrophages[12,40]. IFNγ alone is enough to activate murine peritoneal macrophages and decrease growth of L. donovani[54]. Both GM-CSF and TNFα increased resistance to M. lepraemurium in murine macrophages. Cytokines can also have the opposite effect. For example, recombinant murine IFNγ increases growth of M. lepraemurium in murine macrophages[15].

2. Effects of TNFα on Resistance to Brucella abortus

Another important group of pathogens is the Brucellae family. Brucellae are small, nonmotile, Gram-negative coccobacilli. They are facultative intracellular pathogens that can survive in cells of the reticuloendothelial system, mostly macrophages. It has recently been found that IFNγ enhances the ability of macrophages to kill Brucella abortus strains 2308 and 19[32]. A greater amount of killing is seen with strain 19 which is an attenuated strain. After 72 hours incubation of macrophages activated in vitro with IFNγ and with strain 19, the strain had been eliminated. However using a similar system, some of the more virulent strain 2308 organisms survived[32]. Similar experiments using TNFα and B. abortus have not been reported. Strain 2308 can inhibit phagolysosomal fusion in bovine neutrophils whereas strain 19 cannot[7]. There is evidence of brucellae transferring to the RER and replicating, whereas transfer to the phagolysosomes

results in death of the bacteria in Vero cells[32]. Therefore, it could be that strain 19 is killed within the phagolysosomes, and 2308 moves to the rough endoplasmic reticulum (RER) to replicate. However, B. abortus has not been found in macrophage RER. IFNγ facilitates phagolysosome fusion, possibly minimizing the ability of strain 2308 reaching the RER[32].

B. abortus strain RB51 is a rough derivative of strain 2308 which will protect animals from challenge with virulent strains when used as a vaccine[63]. The strain is highly attenuated and only persists in mice for less than four weeks post-challenge[63]. This is contrary to the clearance of virulent strains which establish chronic infections in mice[50]. The low virulence associated with RB51 may be due to the ability of IFNγ activated macrophages to kill the organism efficiently[5]. The role of TNFα on the killing of strain RB51 by macrophages is unknown.

III. MATERIALS AND METHODS

A. Immunization of Mice

Six week old female Balb/c mice were immunized with 250 μg of monoclonal mouse anti-human-TNFα antibody (Genentech, Inc.) emulsified in Hunter's Titermax adjuvant. Half was injected subcutaneously and the other half intraperitoneally. Mice were bled retrorbitally at 4 weeks and 7 weeks after immunization. The mice were injected again at 11 weeks with 200 μg of anti-TNFα antibody in saline 3 days before performing the fusion.

B. Cytotoxicity Assay

A standard cytotoxicity assay[55] using the TNFα susceptible L929 murine fibrosarcoma cell line, grown in Minimum Essential medium (MEM) supplemented with 2 mM L-glutamine, 1.5X non-essential amino acids, 5 mM HEPES, 50 IU/ml penicillin and streptomycin, and 10% horse serum (HS) was used to check the mouse polyclonal antiserum for TNFα-like activity. L929 cells at a concentration of $1x10^5$ cells/ml were plated 100 μl/well in a flat-bottom 96-well culture plate (Corning). The cultures were incubated at 37°C and 5% CO_2 for eighteen hours. Dilutions of TNFα, antiserum, or affinity purified monoclonal

antibody were made with serum-free MEM containing 1 μg/ml actinomycin-D. The cytotoxic activity is enhanced by the presence of actinomycin-D. Wells with 100 μl of 0.1% Triton-X 100 (positive control) and wells containing medium only (negative control) were used as controls. The media used in the negative control wells also contained actinomycin-D. The plate was incubated for 18 hours at 37°C and 5% CO_2. Twenty microliters of MTT was added to each well and the plate was incubated at 37°C and 5% CO_2 for another four hours[53]. MTT is taken up by living cells, incorporated into mitochondrial DNA, and forms crystals. One hundred microliters of supernatant was slowly removed from each well, not disturbing the crystals. One hundred microliters of DMSO was added to each well to solubilize the crystals. Absorbance was read at 540-550 nm on a 96-well plate reader, Titertek Multiskan MC. The percent cytotoxicity was calculated with the following equation:

$$\% \text{ cytotoxicity} = \frac{A(\text{media})-A(\text{sample})}{A(\text{media})-A(\text{TX-100})} \times 100$$

One unit of TNF-alpha activity equals the amount of protein needed to cause 50% cytotoxicity.

The same cytotoxicity assay was also performed with the WEHI 164 murine fibrosarcoma cell line[22], and the SP2/O and NS-

1 murine myeloma cell lines.

C. Production of Hybridomas

1. Cell Fusion

Three different cell culture media were used. Regular media consisted of RPMI 1640 supplemented with 2mM L-glutamine, 5 mM HEPES, 1.5X non-essential amino acids, 50 IU/ml penicillin and streptomycin, and 10% horse serum. HAT medium consisted of the supplemented RPMI 1640 with 10 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine, and 15% horse serum. HT medium consisted of supplemented RPMI 1640, 10 μM hypoxanthine, 16 μM thymidine, and 15% horse serum.

Standard procedures were followed for cell fusions[67]. Before producing the hybridoma, spleen cells from one mouse and the non-secreting SP2/O murine myeloma cells were prepared. The mouse was euthanized by CO_2 inhalation. The spleen was removed and gently teased through a sterile screen to obtain individual cells suspended in media and the cells were then placed on ice until the SP2/O cells were harvested. SP2/O cells were grown in medium to a density of 10^5 cells/ml with a viability of at least 95% for the fusion. The SP2/O and spleen cells were collected and pelleted in an IEC refrigerated centrifuge at 4°C for 10 minutes at 1,200 RPM

(200 g). The pellets were resuspended in 10 ml serum-free medium, washed twice, and then resuspended in either 5 ml serum-free media (SP2/O cells) or in 3 ml of the serum-free media (spleen cells) depending on the visual amount of cells. The SP2/O cells were counted in a hemocytometer and viability assessed by Trypan blue exclusion. The spleen cells were also counted, without trypan blue, after lysing the red blood cells with 2% glacial acetic acid.

The fusion was performed by mixing spleen cells to SP2/O cells at a ratio of 4:1. The cells were mixed in serum-free medium, centrifuged at 200 G for 10 minutes to form a pellet, and the supernatant was removed keeping the pellet at 37°C in a water bath. A 50% polyethylene glycol (PEG) solution was added over a period of one minute while stirring gently. The amount of PEG in milliliters to be added was calculated by the following equation:

$$\text{fusion factor} = \frac{\text{number of spleen cells}}{1.6\text{x}10^{8}}$$

The number $1.6\text{x}10^{8}$ will keep the final cell concentration at 10^{6} cells/100 μl when the cells are placed in the 96-well tissue culture plates. The minimum amount of PEG that can be used is 1/2 ml. During the second minute, 1 ml serum-free medium warmed to 37°C was added to dilute the PEG, again

stirring gently. Another 1 ml was added over the next minute and thereafter 8 ml medium was added over 3 minutes, stirring gently. The suspension was centrifuged, the supernate was removed, and medium was added to the pellet aiming directly at the pellet to break it up without pipetting up and down. The amount of medium to be added was calculated by the following equation:

$$\text{fusion factor} \times 22\text{ml}$$

This will keep the final cell concentration at $10^6/100\ \mu l$. The suspension was swirled gently and plated at 100 ul/well in 96-well plates. The cells were incubated at 37°C and 5% CO_2.

On the day after fusion (day 1), 100 μl of hypoxanthine-aminopterin-thymidine (HAT) medium was added to each well. On days 2, 3, 5, 8, and 11, half of the medium was removed and replaced with fresh HAT medium. After this time, half the medium was changed every three to four days. This process is known as HAT exclusion and all cells, except the hybridomas of SP2/O-spleen cell should die. SP2/O cells die in the presence of aminopterin, whereas the spleen cells die normally within about seven days. Cells were observed daily for growth by phase contrast microscopy.

The previously described cytotoxicity assay was used for initial screening to find wells producing the proper

antibodies. Once these wells were identified, the cells were transfered to 1 ml cultures in 24-well plates. At this point, the medium was changed to hypoxanthine-thymidine (HT) medium to dilute any remaining aminopterin. After a week, the cells were changed to the regular medium. The most active culture, as determined by testing supernatants in the cytotoxicity assay, was expanded to a 25 cm^2 flask and then to a 75 cm^2 flask. This culture, still positive in the cytotoxicity assay, was used for cloning by limiting dilution.

The cytotoxicity assay was also performed using a non-specific IgG2a antibody (anti-bovine brain clathrin from Balb/c mice, CVC.7) to test for non-specific binding.

2. Cloning by Limiting Dilution

Cells were diluted to 100, 50, 10, and 5 cells/ml in medium. Each was added to half of a 96-well flat bottom plate at 100 μl/well. The remaining cells from the large flask were cryopreserved for later use. The cloned cultures were expanded and tested again for TNFα-like activity using the cytotoxicity assay. The four most active cultures were expanded and cryopreserved.

D. Antibody Purification

Culture supernatants from the most promising hybridomas were collected and frozen at -70°C, lyophilized, and reconstituted with phosphate buffered saline (PBS, pH=7.4) to decrease the volume to one tenth the original volume. Affinity chromatography was carried out with an anti-mouse IgG column (Sigma) to separate out the immunoglobulin G (IgG) fraction from the hybridoma supernatant. The column was first washed with 10 ml of PBS. The sample was applied and allowed to penetrate the column and incubated for 5 minutes. The unbound fraction was eluted with 5 ml of PBS and another 5 ml PBS was added to wash the column, pooling and saving the fractions. To elute the IgG off the beads, 4 ml of 0.2M glycine buffer (pH=2.4) was poured through the column followed by 5 ml of PBS. The eluant was collected and adjusted to pH 7.0 to 7.2 with 1N NaOH. The affinity purified IgG fraction was dialyzed against distilled water, frozen at -70°C, and lyophilized. The IgG was again reconstitute in 1 ml of Hanks's balanced salt solution (HBSS) and tested in the cytotoxicity assay.

E. Determination of Protein Content

Protein content was determined using the extinction coefficient for IgG which is 1.46[44] and the following equation:

$$\frac{(280\ nm)(dilution)}{1.46} = \frac{mg\ IgG}{ml}$$

F. Cytotoxicity Neutralization Assay

A cytotoxicity neutralization assay was performed to see if the TNFα-like activity demonstrated by the Ab2 could be neutralized by the anti-TNFα monoclonal antibody used to immunize the mice. L929 cells were used in this assay. Fifty microliters containing 60 Units/ml (U/ml) of Ab2 IgG were mixed with 50 μl of a serial dilution of monoclonal anti-TNFα and incubated for two hours at 37°C before running the already described cytotoxicity assay. The dilutions were made with serum-free ME medium with 1 μg/ml actinomycin-D. The assay was performed using rHuTNFα (10U/well) alone or neutralized by the anti-TNFα monoclonal antibody to ensure proper functioning of the cytotoxicity neutralization assay.

The neutralization assay performed on rHuTNFα and Ab2β was repeated using a non-specific IgG2a antibody in place of the anti-TNFα antibody for comparison purposes.

G. Isotype and Sub-isotype Determination by ELISA

The isotype and subisotype of the monoclonal antibodies were determined. An indirect ELISA kit (Sigma) was used.

The monoclonal antibody to be tested was diluted to 1 μg/ml using PBS and 100 μl of the antibody was pipetted into each of 12 wells of a microtiter plate. The covered plate was incubated at 37°C for 1 hour and washed 3 times with PBS containing 0.05% Tween 80 (PBST). Goat anti-rabbit isotype specific antibodies of the kit were each diluted 1:1000 in PBS and 100 μl of each reagent was added to the wells in duplicate. The plate was incubated for 30 minutes at room temperature, washed 3 times in PBST, and 100 μl of peroxidase labeled rabbit anti-goat IgG diluted 1:10,000 in PBST was added to all wells. The plate was then incubated for 15 minutes at room temperature, 100 μl/well of the substrate was added, and the plate was incubated for 30 minutes at room temperature in the dark. To prepare the substrate, 10 mg o-phenylenediamine (OPD) was mixed with 1 ml methanol. One hundred microliters 3% H_2O_2 was added to 100 ml distilled water. Right before use, the two parts were added together and mixed. The reaction was stopped by adding a drop of H_2SO_4 per well. The color was inspected visually and read by a 96-well plate reader at 440 nm.

H. Gel Electrophoresis and Western Blotting

Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE)[37] and Western blots[74] were used to further characterize

the antibodies. SDS-PAGE was carried out according to Laemli and co-workers using a 12.5% acrylamide gel[37]. Low molecular weight markers ranging from 14.4 kDa to 94 kDa were used as standards. The samples were prepared by boiling them for 5 minutes in a 2X sample buffer (0.0625M Tris, 2% SDS, 10% glycerol, 0.001% bromophenol blue, pH=6.8) containing 5% 2-mercaptoethanol to reduce the TNFα to monomer and the IgG into heavy- and light-chains. Gels were run at 30 milliamps/gel until the bromophenol blue dye reached near the edge of the gel, about one hour. The gel was removed from the electrophoresis apparatus and placed in Coomassie blue-R for 2 hours. A destainer containing 10% glacial acetic acid and 25% ethanol was used to destain the gel overnight. The gels were preserved by drying. To keep the gels from cracking as they dried, they were first soaked in a solution of 5% glycerol in distilled water for a minimum of 30 minutes. The gel was dried between two sheets of BioGelWrap membrane.

Similar gels were used for Western blots. After electrophoresis, the gel was removed from the elctrophoresis apparatus and equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 30 minutes. The gel was placed next to a piece of nitrocellulose and sandwiched into an electrophoretic transfer chamber (Hoeffer). The power unit was set at 125V for 2 hours making sure the current was running through the gel toward the nitrocellulose. Once the

transfer was completed, the section of nitrocellulose with the molecular weight markers was stained in Ponceau S stain for 5 minutes and destained in distilled water. The remaining nitrocellulose was cut into appropriate strips and blocked with 2% BSA in Tris buffered saline (TBS) for 1 hour. Thereafter, the strips were placed in anti-mouse IgG conjugated with horseradish peroxidase diluted 1:800 in TBS and agitated for 1 hour. The strips were washed by agitating in TBST for 15 minutes and then developed until the positive controls became visible. To make the developer, 60 mg 4-chloro-1-naphthol was mixed with 10 ml methanol and also 600 μl 3% H_2O_2 was mixed with 100 ml TBS. The two solutions were mixed together prior to use. To stop the developing, the strips were rinsed in distilled water and dried between paper towels.

I. Isolation of Peritoneal Macrophages

Female Balb/c mice were injected intraperitoneally with 2 ml of sterile thioglycolate. Four days later, the mouse was euthanized by CO_2 inhalation. Mice under 10 weeks of age were injected with only 1.5 ml thioglycolate with the cells being harvested 3 days later. The peritoneal cavity was washed using three 3 ml aliquots of Ca^{++}- and Mg^{++}-free Hank's balanced salt solution (HBSS). The cells were plated at $5x10^6$

cells/ml in a 24-well tissue culture plate in RPMI 1640 with 10% horse serum without antibiotics and incubated at 37°C in 5% CO_2 to allow for macrophage attachment. Two hours later, the cultures were washed three times with HBSS to remove non-adherent cells. Washing was repeated 3 times, 24 hours later with HBSS to remove all remaining non-adherent cells.

J. Bactericidal Assay

Peritoneal macrophages were treated with 100 U/ml TNFα, IFNγ, Ab2 to TNFα (Ab2β), non-specific IgG2a (anti-bovine brain clathrin isolated from Balb/c mice, CVC.7), or were left untreated for 1 hour. A minimum of three wells was used for each treatment type. During this incubation period, Brucella abortus strain RB51 was adjusted to 4% transmittance (T) at 525 nm in antibiotic-free RPMI 1640 with 10% horse serum. The organisms were diluted 1:10 with a 1:200 dilution of opsonizing monoclonal antibodies, Bru 48. Bru 48, a monoclonal antibody which binds to the surface of RB51, was kept in a stock solution of 40 mg/ml and was adjusted to 20 μg/ml for this purpose. The organisms were incubated for 30 minutes at room temperature and 100 μl of the opsonized strain RB51 was added to each well of macrophages for 30 minutes at room temperature. The media was removed and the infected macrophages were washed 3 times with PBS. The media was

replaced with media containing 50 μg/ml gentamicin and the macrophages were incubated for 1 hour at room temperature to kill any extracellular RB51. Thereafter, the macrophages were washed three times with PBS and the media replaced with media containing 12.5 μg/ml gentamicin in all except three wells to kill any RB51 that may be released from the macrophages during the assay. TNFα, IFNγ, Ab2β, or non-specific IgG2 were added again to the appropriate wells at 100 U/ml. Immediately after this, the three wells of macrophages containing media without the 12.5 μg/ml gentamicin were lysed to determine a baseline of ingested RB51. This was considered time 0. All macrophages were then incubated at 37°C in 5% CO_2 for 24, 48, or 72 hours. At the end of each incubation period, the media was removed and the macrophages were washed three times with PBS. One miliiliter of distilled water was added to each well and incubated for 15 minutes at room temperature. Serial ten fold dilutions down to $1:10^6$ were made from each well. Three drops of 10 μl each were plated onto trypticase soy broth plates from each of the six dilutions. These plates were incubated for 2-3 days at 37°C in 5% CO_2. At the end of the incubation, the number of colony forming units per ml (cfu/ml) was assessed and the number of cfu's per macrophage calculated.

The same experiment was performed using B. abortus strain 2308 using Bru 38 as the opsonizing antibody.

K. Statistical Analysis

The results of the bactericidal assay were statistically analyzed using the Duncan's multiple range comparison[82].

IV. RESULTS

A. Titer Activity

Two known TNFα-sensitive cell lines[22,30], WEHI 164 and L929 cells, were chosen for the TNFα cytotoxicity assay. A dose-dependent cytotoxicity to TNFα was observed for both of the cells lines. However, the L929 cells were found to be more sensitive to TNFα than the WEHI 164 cells. The percent cytotoxicity to 0.2 and 2 Units of TNFα were found to be 40% and 80% for L929 cells and 0% and 60% for WEHI 164 cells, respectively. For all subsequent cytotoxicity assays, L929 cells were used unless otherwise specified.

Mice were immunized with the monoclonal antibody to human TNFα, and production of polyclonal anti-idiotypic antibodies was monitored in blood drawn at 4, 7, and 11 weeks post-inoculation. Detectable amounts of TNFα-like antibody was observed at 1:1,000 dilution of blood obtained at 4 and 7 weeks post-inoculation. The 11 weeks post-immunization sera was able to kill 98% of the L929 cells at a 1:1000 dilution which was similar to the levels reached by a 1:100,000 dilution of rHuTNFα. Sera from mice injected with adjuvant alone showed trivial cytotoxic activity of no more than 10%. These results are presented in Figure 1. Sera or rHuTNFα heated at 56°C for 10 minutes slightly decreased the

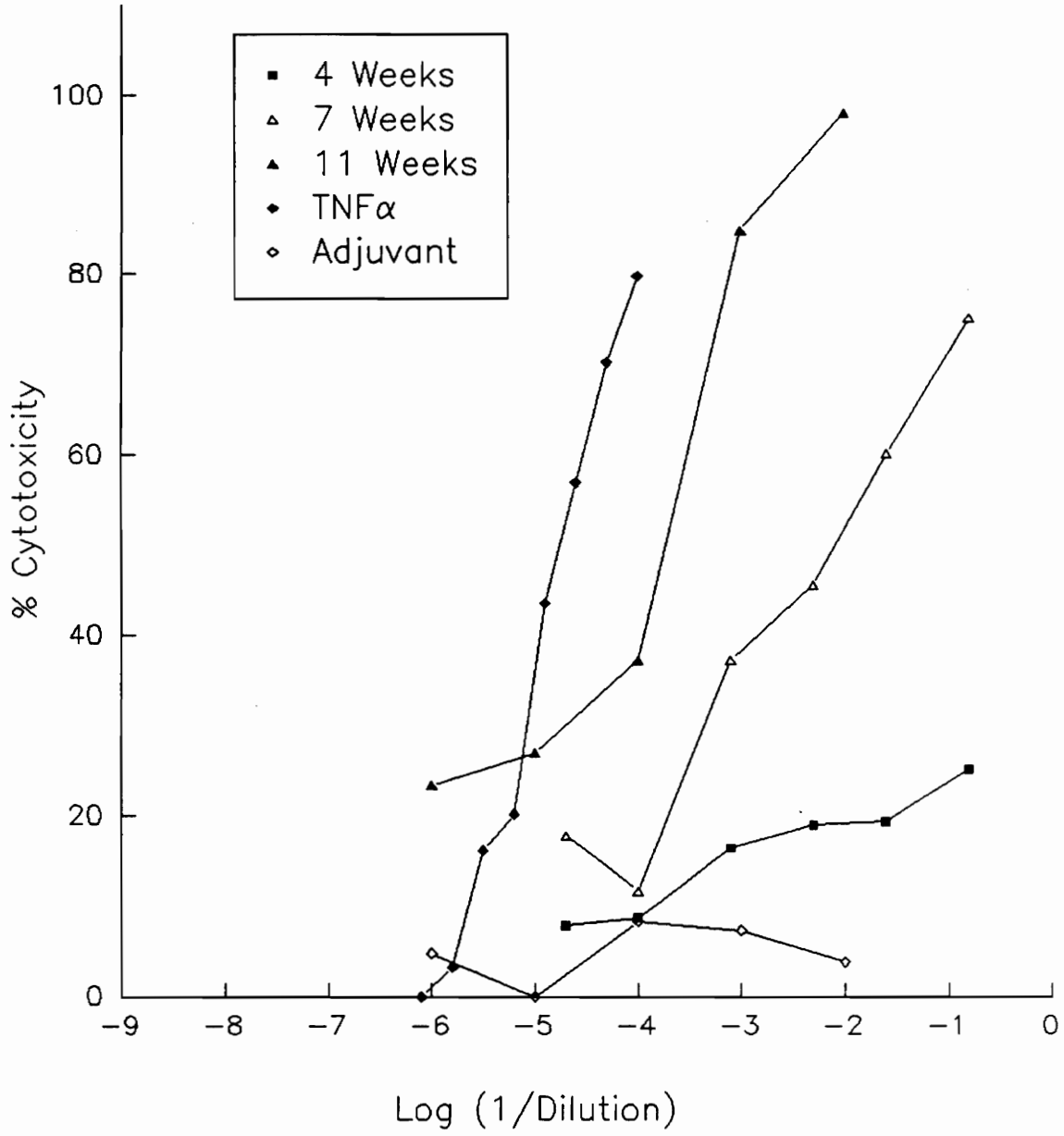

Figure 1

Cytotoxicity assay carried out with the L929 cell line using the mouse antisera to anti-TNFα antibodies obtained at 4, 7, and 11 weeks post-immunization, and for rHuTNFα. The undiluted concentration of rHuTNFα was 10 μg/ml protein. The adjuvant was Hunter's Titermax. The serum from adjuvant injected mice never exceeded 10% cytotoxicity.

cytotoxicity of both preparations.

B. Hybridoma Isolation

The spleen cells from the mouse immunized with the monoclonal antibodies to TNFα and the SP2/O mouse myeloma cells were fused at a 4:1 ratio using PEG. The fusion of spleen cells and SP2/O cells resulted in the appearance of several wells that produced polyclonal antibodies that tested positively according to the TNFα cytotoxicity assay. One well of hybridomas demonstrated production of supernatants with the high cell-killing activity (Figure 2) and good growth characteristics. This cell-killing activity was compared to rHuTNFα action in the standard cytotoxicity assay (Figure 1).

The well that contained the cells with the highest activity and best growth characterisitics was cloned by limiting dilution. From this cloning, 4 wells containing hybridomas producing monoclonal antibodies with significant cell-killing activity were obtained. All four culture supernatants showed equal cell-killing activity, however only one culture, 1C, showed a high rate of growth and was therefore selected for affinity purification. The other three grew at a very slow rate.

Using the standard cytotoxicity, both the supernatant and the affinity purified IgG from the 1C supernatant (Ab2β, i.e.

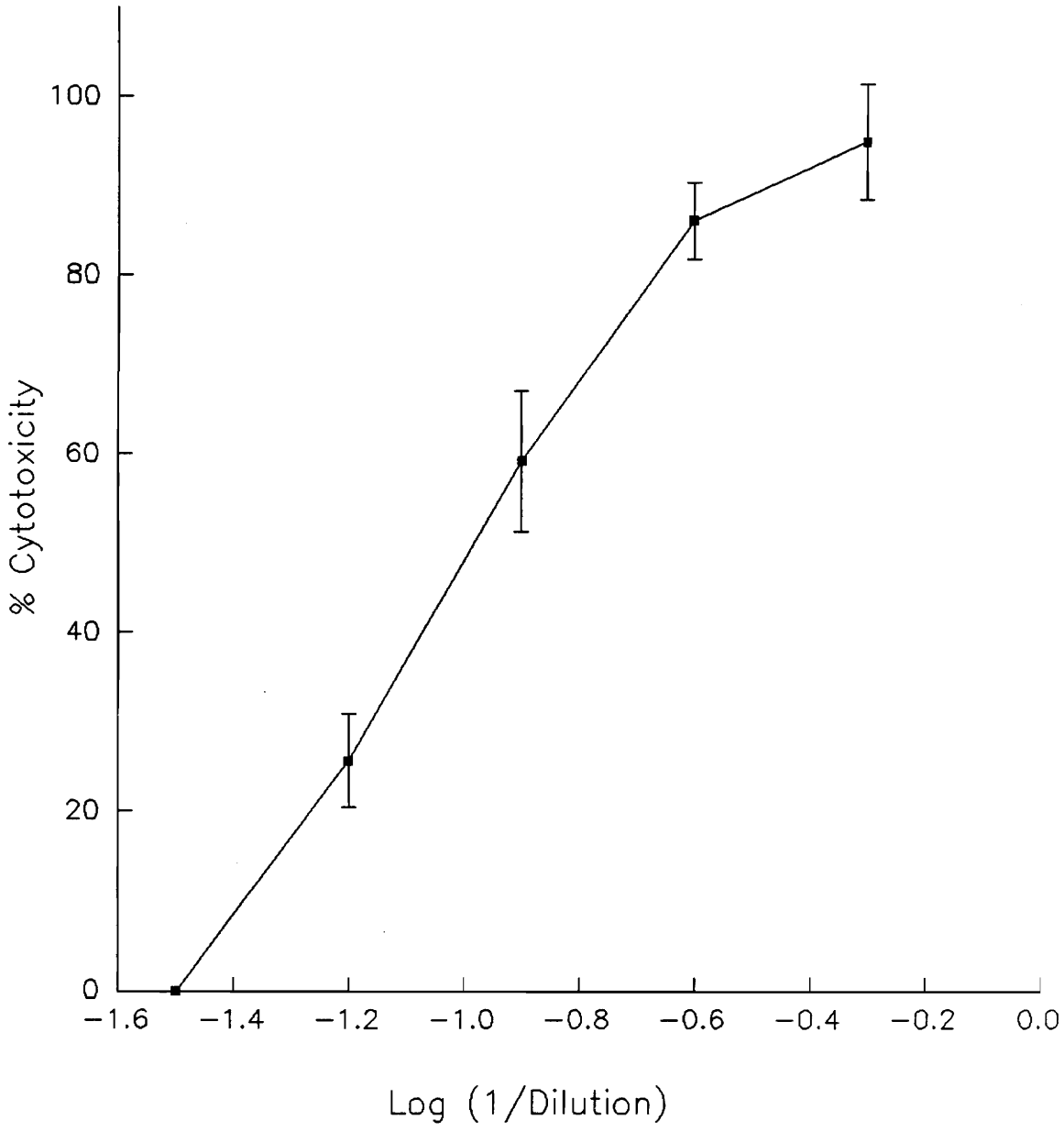

Figure 2

Cytotoxicity assay carried out with the L929 cell line using the polyclonal supernatant from one well containing several hybridomas. Values are reported as mean±SDM.

the group of antibodies bearing the internal image of TNFα) showed cell-killing activity in a dose-dependent manner (Figure 3a). An affinity purified non-specific IgG2a antibody (anti-bovine brain clathrin isolated from Balb/c mice, CVC.7) showed no cell-killing capability using ther standard cytotoxicity assay (Figure 3b).

C. Isotype and Sub-isotype Determination

An indirect ELISA was used to determine the isotype and sub-isotype of Ab2β. Briefly, a 96-well plate was coated with the antibodies to be tested. Goat anti-rabbit isotype specific antibodies were added to the plate. Next, rabbit anti-goat antibodies were added to the plate. A substrate was then added to develop color and determine the isotype. The test showed that the antibodies are of the IgG isotype. More specifically, the ELISA showed the subisotype to be IgG2a.

D. Further Characterization of Ab2β

For further functional characterization of Ab2β, other cell lines, either sensitive (WEHI 164) or resistant (SP2/O, NS-1) to TNFα were used in conjunction with the 1C whole supernatant and the purified Ab2β.

As shown in Figure 4a, the WEHI 164 cells were sensitive

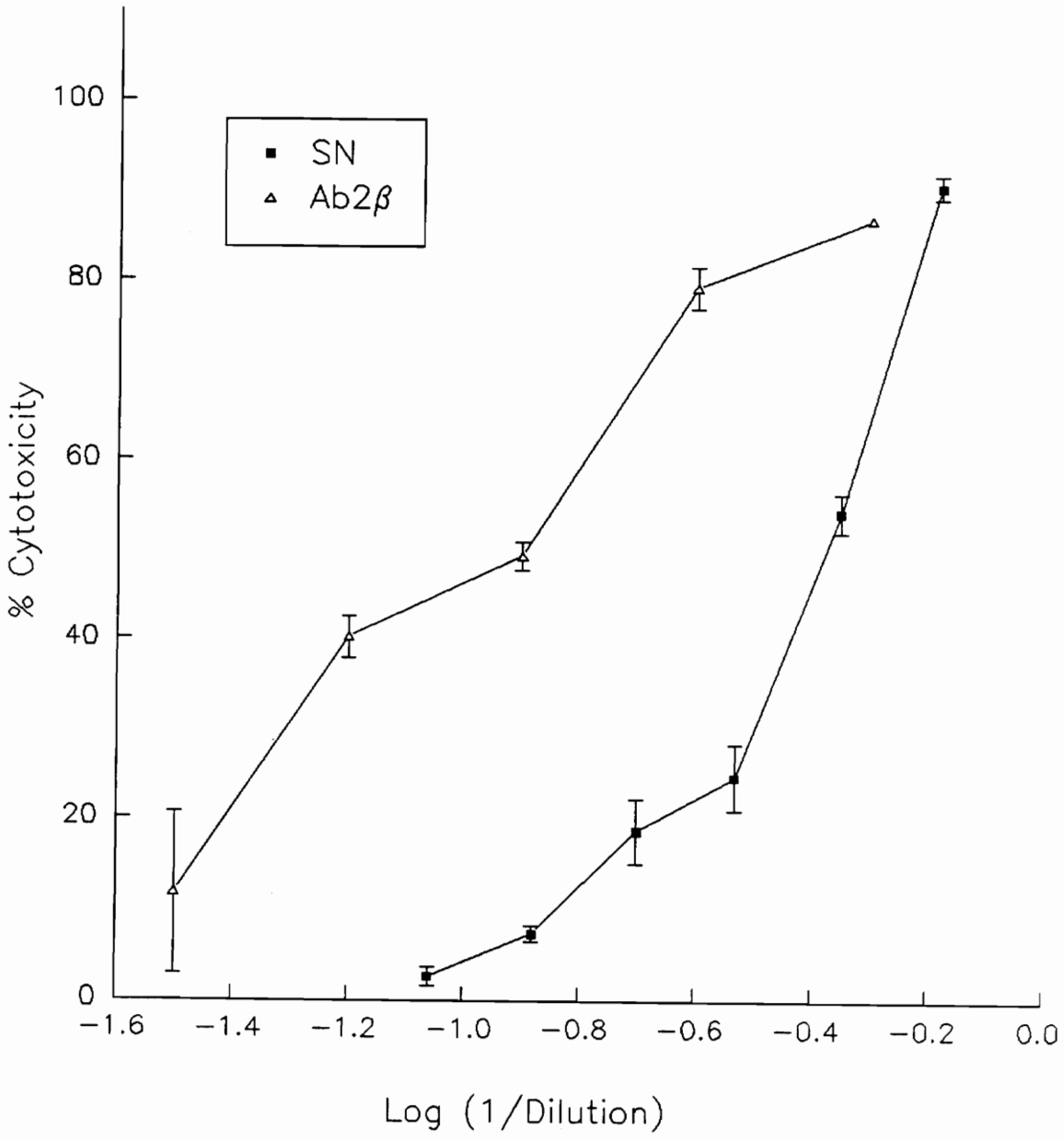

Figure 3a

Cytotoxicity assay carried out with the L929 cell line using the whole supernatant from the well producing the monoclonal antibody 1C and the affinity purified antibody obtained from the same well (Ab2β). The undiluted concentration of Ab2β was 48 μg/ml protein. SN=supernatant. Values are reported as means$\pm$SDM.

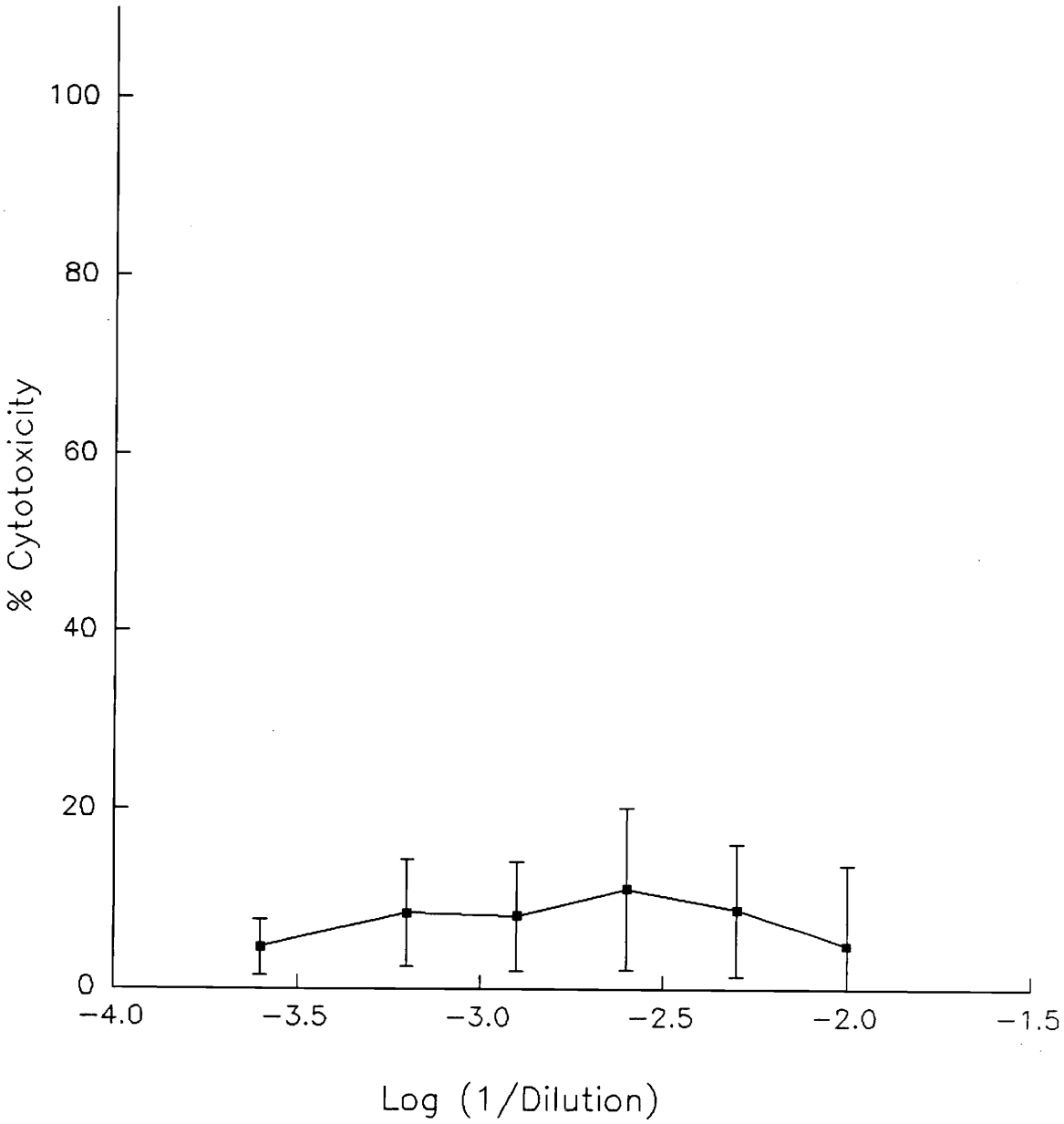

Figure 3b

Cytotoxicity assay carried out with the L929 cell line using a non-specific IgG2a antibody (CVC.7). The undiluted concentration of CVC.7 was 500 μg/ml protein. Values are reported as means±SDM.

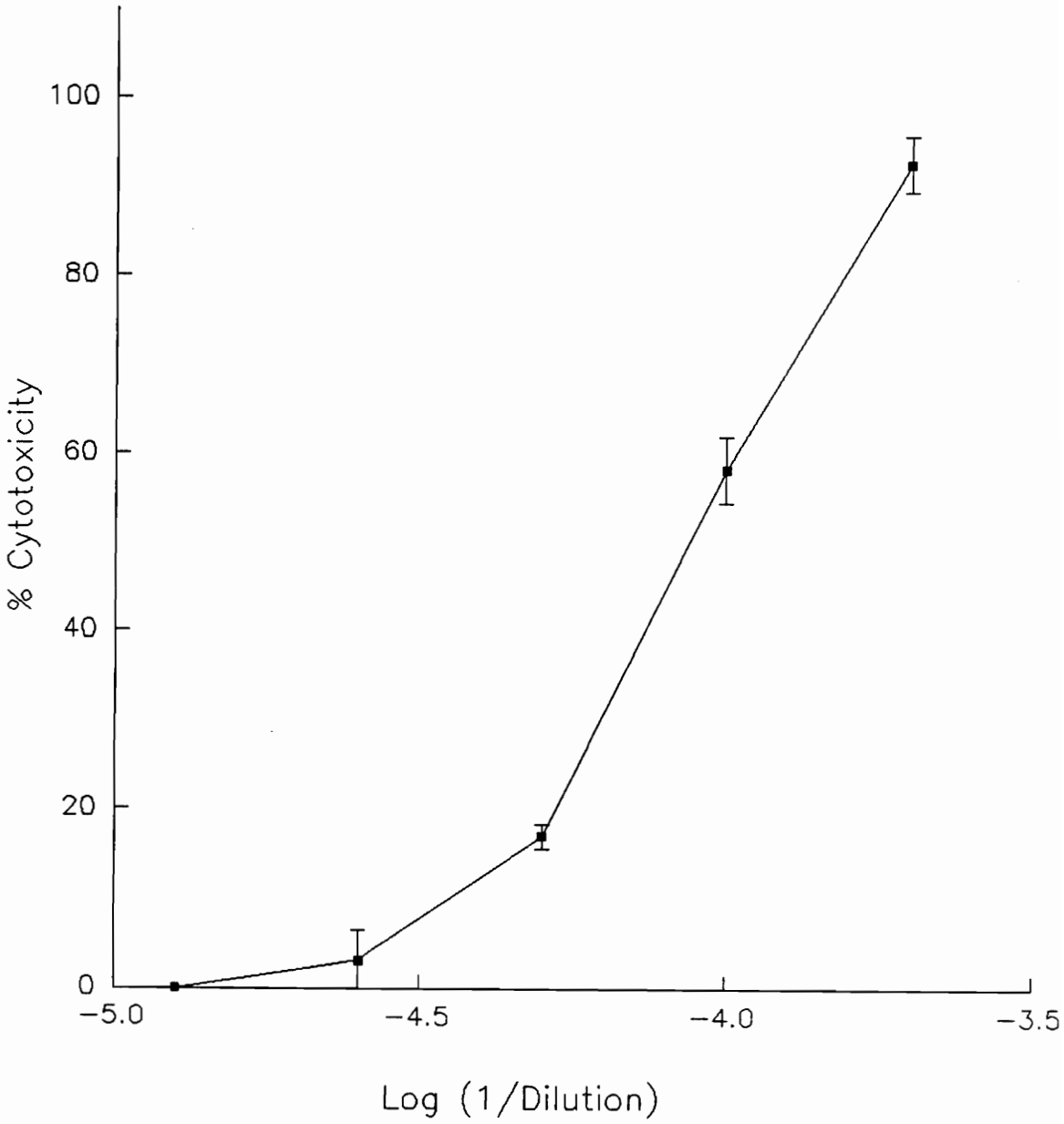

Figure 4a

Cytotoxicity assay carried out with the WEHI 164 cell line using rHuTNFα. The undiluted concentration of rHuTNFα was 10 μg/ml protein. Values are reported as means±SDM.

to rHuTNFα in a dose-dependent manner. The whole supernatant and Ab2β were also found to have a cell-killing effect on WEHI 164, and again these effects were dose-dependent similar to the rHuTNFα (Figure 4b).

The two cell lines that are resistant to rhTNFα were used to compare the cytotoxic activity of Ab2β with rHuTNFα. As shown in Figure 5a, cell death did not exceed 30% when the SP2/O or NS-1 cell lines were treated with rHuTNFα or Ab2β. The non-secreting myeloma cell lines, were found to be less sensitive to the supernatant than the L929 and WEHI 164 cell lines (Figures 5b & 5c) indicating that they may have some defense mechanism or that a different pathway may be triggered by TNFα-receptor binding.

E. Neutralization of Cytotoxic Activity

A neutralization assay was used to further correlate the activities and specificity of rHuTNFα and Ab2β. RHuTNFα can be neutralized by the monoclonal anti-TNFα antibodies used to immunize the mice. As shown in Figure 6a, 10 units of rHuTNFα killed approximately 80% of the L929 cells within 18 hours and addition of a serial dilution of the monoclonal anti-TNFα antibody to the TNFα, 2 hours before addition to the L929 cells, inhibited the cytotoxic activity in a dose-dependent manner. Ab2β was also found to be neutralized by monoclonal

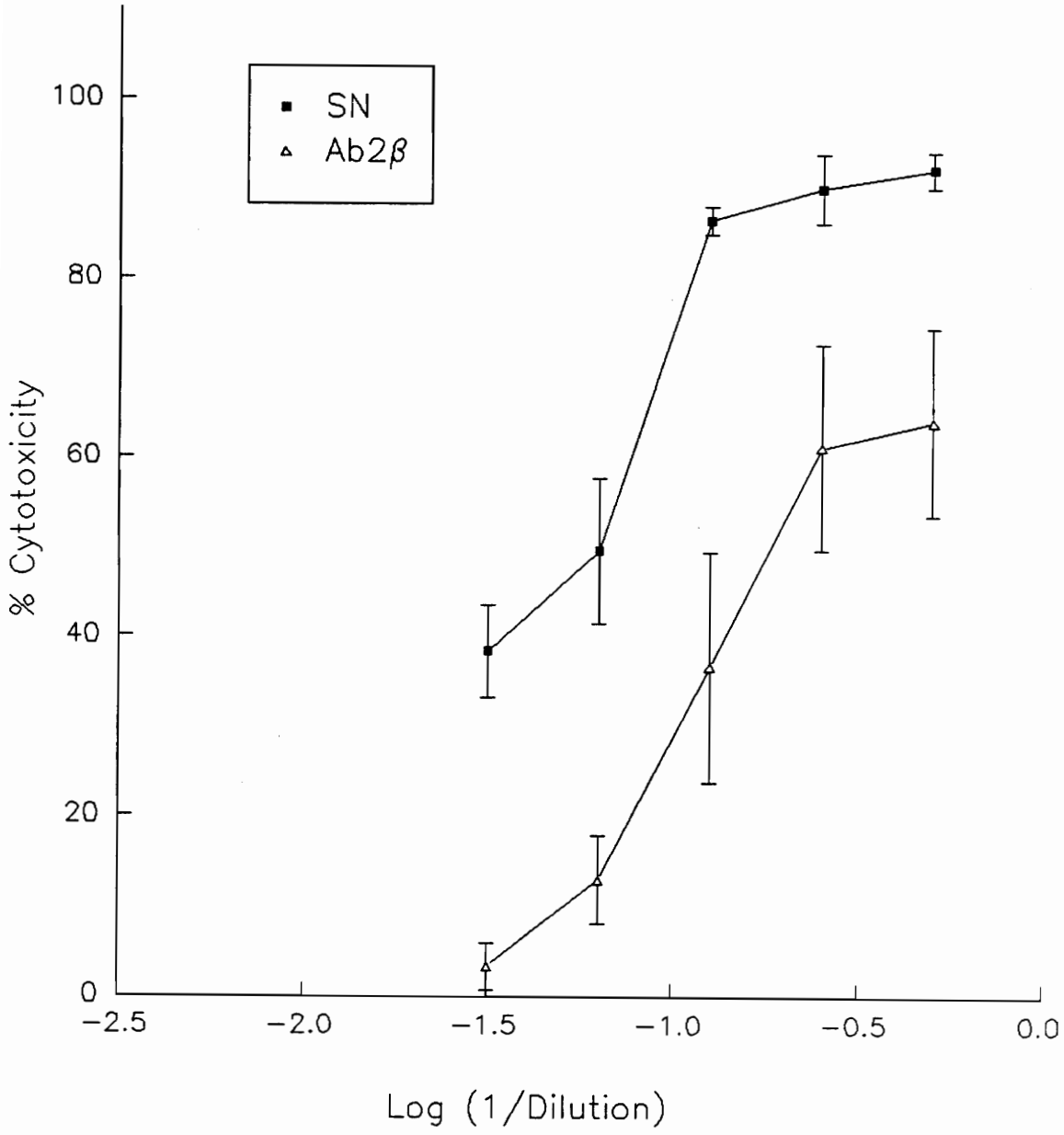

Figure 4b

Cytotoxicity assay carried out with the WEHI 164 cell line using the whole supernatant from the well producing the monoclonal antibody 1C and the affinity purified Ab2β. The undiluted concentration of Ab2β was 48 μg/ml protein. SN=supernatant. Values are reported as means$\pm$SDM.

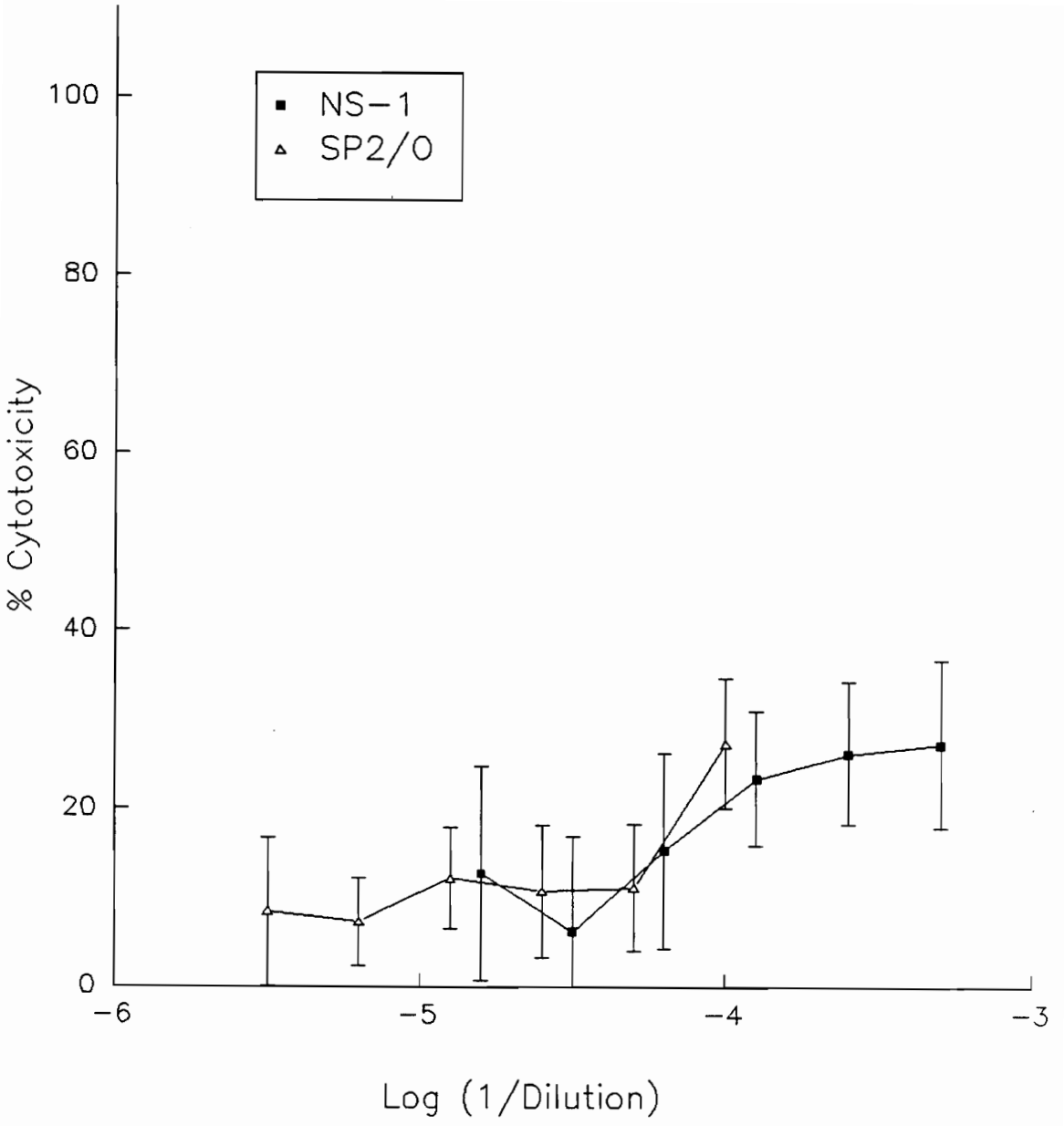

Figure 5a

Cytotoxicity assay carried out with the SP2/O and NS-1 cell lines using rHuTNFα. The undiluted concentration of rHuTNFα was 10 μg/ml protein. Values are reported as means±SDM.

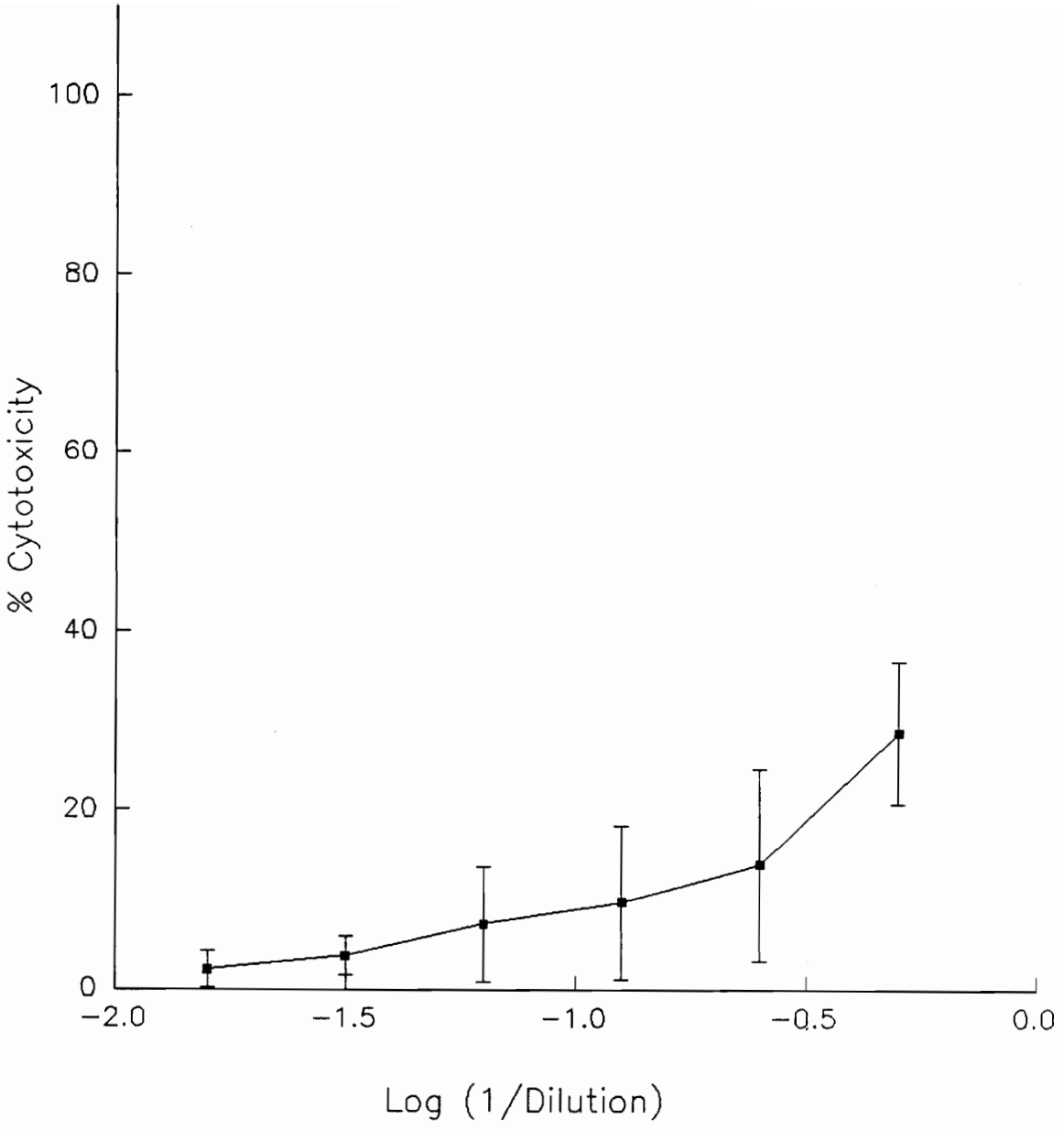

Figure 5b

Cytotoxicity assay carried out with the SP2/0 cell line using the whole supernatant from the well producing the monoclonal antibody 1C. Values are reported as means±SDM.

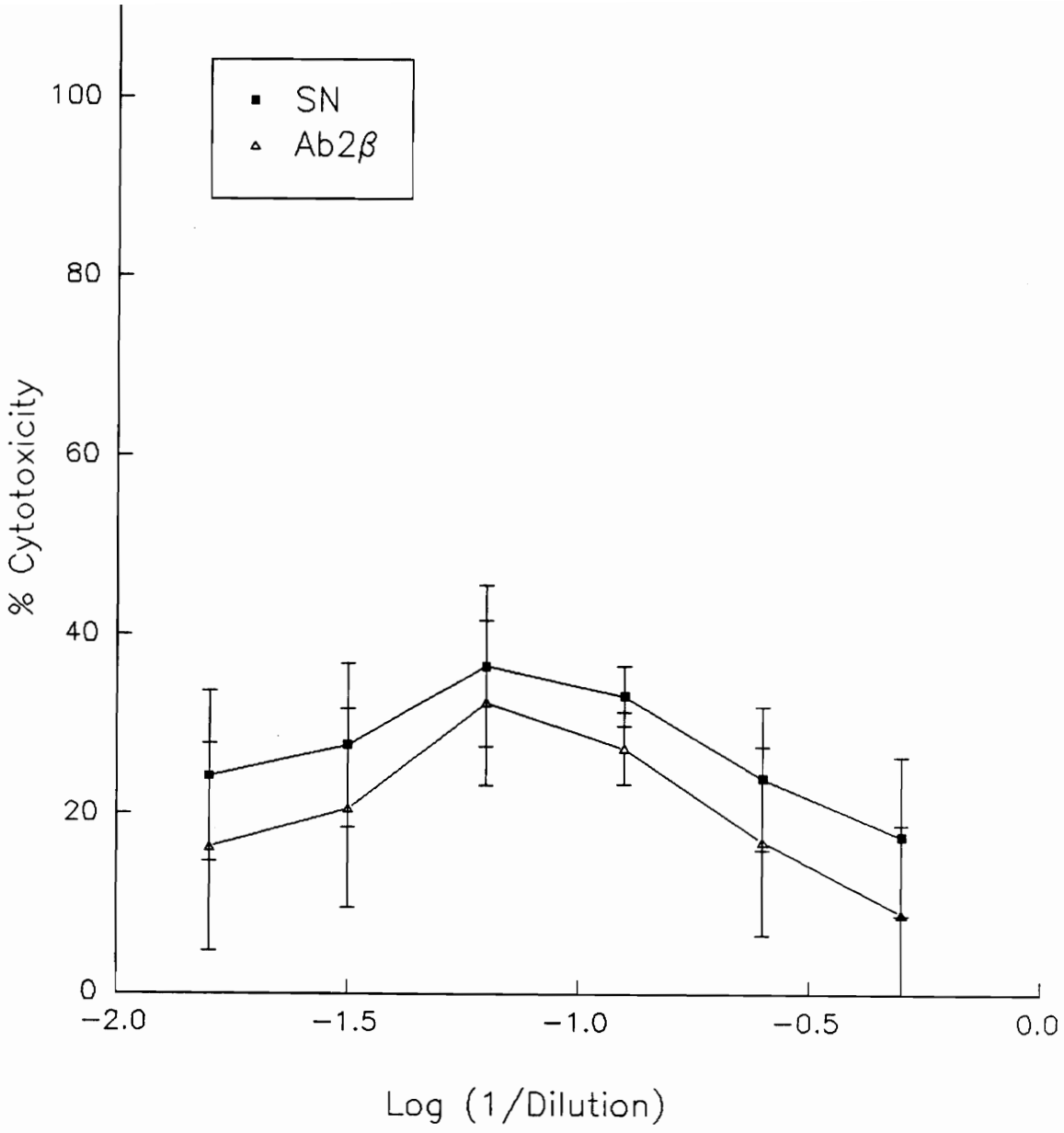

Figure 5c

Cytotoxicity assay carried out with the NS-1 cell line using the whole supernatant from the well producing the monoclonal antibody 1C and the affinity purified Ab2β. The undiluted concentration of Ab2β was 48 μg/ml protein. SN=supernatant. Values are reported as means$\pm$SDM.

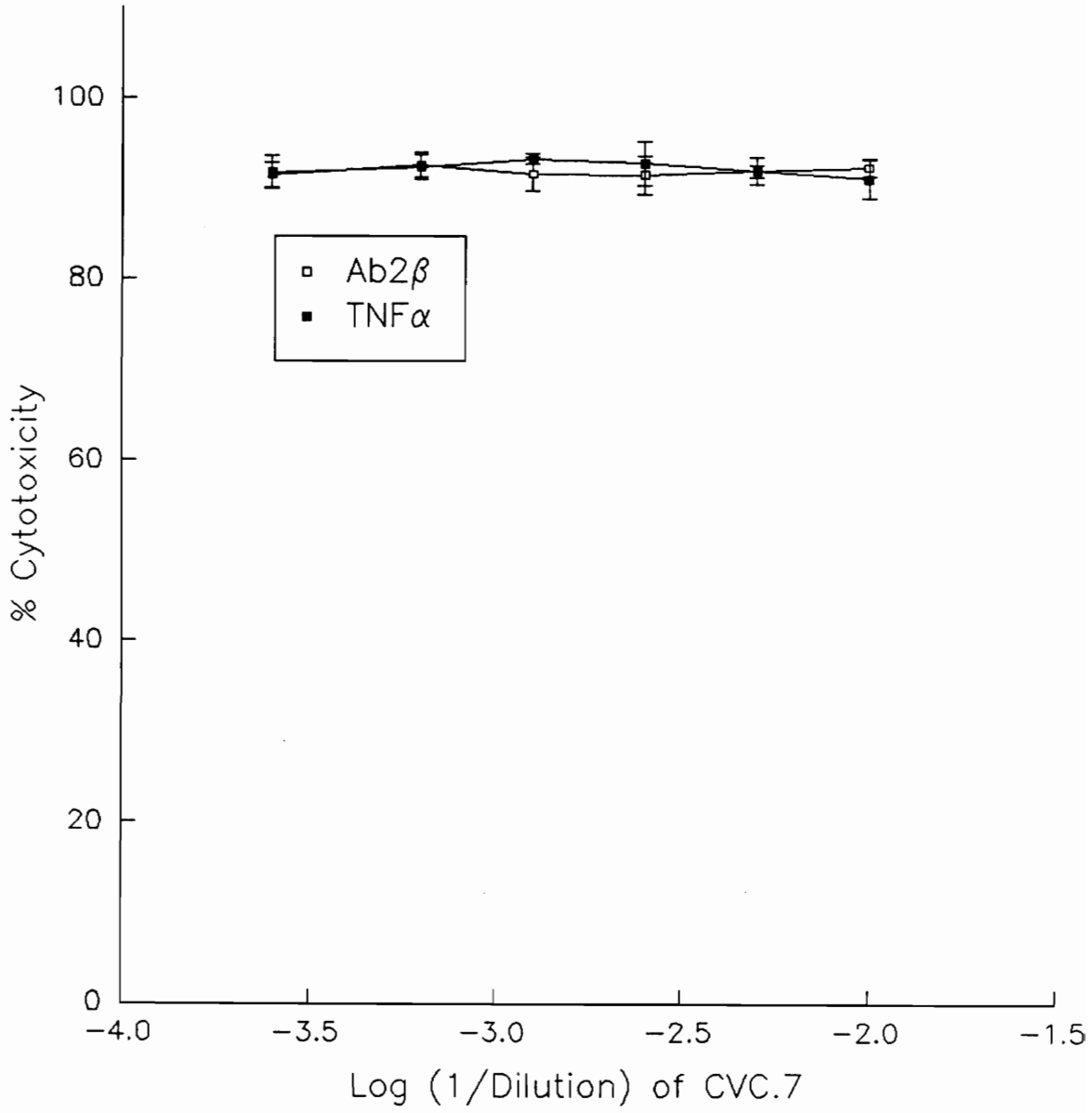

Figure 6a

Cytotoxicity neutralization assay carried out with the L929 cell line using 10 units of rHuTNFα and serially diluted monoclonal anti-TNFα antibodies. The undiluted concentration of the anti-TNFα was 1.2 mg/ml protein. Values are reported as means±SDM.

anti-TNFα antibodies (Figure 6b). When 3 units of Ab2β were added to L929 cells, approximately 80% of the cells were killed in 18 hours and increasing amounts of anti-TNFα antibody decreased the cytotoxic activity of Ab2β (Figure 6b). A non-specific IgG2a antibody (anti-bovine brain clathrin isolated from Balb/c mice, CVC.7) was not capable of neutralizing the cell killing activity of either TNFα or Ab2β (Figure 6c).

F. Molecular Weight Determination

SDS-PAGE, with β-mercaptoethanol, was used to explore the quaternary structure of the Ab2β. The gels were calibrated with the following molecular weight standards: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). The rHuTNFα produced a band at 17 kDa, consistant with the monomer size of rHuTNFα[9,30]. Ab2β, on the other hand, produced bands at 25 and 50 kDa corresponding to the light and heavy chains from the IgG molecule, respectively. A non-related dog IgG also produced similar bands at 25 and 50 kDa. These results are presented in figure 7a.

A Western blot with anti-mouse IgG (HαL) demonstrated that Ab2β is a mouse antibody producing bands at 25 and 50

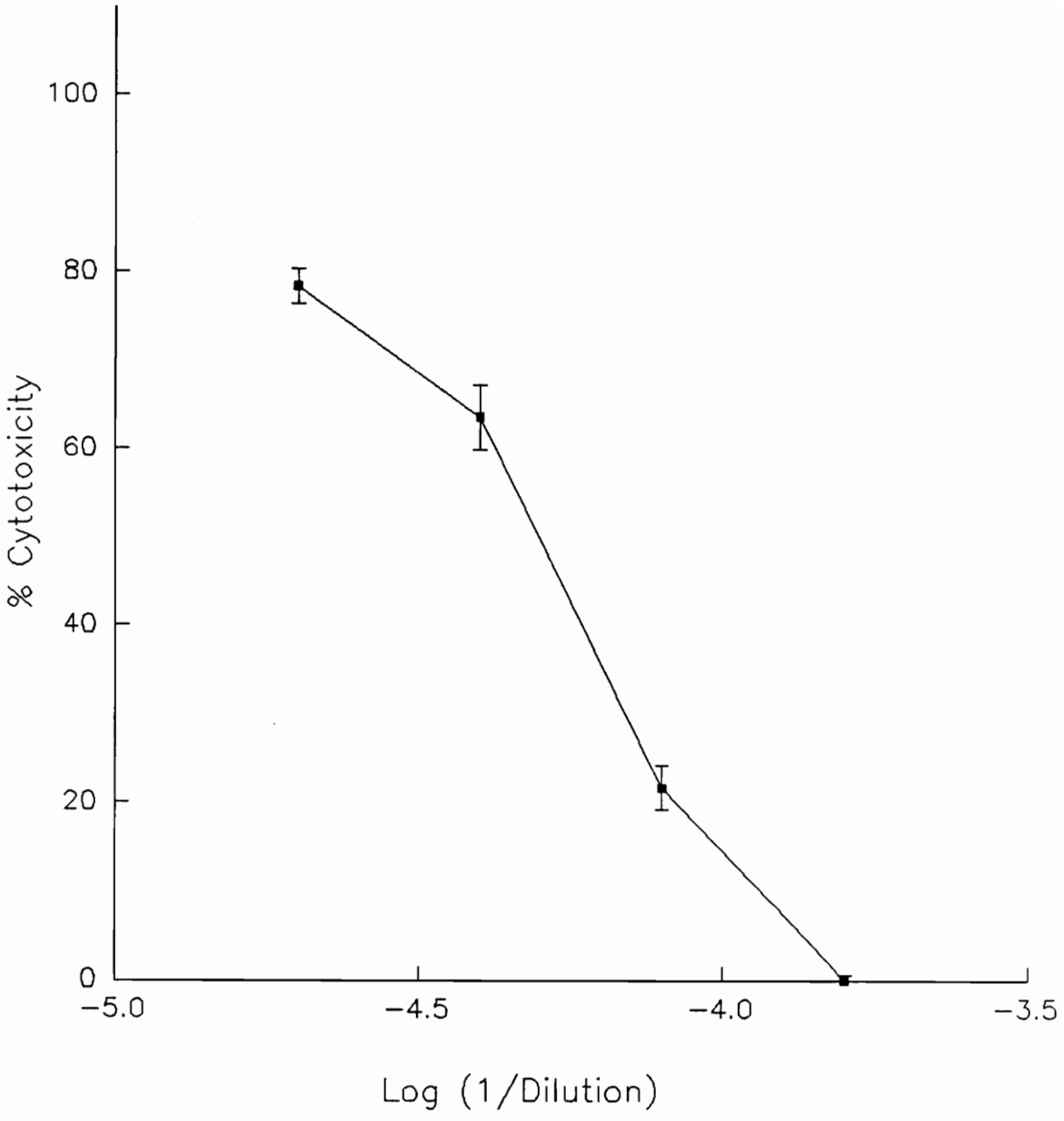

Figure 6b

Cytotoxicity neutralization assay carried out with the L929 cell line using 3 units of Ab2β and serially diluted monoclonal anti-TNFα antibodies. The undiluted concentration of anti-TNFα antibody was 1.2 mg/ml protein. Values are reported as means$\pm$SDM.

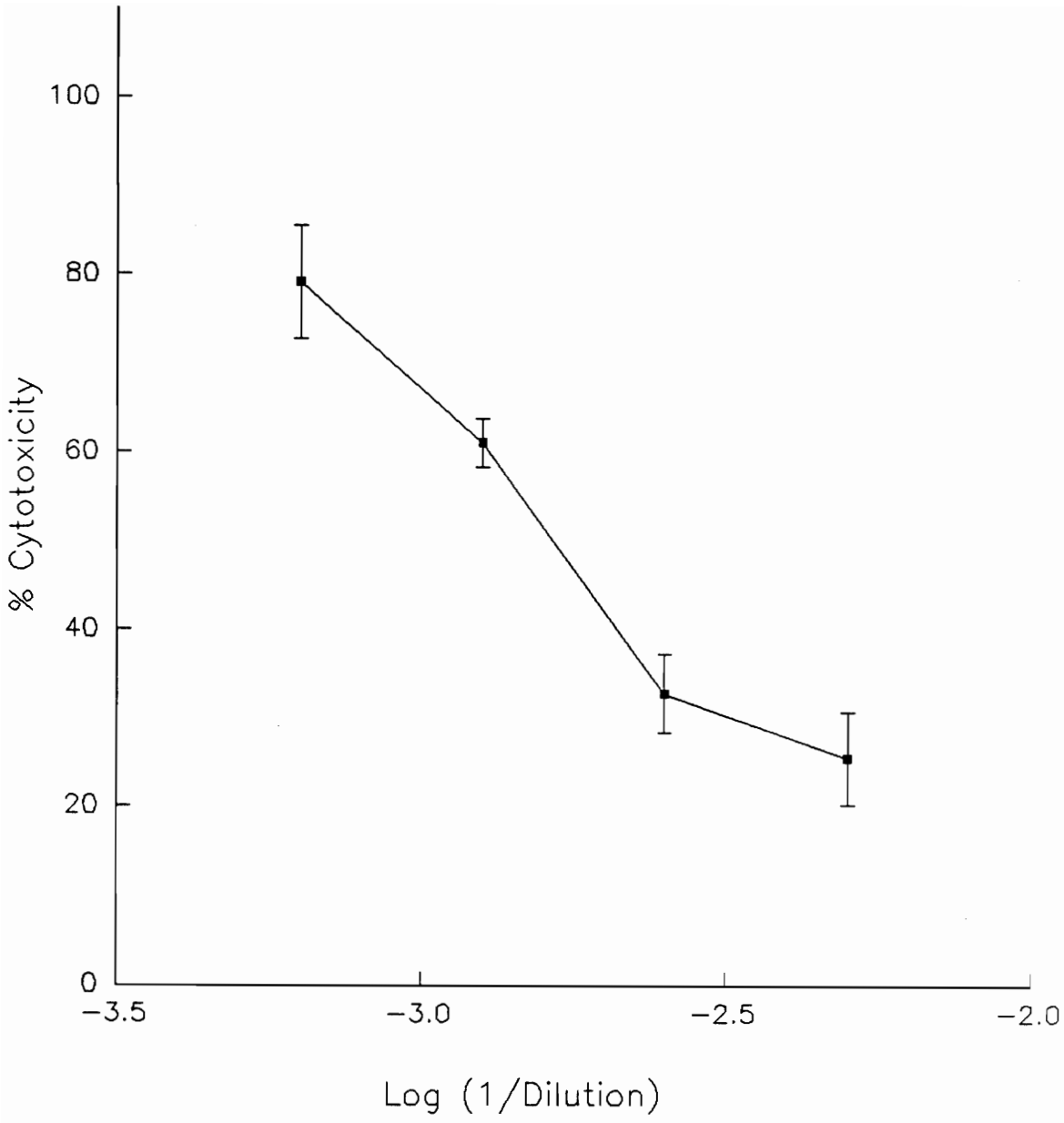

Figure 6c

Cytotoxicity neutralization assay carried out with the L929 cell line using 10 Units of rHuTNFα and the serially diluted non-specific IgG2a antibody (CVC.7). The undiluted concentration of the CVC.7 was 500 μg/ml protein. Values are reported as means±SDM.

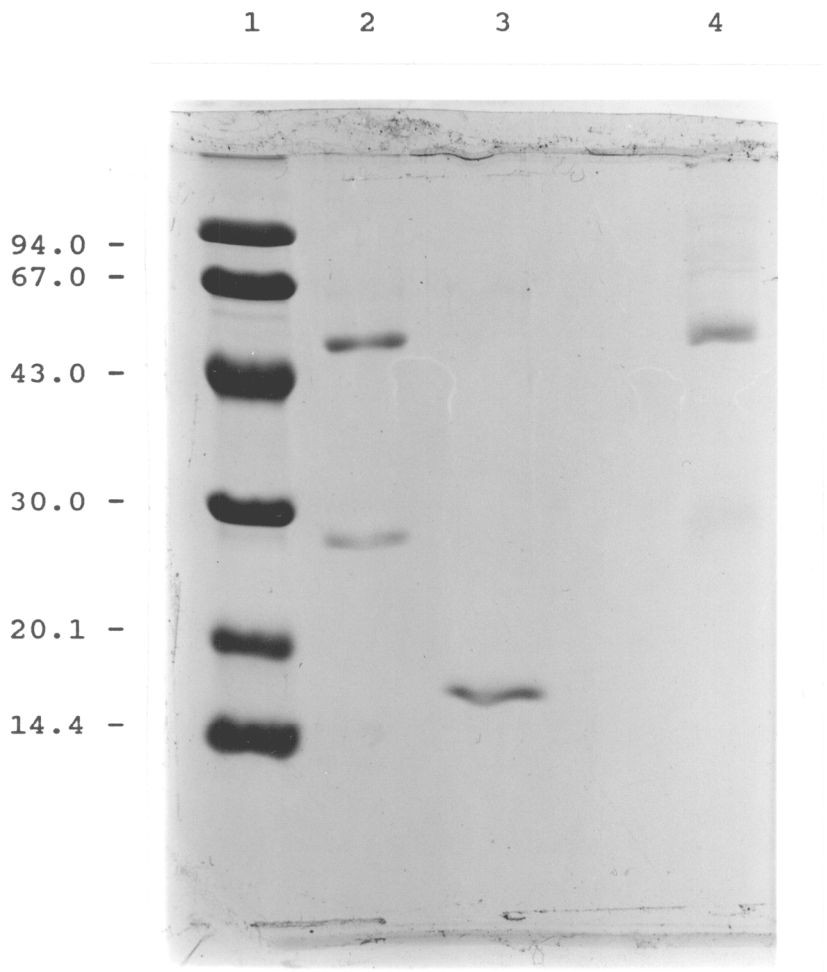

Figure 7a

SDS-PAGE (12.5% acrylamide): Lane 1: molecular weight markers. Lane 2: Ab2β. Lane 3: rHuTNFα. Lane 4: non-specific dog IgG. The gel was stained with Coomassie blue-R. Both IgG molecules (Lanes 2 and 4) showed 25 and 50 kDa bands. A 17 kDa band appeared in Lane 3, corresponds to the TNFα monomer.

kDa, again corresponding to the light and heavy chains from the IgG molecule. No bands appeared on the strip with dog IgG probably because the secondary antibodies coupled to the horseradish-peroxidase were anti-mouse IgG antibodies and did not cross-react with dog IgG (Figure 7b).

G. Bactericidal Assay

Brucella abortus is known to grow inside macrophages[5] and interferon is thought to decrease their replication inside these phagocytes[32]. In this investigation, IFNγ was also found to significantly decrease replication of both strains of B. abortus (strains RB51 and 2308) in the peritoneal macrophages (Figures 8a & 8b) which is consistent with findings by others regarding strain 2308[4,32] and RB51[4]. It has been shown previously that TNFα can stimulate macrophages against other pathogens, however, no such work of this kind has been done using these strains of B. abortus. We tested the effects of TNFα and Ab2β using a macrophage bactericidal assay. TNFα and Ab2β acted in parallel and significantly ($p \leq 0.05$) increased the killing of strain RB51 by macrophages. As shown in Figure 8a, a detectable bactericidal effect was noted at 48 hours, however, the greater increase of macrophage bactericidal activity occurred at 72 hours. Neither TNFα nor Ab2β in significantly ($p \leq 0.05$) increased the killing of strain 2308 by

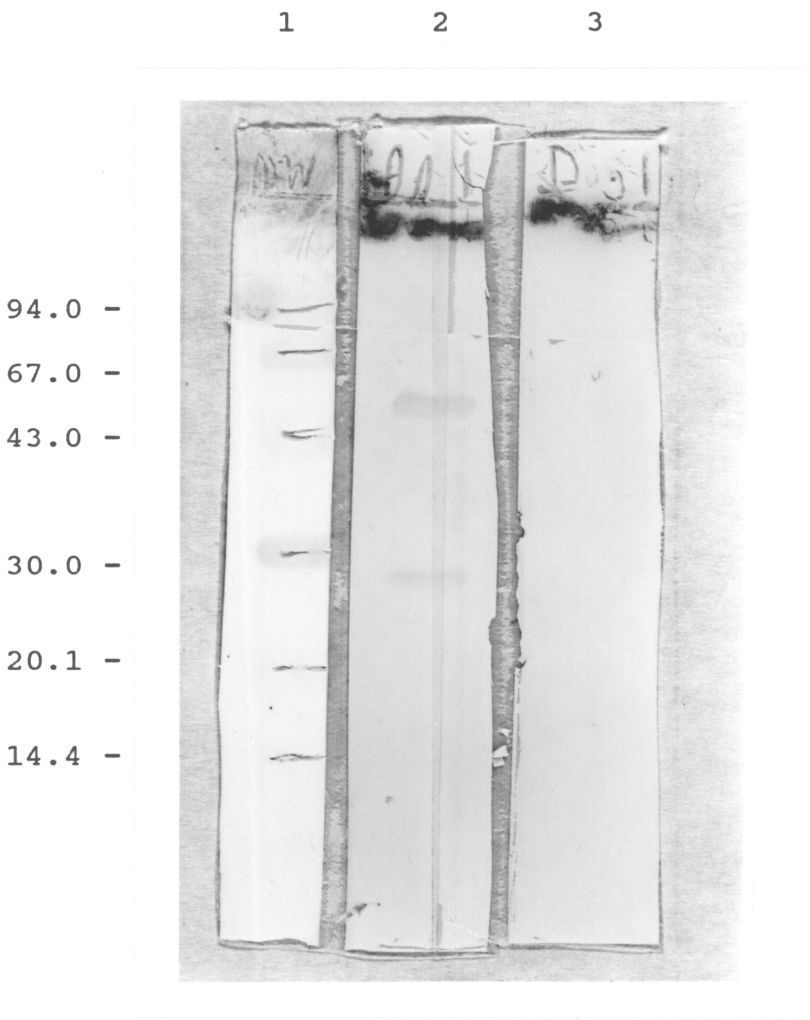

Figure 7b

Western blot of SDS-PAGE: Lane 1: molecular weight markers stained with Ponceau-S stain. Lane 2: Ab2β. Lane 3: rHuTNFα. The primary antibody was anti-mouse IgG coupled to horse-radish peroxidase.

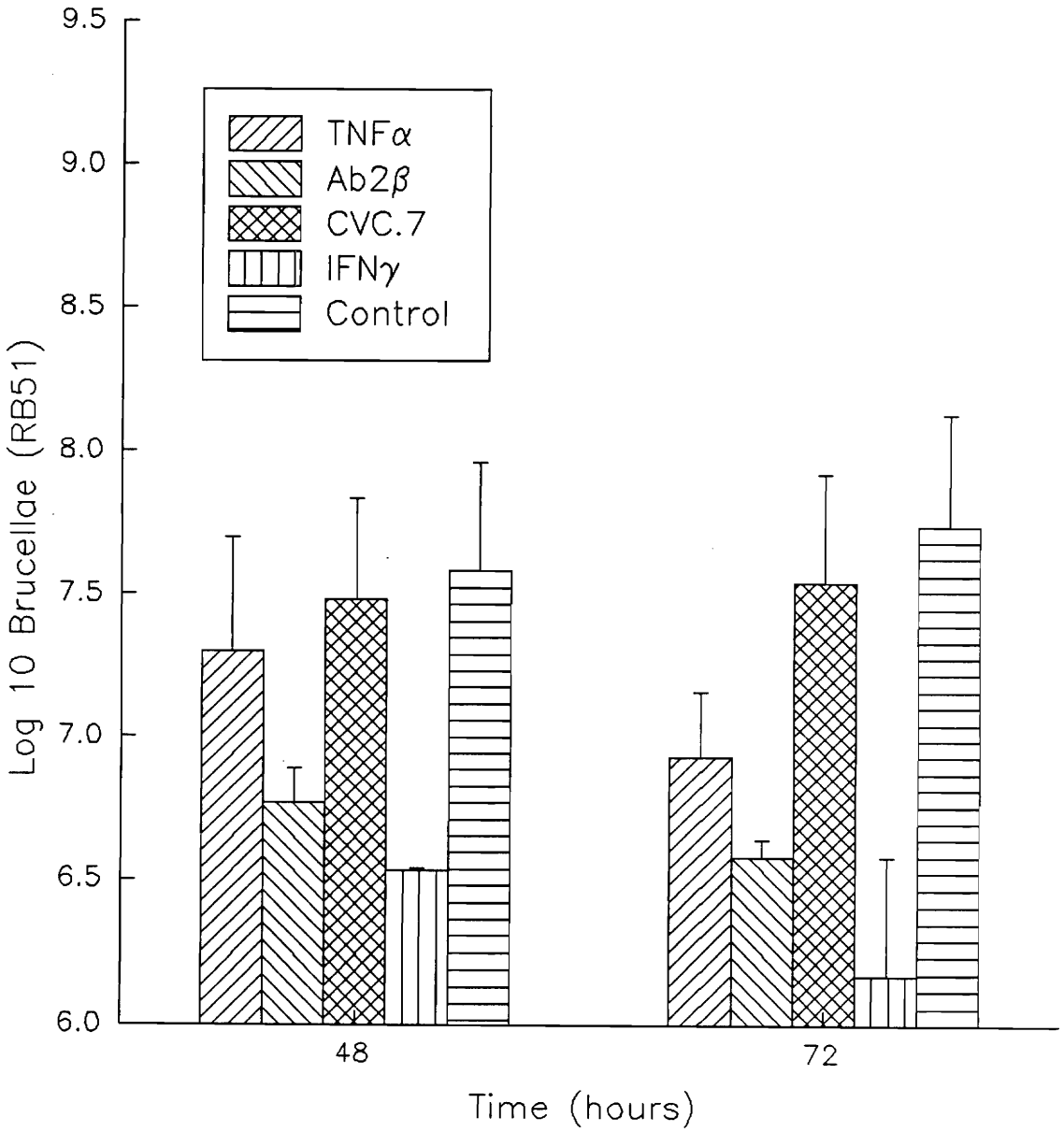

Figure 8a

Bactericidal assay carried out with macrophages pretreated with rHuTNFα, Ab2β, IFNγ, non-specific IgG2a (CVC.7), or no treatment (control). The total number of surviving Brucellae strain RB51 per $1X10^4$ macrophages are depicted 48 and 72 hours after bacterial infection. Values are reported as means±SDM ($P \leq 0.05$). All significnt differences were measured by Duncan's multiple range comparison.

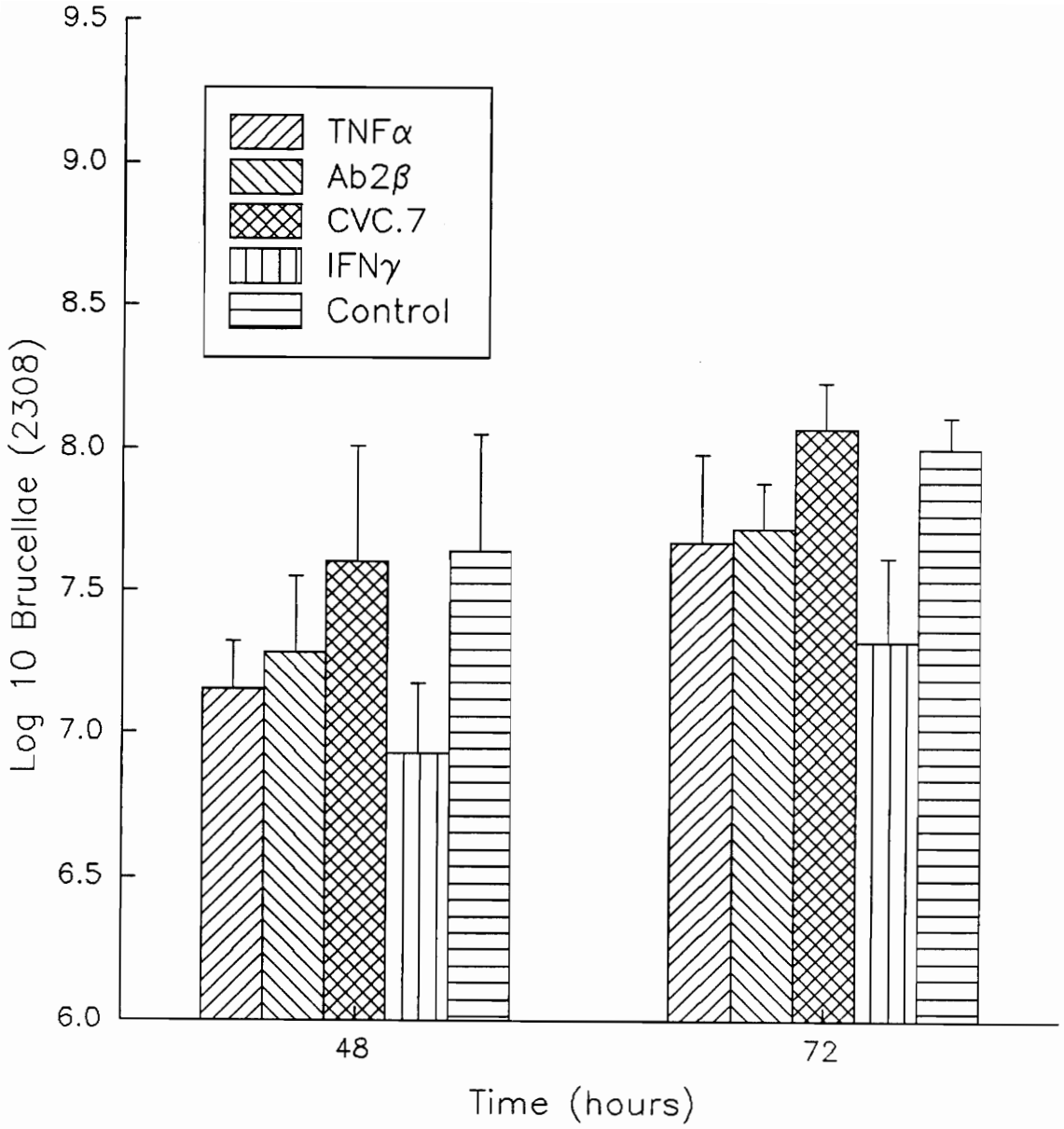

Figure 8b

Bactericidal assay carried out with macrophages pretreated with rHuTNFα, Ab2β, IFNγ, non-specific IgG2a (CVC.7), or no treatment (control). The total number of surviving Brucellae strain 2308 per $1x10^4$ macrophages are depicted 48 and 72 hours after bacterial infection. Values are reported as means±SDM ($p \leq 0.05$). All significant differences were measured by Duncan's multiple range comparison.

macrophages (Figure 8b). In both cases, TNFα and Ab2β acted parallel again demonstrating that Ab2β is capable of mimicking TNFα activity. The number of cfu recovered from macrophages receiving no treatment or were treated with a non-specific antibody (CVC.7) steadily increased over time, whereas TNFα, Ab2β, and IFNγ considerably slowed bacterial growth (Figure 8a & 8b).

V. DISCUSSION

TNFα is a cytokine with pleiotropic properties. It plays an intricate role in a large number of systems[23,76]. One of the areas in which TNFα has been used clinically is in the area of cancer treatment. However, it has met with little success because severe clinical side-effects occur before a high enough dose can be administered to affect the tumors[51,72]. Moreover, due to its short half-life in the circulation, a matter of minutes[9,11,23,60,66], continuous administration of TNFα is required for cancer treatment. The mechanisms by which TNFα mediates its cytotoxic effects are also virtually unknown.

Anti-idiotypic antibodies are used to study the mechanisms of actions of the parent molecules[24,56,57,81]. In this study, an anti-idiotypic antibody apparently bearing the internal image of TNFα was produced and characterized. Its activity on macrophages infected with a facultative intracellular parasite (B. abortus) was also investigated.

After immunization with anti-TNFα, the mice were bled at 4, 7, and 11 weeks to monitor TNFα-like activity. The L929 cells were used in a cytotoxicity assay to screen for the antibodies. The level of antibodies, resembling TNFα action, were found to rise steadily with time while serum from mice immunized with only the adjuvant did not show such activity

(Figure 1). This demonstrated a specific response which increased with time after immunization, as expected.

TNFα, usually found in trimer configuration[9,21,65,69], heated to 56°C for 5 minutes, degrades into monomers, losing some of the biological activity as observed in this study. TNFα activity is most likely triggered by aggregation of the TNFα receptors. A trimer can accomplish this receptor aggregation more efficiently[69]. The antiserum (11 weeks), heated in the same way, also lost some of its cytotoxic activity. It is unlikely that this loss is due to the denaturation or breakdown of the antibodies into two heavy chains and two light chains because of the mild heating conditions. If the chains would have separated, the antibodies would lose their ability to aggregate the receptors on the cell surface and therefore reduce the cytotoxic response. A more likely explanation is that complement contributed somewhat to the cytotoxic activity of Ab2β. Since binding of Ab2β to the receptor had to occur to trigger the response, this reaction has still to be envisioned as an antigen-antibody reaction able to activate complement and therefore increase cytotoxicity levels. Heating at 56°C inactivates complement and would eliminate its cytotoxic effects leaving only the cytotoxic effects obtained after aggregating the TNFα receptors. The absence of complement in the cytotoxic reactions observed with the affinity purified Ab2β indicate

that complement activity is only a minor component in the cytotoxic activity of Ab2β.

After the fusion, one well of hybridomas with high cytotoxic activity was expanded because of its rapid growth characteristics. These hybridoma cells were used for cloning by limiting dilution. After cloning, four hybridomas produced supernatants containing monoclonal antibodies with high cytotoxic activity. One of the hybridomas was expanded due to its high cytotoxic ability and fast growth characteristics and was used for the remaining studies. The antibody was affinity purified and characterized as an IgG2a molecule using an antibody isotyping kit (Sigma). The affinity purified molecules maintained their cytotoxic activities in the absence of complement against the cell lines sensitive to TNFα suggesting the presence of the Ab2β population which was mimicking the functional activities of TNFα. Further, the cell lines, SP2/O and NS-1 murine myeloma cells, known to be resistant to TNFα cytotoxicity, were also resistant to the whole supernatant and affinity purified Ab2β even at high doses which would have caused over 80% killing of the sensitive L929 or WEHI 164 cell lines. This observation further suggested that the monoclonal antibody was mimicking TNFα specific effects.

In order to determine if Ab2β had idiotopes which really mimicked TNFα, the cytotoxicity neutralization assays were

performed on the L929 cells. Ten units of TNFα were first mixed with serially diluted monoclonal anti-TNFα antibodies and then added to the L929 cells after a period of time. These antibodies reacted with TNFα, inhibiting it from binding to the L929 cells and completely eliminating the cytotoxic activity. The same assay was performed using 3 units of Ab2β and the anti-TNFα antibodies. The cytotoxic activity was strongly neutralized by the presence of these antibodies (from 80% cytotoxicity to 22%). This observation is a good indication that Ab2β is an anti-idiotypic antibody containing, at least partially, the internal image of the TNFα binding site. However, even in the presence of neutralization, it is still possible that Ab2β does not bear the internal image of TNFα and a different mechanism accounts for the TNFα-like activities of Ab2β. It is possible that the monoclonal anti-TNFα antibodies were not binding to the active site of Ab2β but were binding to an unrelated site and through steric hindrance of the active site prevented cytotoxicity. A non-specific IgG2a antibody (CVC.7) did not neutralize either TNFα or Ab2β, lowering the possibility that non-specific binding is causing the neutralization of Ab2β. However, if neutralization through steric hindrance is the cause, activity of Ab2β is not due to antigenic mimicry. In order to eliminate this possibility experiments would have to be carried out in which immunization of animals with Ab2β would

lead to a third generation anti-idiotypic antibody (Ab3) with Ab1 (anti-TNFα) activities.

The SDS-PAGE indicated that there was no TNFα contamination of Ab2β as indicated in figure 7a. The TNFα gives a 17 kDa band which is the monomer size of TNFα. No bands appeared at 34 or 51 Kda, the TNFα dimer and trimer, respectively, because the sample buffer contained 2-mercaptoethanol which reduced the TNFα into its monomer subunits. No such band appeared with Ab2β which showed a banding pattern characteristic of an IgG molecule as also indicated by a non-specific dog IgG molecule under the SDS-PAGE conditions in this study. IgG breaks down into heavy- and light-chains showing bands at 25 and 50 kDa.

The Western blot analysis of the SDS-PAGE using anti-mouse IgG (HαL) specific antibodies shows that Ab2β is a mouse antibody with bands at 25 and 50 kDa[44]. Reactions with dog IgG did not occur. These analyses, however, clearly indicated that the active Ab2β is a mouse antibody with no contaminating TNFα or unrelated molecules.

TNFα has been used in studies to stimulate macrophages and increase resistance to certain intracellular pathogens[6,12,15,16,40,59]. In this study, the pathogen used was B. abortus strain 2308, an attenuated rough spontaneous mutant of B. abortus strain RB51. The effects of TNFα on the brucellacidal capabilites of macrophages have not been

reported before. Apparently, TNFα is capable of stimulating macrophage resistance to strain RB51 while not enhancing resistance to Strain 2308. Ab2β had the same effect on the macrophages as did TNFα. Both Ab2β and TNFα significantly increased macrophages resistance to RB51. The number of cfu was inhibited by TNFα, Ab2β, and as expected by IFNγ. The untreated macrophages and the ones treated with a non-specific antibody showed no resistance. The macrophages treated with TNFα or Ab2β did not show an increased resistance to strain 2308. These studies, however, indicate that TNFα and Ab2β exhibit similar responses on these strains of B. abortus. TNFα and Ab2β both afforded some protection against strain RB51 whereas neither enhanced macrophage resistance to strain 2308. Ab2β is exhibiting TNFα-like activity on both strains.

It has been concluded from this study that an anti-idiotypic antibody to TNFα, Ab2β, exhibits functional mimicry by sharing some of the in vitro actions with TNFα, namely its cytotoxic capabilities on the cell lines L929 and WEHI 164 mouse fibro-sarcomas. Also, TNFα and Ab2β share the ability to enhance macrophage resistance to Brucella abortus strain RB51. It is proposed that Ab2β can be used as a tool for studying the mechanisms of TNFα action. Moreover, the structural characterization of Ab2β may shed some light on the active site of the IgG. For example, the ability to produce Fab fragments of Ab2β could be employed to study the role of

cross-linking of TNFα receptors in studying the mechanisms of action of TNFα.

The next step for this project would be to test the effects of Ab2β in vivo. Tumor treatment with TNFα has been somewhat abandoned because of the limitations in administrable doses. It is possible that Ab2β may show the same cytotoxic effects as TNFα on the tumors without the severe side-effects and the limitations imposed by the short half-life of TNFα. However, the clinical applicability of the anti-idiotypic antibody should be considered cautiously. For example, TNFα causes coagulation and advanced tumors have very little vasculature to begin with. Therefore, Ab2β in circulation has less chance of actually reaching a tumor to cause necrosis. Also, the half-life of Ab2β may be longer than the half-life of TNFα, but antibodies in the blood stream can be immunogenic causing an immune response and antibody production against Ab2β. These antibodies may bind to the Ab2β neutralizing any cytotoxic activity that may occur. However, anti-idiotypic antibodies to prolactin cause α-lactalbumin synthesis and the anti-idiotypic antibodies to insulin mimic insulin activity. Therefore, the use of anti-idiotypic antibodies to TNFα in the treatment of cancer shows great promise and may ultimately be the "magic bullet".

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