THE PRODUCTION OF INTERLEUKIN 1 AND TUMOUR NECROSIS FACTOR BY HUMAN MONOCYTES AND EVIDENCE FOR A ROLE IN ARTHRITIS

by

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i

ABSTRACT

Interleukin 1 (IL1 alpha and beta) and tumour necrosis factor (TNF alpha and beta) are inducible cytokines with potent and wide-ranging immunopotentiating, pro-inflammatory and catabolic activities. These activities suggest a potential role in the pathogenesis of inflammatory diseases.

The present study investigated the induction, synthesis and release of IL1 and TNF alpha from human monocytes *in vitro*. This included measurement of kinetics of mRNA accumulation, immunoreactive and bioactive protein production and extracellular translocation. Several microbial products and pro-inflammatory crystals associated with human arthritis were tested for their ability to induce IL1 and TNF production.

All microbial-derived agents tested were potent, concentration-dependent stimuli for both immunoreactive and bioactive IL1 and TNF, but only monosodium urate of three crystal types tested was effective, showing that non-specific membrane perturbation was not a sufficient stimulus for monocyte cytokine induction.

No preformed immunoreactive IL1 alpha, TNF alpha or IL1 beta could be demonstrated in unstimulated cells, while on cellular activation cytokine-peptides accumulated rapidly in the intracellular compartment. This was followed for TNF alpha and IL1 beta, but not for IL1 alpha, by efficient extracellular translocation.

Analysis of mRNA confirmed that IL1 beta or TNF alpha transcripts only accumulated after cellular activation and there was no untranslated mRNA pool or pre-formed intracellular protein in resting cells, indicating that control of cytokine production was predominantly at the stage of gene transcription.

To test *in vivo* the potential role of these cytokines in rheumatic diseases, it was important to demonstrate their presence at the site of inflammation.

Unfractionated synovial exudate fluids from a large number of patients with different rheumatic diseases contained no biodetectable IL1, but high levels of immunoreactive IL1 beta and IL1 alpha were demonstrable in these samples. This discrepancy could reflect the presence of biological inhibitor(s) or the immunodetection of IL1 in a biologically inactive form.

In contrast, bioactive TNF was detectable in approximately 30% of the fluids tested; when assayed in specific immunoassays, excellent agreement with bioactivity was obtained.

Thus, both IL1 and TNF were present at high concentrations in synovial exudate fluids from several rheumatic diseases. Since their production appears to be inducible at transcriptional level, it can be concluded that IL1 and TNF gene expression is activated *in vivo* at the site of inflammation in rheumatic diseases.
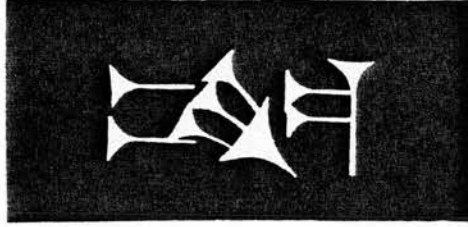
"ummu"

Akkadian cuneiform inscription (600-700 B.C.) adapting an earlier Sumerian pictographic symbol of a flaming brazier to denote in a single ideogram both "fever" and "inflammation" (Majno, 1975).

TABLE OF CONTENTS

DECLARATION

The experiments and composition of this thesis are the work of the author, unless otherwise stated.

Francesco di Giovine

Francesco S. di Giovine

ACKNOWLEDGEMENTS

ORIGINAL ARTICLES

At the moment of submission, three original articles have been published based on the work in this thesis:

F.S. di Giovine, Malawista S.E., Nuki G., Duff G.W.. (1987) "Interleukin 1 (IL1) as a mediator of crystal arthritis. Stimulation of T cell and synovial fibroblast mitogenesis by urate-crystal induced IL1" J. Immunol., 138: 3213-3218.

F.S. di Giovine, Meager A., Leung H., Duff G.W.. (1988) "Immunoreactive Tumour Necrosis Factor alpha and biological inhibitor(s) in synovial fluids from rheumatic patients" Int. J. Immunopathol. Pharmacol., 1: 17-26.

F.S. di Giovine, Nuki G., Duff G.w.. (1988) "Tumour Necrosis Factor in synovial exudate" Ann.Rheum. Diseases, 47: 768-772.

Reprints of these articles are enclosed in the inside back cover.

1.   INTRODUCTION

1.a.   SOLUBLE MEDIATORS OF IMMUNITY: CYTOKINES

The efficiency and discrimination of the "adaptive" immune system are regulated by the selective expansion of lymphocyte clones. When T and B cell surface receptors recognize an exogenous antigen, they become susceptible to activation by soluble polypeptide growth factors, termed "cytokines" which are produced in rapid succession during the inflammatory/immune reaction. If the expansion of antigen-specific clones and the MHC restriction of antigen presentation provide the basis for discrimination, the fine tuning of the proliferative, maturational or functional actions of different cytokines on target cells is mediated by their cellular receptors and by diverse mechanisms that can alter this interaction or its effects.

The description of the different cytokines began with the identification of specific biological phenomena and the demonstration that they were linked to a peptide entity that mediated this action. At this stage, the "factors" were given names reflecting their biological action (and often different actions of the same mediator), more frequently indicating the target rather than the producing cells. The huge impetus provided by molecular cloning in recent years provided new techniques to answer questions about gene regulation, protein structure, processing and translocation, receptor structure and interactions. It became clear that many biologically-described "factors" were, in fact, single molecular species and it was agreed that a known amino acid sequence was an essential criterion for the description of a new cytokine.

Named after particular biological activities, a few families of cytokines have emerged, including **Interferons, Transforming Growth Factors, Colony Stimulating Factors, Tumour Necrosis Factors** and the family of **Interleukins** , to which now virtually all new entries can be assigned. The agreed criteria for designation as an Interleukin (known primary structure, main but not necessarily exclusive production by leukocytes, and action during immune reactions or inflammation) are so broad that many of the previously mentioned cytokines would, if described today, be called Interleukins.

Each one of these mediators plays potent roles in cell activation, differentiation or modulation during the immune response. Much of classical immunology, including "innate immunity" and antibody response is today explicable through a complicated but increasingly defined network of cytokine-receptor interaction and expression of recognition structures on definite cell types. The main features of some families of peptide cytokines (human molecules, unless specified) are summarized in Tables 1.1 to 1.7.

In man, the earliest response to exogenous, pathogenic agents appears to be mediated by two major cytokines, Interleukin 1 (IL1) and Tumour Necrosis Factor (TNF). These mediators can be produced by simple recognition of microbial components, such as bacterial wall constituents or exotoxins, without the need for specific antigen recognition. Their production can preceed antigen presentation and activation of lymphocytes, producing a reaction known as the "acute phase response", characterized by fever, metabolic changes, production of acute phase proteins from the liver and bone marrow activation. These actions are probably the most ancient biological properties of these cytokines, which evolved well before the "adaptive" immune response.

The reasons why IL1 and TNF are of special interest to the study of chronic inflammatory diseases, and rheumatic conditions in particular, are inherent in their biology as outlined in the following sections.

TABLE 1.1   Human Interferons

Definition:	Viral interference (Isaac and Lindemann, 1957)		
Types:	IFN alpha	IFN beta-1*	IFN gamma
Other Names:	Viral IFN, Type I IFN Leucocytic IFN	Viral IFN, Type I IFN Fibroblastic IFN	Immune IFN, Type II IFN MAF
Subtypes:	Products of 14 genes: alpha 1, alpha 2	1 gene	1 gene
Producing cells	Mainly T and B cells; also monocytes and fibroblasts.	mainly fibroblasts; also leukocytes and epithelial cells.	activated T and NK cells; also B cells
Stimuli for production	Viruses, bacteria poly I:C	viruses, bacteria poly I:C IL1	alloantigens, mitogens (via IL1, IL2)
Biological actions:	antiviral antimitotic MHC class II (+) NK activity (+) fever in mice decreased c-myc expression	antimitotic antiviral MHC class II (+) decreased c-myc expression	antimitotic antiviral synergy with TNF FcR1 (+++) expression class II MHC expression (DR +++, SB +++, DC +) enhancement monocytic cytotoxicity inhibits actions of IL4 on B cells inhibition of intracellular parasites "priming" in vivo monocyte chemotaxis

Gene
Location	14 loci, chrom 9	1 gene, chrom 9	1 gene, chrom 12
Structure	no introns	no introns	3 introns

Rec. molec.
mature peptide	165aa(19KDa)	166aa (19KDa)	146aa (17KDa)
glyc. sites	no	no	two
homology	none	none	none

native molecular
structure	monomer	monomer	up to tetramers
glycosylations	-	-	0, 1, 2
MW GF	16 to 27 KDa	20KDa	50-60KDa
MW SDS-PAGE			16, 20, 25 KDa

Receptor
shared	IFN beta-1 and IFN alpha's		no
structure	1 chain, glycoprotein of 95 to 110 KDa		2 subunits 53/75Kd
other	10^{-11} M Kd; RMI; chrom 21		RMI; 10^{-11} M Kd different aggregation (70-92-163-325 KDa)

Main Sources   Trinchieri, Perussia, 1985; Pestka et al, 1987; Friedman, Vogel, 1983.

Abbreviations   MAF = Macrophage Activating Factor
                 RMI = Receptor Mediated Internalization
                 GF = Gel filtration

*NB: IFN-beta 2 (or IL6) is in Table 1.4

TABLE 1.2   Human Colony Stimulating Factors

Definition: Stimulation of haematopoietic colonies in agar culture from progenitor cells. (Bradley, Metcalf 1966)

	M-CSF	GM-CSF	G-CSF	multi-CSF
Types:	M-CSF	GM-CSF	G-CSF	multi-CSF
Other names:	MGF: CSF 1 MGI-1M	MGI-1GM; CSF 2 CSF alpha; NIF-T pluripoietin alpha	MGI-2; DF CSF beta; pluripoietin MGI-1G	IL3; BPA; PSF; MCGF I; HCGF; MEG-CSF; E-CSF; EO-CSF;
Producing cells:	embryonal tissues monocytes, fibroblasts, endothelial cells	T lymphocytes macrophages, endothelial cells fibros, keratinocytes acute myelogenous leukemia	monocytes fibros, endo- thelial cells	only activated T cells
Stimuli for production	LPS, IL1	LPS - IL1 -TNF retroviral infection	LPS, IL1	antigen presentation

Biological actions	macrophage
	  colonies
	induces:
	  PGE_2
	  P. activator
	  IL1
	  IFN gamma
	  TNF alpha
	  antiviral
		granulocyte, eosinophils,
		  macrophage colonies
		radioprotection
		protection from bacterial
		  and parasitic infections
		enhancement eosinophil
		  and neutrophil function
		PGE_2, O_2 induction
		prolif/differentiation
		  of leukemic cells
		IL1 and TNF
			granulocyte
			  colonies
			terminal differen-
			  tiation of
			  myeloid cells
			enhancement
			  neutrophil
			  function
				multiple colonies
				  of each lineage
				enhancement
				  eosinophil
				  functions

Gene				
location	chrom 5	chrom 5	chrom 17	chrom 5
structure	8 exons, at least 1 intron	at least 1 intron	5 exons, 4 introns	5 exons, 4 introns
mRNA	1.5 to 4.5Kb	1Kb	1.6Kb	1Kb
Rec. molecule				
propeptide	224aa (26KDa)	144aa	207aa	152aa
glyc. sites	2	2	no N-glyc	2
mature pept.	127aa (14.5KDa)	127aa (18-24KDa)	177aa (19KDa)	133aa(14.6KDa)
leader seq.	22aa	yes	yes	19 aa
homology	none	none	none	none
Native molec.				
structure	homodimer	monomer	monomer	monomer
MW GF	40-45KDa	22KDa	23KDa (O-glyc)	28KDa
MW SDS	19-22KDa	18-22KDa	19.6KDa	15-25KDa
Receptor	(murine)	(murine)	(murine)	(murine)
shared	no	no	no	no
structure	450aa, 165KDa single chain,	51/130KDa two classes	150KDa 1 chain	60KDa/75KDa two classes ?
homology	c-fms	?	?	?
other	3×10^{-13}M Kd, RMI tyrosine kinase act. transmodulation of other CSF.R's.	10^{-12},10^{-9}M Kd transmod. of other CSF.R's	10^{-13}M Kd transmod. of other CSF.R's	10^{-13}M Kd RMI, transmod. other CSF.R's

Main Sources: Nicola, Vadas, 1984; Metcalf, 1987; Nicola, 1987; Kawasaki et al, 1985; Wong et al, 1985; Souza et al, 1986; Schrader, 1986; Lopez et al, 1987.

Abbreviations used:

MGI	Macrophage/granulocyte inducer	CSF	Colony stimulating factor
BPA	Burst promoting activity	G	Granulocyte
HCGF	Haemopoietic cell growth factor	M	Macrophage
MCGF	Mast cell growth factor	MEG	Megacariocyte
PSF	P-cell stimulating factor	DF	Differentiation factor
NIF-T	Neutrophil inhibiting factor, T-derived	GF	Gel-filtration
RMI	Receptor-mediated internalization	SDS	SDS-PAGE

TABLE 1.3   Human Tumour Necrosis Factors

Definition:	Necrosis of transplantable tumours in vivo. (O'Malley et al, 1962; Carswell et al, 1975	
Type:	TNF alpha	TNF beta
Other Names:	Cachectin Tumour Necrosis Factor	Lymphotoxin
Producing Cells:	Mainly macrophages also T, B cells, endothelial cells, mast cells.	Activated T cells during AP. also monocytes and B cells.
Stimuli	LPS and other microbial agents IL2, GM-CSF, IL1	viruses (herpes, vescic. stomatitis) T cell activation IL2, and gamma IFN + IL2 poly I:C

Biological
  Actions:

TNF alpha	TNF beta

in vivo:
  necrosis of tumours
  endotoxic shock-like syndrome
  cachexia
  fever, IL1, acute phase response
  antiparasitic
 	
in vitro:
  cellular cytotoxicity
  actions on enzyme of lipid and carbo-
    hydrate metabolism
  PGE_2 from several cell types
  antiviral
  induction of IL1, GM-CSF, IL6, TNF
  neutrophil activation
  induction of ICAM 1
  augm. MHC class I and class II expression
  bone, cartilage resorption
  (see also tables 1.11, 1.12)
	cytolytic for several cell types
	mediator of CTL killing?
	necrosis of tumours in vivo
	increases cell mediated cytotoxicity
	synergy with IFN gamma, induces IL6
	augments MHC class II expression and
	  ICAM I expression
	augments PMN phagocytosis
	antiparasitic and antiviral direct
	bone resorption
	differentiation of monocytic lines
	  (HL60, THP-1S)

Gene

	TNF alpha	TNF beta
Location	Chrom 6 (near MHC and TNF beta)	Chrom 6 (near MHC and TNF alpha)
structure	4 exons	4 exons, 3 introns
mRNA	1.6/1.8Kb	1.4/1.6Kb

rec. molecule

	TNF alpha	TNF beta
propeptide	233aa	205aa
glyc. sites	no	one
mature peptide	157aa (17.3KDa)	171aa (18.7KDa)
leader sequence	yes (76 aa)	yes (34 aa)
homology	some, to TNF beta	some, to TNF alpha

native. molecule

	TNF alpha	TNF beta
structure	non-covalent polymers	dimers to trimers
MW GF	39-150KDa; 51KDa	60-70KDa
MW SDS	17.5KDa	20-25KDa

Receptor
  shared   shared by TNF alpha and TNF beta
  structure   at least 2 chains (75KDa and 95KDa) glycoprotein
  other   upregulated by IFN gamma; high (3×10^{-13}M Kd)
                    and low (1.5×10^{-10}M Kd) affinity.
                    Internalization of TNF's and receptor

Main Reviews   (Old, 1985; Beutler, Cerami 1986, 1987, 1988; Le, Vilcek, 1987; Ruddle et al, 1987;
                Aggarwal et al, 1987; Paul, Ruddle, 1988; Cerami, Beutler, 1988.)

Abbreviations Used

AP	antigen-presentation	GF	Gel filtration
ICAM	intracellular adhesion molecule	SDS	SDS-PAGE
PMN	polymorphonuclear cells		

TABLE 1.4   Human Interleukins

	IL1		IL2
Definition:	Known sequence, production also by leukocytes in inflammation or immune response.		
Type:	IL1		IL2
Also known as:	BAF; ETAF; LEM; EP; LAF; HPI.1		TCGF I; KHF; TRF; TSF
Subtypes	IL1 alpha	IL1 beta	1 gene IL2
Producing cells:	monocytes and many others (see also Table 1.10)		T cells (TH$_1$ mainly), NK cells, cord blood lymphocytes, some CD8$^+$ T cell lines
Stimuli:	microbial products; TNF, GM-CSF, IL2; atg presentation		antigen presentation; IL1; IL2
Biological actions:	immunoregulation (induction of IL2, IL4, IL6, IL1, TNF) Fever, acute phase response hypotension, slow wave sleep induction of PGE$_2$ and collagenase from mesenchymal cells bone and cartilage resorption in organ culture (see also tables 1.8, 1.9)		Proliferation of T, B, TIL, LAK cell increased cytotoxicity of NK cells maturation and proliferation of CTL induces IFN gamma, IL1, IL4 TNF alpha, TNF beta increased expression of c-myb; c-myc reduction of tumour metastasis and lymphoid expansion in vivo inhibited by PGE$_2$, TGF beta and itself (down reg. receptor)

Gene

Location	unpublished (2)	chrom 2	chrom 4
structure	7 ex, 6 in	7 ex, 6 in	4 ex, 3in
mRNA	2.0/2.2Kb	1.6/1.8Kb	0.65/1.1Kb

Rec. Molecule

propeptide	271aa (30.6KDa)	269aa (30.7KDa)	153aa
glyc. site	no	potentially one	O-glycosylation (1)
mature p.	159aa (17.5KDa)	153aa (17.3KDa)	133aa (15.4KDa)
leader seq.	no	no	20aa
homology	some, to IL1 beta	some, to IL1 alpha	no

Native molec.

structure	monomer or non-covalent polymer; propeptide, mature peptide or cleavage products	monomer or non-covalent polymer; propeptide, mature peptide or cleavage products	monomer
MW GF	38,31,17,15, 4.2KDa	38,31,17,15,4, 2KDa	19-22KDa
MW SDS	31, 17.5KDa	31, 17.5KDa	15-17KDa

Receptor

shared	IL1 alpha and IL1 beta mature peptides, IL1 alpha propeptide	no
structure	alpha (80KDa) and beta (100KDa) chains. Alpha (murine) 576aa	alpha (55KDa, Tac protein) beta (75KDa) chains
homology	Ig superfamily (murine)	no
other	0.2-2.5x10^{-10}M Kd	alpha chain (=Tac) gene: chrom 10 8 ex, 7 in; 1.5-3.5Kb mRNA down-regulated by IL2 and TGF beta (beta = 1x10^{-8}M Kd; alpha + beta = 1x10^{-11}M Kd)

Main Reviews Oppenheim, Gery, 1982; Mizel, 1982; Dinarello, 1984; Durum et al, 1985; Oppenheim et al, 1986; Dinarello, 1986; Le, Vilcek, 1987; Dower, Urdal, 1987; Smith, 1984; Robb, 1984; Greene, Leonard, 1986; Smith, 1987; Siegel et al, 1987; Fletcher, Goldstein, 1987; Sims et al, 1988.

Abbreviations Used:

BAF	B cell activating factor	TCGF	T cell growth factor	LAK	lymphokine activated killer
LEM	Leukocytic endogenous mediator	KHF	Killer cells helper factor	CTL	Cytotoxic T lymphocytes
EP	Endogenous pyrogen	TRF	T cell-replacing factor	NK	Natural killer
LAF	Lymphocyte activating factor	TSF	T cell-stimulating factor	HP1	Hemopoietin 1
TIL	Tumour infiltrating lymphocytes	ETAF	Epidermal-cell derived T cell activating factor		

Type:	IL3	IL4	IL5
Other Names:	multi CSF, MCGF I ... (see also CSF's)	TCGF II, BCGF I, BSF-I BCDF epsilon, BCDF gamma, MCGF II	TRF, BCGF II, BCDF alpha EDF, KHF, BCDFmi
Producing cells:	activated T cells (TH$_2$)	activated T cells (TH$_2$)	activated T cells (TH$_2$)
Stimuli:	atg presentation	atg presentation	atg presentation
Biological actions:	multiple colonies for each lineage enhancement of eosinophil functions	T cells: TCGF activity (++) thymocyte prol. (atg) LAK activity B cells: BCGF (atg) (++) MHC class II (+) IgG$_1$ enhancer (+++) IgE^1 " (+++) FCe RII " (+++) IL4R " (+++) (all blocked by IFN gamma) synergy on actions by: G-CSF; M-CSF; IL3 Mast cell growth factor	B cells: BCGF (atg) (++) IgA enhancer (+++) IgM " (++) BCL 1 growth (+++) Eosinophils: maturation growth Synergy on actions by: IL4 for IgE IL2 for CTL genes and IL2R
Gene location	chrom 5	chrom 5	chrom 5
Rec. molecule propeptide glyc. sites mature p. leader seq. homology	152aa 2 133aa (14.6KDa) 19aa no	153aa 2 129aa (15KDa) yes no	133aa 2 125aa (13.2KDa) yes no
mat. molecule structures MW GF MW SDS	monomer 28KDa 15-25KDa	monomer 20KDa 15-19KDa	dimer, trimer 45,60KDa 18,20,22 KDa
Receptor shared structure	(murine) no 60/75KDa	no 140/70KDa	no 92.5KDa complex
other	10^{-13}M Kd internalized	7×10^{-11}M Kd internalized	10^{-11} and 10^{-9}M Kd

Main Sources   Nicola, 1987; Yang et al, 1986; Schrader, 1986; Sanderson et al, 1988; Swain et al, 1988; Takatsu et al, 1988; Yokota et al, 1988; Kishimoto, Hirano, 1988; Paul, Ohara, 1987)

Abbreviations Used:

CSF	Colony stimulating factor	BCDF	B-cell differentiation factor
MCGF	Mast-cell growth factor	TRF	T-cell replacing factor
TCGF	T-cell growth factor	EDF	Eosinophil differentiation factor
BCGF	B-cell growth factor	KHF	Killer-helper factor
BSF	B-cell stimulating factor		

TABLE 1.4 (cont)   Human Interleukins

Type:	IL6	IL7 (murine)
Also known as	BCDF, BSF-2, IFN beta 2; HPGF, HSF, CDF	LP-1
producing cells:	activated T cells, glioblastoma cells, fibroblasts, cardiac mixoma cells, cervical adeno-carcinoma lines, monocytes	stromal adherent cells from bone marrow
Stimuli	IL1, TNF, PDGF, poly I:C autocrine in some lines	LPS, ?

Biological actions:	proliferation
	  plasmacytoma lines
	  hybridoma lines
	  haemopoietic cells
	  (IL3 and GM-CSF-like action)
	  EBV-transformed cells
	  thymocytes (atg)
	T cells:
	  induce IL2R
	  with IL2, CTL induction
	B cells:
	  induction of Ig production
	  (differentiation without growth)
	antiviral (??)
	induction of acute phase
	  reactions in liver cells
	fever in vivo
		long-term growth of pre-B cells and pro-B cells
		
		T-cell activation in presence of lectin

Gene		
Location	chrom 7	
structure	5 exons, 4 introns	
mRNA	1.3Kb	1.5-2.9Kb
homology	G-CSF	

Rec. Molecule		
propeptide	212aa	
glyc. sites	2	2
mature p.	184aa (20.7KDa)	129aa (14.9KDa)
leader	yes	yes

Native Molecule		
structure	monomer	monomer
MW SDS	21KDa	25KDa

Receptor	55;75;95KDa	
	$10^{-10}/10^{-11}$M Kd	

Main Sources   Hirano et al, 1986; Kishimoto, Hirano, 1988; Wong, Clark, 1988; Billiau, 1987; Namen et al 1988a; Namen et al, 1988b

Abbreviations Used:

BCDF: B-cell differentiation factor   HSF: Hepatocyte-stimulating factor
BSF-2: B-cell stimulatory factor   CDF: Cytotoxic cell differentiation factor
IFN: Interferon   LP: Lymphopoietin
HPGF: Hybridoma/plasmocytoma growth factor

TABLE 1.5   Human Transforming Growth Factors

Definition:	Phenotypic transformation of rat fibroblasts (Roberts et al, 1980).		

	TGF alpha	TGF beta	
Types:	TGF alpha	TGF beta	
Related Molecules:	very related but distinct to EGF and VGF	related, but distinct to ACTIVINS, INHIBINS, MIS, DPP.C, Vg1	
Subtypes:	TGF alpha	TGF beta 1	TGF beta 2	TGF beta 1/beta 2

Producing Cells:

TGF alpha: most tumoral lines but not leukaemias retroviral infected cells, fetal cells

TGF beta: macrophage, lymphocytes B/T Megacarinocytes, then stored in alpha granules of platelets, bone

Biological actions:

TGF alpha:
mitogenic in vitro
epidermal regeneration in vivo
angiogenesis in vivo
bone resorption

TGF beta:

Immunosuppressive
    inhibition of all known IL2 effects including IL2R expression
    ? activation of monocytes: IL1, TNF alpha
    chemotactic for monocytes.
Connective Tissues
    fibrosis and wound healing in vivo
    increases: collagen I, III, IV gene expression, fibronectin, TIMP, PGE_2 and Ca release from bone, FSH induced steroids, osteoblast proliferation, fibroblast chemotaxis, fibroblast glycolysis, fibroblast fibroblast collagenase
    decreases: production of serum/metalloproteinases P.A. secretion, ACTH effects on adrenal glands.
General Cellular Effects
    anti-proliferative: hepatocyte, epithelial cells, keratinocytes, T, B cells.
    Control of differentiation in vitro, via expression of INTEGRINS (fibronectin receptors), and influencing cell adhesion/cytoskeleton interaction.
Inhibition of Other Cytokines
    EGF induced proliferation in monolayers
    PDGF   "   "
    IL2   "   "
    FGF   "   "

Gene

	TGF alpha	TGF beta	
location	Chrom 2	Chrom 19	
mRNA	4.5/4.8Kb	2.5Kb	

Rec. Protein

	TGF alpha	chain beta 1	chain beta 2
precursor	160aa (20KDa)	391aa	391aa
mature	50aa (6-10KDa)	112aa (12.5KDa)	112aa (12.5KDa)
homology	EGF, VGF	75%, chain beta 2	75%, chain beta 1

Native molecule

	TGF alpha			
structure	monomer	homodimer beta 1/beta 1	homodimer beta 2/beta 2	heterodimer beta 1/beta 2
MW	6KDa	25KDa	25KDa	25KDa

Receptor

TGF alpha: shared with EGF (see) 170KDa c-erb B molecule

10^{-10}M Kd

TGF beta: 10^{-11}M Kd, three order of receptors:
1) 280KDa receptor, most biological actions, used by the three forms of TGF beta.
2) 65KDa and 3) 80KDa receptors, used only by TGF beta 1.

Main Sources   Todaro, 1987; Sporn et al, 1986; Cheifetz et al, 1987; Massague, 1987; Assoian et al, 1987; Ignotz, Massague, 1987.

Abbreviations Used

VGF	Vaccinia growth factor	DPP.C	Decapentaplegic transcript
MIS	Mullerian inhibitory substance	Vg1	Xenopus Vg1 material mRNA product

TABLE 1.6   Human Heparin-Binding Growth Factors (HBGF)

Definition:	Based on the biochemical purification (Lobb et al, 1986A)	
Type:	acidic FGF	basic FGF
Similar molecules:	class 1 HBGF's: ECGF EDGF-II; alpha-RDGF aHDGF; BDGF: AGF-I	class 2 HBGF's: TAF; EDGF-1 beta-RDGF; cHDGF; CDGF AGF; HDGF; MDGF
Producing cells:	mainly neural tissue	neural tissue, kidney, corpus luteum, macrophages, adrenal glands, endothelial cells, cartilage, bone.

Biological
actions: myoblast and cortical neuron growth
                  endothelial cells chemotaxis and growth
                  induction of IFN gamma
                  proliferation of neuroectodermal derived cells
                  angiogenesis in vivo, neovascularization
                  induction of proteases from mesenchymal cells

Gene		
location	chrom 5	chrom 4
mRNA	4.8Kb	2.2; 5Kb

rec. protein		
propeptide	155aa	155aa
glyc sites	yes	yes
mature p.	140aa (18KDa)	146aa (18KDa) no leader sequence

natural protein		
structure	monomer	monomer
MW	16-19KDa	16-19KDa

Receptor   shared, 140/160KDa, high affinity
                  autophosphorylating-tyrosine kinase

Main Sources   Lobb et al, 1986B; Deuel, 1987; Lobb et al, 1986A.

Abbreviations Used:

FGF	Fibroblast growth factor	BDGF	Brain-derived growth factor
ECGF	Endothelial cell growth factor	AGF	Astroglial growth factor
EDGF	Eye-derived growth factor	TAF	Tumor angiogenesis factor
RDGF	Retina-derived growth factor	CDGF	Cartilage derived growth factor
HDGF	Anionic (a) or cationic (c) hypothalamus-derived growth factor	MDGF	Macrophage-derived growth factor

TABLE 1.7   Some Other Human Cytokines

Type:	PDGF		EGF
Other names:	PDGF I, PDGF II (diff. glycosilations, homo or heterodimer)		Beta-urogastrone
Producing Cells:	endothelial, smooth muscle cells monocytes, megakariocytes (alpha granules platelets) developing placenta		submaxillary gland ectodermic cells monocytes
Stimulus	activated by thrombin, coagulation, LPS, lectins, zymosan		

Biological actions:	PDGF		EGF
	chemotaxis and proliferation of mesenchymal cells		proliferation and differentiation of basal cell layer in epithelia:
	induction of IL1, IL1R, IFN gamma and IFN beta		cornea skin
	induction of: PGE_2, PGI_1 LDL receptor c-myc; c-fos aa transport		lung and trachea mammary gland neonatal development
	intracellular Ca and Na, acidosis intracellular actin reorganization neutrophil activation augment synthesis collagen V, III, IV decreases EGF binding		in vivo: angiogenic, wound healing, augmentation of disulphide bonds in skin, hepatic hypertrophia and hyperplasia induces IFN gamma, PG's, hCG secretion inhibition of gastric secretion augmented glycolysis, ODC activity, protein synthesis
	in vivo: vasoconstriction wound healing, vascular permeability		augmented hyaluronidase acid production enhanced by insulin suppressed by TGF beta, PDGF

Gene	A chain	B chain	
location	chrom 7	chrom 22	chrom 4
homology	B chain	p28^sis, product of c-sis gene	TGF alpha
mRNA	1.9/2.3/2.8Kb	4Kb	4.9Kb

Rec. Molecule			
propeptide	211aa(23KDa)	226aa(28KDa)	1217aa
mature peptide	125aa (14KDa)	160aa (18KDa)	53aa (6KDa)
glyc. sites	one		

Native Molecule		
structure	A,B hetero/homodimer	monomer
glycosilation	variable	
MW gel filt.	28-35KDa	6KDa
MW SDS	28-35KDa	6KDa

Receptor		
shared	no	with TGF alpha
structure	single chain, 164KDa	single chain, 170KDa
homology	c-kit (chrom 5) and c-fms (M-CSF/R) molecule	c-erb B molecule
characteristics	autophosphorylating tyrosine kinase 10^{-11} M Kd	autophosphorylating tyrosine kinase product of a gene in chrom 7 (like c-erb B) 1186aa, 132KDa glycosilated to 170KDa; 10^{-10} M Kd

Main Sources: Deuel, 1987; Ross, 1987; Johnson, Torres, 1985; Carpenter, 1987; Carpenter, Cohen, 1979.

Abbreviations Used:

PDGF	Platelet-derived growth factor	hCG	Human corion-gonadotropin
EGF	Epidermal growth factor	ODC	Orhithin-decarboxylase

1.b INTERLEUKIN 1

Products of activated macrophages have attracted great interest as mediators of host responses to infection, injury and immune reactions. One family of these peptides is Interleukin 1 (IL1). IL1 has been studied for decades for its ability to induce the acute phase response, namely fever (Endogenous Pyrogen [EP] activity: Beeson, 1948; Atkins, 1960) and synthesis of acute phase reactants with redistribution of metal ions and neutrophilia (Leukocytic Endogenous Mediator [LEM] activity: Homburger, 1945; Kampschmidt, 1978). More recently described are its action in the immune system (Lymphocyte Activating Factor [LAF] activity; Gery et al, 1972), its effects on mesenchymal cells with induction of PGE_2 and collagenase (Mononuclear Cell Factor [MCF] activity: Dayer et al, 1976) and its catabolic effects on cartilage (Catabolin: Saklatvala, 1981).

Following demonstration of the substantial biochemical similarity of these factors (reviewed by Dinarello, 1984), it was agreed (Aarden et al, 1979) to use the term Interleukin 1 to refer to the monocyte product(s) responsible for these activities. The cloning of IL1 (Lomedico et al, 1984; Auron et al, 1984; March et al, 1985) confirmed earlier observations on the biochemical heterogeneity of IL1 peptides (Lachman et al, 1977; Murphy et al; 1981): IL1 consists of two different, acidic (pI5) and neutral (pI7) proteins, referred to as IL1 alpha and IL1 beta respectively. The availability of recombinant proteins has now made it possible to assign to both IL1's the extensive range of biological properties (Table 1.8 and Table 1.9) previously reported. These include direct action on human liver, supporting a role for IL1 in the induction of acute phase proteins, and on T cell activation, with an increase in expression of IL2 and IL2 receptor followed by proliferation. Rabbits and mice injected intravenously with IL1 develop typical monophasic fever, confirming the role of IL1 as an endogenous pyrogen. Interestingly, recombinant IL1 has been shown to induce IL1 gene expression, suggesting a mechanism by which IL1 production could amplify and sustain local inflammatory responses. The confirmed catabolic actions of IL1 include bone and cartilage resorption, with proteoglycan loss from the cartilage, and osteoclast activation with calcium release from the bone. Most of these biological activities are not exclusive to IL1: the availability of pure, recombinant reagents has demonstrated that Tumour

Necrosis Factor (TNF) and other cytokines share some of the actions of IL1. TNF alpha (Dinarello et al, 1986B), Interferon alpha (Dinarello et al, 1984) and IL6 (Dinarello et al, 1988) are all able to induce fever when injected in rabbits, and could be responsible for some of the activity of EP, but their specific activity (10-1000 times less than IL1 *in vivo*) indicates that most of this activity is probably attributable to the presence of IL1. Similarly, MCF and catabolin-like activities are exhibited by TNF alpha (Dayer et al, 1985; Saklatvala, 1986) but at concentrations 10-100 times greater than IL1.

Interleukin 1 is classically described as a macrophage product, but in keeping with its essential role in host response, many different cell types have been reported as sources of IL1 (Table 1.10).

Interleukin 1 production is activated by the recognition of microbial components (reviewed in 2.c.1, pp 67-69) or during antigen-mediated immune-responses. During MHC-restricted T cell activation, production of IL1 by the antigen-presenting cell (APC) seems important.
This was demonstrated by showing that addition of IL1 could achieve T cell activation to specific antigen, when mononuclear cells were depleted of APC by density centrifugation (Smith et al, 1980) or by anti Ia treatment (Durum, Gershon, 1982; Durum et al, 1984). Addition of IL-1 restored antigen presentation when APC were paraformaldehyde-fixed (Scala and Oppenheim, 1983) or UV-treated (de Freitas et al, 1983). Interestingly, all the cells reported to perform antigen presentation have been shown to be IL1 producers: these include monocytes/macrophages (Gery et al, 1972) B cells (Matsushima et al, 1985), endothelial cells (Wagner et al, 1985) and dendritic cells (Duff et al, 1985).

Cytokines produced during T cell activation (lymphokines) are able to induce IL-1 or synergize with other cytokines in triggering such production. These interactions are now increasingly being characterized: IFN gamma synergizes with minute quantities of LPS or TNF in inducing IL-1 (Arend et al, 1985; Boraschi et al, 1984; Arenzana-Seisdedas et al, 1985) and enhances MHC class II expression, in absence of which IL-1 production is greatly reduced (Gilman et al, 1983). Other cytokines produced during immune response that are able to induce IL-1 include IL2 (Numerof et al, 1987), TNF alpha (Dinarello et al, 1986; Libby et al, 1986), CSF's (Moore et al, 1980; Ralph et al, 1987), IL-1 itself (Warner et al, 1987), and a low molecular

14

weight newly characterized cytokine (Takacs et al, 1987).

In response to membrane IgM perturbation (Matsushima et al , 1985) B-lymphoblasts release IL1, and immune complexes formed in the presence of excess antigen will induce monocyte IL-1 production (Duff et al, 1982; Arend et al, 1985). The multiple roles of IL1 in the immune system confer powerful adjuvant effects *in vivo* (Staruch et al, 1983)

The human IL1 alpha gene (12kb) has seven exons and six introns. The first and seventh exons code for 5' and 3' untranslated mRNA regions, the last containing the octameric sequence probably related to mRNA instability (Shaw, Kamen, 1986). The IL1 alpha transcript codes for a propeptide of 271aa (MW 30.6 KDa) that is then processed to a mature acidic (pI5) peptide of 159aa (MW 17.5 KDa) (March et al, 1985).

The human IL1 beta gene, located on chromosome 2, also has seven exons; the transcript codes for an acidic propeptide of 269aa (MW 30.75 KDa) that is cleaved to a mature neutral peptide of 153aa (MW 17.3 KDa) (March et al, 1985). Processing of both IL1 propeptides is believed to happen near or within the plasma membrane. The mechanism by which IL1 leaves the cytosolic compartment is not known; unlike other secretory proteins, IL1 alpha and IL1 beta lack classical hydrophobic signal sequences. It has been suggested that IL1 is only released from damaged cells (Gery, Lepe-Zuniga, 1983) or that intracellular processing can provide a release mechanism different from other secretory proteins.

Both mature IL1 forms and the IL1 alpha propeptide compete equally for a single class of high affinity receptor on target cells. The ability of IL1 alpha and IL1 beta to bind to the same receptor and mediate similar biological actions *in vitro* is surprising considering the low degree of homology between the two molecules. When the two IL1 proteins are compared, only a few regions of amino acid homology exist (approximately 26% overall). Protein domains have been termed A-E (Auron et al, 1985). Since the first two (A,B) are contained in the precursor sequence that is missing from the mature form of IL1, regions of homology in C, D and E are likely to represent the conformational "active site" of the IL1 molecule. The other possibility is that the two molecules, even with different structures, are able to interact successfully with a complex receptor molecule(s) assembled in such a way that both IL1 molecules are recognized. In fact, no

15

convincing report exists of major differences in bioactivity between IL1 alpha and beta, even if it has been recently suggested that IL1 alpha is preferentially expressed on the cell membrane, predicting a role in cell-cell interactions (Conlon et al, 1987). The wide range of biological activities of IL1 involves an enormous number of different cell types. All of these cells have been shown to have specific cell surface receptors for IL1 (reviewed by Dower, Urdal, 1987). The number of receptor ranges from less than 50 in some T cell lines to about 5000 in some fibroblast lines. Competitive binding studies indicate that IL1 alpha and IL1 beta share the same receptor (Dower et al, 1985; Bird, Saklatvala, 1986; Matsushima et al, 1986; Mizel et al, 1987, Mosley et al, 1987).

The IL1 receptor has been characterized in human and murine cells by covalent cross-linking and ligand blotting, as a moiety of around 80KDa (Dower et al, 1985; Bird, Saklatvala, 1986) and 60-70KDa IL1 binding proteins have been reported on the surface of B cells (Matsushima et al, 1986A; Horuk et al, 1988). The murine receptor appears to be a heavily glycosylated protein: N-glycanase treatment reduces its MW from 80KDa to 62KDa and molecular cloning (Sims et al., 1988) revealed a 64.6KDa transmembrane protein, part of the Ig supergene family. There is some evidence consistent with the presence of a second chain within the IL1 receptor: covalent cross-linking studies have reported molecular complexes of different sizes, indicating that IL1 also associates with polypeptides of 43KDa (Martin et al, 1988) or 100 KDa (Bird et al, 1987) on murine cells.

Of the many studies investigating the post-receptor events after IL1 binding, IL1-dependent phosphorylation has been reported for 27KDa (Saklatvala, 1988), 41KDa (Martin et al, 1986) and 65KDa (Matsushima et al, 1987) polypeptides.

The wide range of actions that makes IL1 a principal mediator of host defence against infection, injury or antigenic challenge is outlined in Tables 1.8 to 1.9. These powerful effects also confer on IL1 a major pathogenetic potential if its production or target cell responsiveness is dysregulated in inflammatory and immune diseases.

TABLE 1.8   Some biological effects of IL1 on target cells IN VITRO

1.   IMMUNE SYSTEM

- T cells
      Co-mitogenic with atg   (Gery et al, 1972, Maizel et al, 1981)
      IL-2 induction   (Smith et al, 1980: Hagiwara et al, 1987)
      IL-2R expression   (Kaye et al, 1984)
      IL-4 production   (Ho et al, 1987)
      IFN gamma induction   (Kasahara et al, 1985)
      IL3 induction   (Hagiwara et al, 1987)
      c-fos/c-myc induction   (Kovacs et al, 1986)
      chemotaxis   (Miossec et al, 1984)

- B cells
      BCGF activity (proliferation)   (Wood et al, 1976; Leibson et al, 1982;
          -cofactor ?   Chiplunkar et al, 1986)
      BCDF activity (ab production)   (Hoffmann et al,1979; Giri et al, 1984;
          -cofactor ?   Chiplunkar et al, 1986)

- Monocytes
      Chemotaxis   (Luger et al, 1983; Sauder et al, 1984)
      Cytotoxicity enhancement   (Onozaki et al, 1985; Lovett et al, 1986)
      PGE_2 synthesis   (Dinarello et al, 1983)
      IL1 induction   (Dinarello et al, 1987)

- NK Cells
      Enhancement in NK activity   (Herman et al, 1985)
          (with IL2 ?)   (Dempsey et al, 1984)

- Basophils
      Degranulation, histamine
          release   (Subramanian, Bray, 1987)

- Neutrophils
      Chemotaxis   (Luger et al, 1983; Sauder et al, 1984)

- Eosinophils
      Degranulation   (Pincus et al, 1986)

- Fibroblasts
      Induction of:
          IFN beta 2/IL6   (Van Damme et al, 1985)
          GM-CSF   (Zucali et al, 1986)
          PGE_2 and proteases   (see Musculo Skeletal Tissues)

- Endothelial Cells
      Induction of:
          GM-CSF   (Seelentag et al, 1987; Zuckermann et al, 1985;
                                             Broudy et al, 1987)
          G-CSF   (Broudy et al, 1987; Seelentag et al, 1987)
          M-CSF   (Broudy et al, 1987; Seelentag et al, 1987)
          IL1   (Wagner et al, 1985)
          ICAM 1 protein   (Pober et al, 1986)

- Vascular Smooth Muscle Cells
      Induction of IL1   (Warner et al, 1987; Dustin et al, 1986)

2.   CENTRAL NERVOUS SYSTEM

      Astrocyte proliferation   (Lachman et al, 1987)
      Production of PGE_2 in
          hypothalamic cells   (Dinarello, Bernheim, 1981)

17

TABLE 1.8 (cont.)

3. LIVER

Decreased lipoproteinlipase
    activity   (Beutler, Cerami, 1985)
Decreased albumin synthesis   (Ramadori et al, 1985; Perlmutter et al, 1986;
                                        Darlington et al, 1986; Gauldie et al, 1987)

Increased synthesis of:
  alpha 1 acid-
  glycoprotein   (Darlington et al, 1986; Gauldie et al, 1987)
  C3   (Darlington et al, 1986; Perlmutter et al,1986)
  Factor B   (Perlmutter et al, 1986)
  CRP   (Darlington et al, 1986)
  Haptoglobin   (Darlington et al, 1986)

4. MUSCULO-SKELETAL TISSUES

Bone
  Resorption (organ culture)   (Gowen et al, 1983)
  Osteoclast activation   (Rifas et al, 1984)
  Osteoblast mitogenesis   (Gowen et al, 1983)

Cartilage
  Resorption (organ culture)   (Dingle et al, 1978; Saklatvala, 1981; Gowen et al, 1984)
Chondrocytes
  release of collagenase   (Saklatvala et al, 1984)
  release of plasminogen
    activator   (Richardson et al, 1985)
  release of neutral
    proteases   (Herman et al, 1984; Gowen et al, 1984; Saklatvala et al, 1985).
  activation of PLA_2 (Chang et al, 1986)
  decreased collagen synthesis (via PGE_2?) (Pujol et al, 1984; Tyler, 1987)
  decreased proteoglycan synthesis   (Tyler, 1985A; Tyler, 1985B)

Fibroblasts
  Proliferation   (Schmidt et al, 1982)
  PGE_2 production   (Korn et al, 1980; Postlewaite et al, 1983)
  Collagenase induction   (Mizel et al, 1981)
  Cytokine production   (see Immune System)
  increased collagen synthesis   (Kahari et al, 1987)
  decreased collagen synthesis (via PGE?) (Bhatnagar et al, 1986)

Synoviocytes
  PGE_2 production   (Dayer et al, 1976,; Dayer et al, 1980; Mizel et al, 1981)
  Collagenase induction   (Dayer et al, 1977; Mizel et al, 1981; Krane et al, 1982)

5. VASCULAR TISSUES

Endothelial Cells
  Proliferation   (Ooi et al, 1983)
  Thromboxane production   (Rossi et al, 1985)
  PGI_2 production   (Rossi et al, 1985)
  Procoagulant activity   (Bevilacqua et al, 1984)
  PGE_2 production   (Albrightson et al, 1985)
  Increased adhesiveness   (Bevilacqua et al, 1985; Pober et al, 1986)
  Cytokine production   (see Immune System)

6. CYTOSTATIC/CYTOTOXIC EFFECTS

Human pancreatic islet beta
    cells   (Bentzen et al, 1986)
Some transformed cell lines   (Onozaki et al, 1985 B)
Melanoma cells   (Lachmann et al, 1986)

18

TABLE 1.9   Some biological activities of IL1 IN VIVO

- Hypotension   (Dinarello, 1987; Okusawa et al, 1988)
- Fever   (Beeson, 1948; Atkins, 1960; Murphy et al, 1980;
                                        Dinarello et al, 1986)
- Slow wave sleep   (Kreuger et al, 1984)

- Anorexia, weight loss   (McCarthy et al, 1984)
- Neutrophilia   (Kampschmidt, Pulliam, 1978)

- Acute phase proteins   (Kampschmidt et al, 1973; Sipe et al, 1979; Sztein et
  (SAA, SAP, Fibrinogen,   al 1981; Dinarello, 1984)
  CRP, alpha 1 AT, C3)

- Plasma metal levels   (Kampschmidt and Pulliam, 1978)
    hypozincaemia
    hypoferraemia
    hypercupraemia

- Increased hormone levels
    corticosterone, ACTH   (Besedowsky et al, 1986)
    insulin, glucagon   (Besedowsky, del Rey, 1987)

- Radioprotection   (Neta et al, 1986)
- Bone marrow cell-cycling   (Neta et al, 1987B)
- Induction of circulating CSFs   (Vogel et al, 1987)

- Adjuvant effect   (Staruch and Wood, 1983)

- Decreased cytocrome P450
    enzyme activity   (Ghezzi et al, 1987)
- Neutrophils accumulation in
    joints   (Granstein et al, 1985; Pettipher et al, 1986)
- Cartilage proteoglycan release   (Pettipher et al, 1986)
- Enhanced resistance to bacterial
    infection   (Ozaki et al, 1987)

TABLE 1.10   Cells reported to produce Interleukin 1

Blood monocytes   (Gery et al, 1972; Gery and Lepe Zuniga, 1983)
Placental monocytes   (Flynn et al, 1982)

Tissue macrophages
    Alveolar macrophages   (Simon and Willoughby, 1981)
    Kupffer cells   (Dinarello et al, 1968)
    Synovial cells   (Wood et al, 1985)
    Peritoneal macrophages   (Murphy et al, 1980)

Lymphocytes
    Helper T cell clones   (Tartakowsky et al, 1986, Acres et al, 1987)
    EBV transformed B cells   (Matsushima et al, 1985)
    B cells   (Matsushima et al, 1985; Chiplunkar et al, 1986)
    NK/LGL cells   (Scala et al, 1984)
    Adult T cell leukemia
    cell lines   (Yamashita et al, 1987)

Vascular cells
    Smooth muscle cells   (Libby et al, 1986)
    Endothelial cells   (Libby et al, 1986; Wagner et al, 1985)

Brain cells
    Astrocytes   (Fontana et al, 1982)
    Microglia   (Giulian et al, 1985)
    Glioma lines   (Fontana et al, 1984)

Skin cells
    Keratinocytes   (Sauder et al, 1982; Luger et al, 1982)
    Langerhans cells   (Sauder et al, 1984)

Miscellaneous
    Synovial dendritic cells   (Duff et al, 1985)
    Kidney mesangial cells   (Lovett et al, 1983)
    Neutrophils   (Goto et al, 1984)
    Fibroblasts   (Iribe et al, 1983)
    Chondrocytes   (Ollivierre et al, 1986)
    Corneal epithelium   (Grabner et al, 1982)
    Thymic epithelial cells   (Le et al, 1987)
    Epithelial cells   (Gahring et al, 1984)

20

1.c.   TUMOUR NECROSIS FACTOR

Of the many effects induced *in vivo* by endotoxin, one of the most impressive is haemorrhagic necrosis of tumours. This phenomenon, initially observed at the end of the last century (Coley, 1893), was later characterized (Shear, 1944) by isolation of the active moiety (lipopolysaccharide, LPS) from culture broths, which had been used in the previous experimental work (Shear, Andervout, 1936). However, the lethal effects of LPS ("endotoxic shock": profound hypotension, coagulopathy and multiple organ failure) precluded its use in therapy and directed the interest of clinical cancer research in other directions.

New interest arose two decades ago, when it was demonstrated that the phenomenon of tumour necrosis was related to a transferrable factor (O'Malley et al, 1962) in the serum of animals in endotoxic shock. Later, it was reported (Carswell et al, 1975) that BCG priming of the mouse before LPS stimulation led to a higher yield of transferrable anti-tumour activity. Tumour necrosis in the recipient animal was dose-dependent and not related to LPS carry-over. This factor was produced by the macrophage population (Matthews, 1978) and tumour necrosis *in vivo* was related to cytotoxicity for certain cell lines *in vitro* (Carswell et al, 1975).

The identification of a bioassay *in vitro* led to the isolation of human cell lines producing high yields of TNF activity. This allowed biochemical purification of TNF (Aggarwal et al, 1985) and cloning of the human TNF cDNA and genomic sequence (Pennica et al, 1984; Shirai et al, 1985; Wang et al, 1985; Marmenout et al, 1985). The molecular cloning of human TNF led immediately to two important observations:

a.   *"Tumour Necrosis Factor" and "Cachectin" are the same molecule*

As the name implies, "Cachectin" is a molecule isolated in the course of studies on the metabolic changes occurring in animals with chronic infectious diseases and leading to wasting diathesis (Rouzer, Cerami, 1980). Cachexia could be reproduced by injection of LPS in an animal model, and it was related to a transferrable factor, termed "Cachectin", with multiple biological actions including the inhibition of lipoprotein lipase activity (Kawakami, Cerami 1981). This biological activity was the basis for the

21

biochemical purification of the molecule and an N-terminal sequence of cachectin was obtained. This was found to be identical to that of TNF, which had been cloned a few months before (Pennica et al, 1984). Immunological identity (Beutler et al, 1985) and subsequent molecular cloning of cachectin (Caput et al, 1986) confirmed that this and tumour necrosis factor were the same molecule.

b.   *"Tumour Necrosis Factor" and "Lymphotoxin" are different but related polypeptides*

Another cytotoxic molecule, lymphotoxin (LT), had been described as a factor produced by T lymphocytes in the presence of antigen (Ruddle, Waksman, 1967; Granger, Williams, 1968). The relationship between LT and TNF was unclear because of their similar biological activities. The molecular cloning of human LT (Gray et al, 1984) and of TNF (Pennica et al, 1984) demonstrated that the two proteins were separate entities. Regardless of the low degree of homology (28% homology at a.a. level; Nedwin et al, 1985), the two proteins bind and compete for the same receptor (Hass et al, 1985), mediating similar actions in vitro.

Following these observations it was proposed (Shalaby et al, 1985) to adopt the name of TNF alpha for TNF and Cachectin and to use TNF beta to indicate lymphotoxin.

The predominant cell source of TNF alpha is the macrophage (Old, 1985). Other human cell types reported to synthesize TNF alpha include T cells (Cuturi et al, 1987) and NK cells (Koyabashi et al, 1986), as well as B lymphoblasts (Williamson et al, 1983), basophils (Jadus et al, 1986) and mast-cells (Steffen et al, 1987).

TNF production by these cells is activated by recognition of microbial components, either by direct interaction or possibly *via* an antigen mediated mechanism. TNF alpha seems to be implicated in some aspects of cell-mediated immune responses: TNF alpha production occurs during the early phase (4hr) of the MLR reaction (Ranges et al, 1987), treatment with anti-TNF alpha antibody dramatically modifies GVH disease (Piguet et al, 1987) and the presence of TNF alpha is thought to be related to several features of the Schwartzmann reaction (Palladino et al, 1987).

22

Activation of T cells with PHA or Con A within a mononuclear cell population leads to production of TNF alpha (Debets et al, 1987) probably due to action of T cell products on monocytes, as separated populations do not produce TNF in these conditions (Cuturi et al, 1987). In fact, IL-2 has been shown to induce TNF (Numerof et al, 1987; Nedwin et al, 1985), and IFN gamma synergizes with minute quantities of LPS in TNF induction (Gifford, Lohmann-Matthes, 1987). Both lymphokines up-regulate TNF receptor in target cells (Scheurich et al, 1987) and IFN gamma enhances TNF alpha translation in murine macrophages (Beutler et al, 1986). Colony stimulating factors have also been reported to induce TNF production (Gao et al, 1987; Warren, Ralph 1986) and TNF itself is able to induce further TNF synthesis (Old, 1987). Another potentially relevant amplification circuit could be the induction of IL1 (Dinarello et al, 1986) which has also been shown to induce TNF activity (Philip, Epstein 1986).

The human TNF alpha gene is located on chromosome 6, within the MHC region, between HLA DR and HLA-A (Spies et al, 1986) and 1.1Kb upstream from the TNF beta gene. It is composed of 4 exons and 3 introns (3.3Kb). In the 5' untranslated region there is significant homology with TNF beta and, in common with other cytokines, the 3' untranslated region contains overlapping repeats of an octameric sequence thought to be related to mRNA instability (Shaw, Kamen, 1986). This duplication of functions and tandem arrangement in the genome of different proteins able to compete for the same receptor is not unique to TNF alpha and TNF beta within cytokines, as human IFN alpha and beta (chromosome 9) and murine IL1 alpha and IL1 beta (chromosome 2), also share these characteristics.

Translation of the TNF alpha mRNA produces a 233 a.a. polypeptide containing a highly hydrophobic leader sequence of 26aa. The first 76 a.a. of the N-terminus are cleaved during secretion, leaving 8 smaller fragments and the mature TNF alpha peptide (157 aa). The calculated MW of the mature peptide (17.3KDa) is in agreement with SDS-PAGE analysis of human purified protein (Aggarwal et al, 1985). TNF alpha does not appear to be glycosylated and aggregates to form biologically active oligomers.

Mature TNF alpha protein can represent up to 1% of total secreted protein in activated macrophages (Beutler et al, 1985C) and concentrations of up to 0.3uM are obtainable in the blood of endotoxaemic mice (Abe et al, 1985). When injected (Beutler et al, 1985D), the half life of TNF alpha in

plasma was calculated as about 6 minutes, with the protein accumulating in organs such as liver, kidneys, lungs and GI tract.

The TNF alpha receptor binds both TNF alpha and TNF beta with equal affinity (Hass et al, 1985). The receptor is composed of at least two chains of 75 KDa and 95KDa (Nedwin et al, 1985) and is internalized with the ligand (Tsujimoto et al, 1985). The TNF receptor can be expressed at relatively high levels, especially in the presence of IFN gamma (1000-10000 molecules/cells) (reviewed by Aggarwal et al, 1987).

By interaction with its receptor, TNF alpha exhibits a considerable range of biological activities, summarized in Tables 1.11 and 1.12. Of the many actions of TNF, its antitumoural activity *in vitro* and *in vivo* has received the greatest attention. After experimentation in animals, recombinant human TNF alpha is now being tested in phase I clinical trials.

A group of biological actions of TNF, on the basis of which "Cachectin" had been isolated, are those related to endotoxin shock and wasting diathesis (cachexia). The observation that lipaemia in cachectic animals with chronic parasitic infections (Rouzer, Cerami, 1980) was reproducible by LPS injection in mice led to the identification of a factor that reduced lipoprotein-lipase activity (Kawakami, Cerami, 1981). Later experiments demonstrated that wasting was related to the anorectic actions of TNF (Cerami et al, 1985) and to inhibition by TNF of the expression of enzymes involved in fat and glucose metabolism (Torti et al, 1985; Min, Spiegelmann, 1986) leading to transient hyperglycaemia and terminal hypoglycaemia (Lee et al, 1987). An elegant study (Oliff et al, 1987) demonstrated that TNF alpha could produce *in vivo* a severe wasting diathesis, reminiscent of cancer cachexia, if the exposure was at low but constant dosage. On the contrary, high doses of TNF alpha injected i.v. in rats (Tracey et al, 1986), produced a lethal series of systemic manifestations, indistinguishable from those of endotoxic shock (reviewed by Beutler and Cerami, 1987). The hypothesis of a primary role for TNF alpha in endotoxic shock was supported by experiments in mice (Beutler et al, 1985) and primates (Tracey et al, 1987) in which passive immunization with anti TNF alpha antibodies prevented death from a lethal dose of LPS. Anti TNF alpha monoclonal antibodies are currently being tested in human phase I

24

trials.   Both the general anti-tumoural activities of TNF and its role in the body's response to microbial invasion point to a role of TNF in maintenance of homeostasis.

It would be predictable that a factor with a first-line role in host defence should be able to elicit two of its most basic mechanisms, i.e. fever and inflammation.   With the availability of recombinant reagents, a role for TNF alpha in inflammation has been established.   TNF alpha administration *in vivo* induces fever (Dinarello et al, 1986), acute phase response (Mortensen et al, 1987), neutrophilia (Ulich et al, 1987) and potentiation of the immune response (Ghiara et al, 1987).   Other actions potentially important in chronic inflammation include induction of MHC class I and class II molecules, angiogenesis and induction of adhesion molecules such as ICAM 1. TNF also induces the proinflammatory and catabolic (MCF-like) activities of IL1 on musculo-skeletal tissues, such as induction of PGE_2 and collagenase release from mesenchymal cells and bone and cartilage resorption (see Tables 1.11 and 1.12).

TNF alpha also interacts more widely in the cellular cytokine network: it has been shown to induce directly IL1, GM-CSF, IL6, G-CSF and M-CSF and to be induced by itself, IL1, M-CSF, GM-CSF and IL2.

The striking similarities between TNF and IL1 have recently been reviewed (Dinarello, 1986; Dinarello, 1987; Le, Vilcek, 1987).   The mechanism by which structurally dissimilar molecules can achieve very similar actions is unknown and further studies are needed to elucidate why such redundancies should have been maintained through evolution.

TABLE 1.11   Some biological effects of TNF alpha IN VITRO

1. IMMUNE SYSTEM

        T Cells (previously activated)
            enhancement of proliferation   (Yokota et al, 1988)
            enhancement of HLA DR, IL2 receptor
                IFN gamma production   (Scheurich et al, 1987)

        B Cells (previously activated)
            enhancement of proliferation and ab
            production in presence of IL2 or
                anti IgM   (Kashiwa et al, 1987, Jelinek, Lipsky, 1987)

    Monocytes
        induction of IL1   (Dinarello et al, 1986; Nawroth et al, 1986)
        induction of GM-CSF   (Munker et al, 1986)
        inducer and mediator of
            macrophage cytotoxicity   (Philip, Epstein, 1986; Feinman et al, 1987)
        induction of TNF   (Philip, Epstein, 1986)

    Neutrophils
        enhancement of O_2-production   (Berkow et al, 1987; Tsujimoto et al, 1986)
        phagocytosis, degranulation,   (Shalaby et al, 1985; Klebanoff et al, 1986;
            increased adhesiveness   Gamble et al, 1986)
        augment. cytotox. for Candida A   (Djeu et al, 1986)

    Eosinophils
        enhanced cytotoxicity to parasites   (Silberstein, David, 1986)

    Fibroblasts
        induction of IL6   (Kohase et al, 1986)
        increased class I expression   (Collins et al, 1986)
        inhibits fibroblast accessory function   (Le, Vilcek, 1987)
        induction of IL1   (Le et al, 1987)

    Endothelial cells
        increased class I expression   (Collins et al, 1986)
        induction of GM-CSF   (Munker et al, 1986; Seelentag et al, 1987)
                    G-CSF   (Seelentag et al, 1987)
                    M-CSF   (Seelentag et al, 1987)
                    IL1   (Libby et al, 1986; Nawroth et al, 1986)
                    ICAM 1   (Pober et al, 1986)

    Other actions in vitro
        augmentation of MHC class 2
            on islet cells   (Pujol-Borrel et al, 1987)
        inhibition of haematopoiesis in
            vitro (decreased generation of
            GM-CSFU; E-BFU; GEMM-CFU)   (Degliantoni et al, 1985; Broxmeier et al, 1986)
        terminal differentiation of
            myeloid lines   (Takeda et al, 1986; Trinchieri et al, 1987)
        antiviral   (Wong, Goeddel, 1986; Mestan et al, 1986)
        antibacterial   (Paraut et al, 1980)
        generation of specific
            antitumor immunity   (Palladino et al, 1987)

2. CENTRAL NERVOUS SYSTEM

        astrocyte proliferation   (Lachman et al, 1987)
        hypothalamic cell PGE_2 production   (Blatteis et al, 1987)
        enhancement of MHC class II
            expression of glioma cells   (Zuber et al, 1987)

26

TABLE 1.11 (Cont.)

3. LIVER

acute phase proteins in human hepatoma lines	(Darlington et al, 1986; Perlmutter et al, 1986)
inhibition adipocyte gene expression:	
lipoproteinlipase	(Torti et al, 1985; Kawakami et al, 1982)
glycerophosphate-dehydrogenase	(Torti et al, 1985; Min, Spiegelmann, 1986)
adipsin	(Min, Spiegelmann, 1986)
increased glycogenolysis (augm. lactate and fructose biphosphate, decreased glycogen storage)	(Lee et al, 1987)

4. VASCULAR TISSUES

Endothelial cells

increased adherence to PMN	(Gamble et al, 1985)
increased ICAM 1 expression	(Pober et al, 1986)
increased procoagulant activity and decrease of thrombomodulin	(Bevilacqua et al, 1986; Stein and Nawroth, 1986)
endothelial cells rearrangement	(Stolpen et al, 1986)
angiogenesis	(Leibovich et al, 1987)

5. MUSCULO-SKELETAL TISSUES

Bone

bone resorption and inhibition of bone formation	(Bertolini et al, 1986; Stashenko et al, 1987)
induction of OAF activity from osteoblasts	(Thomson et al, 1987)

Cartilage

cartilage resorption and inhibition of PG synthesis	(Saklatvala, 1986)

Fibroblasts

proliferation	(Vilcek et al, 1986)
collagenase, PGE_2 production	(Dayer et al, 1985)
synergy with IL1	(Elias et al, 1987)

Synoviocytes

Plasminogen activator	(Mochan, Armor, 1987)
Collagenase, PGE_2 production	(Dayer et al, 1985)

Muscle

Reduction of resting transmembrane potential difference	(Tracey et al. 1986)

6. CYTOSTATIC-CYTOTOXIC EFFECTS

several tumoral lines	(Helson et al, 1975; Old, 1985; Ruggero, Baglioni, 1987)
inhibition of c-myc expression (HL60, HeLa cell lines)	(Kronke et al, 1987; Yarden, Kimchi, 1986)

TABLE   1.12   Some effects of TNF alpha IN VIVO

necrosis of transplantable tumours
    in mice   (O'Malley et al, 1962; Carswell et al, 1975)

endotoxic shock-like syndrome:
    piloerection, diarrhoea,
    hypotension, tachipnoea,
    metabolic acidosis, final hypo-
    glycaemia, hyperkaliaemia, death,
    diffuse visceral necrotic lesions   (Tracey et al, 1986)

fever and induction of IL1   (Dinarello et al, 1986)

systemic suppression of
    lipoproteinlipase activity   (Kawakami, Cerami, 1981)

tumoral cachexia   (Oliff et al, 1987)

anorexia   (Cerami et al, 1985)

anaemia   (Wei et al, 1987)

neutropenia, followed by
    neutrophilia and lymphopenia   (Ulich et al, 1987)

antiparasitic actions   (Reviewed by Playfair, 1987)

enhancement of in vivo response to
    T cell dependent antigens   (Ghiara et al, 1987)

induction of circulating CSF   (Vogel et al, 1987)

acute phase proteins   (Mortensen et al, 1987)

slow wave sleep   (Kreuger et al, 1987)

hypotension   (Okusawa et al, 1987)

decreased p450 cytochrome activity   (Ghezzi et al, 1987)

1.d. RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a chronic condition affecting 2-3% of the population, with prevalence and clinical manifestations being slightly variable in different countries. It is a disease with major social consequences because it predominantly affects adults during their working life. The natural history of the disease is variable, but it is characterised by remissions and exacerbations with progressive joint deformity.

RA is a sterile inflammation of both small and large joints, often involved symmetrically. In addition, extra-articular manifestations such as rheumatoid nodules, vasculitis, neuropathy, scleritis, pericarditis, lymphadenopathy and splenomegaly may occur. The articular features of classical RA include pain and stiffness that improve with motion, joint swelling due to synovial proliferation and production of inflammatory exudate (synovial fluid) within the joint cavity. The disease can have an insidious or acute onset with an unpredictable course and prognosis. Some patients have an unrelenting progression to deformities: more typically episodes of acute arthritis are interspaced with periods of low activity or occasional complete remission.

In the majority of patients, cartilage and bone involvement becomes evident at radiographic examination as narrowing of the joint space (usually occupied by radio-translucent cartilage) and subchondral osteopaenia which is frequently followed by the appearance of bone erosions. The progressive damage of the articular structures (bone, cartilage, tendons and ligaments) by this inflammatory process, in conjunction with mechanical forces, frequently lead to characteristic deformities of RA. These result in varying loss of functional capacity.

The histopathology of RA in the joint consists of an initial hyperaemic swelling of the normally oligocellular synovial membrane, followed by hyperplasia and hypertrophy of the lining cells, extensive proliferation of small blood vessels together with massive infiltration by mononuclear cells. Many of these cells (mainly T-lymphocytes) are aggregated into follicles, particularly around small blood vessels. Transitional areas consist of a mixture of macrophages , B and T lymphocytes, plasma cells and fibroblasts in the connective tissue stroma.

This hypercellular tissue forms villous projections and is known as "pannus". This is able to invade adjacent tissues and leads to the destruction of articular cartilage, ligaments, bone and tendons. The synovial exudate, that frequently accumulates in the joint space, mainly contains polymorphonuclear cells, which are usually sparse in the hyperplastic synovial tissue.

Symptomatic treatment relies on the use of analgesic/anti-inflammatory drugs (NSAIDs). If the disease remains active and particularly if progressive joint deformity is occurring, "disease modifying drugs" are often introduced. These drugs, which include anti-malarials, gold salts, d-penicillamine and various immunosuppressive agents may well affect the course of the disease and occasionally induce clinical remission. Corticosteroids (local and systemic) are also used to suppress inflammation.

Although these various treatments often improve the symptoms of RA, they do not offer a cure. This possibility seems more likely to follow an understanding of the aetiology and pathogenesis of the disease.

Several types of association of arthritis with infections are well recognized (viral/bacterial arthritis, Lyme disease, rheumatic fever, Reiter's syndrome). Despite considerable experimental effort (reviewed by Saag, Bennett, 1987; Phillips, 1988) no clear association with infectious agents has been shown in RA.

The expression of MHC class II products on virtually all cells present in the synovial infiltrate (T, B cells; macrophages, dendritic cells), the hypergammaglobulinaemia often present and the dramatic response to "immunosuppressive" drugs such as steroids, suggest that immunopathogenic mechanisms operate in RA. A clear manifestation of autoimmunity is the presence of "Rheumatoid Factor", an autoantibody (usually IgM/anti-IgG) that occurs in a majority of patients ("seropositive RA"). Ig immune complexes are, in theory, able to activate a cascade of immune reactions, such as the complement pathway, and induce cytokine production (IL1 included). There is no strong evidence, however for a major pathogenetic role of rheumatoid factors and their occurrence is mainly of diagnostic value.

An association between RA and HLA-DR4 was recognized from epidemiological studies. Seventy per cent of UK patients with RA have this

haplotype and an individual with HLA-DR4 is about 4 times more likely to develop the disease. The association between class II molecules and immunity is related to the MHC restriction of antigen presentation. It is noteworthy that within the MHC regions (human chromosome 6) there are complement (C2, C4, factor B) and TNF beta genes, with the TNF alpha gene only 1100 nucleotides upstream.

This association with HLA-DR is at the moment being extensively studied in the hope that the genetic structure might elucidate possible pathogenic mechanisms. However, the observation that of individuals bearing HLA DR4 only 5% actually develop RA suggests that a DR4-related mechanisms might be one of many cofactors that can influence, but probably not determine, the initiation of the disease (reviewed by de Vries, 1988).

Of particular interest in the pathogenesis of RA has been the potential role of cytokines and IL1 in particular. The pro-inflammatory, catabolic and systemic actions of IL1 seem very relevant to the pathology of RA. At the time work for this thesis was commenced, the first reports of IL1 in synovial fluids, showing presence of such mediator at the site of the rheumatoid lesion, were being published.

1.e. GOUT

This is a metabolic disease, with predominantly articular manifestations, associated with the presence of high levels of uric acid in the blood. In humans, uric acid is the major end product of purine catabolism and is mainly excreted in the urine.

The elevation of uric acid levels can occur as a consequence of overproduction or underexrection of urate or a combination of both. Overproduction may be secondary to an increased turnover of nucleic acids as occurs in a number of diseases such as myeloproliferative and lymphoproliferative disorders, multiple myeloma and pernicious anaemia. Rarely, the overproduction occurs as a consequence of an X-linked inherited enzyme deficiency (hypoxanthine-guanine phorphoribosyl transferase [HG-PRT]) of the purine metabolic pathway or of a similarly inherited hyperactivity (phosphoribosyl pyrophosphate synthetase [PRPPs]). More commonly, the molecular defects resulting in overproduction are undefined, but are probably polygenic. A defect in the renal excretion of uric acid can be demonstrated in a high percentage of gouty patients but the precise renal abnormality in the handling of urate is often undefined and it is, again, believed to be of polygenic inheritance. Other conditions which can interfere with renal excretion of uric acid include therapy with most diuretic agents or low dose aspirin and renal insufficiency itself.

Gouty arthritis typically begins with an acute monoarthritis in the lower limbs, often the first metatarsophalangeal joint. Unlike most other types of arthritis the onset of joint inflammation is characteristically very abrupt, often occurring at night with the involved joint being swollen, painful and tender. The attacks can be polyarticular and occasionally joints may become involved successively over a period of hours.

The gouty attack is usually accompanied by fever, neutrophilia and elevated acute phase proteins. Compensated polarized light microscopy of synovial fluid taken from an involved joint will reveal characteristic needle-shaped negatively birefringent crystals of monosodium urate monohydrate.

These acute attacks of arthritis will resolve spontaneously over several days or can be effectively controlled by treatment with NSAIDs or colchicine. Between attacks the patient frequently remains asymptomatic, usually maintaining elevated serum uric acid levels. Some individuals have no more than a few attacks in a lifetime: when they are frequent, however, and no exacerbating factors can be removed (e.g. cessation of diuretic therapy), therapy is commenced with uricosuric agents or by using allopurinol which inhibits the production of uric acid. In absence of treatment, paroxysms of arthritis can recur with increasing frequency and are associated with gradual degeneration of involved joints: loss of articular cartilage, erosion of subchondral bone and infiltration of periarticular structures by deposition of crystals. In the most severe cases these deposits stimulate a fibrotic reaction and become clinically or radiographically detectable ("tophaceous gout").

The acute attack of gouty arthritis is thought to be an inflammatory response triggered by microcrystals of monosodium urate formed within the joint as a consequence of hyperuricaemia or by shedding from pre-existing intra-articular urate deposits.

Experimental intra-articular injection of MSU crystals in human volunteers produced an inflammatory response very similar to gout (Seegmiller et al, 1962). However, not all of the volunteers injected developed the attack, suggesting that the presence of the crystals themselves is a necessary but not sufficient stimulus for the inflammatory sequelae.

It has been proposed that a direct effect of MSU crystals on polymorphonuclear cells, induces release of pro-inflammatory enzymes and chemotactic molecules in relation to phagocytosis (Phelps, 1969) or to the membranolytic effects of these crystals (Wallingford, McCarty, 1971). Other reported effects of MSU crystals, independent of phagocytosis or cell-lysis include the induction of PGE_2 and collagenase from fibroblast and monocytes (McMillan et al, 1981; Hasselbacher et al, 1981) providing perhaps the grounds for local inflammation but not explaining the systemic disturbance observed in a patient with acute gout (fever, acute phase proteins, neutrophilia). In a classical experiment (Phelps, McCarty, 1966) it was proved that the presence of leukocytes was needed for gouty inflammation, as leukopenic animals when challenged with intra-articular injection of MSU

crystals, only developed a full attack after leukocytic reconstitution.

On theoretical grounds, a more satisfactory explanation for the initiating mechanism of acute gouty arthritis would invoke the participation of a *soluble mediator* (to explain the systemic involvement) of *leukocyte origin*, *released in presence of MSU crystals*, with *powerful inflammatory consequences*. These considerations identify pro-inflammatory cytokines as potential candidates for this role.

1.f. OTHER CRYSTAL-RELATED ARTHRITIDES

Pseudogout

The deposition of crystals of calcium pyrophosphate dihydrate (CPPD) is a very common x-ray observation (visible as articular cartilage calcification) but these crystals only rarely cause arthritis. In those patients that develop articular symptoms, the clinical pattern is varied. An acute arthritis resembling an attack of gout is the commonest form and is known as "pseudogout". This is usually a monoarthritis, especially affecting the knee but more than one joint can be involved. Although to locate the crystals may be difficult, they differ from MSU crystals because of their irregular or rhomboid shape and positive birefringence.

A small percentage of patients have multiple joint involvement with low grade inflammation, often mistaken for subacute RA.

Hydroxyapatite arthropathy

Hydroxyapatite (HA) is the crystal configuration in which calcium is found in the human body, both in bones and in the majority of dystrophic calcifications. Its commonest rheumatological association is in clinical syndromes of calcific periarthritis, particularly affecting the subacromial rotator cuff tendons of the shoulder.

Aggregates of apatite crystals (visible by electron microscopy) have been found in a high percentage of osteoarthritis effusions, but HA crystal deposition seems to be more clearly associated with an uncommon syndrome named "Milwaukee Shoulder". This is a dramatic form of glenohumeral osteoarthritis with bilateral rotator cuff defects (McCarty et al, 1981) and high levels of collagenase and neutral protease in the synovial exudate, where clumps of HA crystals can be found. In contrast to acute gouty arthritis or RA, however, this synovial fluid is relatively acellular and the synovial histology does not show any mononuclear cell infiltrate.

35

1.g. Aims of the Study

At the start of this work (November 1984), there was only circumstantial evidence for a pathogenic role of soluble mediators in human arthritis.

Because of the potential role of IL1 in the genesis of chronic inflammation and including the recently characterized TNF alpha (with its similar pro-inflammatory activities) it was decided to investigate the production of these cytokines. The experiments included initially the induction of their release from human monocytes following different stimulation and, in a second phase, the study of their gene expression and cellular distribution.

The stimuli used in these studies included a variety of microbial products as well as different crystals associated with human arthritis such as Monosodium Urate, Hydroxyapatite and Calcium Pyrophosphate Dihydrate. Monosodium Urate crystal deposition is clearly associated with the acute attack of gout, but the events linking crystal precipitation and acute inflammation are unclear. The inflammatory nature of acute gouty arthritis and the systemic features of the disease seemed to be compatible with a local overproduction of IL1/TNF following stimulation by MSU crystals.

In parallel studies, the presence of monocytic cytokines was tested at the site of inflammation, i.e. in synovial fluids from arthritic patients. This had not previously been done for tumour necrosis factor and only in limited, non-quantitative studies for IL1.

2. SECTION I :

Induction, synthesis and release of IL1 and TNF in human
monocytes in vitro

2.a. INTRODUCTION

The response of the host to microbial invasion involves several pathophysiological mechanisms, including fever, release of acute phase reactants from the liver, augmentation of circulating phagocytes, and considerable changes in the functions of cell-mediated immunity. All of these mechanisms ("acute phase response") are targeted to the removal of the causative agent (Dinarello, 1984) and eventual activation of repair mechanisms with tissue remodelling.

A variety of experimental evidence suggests that IL1 and TNF can play a central role in mediating such responses through their wide range of biological activities and with the cascade of soluble mediators triggered by their actions on responsive cells population. The direct recognition of microbial constituents is sufficient to induce IL1 and TNF from monocytes as an early event in "innate immunity".

In parallel, IL1 production following antigen presentation potentiates several aspects of the "adaptive" immune response.

Both of these host defence responses rely on recognition of the microbial product to trigger a tightly regulated chain of events leading to re-instatement of homeostasis. The work presented in this section studies the induction, synthesis and release of IL1 and TNF in human monocytes *in vitro*. This includes measurement of kinetics of mRNA accumulation, immunoreactive and bioactive protein production and extracellular translocation. In keeping with a putative role of IL1 and TNF in the host response to microbial invasion, the stimuli tested included products of bacterial and fungal origin, such as *Staph. Aureus* and *Sacch. Cerevisiae* cell walls, *E. coli* endotoxin and Toxic Shock Syndrome *Staph. Aureus* exotoxin.

To test the possibility that IL1 and TNF production could be induced by non-microbial, non-antigenic, inflammatory particles, we tested the inducing properties of three sterile, LPS-free synthetic crystal types associated with human pathophysiology, i.e. MSU, HA and CPPD.

38

2.b.   MATERIALS AND METHODS

2.b.1. CELL CULTURE

2.b.1.1. Media

RPMI 1640 medium was bought as a sterile 10X liquid (Gibco Ltd, Paisley, Scotland). Concentrated medium was reconstituted in sterile conditions with LPS-free distilled water (Travenol) and buffered with sodium bicarbonate (2.2g/l; Gibco). After addition of penicillin (100u/ml), streptomycin (100ug/ml) and Glutamine (2mM), pH was adjusted to 7.4 with NaOH 6M (Sigma, Dorset, UK).

Foetal calf serum (FCS) (Gibco) was used at 5% unless otherwise stated. Prior to purchase, samples of reserved batches were tested for LPS content (Limulus Amebocyte Lysate- LAL test), for proliferation experiments (murine fibroblasts and murine T cells) and murine fibroblast cytotoxicity. Heat inactivation of FCS was carried out at 56° C, for 1 hour.

MEM (Eagle's Minimal Essential Medium; Sigma) was obtained as powdered medium and reconstituted in sterile water, with 2.2g/l sodium bicarbonate. The fluid was filter sterilized (Millipore, 0.22um sterile filters). Antibiotics and glutamine were added as described for RPMI medium, and the pH adjusted to 7.4 with NaOH.

2.b.1.2. Cell lines

Non-adherent cells not requiring defined exogenous growth factors included the murine thymoma cells EL4.NOB.1 and human myelocytic leukemia U937 cells. These were routinely cultured in RPMI, 1-5% FCS and divided every 3-4 days according to their doubling times. EL4.NOB.1 was cultured at low density ($<1 \times 10^6$/ml) in order to maintain responsiveness to IL1.

Non-adherent cells with special requirements for cell growth such as the murine antigen-specific T-helper clone D10.G4.1 and the cytotoxic T lymphocyte lines (CTL's), were cultured at low density and split every 2-4 days, when medium (RPMI 1640, 10% FCS) was replaced and 3-10% crude rat TCGF preparation added. D10.G4.1 also required mitomycin-treated splenic antigen presenting cells (H-2K mice) once every 7-10 days, and specific antigen (Hen's Conalbumin) every 3-4 days. Protocols for the generation of crude rat TCGF and feeder cells for the long term culture of D10.G4.1 are presented in Appendix (p 209).

Adherent cells used included a number of lines of human synovial fibroblast origin and L929 cells (murine nipple fibrosarcoma fibroblasts), grown in RPMI and MEM respectively, 10% FCS. The human synovial fibroblasts were raised by culturing synovial fluid cells from patients with rheumatoid arthritis in 10% FCS RPMI. After 2 weeks culture, all the cells were of fibroblast morphology and the cultures were maintained with the addition of 0.01% of a highly purified IL1 peptide ("22KDa factor" from J. Van Damme, Univ. of Leuven, Belgium) in fresh medium once weekly. These lines grew continuously to a maximum of 22 weeks.

Adherent cells were grown to a subconfluent status, when the spent medium was removed, the cell monolayer washed with saline and cells loosened by Trypsin/EDTA (Gibco) treatment. Cells were removed with vigorous pipetting in 10% FCS medium, and used for experiments or subculture.

2.b.1.3.   Preparation of cells for induction experiments

Peripheral blood from normal volunteers was taken into preservative-free heparin (20u/ml, Unihep-Leo Labs, UK) and mononuclear cells obtained by density centrifugation (Lymphoprep, pyrogen/tested, 1077 density, Nyegaard, Oslo). Mononuclear cells were washed twice in serum-free RPMI and allowed to adhere to multiwell plates (24 well plates, Linbro-Flow Labs, UK; Nunc, Denmark) or petri-dishes (4cm diameter, Nunc, Denmark). After 40 mins adhesion at 37° C, 5% CO_2, non-adherent cells were harvested and by comparison of differential and total cell counts before and after adherence the volume of medium to be added to the adherent cells to obtain 1×10^6 monocytes/ml was estimated. In a few experiments, U937 human monocytic leukemia cells were used, harvesting them at the third day of subculture.

Following incubation of the cells at 37° C 5% CO_2 in the presence or absence of stimuli for various lengths of time, supernatants were clarified by centrifugation at 1800g for 15mins and stored at -70° C. Supernatants represent the "extracellular" fraction. In some experiments, a volume of fresh medium equal to the supernatant was used to wash the centrifuge tube and recover cells that had become non-adherent. This was returned into the relevant well containing the adherent population. After 3 cycles of freeze-thawing the whole preparation will represent the "cell-associated" fraction. In time course experiments, cells were prepared in petri-dishes and samples individually harvested at the different time points.

The protocol used to study the kinetics of cytokine cellular distribution is described in more detail elsewhere (2.c.2.6., pp 71-72).

Adherent synovial exudate mononuclear cells were also used in induction experiments. These were obtained from fresh synovial fluid from patients with rheumatoid arthritis, treated with hyaluronidase (Sigma, bovine testicular hyaluronidase, 150U/ml) for 45 mins at 37° C. Cells were washed twice in serum-free medium before density centrifugation and selection by plastic adherence.

2.b.2.   STIMULI

Stimuli tested in this section for their ability to induce IL1 or TNF production in human mononuclear cells, included:

Lipopolysaccharide (LPS)

Lipopolysaccharide (E. coli 0127:B8 strain, TCA extracted, Sigma) was resuspended at 1mg/ml in saline and stored at 4° C until used.

Heat killed Staph. Aureus cells walls (HKS)

These were kindly donated by Dr E. Atkins (Dept. Internal Medicine, Yale University, New Haven, CT) and prepared in his laboratory according to the method described by Morse (1962). Autoclaved, acetone-dried bacteria (Staph. Aureus, NYH-6, 80/81 strain) were disrupted in a Mickle disintegrator. Cell walls were prepared by centrifugation, treated with RNase, repeatedly washed in LPS-free saline, and resuspended at 500ug = $4x10^9$ cell walls/ml saline.

Toxic shock syndrome exotoxin (TSS)

This was a gift from Dr D. Shapiro (Dept. Pathological Biochemistry, WGH, Glasgow), who also tested for the presence of contaminating LPS (<20pg/ml by LAL assay). Bacterial broth of a Staph. Aureus isolate from a patient with Toxic Shock Syndrome was concentrated x5 by YM-10 ultrafiltration (Amicon, UK) and stored at -20° C until used. Staph. Aureus strains from patients in Toxic Shock Syndrome produce a 20-30KDa exotoxin, which has been described as one of the most powerful inducers of IL1 synthesis (Ikejima et al, 1984).

Saccharomyces Cerevisiae cell walls (Zymosan A)

Fungal cell wall extracts were purchased from Sigma (Zymosan A, Cat. No. Z4250). This is prepared by trypsin digestion of Saccharomyces Cerevisiae and purified by solvent extraction and acid-washing.

Proinflammatory crystals

Crystals of **monosodium urate** (MSU), **hydroxyapatite** (HA) and **calcium pyrophosphate dihydrate** (CPPD) were kindly given by Dr S.E. Malawista (Dept. of Medicine, Yale University, New Haven, CT) and prepared in his laboratory according to published methods (Malawista et al, 1985). Additional crystals of each type were prepared and donated by Dr Paul Dieppe (Dept. of Rheumatology, Bristol University, UK). Crystals of MSU (8-40um), HA (aggregates of 3-15um), CPPD (15-50um) were of comparable size, as assessed under incident-light microscopy (by courtesy of Dr Webb, Department of Pathology, Western General Hospital, Edinburgh). Crystal batches were tested for LPS content by L.A.L. assay, mixed, resuspended at 10mg/ml in LPS-free PBS and stored in 500uls aliquots at -30° C until used.

Before use, some crystal aliquots were pre-treated (1hr, 37° C, shaking incubation) with heat-inactivated fresh human serum (10mg crystals/1ml serum). After two rapid washes in PBS, these were used for induction experiments.

Crystals were shown to be LPS-free by three different approaches:

(i)   *Limulus gelation test*

This test is based on the clotting of a lysate from amebocytes of limulus polyphemus (E-TOXATE L.A.L. test, Sigma) in the presence of LPS (details in Appendix, pp 209). Crystals assayed on four different occasions contained less than 25pg LPS/mg crystals. PBS in which the crystals had been suspended at 10mg/ml also scored below the detection limit, indicating that soluble factors from 1mg of crystals contained less than 2.5pg/ml LPS. When tested for the presence of inhibitors of the gelation test, 0.5mg/ml of crystals failed to inhibit L.A.L. gelation by 250pg/ml LPS.

(ii)   *Polymyxin B experiments*

Polymyxin B is an anionic molecule which strongly binds LPS and inhibits its biological activities (Duff, Atkins, 1982A). When tested, 250u/ml PMB failed to inhibit MSU-induced IL1 release (crystals at

43

0.4mg/ml). In the same experiment 250U/ml PMB was able to inhibit the activity of 100ng/ml LPS. This experiment (shown in Appendix, p. 210) strongly suggests that MSU activity is not related to the presence of contaminating LPS.

(iii)   *Soluble activators of IL1 production*

One of the most sensitive tests for the presence of LPS is the induction of IL1 from monocytes (Duff, Atkins 1982B). Crystals of MSU were suspended at 10mg/ml in PBS, thoroughly mixed for 15 mins, and supernatants were tested for IL1 induction. Failure to induce detectable IL1 induction from $5x10^5$ adherent mononuclear cells (experiment shown in Appendix, p. 210), indicates that no soluble activators of IL1 production (including LPS) were released from the crystal surface.

2.b.3.   MEASUREMENTS OF INTERLEUKIN 1

2.b.3.1   Murine thymocyte assay

This assay is based on the co-mitogenic activity of IL-1 on murine thymocytes in the presence of suboptimal doses of lectins (Gery et al, 1972).

The murine strain C3H/HeJ is 100-1000 times less sensitive to LPS effects than other H-2K mice, and use of this strain minimizes the influence of contaminating LPS in the assay. We used C3H/HeJ mice from our own breeding colony (initial stock from Harlan Olac, Oxon, UK). In some experiments, we have however successfully used CBA/Ca mice.

To obtain thymocytes, 6 to 8 week old mice were killed by cervical dislocation. The thymuses were removed under aseptic conditions, gently washed in a petri-dish containing serum-free RPMI, and carefully dissociated between 2 sterile ground glass slides. Dispersed cells in the media (about 10^8/thymus) were gently aspirated, washed and the pellet resuspended in serum-free RPMI.

Concanavalin A, at a suboptimal concentration (suboptimal activity varies between batches: usually 0.5-2.5ug/ml) was dispensed 50ul/well in a microtitre plate. One triplicate received 100ul of medium only ("cell control").

44

A standard preparation of IL-1 or samples were added (50ul/well) in triplicate and in dilutions covering a range of 10-100 fold, to ensure that comparisons between samples could be made in an appropriate part of the dose-response curve. One triplicate received medium only ("cells + Con A" triplicate).

Thymocytes freshly prepared as previously described were gently spun, resuspended in fresh RPMI 10% FCS and 100ul aliquots ($1x10^6$ cells) were dispensed into the wells. Following 65-70 hours culture (5%CO_2, 36.5° C, 95% humidity) wells were pulsed with 20ul/1uCi/well of ^{3}H thymidine (TRK296, Amersham, Bucks, UK; specific activity 95-105mCi/mg). After 4-6 hours, cells were harvested (Ph.D cell harvester, Cambridge Technology Ltd, Cambridge, MA) and automatically deposited onto glass fibre filters. Dried filters were counted by beta scintillation (Filter-count scintillation fluid, Packard; Packard TRI Carb 4000). Using a purpose-made programme, average and SEM of triplicate, row data and histograms were then plotted and/or stored on an IBM PC. Typical data from a C3H/HeJ thymocyte assay is shown in Table 2.1.

It has become evident that the C3H/HeJ thymocyte assay, previously the standard for IL1 biological activity (LAF) measurements, responds to other T cell growth factors (IL2, IL4, IL6). Interleukin 1-inducing cytokines (such as TNF) could, in theory, induce IL1 production from the resident thymic macrophages. The possibility of false positives must be considered, especially when testing mixed cell population supernatant. However, controls can be obtained by specific anti-IL1 antibody neutralization and the absence of TCGF activity (as measured by spleen T-lymphoblast assays) in the supernatants can be easily checked.

2.b.3.2.   D10.G4.1. T cell assay

D10.G4.1. is a murine helper T cell clone, specific for hen conalbumin, from Drs C. Janeway and J. Kaye (Howard Hughes Institute for Immunology, Yale University, New Haven, CT). These non-transformed cells (Kaye J. et al, 1984) will clonally expand in the presence of IL-1 *and* antigenic stimulation (Conalbumin, Concanavalin A or anti-CD3 monoclonal-antibody).

For the assay, cells were used *at least* 8 days after the addition of mitomycin-treated feeder cells and not less than 3 days after rat crude TCGF preparation. At this time, cells are free of feeder cells, and express low levels of IL-2R (Kaye et al, 1984). These produce no IL-2 and are unresponsive to IL-1 unless presented with antigen. Also, D10.G4.1 proliferate only minimally to Con A in the absence of IL-1 or feeder cell, explaining proliferation units in the D10 assay being 10-50 times higher than in a thymocyte assay (Fig. 1). Cells with viability in excess of 60%, were used in the assay, which is very similar to the thymocyte assay with a few exceptions:

1) $2x10^4$ cells/well are used, and the assay medium consists in RPMI 1640 with 5% FCS and 60uM mercaptoethanol.

2) Concanavalin A used at 2.5ug/ml (final conc.)

3) Supernatants tested at higher dilutions due to increased sensitivity of the assay.

4) The cells are much more sensitive than thymocytes to environmental changes in handling and incubation.

The assay, including cell harvesting and expression of results (in Proliferation Units ± SEM of triplicates) is otherways similar to the thymocyte assay. Proliferation Units were defined as the ratio of cpm obtained with cells+lectin+sample and cpm with cells+lectin. Several control experiments were carried out using this line:

D10.G.4.1 proliferation requires presence of both IL1 and lectin

In the experiment shown in Fig. 2, requirements for D10 growth were studied. Optimal D10 cell proliferation was obtained only when Conanavalin A *and* IL1 were present.

Presence of LPS and D10.G4.1 assay of IL-1

When tested, LPS in doses from 10pg/ml to 100ng/ml did not influence detection of IL-1 in D10 assay. Data are shown in Fig. 3.

Presence of human IFN alpha and D10.G4.1. assay for IL-1

Human leukocyte-purified IFN alpha (from Celltech, Slough, Berkshire) was tested in absence or presence of human recombinant IL-1 beta in D10.G4.1 assay. The preparation, in doses from 2 to 2000u/ml did not cause D10.G4.1 cell proliferation and, when tested at 200u/ml in presence of hr IL-1, showed, if any, an anti-mitogenic effect (Fig. 4).

46

Presence of human TNF alpha and D10.G4.1 assay for IL-1

Human recombinant TNF alpha (from Genentech Corp, San Francisco, US) was tested for activity in the D10.G4.1 assay. In a series of experiments, hr TNF alpha in doses from 0.1pg/ml to 1ug/ml failed to induce D10.G4.1 proliferation in absence of IL-1 (data not shown). In Fig. 5, experiments are shown in which human TNF alpha did not synergize with different recombinant or purified IL-1 preparations for D10.G4.1 proliferation.

Anti IL1 antiserum

A rabbit antiserum raised against pI7 IL1 (from Dr C A Dinarello, Tufts University, Boston) was used in neutralizing experiments to confirm the identity of LAF activity as IL1. To check if this antibody was reacting with IL2 or causing nonspecific cytotoxicity, it was tested in IL 2-driven CTL proliferation (Fig. 6) and in D10.G4.1 cell proliferation stimulated by hr IL1 beta or purified IL2 (Fig. 7). Only IL1 driven D10 proliferation was inhibited by this antiserum.

The specific activity of the antiserum was approximately 400 neutralizing units/ml (one neutralizing unit defined as the antibody needed to neutralize LAF activity by 1ng hr IL1 beta) and it was ineffective in neutralizing D10.G4.1 proliferation by IL1 alpha (Fig. 8). In fact, at the highest IL1 alpha concentrations tested, the antiserum (1:400) enhanced D10 proliferation (up to 220% with mrIL1 alpha). The reasons for this were unclear, but it was never observed when IL1 beta was used.

2.b.3.3.   EL4.NOB.1/CTL conversion assay

EL4 is a murine thymoma line from which the subclone EL4.NOB.1 was derived (Gearing et al, 1987). EL4.NOB.1, if cultured at low density, will produce only low levels of IL-2 unless stimulated by IL-1. The IL-2 produced by the cells in response to IL1 can be assayed in a CTL assay, CTL proliferation being proportional to the initial concentration of IL1 to which EL4 cells had been exposed.

Briefly, resting EL4.NOB.1 cells ($2x10^5$/100ul/ml well) were washed and resuspended in fresh medium (RPMI, 5% FCS) in presence of dilutions of the samples, hrIL1 or medium control. After 20hrs incubation (37° C, 5% CO_2), supernatants from EL4 cultures are added to CTL cultures ($4x10^4$/100ul/well).

Proliferation of the CTL cultures (20hrs) was assessed by a colorimetric method according to Mossmann (1983). This allows cell proliferation to be assessed by MicroELISA scanning spectophotometry. In the final stages of the assay, cellular mitochondrial enzymes will cleave the substrate (MTT, a tetrazolium salt) to a dark formazan product, giving a

colorimetric estimate of cell number when plates are scanned at 570-630nm wavelength.

Interleukin 1 biological activity was calculated by comparison of the O.Ds in the wells containing the sample (tested in triplicate, four dilutions) with those of the standard dose-response curve of hr IL1 beta (triplicate wells, 0.01 to 10ng/ml) present in each plate. The results are expressed in units/ml where 1 unit is arbitrarily defined as the amount of IL-1 in the sample giving CTL proliferation comparable to 1 ng/ml hr IL-1 in the standard dose-response curve.

2.b.3.4.   Interleukin 1 immunoassays

Commercially available immunoassays were used to assess specific immunoreactive human IL1 alpha or IL1 beta. Interleukin 1 beta RIA (Cistron Biotech./Laboratory Impex) and interleukin 1 alpha RIA (Amersham, UK) are based on the competition for specific antibodies ("sequential saturation") between ligand in the sample or dilutions of standards and exogenously added, ^{125}I labelled human recombinant ligand. Antibody-bound ligand was precipitated with a polyethylene glycol-second antibody solution. After centrifugation, radioactivity in the precipitates was assessed by gamma counting. Detection limits are 0.25-5ng/ml for the IL1 beta RIA and 0.075-2.5ng/ml for the IL1 alpha RIA. For the IL1 beta ELISA (Cistron Biotech/Laboratory Impex), 96 well microtitre plates are coated with IL1 beta-specific monoclonal antibody. Samples of IL1 beta standards were added and incubated for 2 hours at 37° C. Following extensive washing, a polyclonal rabbit anti-human IL1 beta was added and incubated for a further 2 hours. After a final wash, horseradish peroxidase-labelled goat anti-rabbit IgG is added, followed by enzyme substrate (O-phenylenediamine) to produce a chromogenic reaction. Plates were scanned at 405nm in a microELISA reader (Dynatech MR700) and the concentration of IL1 beta in the samples derived from a standard curve obtained with hr IL1 beta. Detection limits of the assay were 0.020-2.5ng/ml. Samples were tested in triplicate wells in ELISA assays, and as single determinations in RIA assays, unless otherwise stated.

2.b.4.   MEASUREMENTS OF INTERLEUKIN-2
2.b.4.1   Spleen murine lymphoblast assay

This assay is based on the property (TCGF activity) of IL2-containing supernatants to maintain T cell blast growth (Larsson et al, 1980). Whole spleen cell populations were prepared from C3H/HeJ mice, lysing red blood

48

cells by hypotonic shock. After two washes in serum free medium, cells were resuspended at 10^6cells/ml in presence of 2.5ug/ml Concanavalin A (Sigma) in RPMI 1640, 10% FCS. Following 72 hours incubation (37° C, 5% CO_2), cells were washed and 10^4 cells/100ul/well were incubated in a microtitre plate with 100ul of supernatant at various dilutions. Standard rat TCGF was used to standardize the assay. After a further 72 hours incubation, proliferation was assessed by ^{3}H thymidine incorporation (5 hours, 1uCi/well).

This assay is very sensitive but is not specific for IL2, as TCGF activity is shared at least with IL4 and IL6. For these reasons, it is probably the ideal control to exclude the presence of these T cell growth factors in crude supernatants active in the LAF/IL1 biological assay.

2.b.4.2   Cytotoxic T lymphocyte proliferation

This biological assay is based on the ability of IL2 to support the growth of murine cytotoxic T lymphocyte clones, selected by long term culturing and subcloning in IL2 (Gillis et al, 1978). In this laboratory, different clones have been used (CTLL, CTL-D, CTL-1, CTLL-2) from various sources with very similar characteristics.

For the assay, CTL cells were harvested and washed 3 to 4 days after the last addition of TCGF to the cultures: $1x10^3$ to $5x10^3$ healthy CTL cells/100ul/well were cultured in different dilutions of sample, medium or IL-2 (BRMP standard or human recombinant IL-2). Cell proliferation can be assessed in 24 hours incubation followed by 24 hours pulsing with ^{3}H thymidine (0.5uCi/well). An alternative to monitor clonal expansion is the use of colourimetric methods such as MTT reduction (Mosmann et al, 1983) or NAG activity assessment (Landergren, 1984).

2.b.5   FIBROBLAST PROLIFERATION ASSAY

Interleukin 1 has been reported to induce fibroblast proliferation (Schmidt et al., 1982), as have been other factors and cytokines including TNF alpha (Vilcek et al, 1986). Supernatants from human monocytes, induced by an arthritogenic stimulus (MSU crystals), were tested on human fibroblast lines established from joint fluid exudate cells from patients with rheumatoid arthritis.

Fibroblasts were cultured in RPMI 1640 or MEM with 10% FCS, glutamine and antibiotics. Medium was changed twice weekly and cells were passaged

1:3 to 1:10 when subconfluent by loosening the cells with trypsin/EDTA.

For the proliferation assay, fibroblasts were washed and resuspended in fresh medium at $1x10^5$/ml. Aliquots of 100ul were dispensed in the wells of a microtitre plate ($1x10^4$ fibroblast/well) and incubated overnight (to let them re-express membrane structures that could be damaged by trypsin treatment). On the second day, the spent medium was replaced by 100uls of IL-1 and sample dilutions in fresh medium. The plate was then incubated for a further 48 hours. On the fourth day, the wells were pulsed with ^{3}H thymidine (1uCi/well/20ul; TRK 296, Amersham, UK). After 4 to 8 hours incubation, the medium was removed, the wells washed with PBS, and 100ul of trypsin/EDTA solution (1X, Gibco) were dispensed into the wells. After 30 mins at 37° C, cells were harvested and thymidine incorporation assessed by liquid scintillation counting as previously described.

2.b.6.   MEASUREMENTS OF TUMOUR NECROSIS FACTOR

2.b.6.1   L929 cell cytotoxicity assay

L929 is a mouse nipple fibrosarcoma line (ATC CCL1) and it was one of the first cell lines established.

TNF cytotoxicity *in vitro* for L cells was an early observation (reviewed by Old, 1985) but many other human and murine tumoral lines are susceptible to lysis by TNF and thereafter suitable for TNF cytotoxicity tests. Among these, WEHI 164 and the subclones WEHI 164.13 (Espevik, Nissen-Meyer, 1986), WEHI 3 (Green et al, 1982), EMT-6 (Williamson et al, 1983), FELC cells (Suyama et al, 1985), U937 and A673/6 cells (Feinman et al, 1987) have been used, quantifying cytotoxicity by photometric measurement of neutral red, crystal violet uptake or MTT dye reduction or by measuring the release of ^{3}H-thymidine or ^{51}Cr from labelled target cells. As none of these assays distinguishes between TNF alpha and TNF beta, subsequent specific antibody neutralization of either cytokine is needed for identification. L929 are undemanding cells in culture, the supply of cells is steady and in the conditions that we used (Kramer et al, 1986) the assay is sensitive, inexpensive and reproducible.

L929 cells, grown in non-confluent status for at least four passages, were detached by Trypsin/EDTA treatment, washed, resuspended in MEM medium 10% FCS and seeded at $3x10^4$cells/well in the 60 internal wells of a microtiter plate (100ul/well). The remaining wells were filled with MEM

medium, and plates gently shaken to achieve an even distribution of the cells. After 24 hours incubation at 37° C the medium was replaced in all wells with 100uls of fresh MEM containing 1ug/ml Actinomycin D (Sigma). Wells in column 2 received 50ul of medium containing 2ug/ml Actinomycin D and 50ul of sample, medium control or hr TNF as a standard. Samples were then double diluted in the different rows, and plates incubated for 18 to 20 hours at 38.5° C, a temperature shift that will enhance TNF-mediated cell killing (Kramer et al, 1986). On the third day, medium was removed and adherent, viable cells stained and fixed with a solution of crystal violet (0.5% in 20% methanol). After a minimum of 2 minutes, plates were washed vigorously in water, dried and the absorbance at 470nm measured by microELISA scanning spectrophotometry. The half-maximal cytotoxicity is assessed by the average optical density of column 1 (no cells, equivalent to 100% killing) and the row that only received medium and Actinomycin D (100% viable cells). By plotting dilution against O.D. and using interpolation, the dilution of the sample giving half-maximal killing can be derived. The readings of the standard TNF preparation express the sensitivity of the assay.

Assay sensitivity

Scanning spectrophotometry from a TNF assay plate is shown in Fig. 9. Under optimal conditions, the assay is able to detect (4u/ml) 100 to 200pg/ml TNF alpha or LT and the 1/2 maximal cytotoxicity unit is usually obtained by a concentration of hr TNF alpha in the order of 25-50pg/ml.

Influence of Temperature

An increase in sensitivity (Kramer et al, 1986) can be obtained by incubation of the plate at 38.5° C after addition of the samples (Fig. 10). This does not affect reproducibility between samples which are assayed on the same day using homogenous L929 cells.

Influence of cell density

Sensitivity of L929 cells to TNF-mediated cytotoxicity is related to the cell density at which the cells are cultured. To obtain maximum sensitivity (Fig. 11), cells were cultured for 10-14 days in a non-confluent status and seeded in the assay plate at 3.5-4x10^4 cells/well. Lower or higher concentrations result in diminished sensitivity (Kirstein et al., 1986).

51

L929 assay specificity: the effects of other cytokines

To test the specificity of L929 cytotoxicity, an extensive range of recombinant, purified and cell-derived cytokine preparations were tested (Table 2.2). At the concentrations tested, none of the cytokine preparations used were cytotoxic for L929 cells. In subsequent experiments, IL-1 proteins were tested at lower concentrations, but no L929 cytotoxicity was observed. This is apparently in contrast with previous observations, obtained however with purified IL1 and achieving cytolysis only after a long time in culture (Onozaki et al, 1985B). All the cytokines used were active when tested for their specific activity.

L929 assay specificity: lack of synergy between human IL-1 and human TNF

Synergy of IL-1 and TNF has been described in a number of human systems (Elias et al, 1987; Stashenko et al, 1987). To check if human IL-1 could synergize with human TNF in L929 cell cytotoxicity, different concentrations of hr TNF alpha were assayed in the presence of constant amounts of hr IL-1 beta (Fig. 12A) or vice-versa (Fig. 12B). Identical experiments were performed in which hr IL-1 alpha instead of IL-1 beta was used (data shown in Appendix, p. 211). IL-1 alpha and IL-1 beta failed to demonstrate L929 cytotoxicity, and did not synergize with TNF alpha in this biological activity on murine cells. Biological activity of both IL1 preparations was confirmed by EL4.NOB.1 assay.

Neutralization of TNF biological activity by specific monoclonal antibody

Murine purified monoclonal antibody to hr TNF alpha ("TNF-E" 1.4mg/ml) was kindly donated by Dr G. Adolf (Boehringer Institute, Wien). This antibody exhibited no effect on TNF beta-mediated L929 cytotoxicity (Fig. 13) and was used as a specificity control in the L929 cytotoxicity assay. Cytotoxicity completely neutralized by the mab was interpreted as due to the presence of TNF alpha. The specific neutralizing activity of this antibody in the L929 cytotoxicity assay was approximately 300ng hr TNF alpha/mg antibody (Fig. 13).

2.b.6.2.   TNF alpha radioimmunoassay

This radioimmunoassay (kits were kindly donated by Medgenix, Belgium) is based on the competition for TNF alpha-specific antibodies between unlabelled TNF contained in the sample or in the standard tube, and

exogenously added ^{125}I-labelled human TNF alpha.

After precipitation of the immune complexes (by adding anti rabbit Ig antiserum and PEG) the radioactivity in the pellet will be inversely related to the amount of immunoreactive TNF alpha in the samples. These last titers are calculated by interpolation from the standard curve. All samples tested as single determinations unless otherways stated.

The sensitivity and limits of the assay were 0.1-5ng/ml hr TNF alpha .

Comparison of TNF alpha immunoreactive protein and biological activity in supernatants from stimulated adherent mononuclear cells

Immunoreactive TNF alpha levels were tested in the supernatants of cultures from different experiments. These were generated by stimulation of human adherent mononuclear cells $(1x10^6/ml)$ with a variety of microbial agents and tested for L929 cytotoxicity. Such bioactivity could be completely neutralized by a specific mab to human TNF alpha. Biological activity on L929 cells (normalized for 10ng/ml hr TNF alpha = 256u/ml; range 270.3±20.8u/ml) was highly correlated with immunoreactive TNF alpha (Fig. 14). This is in accordance with the report that no inactive propeptide is present in extracellular supernatant (Beutler, Cerami, 1986) and suggests that in 12-18 hour stimulated monocyte cultures produce no major inhibitors of TNF biological activity.

	IL 1 alpha (A$_1$)		IL 1 alpha (A$_2$)		IL 1 beta (B$_1$)		IL 1 beta (B$_2$)	
	cpm	p.u.	cpm	p.u.	cpm	p.u.	cpm	p.u.
10 pg/ml	15466±1266	1.40±0.11	17186±3074	1.56±0.28	21238±3348	1.93±0.30	14316±784	1.30±0.07
100 pg/ml	25365±1034	2.31±0.09	41374±3359	3.76±0.31	31924±1535	2.91±0.14	43284±1961	3.94±0.18
1 ng/ml	45779±4731	4.17±0.43	43411±2024	3.95±0.18	46435±3137	4.23±0.28	50328±4062	4.58±0.37
10 ng/ml	35750±4339	3.25±0.39	42131±3993	3.83±0.36	47796±5321	4.35±0.48	48624±2679	4.42±0.24
100 ng/ml	39532±3322	3.60±0.30	48518±5081	4.42±0.46	37335±1441	3.40±0.13	59774±3382	5.44±0.31
half-max. prolifer.	~ 100 pg/ml		~ 10 pg/ml		~ 80 pg/ml		~ 20 pg/ml	

TABLE 2.1   **Application of LAF assay to measurement of IL1 biological activity**

Shown are results from a C3H/HeJ thymocyte assay to test long-term storage of human recombinant IL1 (Immunex Corp., Seattle, US; 1ug/ml hrIL1 in PBS/0.3% HSA, ±0.1% trehalose). Results were expressed in proliferation units (p.u. = cpm cells+lectin+sample / cpm cells+lectin) and activity compared by comparison of half-maximal proliferation.

It was clear that IL1 alpha and IL1 beta stored in absence of trehalose (**A1** and **B1** in the table) had comparable activity (half-max. U = 80-100pg/ml) suggesting that IL1 alpha and IL1 beta had similar tolerance to long term storage. However, when compared to storage in 0.1% trehalose (**A2** and **B2** in the table) the latter showed higher specific activity (half-max. U = 10-20pg/ml) reflecting a protective action of trehalose on long-term IL1 storage (cells + Con A = 10981 ± 702 cpm).
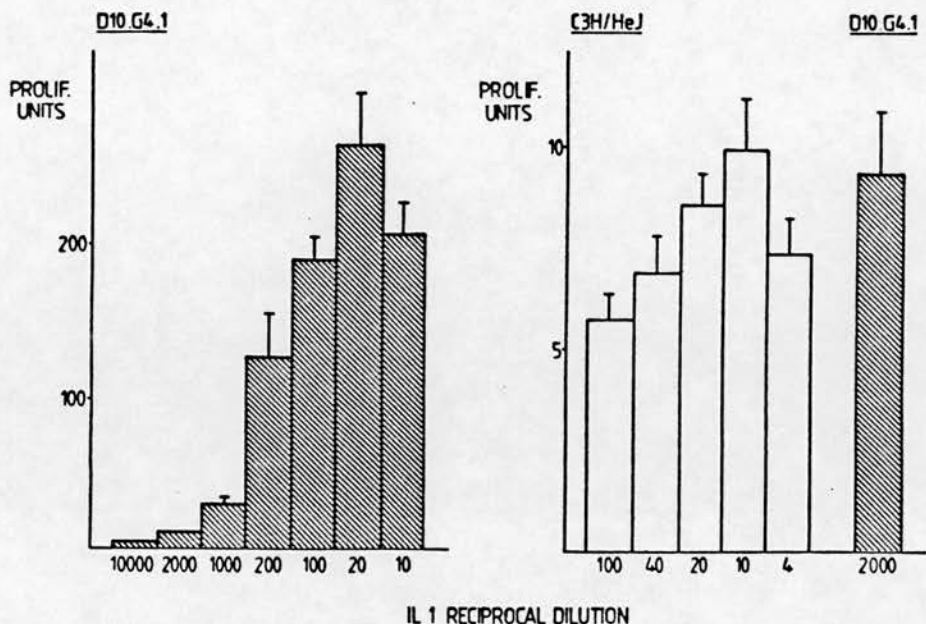
Fig. 1 Comparison of thymocytes and D10/G4.1 T cell proliferation

Thymocyte and D10.G4.1 cells were tested for ability to proliferate in response to "standard" crude IL1 preparation (monocytes supernatant, LPS 100ng/ml, 20hrs). To achieve the same proliferation units D10 cells require 100-200 times less IL1 (note the difference in scale) indicating a higher sensitivity of this assay at low IL1 concentrations. Even with the different background (thymocyte + Con A = 2318 ± 160cpm; D10 cells + Con A = 347 ± 21cpm), D10.G4.1 proliferation (as measured by ^{3}H thymidine incorporation) appears to be 15-30 times in excess to C3H/HeJ thymocytes.

Cells + ConA = 157.67 ± 15 cpm
$D_{10}G_4.1$ T cell proliferation
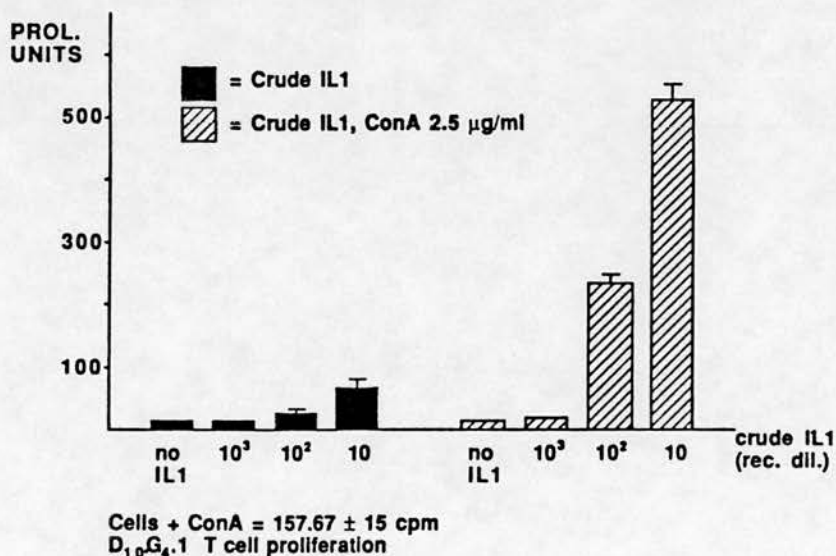
Fig. 2 D10.G4.1 proliferation requires a double signal

D10 proliferation in presence (hatched bars) or absence (closed bars) of 2.5ug/ml Concanavalin A. Results, expressed in proliferation units, are means of triplicate wells. Crude IL1 reciprocal dilutions as indicated on the horizontal axis.
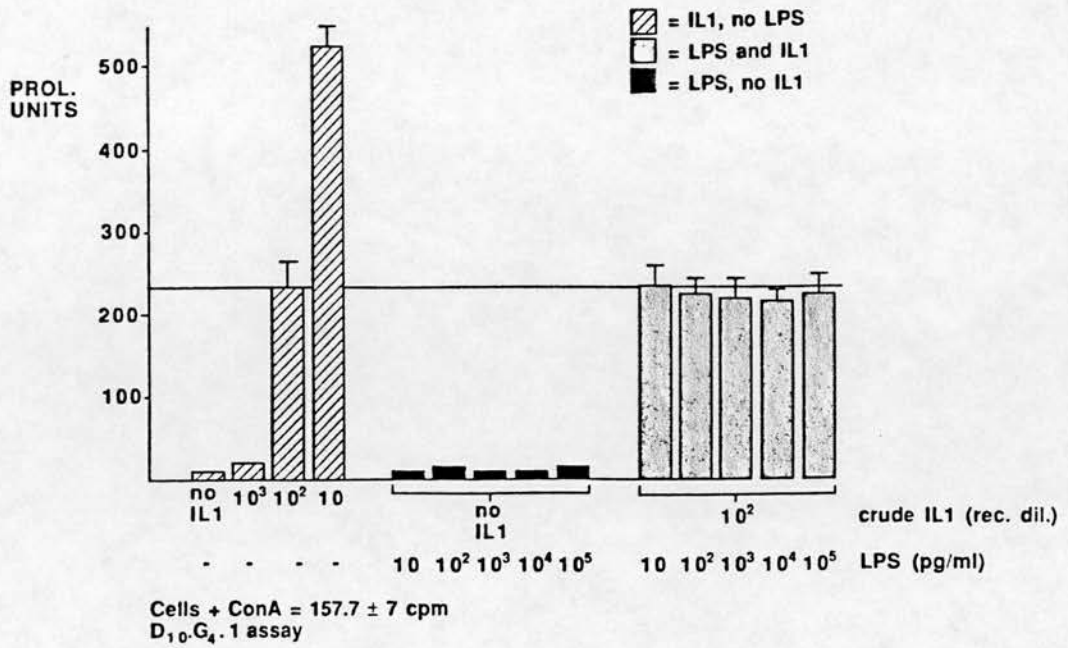
Fig. 3   Effects of lipopolysaccharide on D10 proliferation

Lipopolysaccharide was tested in different concentrations in presence (shaded bars) or absence (closed bars) of crude IL1 for its effect on D10 proliferation in presence of Concanavalin A (2.5 ug/ml).   LPS was not mitogenic for D10 cells nor influenced IL1-driven T cell proliferation.
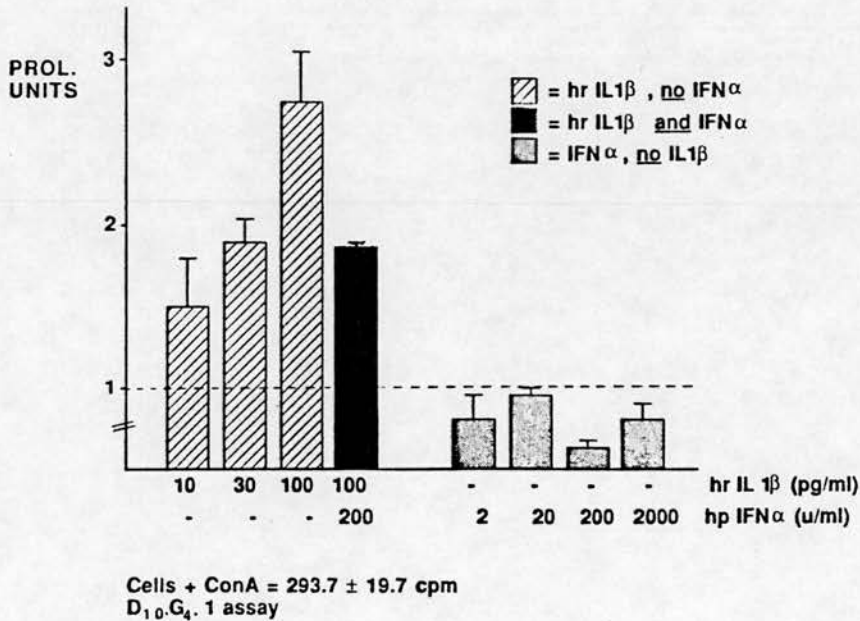
Fig. 4   Effects of IFN alpha on D10 proliferation

Human purified IFN alpha was tested for D10 cell proliferation in absence (shaded bars) or presence (closed bar) of hr IL1 beta.   IFN alpha was not mitogenic on D10.G4.1 cells and did not synergize with IL1 in this effect.

56
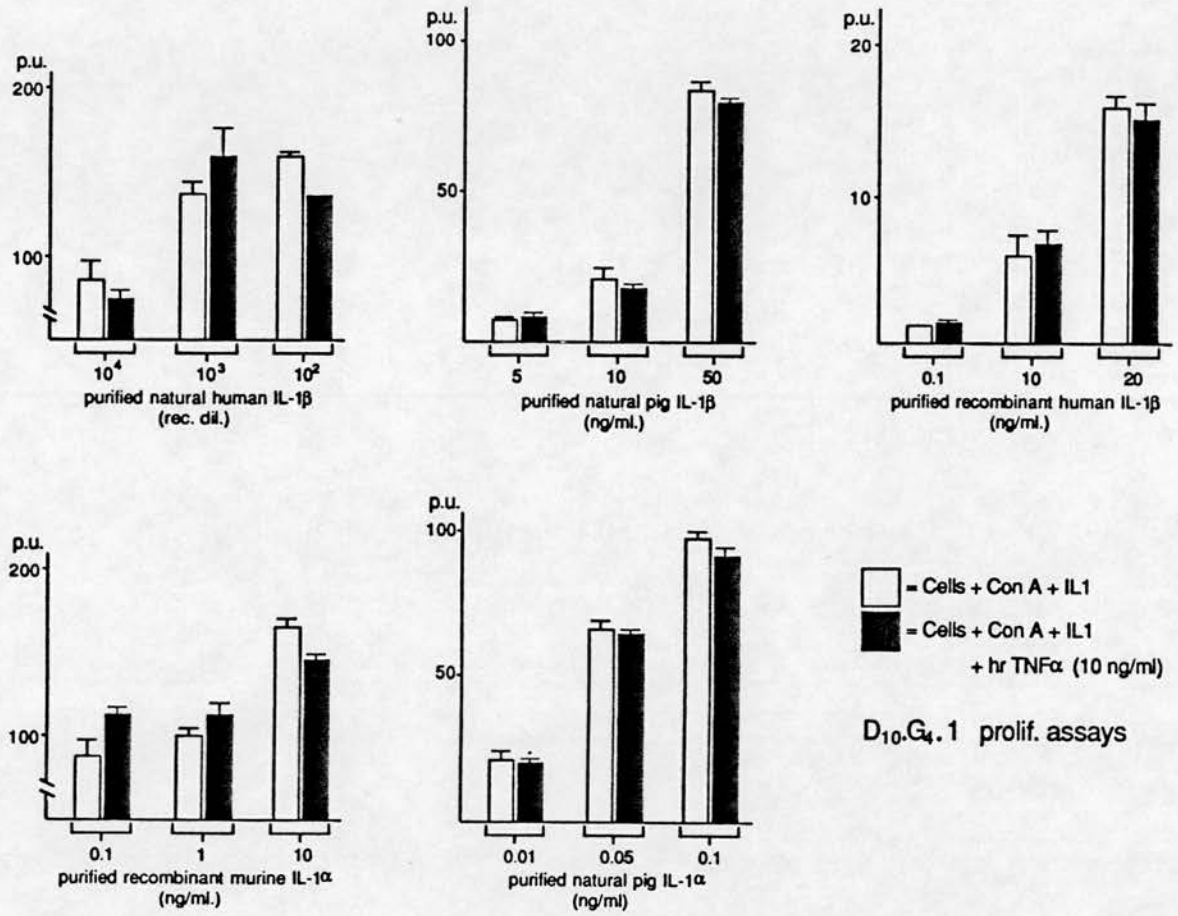
Fig. 5   Effects of human recombinant TNF alpha on D10 proliferation

After several experiments in which hrTNF alpha failed to stimulate D10.G4.1 cell proliferation in presence or absence of Concanavalin A, it was tested if it could synergize with human IL1 in D10.G4.1 proliferation.   A fixed amount of hr TNF alpha (10ng/ml) was added to different concentrations of IL1 (5 different highly purified or recombinant proteins) and tested for LAF activity.   No apparent synergy could be seen in these experiments.

57

PROL. UNITS

100

50

▨ = CTLL + IL2
■ = CTLL + IL2 + anti IL1 ab (1:400)

1   5   25   125

BRMP IL2 (U/ml)

CTLL bkg = 2310 ± 190 cpm
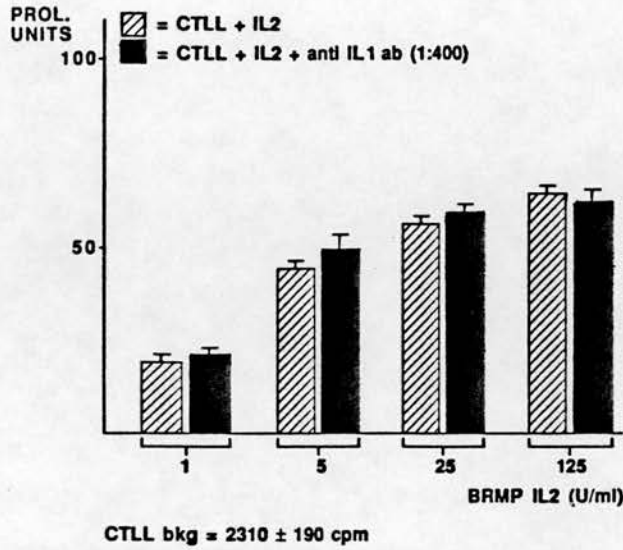
Fig. 6   Effects of anti-IL1 antiserum on IL2-driven CTL proliferation

Anti IL1 antiserum did not show any neutralization of IL2 biological activity nor nonspecific cytotoxicity for T cells. BRMP (Biological Response Modifier Program) standard IL2 was obtained from Dr S K Durum (N.C.I. Frederick, MD).

100

PROL. UNITS

▨ = D.10 cells + cytokine
■ = D.10 cells + cytokine
    + anti IL1 ab (1:400)

10

1

30   100 100   25   25   125 125

hr IL1 β (pg/ml)   BRMP IL2 (u/ml)

(BKG = 321 ± 36 cpm)
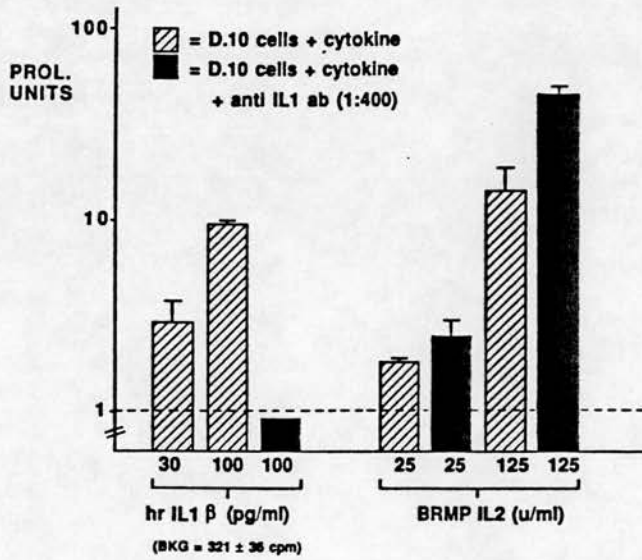
Fig. 7   Effects of anti-IL1 antiserum on IL2 or IL1 driven D10 proliferation

In this experiment, anti IL1 antiserum neutralized hrIL1 beta-D10 proliferation, but failed to inhibit the action of BRMP IL2 on the proliferation of the same cells.

58
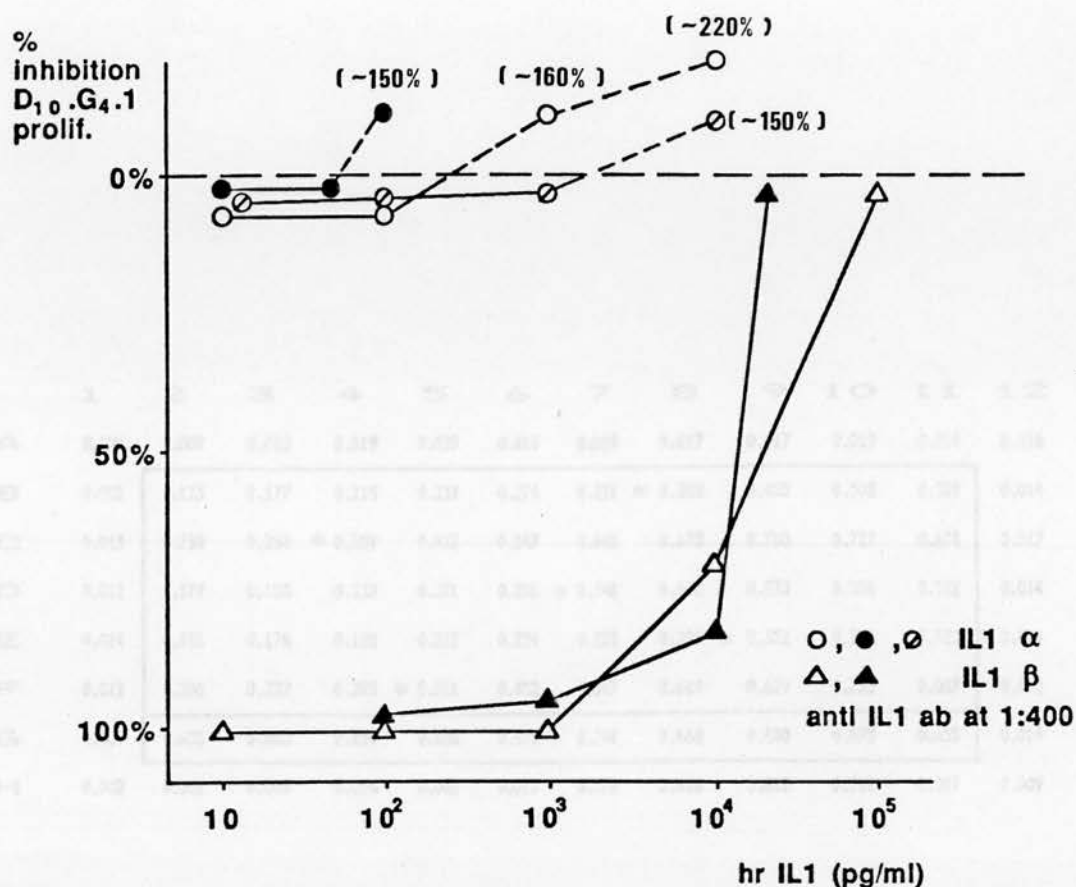
Fig. 8   Effects of anti IL1 antiserum on D10.G4.1 proliferation

Antiserum to human pI7 IL1 (a gift from Dr C A Dinarello, Boston) was tested (1:400 dilution) for its ability to inhibit LAF activity of several IL1 preparations.

The antibody at the dilution used was able to neutralize LAF activity by hr IL1 beta preparations but did not inhibit the action of IL1 alpha proteins on D10.G4.1 proliferation. In fact, proliferation of D10 cells was enhanced at the highest concentration of IL1 alpha tested (degree of enhancement indicated in brackets): the interpretation of this phenomenon is unclear but it was reproducible.

▲   hr IL1 beta from Dr C A Dinarello, (Tufts Univ, Boston, US)
△   hr IL1 beta from Dr A Shaw (Biogen, Geneva, CH)
∅   hr IL1 alpha from Dr S Gillis (Immunex Corp, Seattle, US)
○   mr IL1 alpha from Dr P Lomedico (Hoffman La Roche, Nutley, US)
●   pig purified IL1 alpha from Dr J Saklatvala (Strangeways Labs, Cambridge , UK)

Protein conc.		TEMPERATURE	
		36.5 °C	38.5 °C
1 ng/ml	hr TNF	8.13 *	29.69
	hr LT	7.76	25.27
10 ng/ml	hr TNF	134.73	195.04
	hr LT	107.60	246.49
100 ng/ml	hr TNF	751.30	>1024
	hr LT	972.10	>1024

Fig. 10   Effects of temperature on L929 cytotoxicity

Two identical plates were prepared with titrations of 3 doses of hr TNF alpha and hr TNF beta. The plates were incubated for the second 24 hours at 38.5° C or 36.5° C.
[*] = L929 cytotoxicity expressed as U/ml.

cells/well	$\frac{1}{2}$max U. (O.D.)	protein (1ng/ml)	U/ml
2×10^4	0.143	hr TNF	50.66
		hr LT	39.37
3×10^4	0.238	hr TNF	59.42
		hr LT	46.74
4×10^4	0.322	hr TNF	77.77
		hr LT	86.43
5×10^4	0.348	hr TNF	74.15
		hr LT	78.22

Fig. 11   Effects of cell density on L929 cytotoxicity
Influence of cell density on L929 cytotoxicity by TNF alpha or LT. Highest specific activity is obtained by seeding 4×10^4/well.

61

Sample	Cytokine specific bioassay	Cytokine specific activity (U/ml)	Cytokine concentration	L929 cells bioassay activity (U/ml)
hr IL 1α	D10.G4.1 [³H] TdR incorporation	3000	100 ng/ml	<4
pig purified IL 1	"	2500	100 ng/ml	<4
hr IL 1 β	"	3000	100 ng/ml	<4
hr IL 1 β	"	3000	100 ng/ml	<4
hr IL 1 β	"	3000	100 ng/ml	<4
pig purified IL 1 (CATABOLIN) pI 8	"	10	100 ng/ml	<4
22K FACTOR	D10.G4.1 prolif./ antiviral	1000/ 1000	n.a.	<4
hr IFNγ	antiviral	3000	300 ng/ml	<4
human leukocyte purified IFNα	antiviral	3000	n.a.	<4
IL 2 (MLA.144 cell derived)	IL-2 dependent cells [³H] TdR incorporation	100	n.a.	<4
IL 3 (WEHI-3B cell derived)	DA.1 cells [³H] TdR incorporation	120	n.a.	<4
hr TNFα	L929 cells cytotoxic assay	256	10 ng/ml	256
hr TNF β (Lymphotoxin)	L929 cells cytotoxic assay	256	10 ng/ml	256

TABLE 2.2.   **Different purified and recombinant cytokine preparations tested for L929 cytotoxicity**
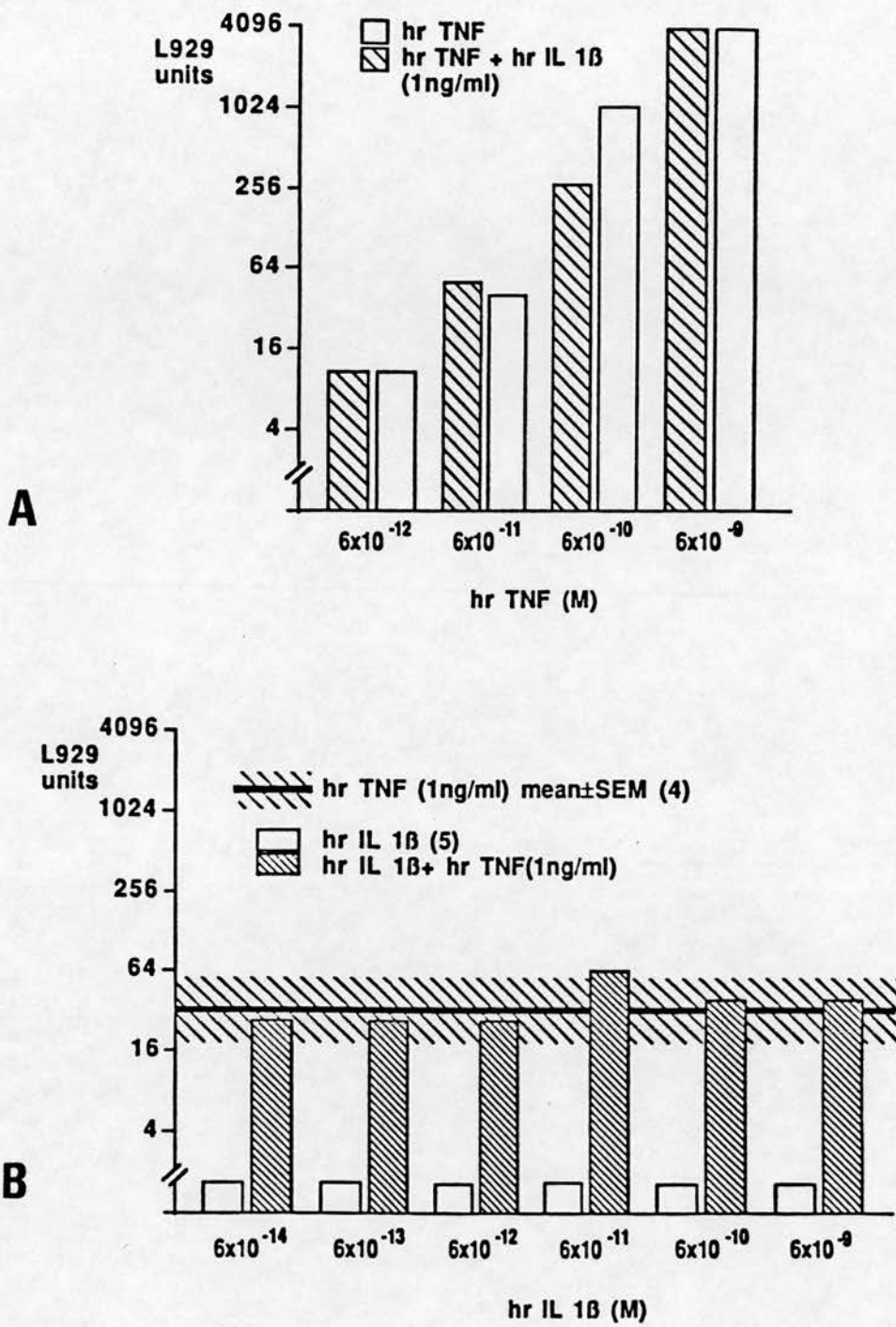
Fig. 12 Effects of hr IL1 in L929 cytotoxicity

Human recombinant TNF alpha and hr IL1 beta were tested for synergy in L929 cytotoxicity assay. (a) different concentrations of hr TNF alpha were tested in presence or absence of hr IL1 beta (1ng/ml); (b) hr TNF alpha (1ng/ml) was tested in the presence or absence of different concentrations of hr IL1 beta. No synergy in L929 cytotoxicity was observed and hr IL1 beta alone in a wide concentration range was inactive in this assay.
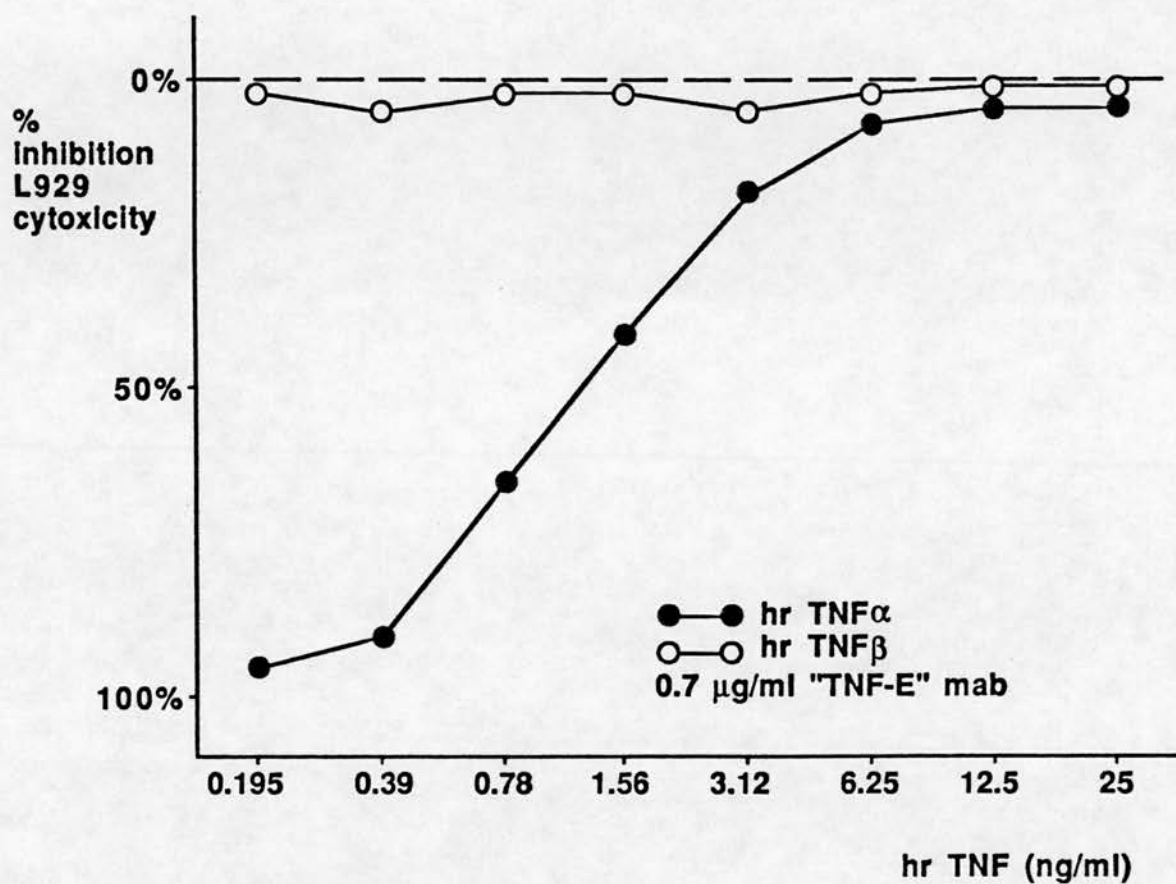
Fig. 13 Inhibition of L929 cytotoxicity by "TNF-E", murine monoclonal antibody

Murine monoclonal anti hr TNF alpha ("TNF-E") was tested for inhibition of L929 cytotoxicity. This antibody neutralized hr TNF alpha induced cytotoxicity but not hr TNF beta biological activity. Specific activity was about 300ng hr TNF alpha/mg antibody.

64

Stimulus	L929 cytoxicity in supernatants (U/ml)	TNF alpha R.I.A. in supernatants (ng/ml)
10 HKS/mo	7306	6.31
1mg/ml Zym.A	1696	5.33
0.1 HKS/mo	277	3.18
1% TSS	149	2.77
10ug/ml Zym.A	45.23	1.77
1ng/ml LPS	42.24	1.22
none	<4	<0.05
0.001 HKS/mo	<4	<0.05
none	<4	<0.05
1ug/ml Zym.A	<4	<0.05
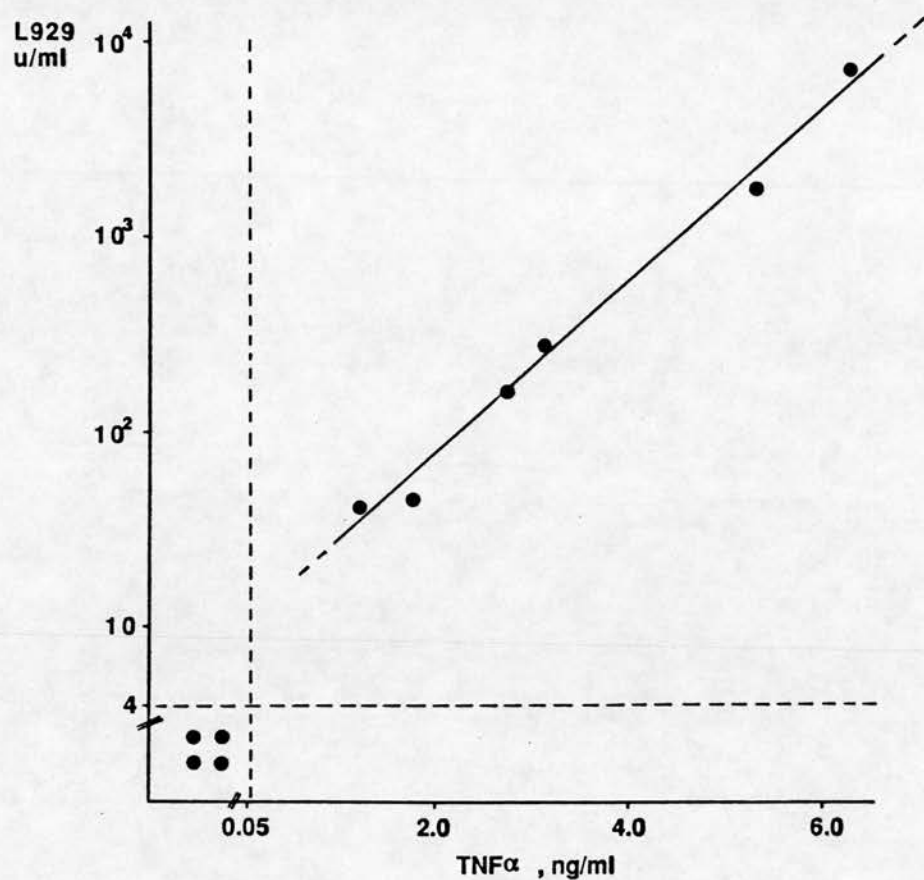
Fig. 14   Comparison of extracellular TNF alpha immunoreactive protein and biological activity

Supernatants from several experiments in which human adherent peripheral blood mononuclear cells (1×10^{6} monocytes/ml) were stimulated with different microbial agents were tested for L929 cytotoxicity and subsequently in a TNF alpha RIA. The values obtained by TNF alpha RIA highly correlated with TNF biological activity (semi-log scale).

2.c.   **CHAPTER 1**

Induction of IL1 by microbial Products

2.c.1 INTRODUCTION

Early-acting mediators of host defence such as IL1 and TNF are produced promptly upon cellular recognition of microbial products. This feature of IL1 induction has been studied for decades as "Endogenous Pyrogen" (EP) activity. As Interleukin 1 is the main, if not exclusive, mediator of E.P. activity, it can be assumed that agents reported to induce E.P. are also IL1 inducers. Some microbial agents reported to induce directly EP or LAF biological activities are indicated in Table 2.3.

In this study, different microbial products have been tested as stimuli for IL1 release from adherent human mononuclear cells, measured as LAF activity. The stimuli included three microbial cell wall products: E. Coli Endotoxin (LPS, from gram negative bacteria); Saccharomyces Cerevisiae cell walls (Zymosan A, from yeasts); and staphylococcal cell walls (HKS, from gram positive bacteria). An extracellular bacterial toxin, the exotoxin from Staph. Aureus strains that cause Toxic Shock Syndrome was also tested. Lipopolysaccharide (LPS) is the external part of the cell wall of gram negative bacteria. It is responsible for most effects of gram negative bacteria, and its enormous range of activities *in vivo* has been reviewed by Morrison & Ryan (1979). However, it is now clear that most of its effects can be explained by induction of IL1 and TNF (Morrison, Ryan 1987). The macromolecule is formed of a polysaccharide moiety ("O" antigen), an internal ("R") core and a lipid moiety ("Lipid A"). The composition of polysaccharides in the "O" moiety determines the antigenicity of the bacterial strain (Elin, Wolff, 1973). The "R" core is composed of hexoses, glycosamines and KDO (2-keto-3-deoxyoctulonic acid). "Lipid A" is composed of glycosamines linked to KDO and long-chain hydrophobic fatty acids (Rietschel et al, 1973).

LPS has been shown to induce high levels of IL1 from a number of cell types such as monocytes or macrophages (Gery et al, 1972; Gery, Lepe-Zuniga, 1983) and many others (Table 2.3). Early work showed that "Lipid A" was responsible for toxicity and induction of fever by LPS (Tripoly, Nowotny, 1966; Konno, Yoshioka, 1978). Recent reports suggest however, that the antigenic "O" part may be involved in some of the biological actions of LPS (Vukajlovich et al, 1987) and some components of the polysaccharidic region (glycolipid M9) might be more efficient than LPS or Lipid A in stimulating IL1 release and equally effective in stimulating IL1 synthesis (Lasfargues

et al, 1987). A monosaccharidic component of LPS termed "Lipid X", has also been described as a powerful macrophage activator (Amano et al, 1986).

At the time when these studies started, there was growing evidence (Gery, Lepe-Zuniga, 1983) that cellular distribution of IL1 and the existence of a cell-membrane form (Kurt-Jones et al, 1985) could have significance beyond the simple processing of the protein for secretion.

In the present experiments, the kinetics of cellular distribution of IL1 bioactivity and immunoreactivity were determined after stimulation of human monocytes with LPS: the aim was to test if specific patterns existed for distribution of either of the two IL1 proteins. The methods followed have previously been described in detail in the relevant sub-section (2.b.: Material and Methods, p. 39)

<u>TABLE 2.3</u>   <u>Direct induction of IL1 by microbial agents</u>

<u>Agent</u>	<u>Target cells</u>	<u>Activity induced</u>	<u>Author(s)</u>
VIRUSES			
Influenza	leukocytes	EP	Atkins et al, 1964
			Kanoh, Kawasaki, 1966
			Atkins, Bodel, 1974
EBV	B cells	LAF	Scala et al, 1984
HTLV I	T cell lines	LAF	Okada et al, 1986
BACTERIA			
Gram positive			
Peptidoglycans	Leukocytes	EP	Atkins, Morse, 1967
Muramyl-dipeptide	Monocytes	EP	Dinarello et al, 1978
	Monocytes	LAF	Togawa et al, 1979
Staph. Aureus:			
intrac. toxin	Leukocytes	EP	Bodel, Atkins, 1965
28KDa enterotoxin	Leukocytes	EP	Shantz et al, 1972
12KDa exotoxin	Leukocytes	EP	Bruson, Watson, 1974;
			Schlievert et al, 1979
TSS exotoxin	Monocytes	LAF	Ikejima et al, 1984
Streptococcus:			
Dick's toxin(s)	Leukocytes	EP	Watson, Kim, 1970
Gram negative			
Endotoxin	Leukocytes	EP	Atkins, Wood, 1955
	Monocytes	LAF	Gery et al, 1972
			Gery, Lepe-Zuniga, 1983
	B cells	LAF	Matsushima et al, 1985
	Langerhans cells	LAF	Sauder et al, 1984
	Dendritic cells	LAF	Duff et al, 1985
	NK cells	LAF	Scala et al, 1984
	Endothelial cells	LAF	Wagner et al, 1984
	Astrocytes	LAF	Fontana et al, 1982
	Mesangial cells	LAF	Lovett et al, 1983
SPIROCHETAE			
Borrelia Burgdorferi	Monocytes	LAF	Habicht et al, 1985
FUNGI	Leukocytes	EP	Butler et al, 1982
S. Cerevisiae	in vivo	EP	Koyabashi, Friedmann, 1964

2.c.2. RESULTS

2.c.2.1. LPS induced IL-1 activity from blood monocytes

Lipopolysaccharide at concentrations from 100pg to 1ug/ml induced extracellular release of IL-1 activity from human monocytes (Fig. 15). Different doses (1ng to 1ug/ml) of LPS administered to the thymocytes in the presence of lectin did not stimulate proliferation, excluding a possible carry-over of LPS in the thymocyte assay. In the experiment shown in Fig. 15, a dose of 250u/ml of Polymyxin B was able to neutralize the IL-1 inducing activity of 10ng/ml LPS.

2.c.2.2. Induction of IL-1 activity from monocytes by Saccharomyces Cerevisiae cell walls (Zymosan A)

Zymosan A was tested for IL-1 inducing activity in concentrations ranging from 100ng to 10mg/ml. In doses from 100ug to 10mg/ml zymosan induced release of IL-1 activity (Fig.16). Carry-over of Zymosan could not account for the thymocyte proliferation, and anti IL-1 antiserum (1:400) with one of the supernatants completely abolished C3H/HeJ thymocyte proliferation. This action of the antiserum could not be attributed to cytotoxic effects or IL-2 neutralizing activity (experiments previously shown in fig. 6 and 7, p. 58).

2.c.2.3. Heat-killed staphylococci induction of IL-1 activity from monocytes

Heat killed staphylococci were a dose dependent stimulus (Fig. 17) for extracellular IL-1 release, in a concentration from 10^5 to 10^8 cell walls/ml (at 10^6/ml monocytes = 0.1 to 100 bacteria/monocyte). The cell walls, incubated in absence of monocytes did not stimulate C3H/HeJ thymocyte proliferation when tested in the assay; the most active supernatant (6.96 p.u.) was reduced to background levels (1.689 $\pm$ 0.135) by 1:400 dilution of specific IL1 antiserum added in the thymocyte assay.

2.c.2.4. Induction by TSS exotoxin of IL1 bioactivity from human monocytes

The Staph Aureus/Toxic Shock Syndrome exotoxin (TSS) was tested as a stimulus for release of IL1 extracellular activity. TSS was a powerful, dose dependent stimulus (FIG.18) in dilutions from 1:10,000 to 1:100 (maximum tested). The TSS preparation itself was unable to stimulate D10.G4.1 proliferation and the IL1-inducing activity of a 1:100 dilution of

70

TSS was comparable to that of 10ng/ml LPS (FIG.14). This TSS preparation had been tested in LAL assay for LPS and contained <20pg LPS/ml (Dr D Shapiro, personal communication).

2.c.2.5. Induction of biodetectable extracellular IL-1 from U937 cells

Several attempts were made to induce release of IL1 activity from U937 cells, using a wide range of LPS concentrations. No bioactive IL-1 could be detected in these experiments (data not shown).

Toxic shock syndrome exotoxin was therefore tested for ability to induce IL-1 from U937 cells (Fig. 19). TSS was incubated for 18hrs with $2x10^6$ U937 cells/ml with or without the use of "inducing" protocols, such as coincubation with the Ca Ionophore A23187 (10^{-6}M) or incubation at 38.5°C. As shown in Fig. 19, TSS induced IL-1 bioactivity in dilutions from 1:10 to 1:100 at 36.5°C, and this was not significantly augmented by incubation at 38.5°C.

The use of A23187 (10^{-6}M) in conjunction with TSS, induced optimal response at 1:100 dilution of TSS at 36.5°C, which was not increased by incubation at 38.5°C. When tested in the IL1 assay, A23187 (10^{-6}M) was unable to stimulate D10 proliferation. TSS itself had already been demonstrated to be inactive on D10 cell proliferation (Fig. 18).

In the same IL-1 assay was tested a crude supernatant from 10^6/ml human monocytes, incubated for 18hrs with 100ng/ml LPS (data not shown). The proliferation units obtained by 1:500 of this supernatant (62.08 ± 1.78) matched the 1:100 dilution of the optimal response of U937 with TSS and A23187 (63.48 ± 4.084). Since this IL1 activity was generated by twice as many U937 cells, it can be calculated that this cell line is about 10 times less efficient in producing extracellular IL-1 activity than a comparable human monocyte preparation stimulated with LPS. Without "induction" the release of IL1 biological activity appears to be 30-50 times lower.

2.c.2.6. Time course of IL-1 production and release by human monocytes stimulated by LPS

Human adherent blood monocytes(10^6/ml) were tested for IL-1 production and release at different time points.

Cells were prepared from blood of healthy donors and before adherence a lysate of 10^6 monocytes ($3x10^6$ mononuclear cells) was stored (cell-associated IL-1 before adherence). After 40mins of plastic adherence non-

adherent cells were pelleted and supernatant stored for IL-1 assay (extracellular IL-1 after adherence). One sample of adherent cells was also stored for IL-1 assay after reconstitution in the same volume of medium (cell-associated IL-1 after adherence). To the adherent cells was added a volume of medium such that the final monocyte count was 10^6/ml. A variety of stimuli was given to cultures in different Petri dishes (MSU, HA, CPPD time course were performed at the same time) and immediately one supernatant of each series was harvested (extracellular IL1 at time point zero) and cells reconstituted and stored (cell-associated IL-1 at time zero). At different time points, cell associated and extracellular fractions were separated from cultures stimulated with the four different stimuli or incubated in absence of stimulus: in each Petri dish, the clarified supernatant, represented the "extracellular" fraction. The adherent cells, reconstituted to the original volume plus the cells that had become non-adherent were lysed by three cycle of freeze-thawing ("cell-associated" fraction). Each sample was stored at -80° C until use.

These fractions were first tested in the EL4/CTL assay for IL-1 bioactivity. This is a very sensitive bioassay suitable for large numbers of samples (i.e. in this experiment 140 samples assayed in triplicate at four dilutions). Results are shown in Fig. 20. All supernatants and cell associated fractions did not contain significant IL1-like activity before or at the end of plastic adherence. The monocytes incubated with medium alone did not accumulate significant IL1 bioactivity in the supernatant or in the cells: the highest production was at 6 hours (comparable to 52pg/ml IL-1), at least 1000 times less than a maximally stimulated macrophage population.

Cell associated IL-1, as in previous experiments, started to accumulate at 45 mins, with a sharp increase to reach 280U/ml by 6hrs and 380-390U/ml by 12 and 18 hrs. Extracellular IL1 content was detectable at 2hrs, with a plateau at 9-18hrs (67u/ml), never however reaching the concentrations of bioactive IL1 found within the cells.

Since IL1 biological activity is related to both IL1 alpha and IL1 beta, the fractions from the above and other similar experiments were also tested with specific immunoassays for IL1 alpha (Fig. 21) and IL1 beta (Fig. 22).

72

Intracellular proteins, as could be expected, accumulated earlier (IL1 alpha: 30 min; IL1 beta: 2hrs) than in the extracellular compartment (IL1 alpha: 2hrs; IL1 beta: 3hrs), with IL1 alpha constantly preceeding IL1 beta. On a quantitative basis, it appears that IL1 alpha protein (Fig. 21) mainly accumulates in the cell-associated compartment, while IL1 beta (Fig. 22) achieves around 50% extracellular translocation by 6-18 hours.
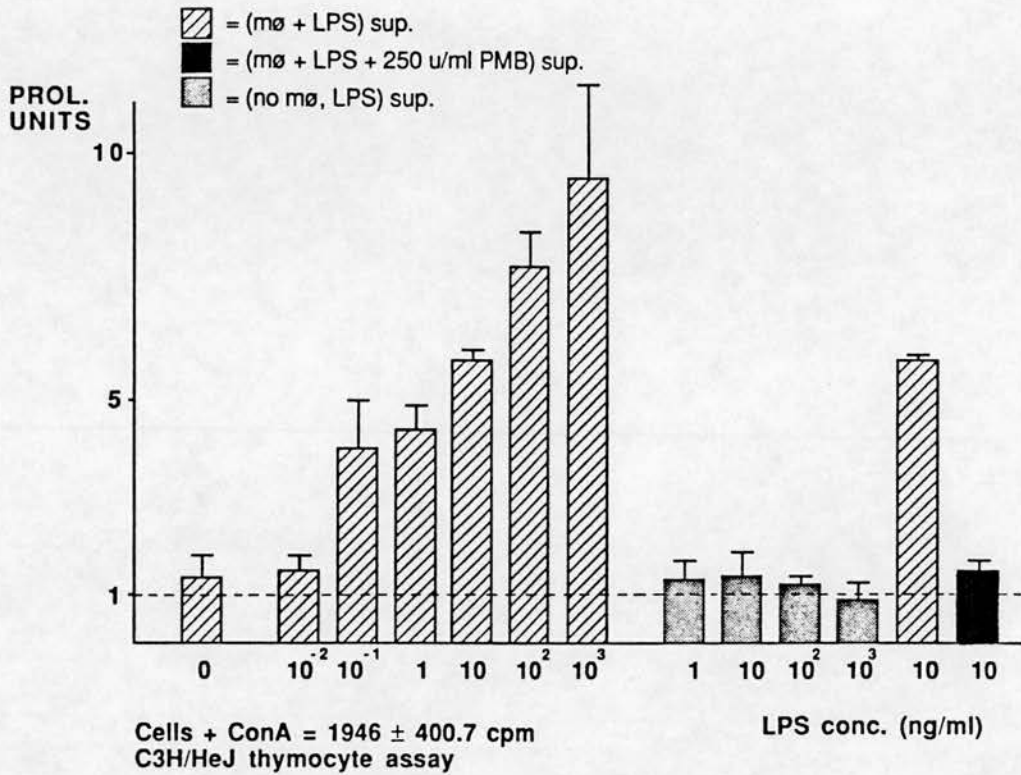
FIG. 15   Induction of extracellular IL1 biological activity by LPS

Lipopolysaccharide   was   added   at   different   concentrations   (horizontal axis) to human blood monocytes (1×10^6/ml, hatched bars) or incubated in absence of cells in complete medium (dotted bars).   After 18hrs (37°C, 5% CO_2) supernatants   were   collected   and   assayed   in   triplicate,   in   three dilutions   (1:100, 1:20, 1:4)   in   a   C3H/HeJ   thymocyte   assay   for   IL-1   activity. The   figure   shows   the   results   (in   proliferation   units   [p.u.]   =   cpm   with sample plus Con A divided by cpm with Con A only) for 1:20 dilution (in complete medium) of supernatant.   To check that IL-1 inducing activity was due to LPS, polymyxin B at 500u/ml was added to 10ng/ml LPS prior to incubation (18 hrs) with 1×10^6 monocytes/ml.   Result of the IL-1 assay (1:20 dilution)   on   the   supernatant   is   shown   in   the   solid   bar.   Supernatant   from monocyte   cultures   incubated   in   absence   of   stimulus   did   not   release   IL-1 activity   (1.344 p.u. $\pm$ 0.337, BKG = 1 p.u.)   and   PMB   had   no   effect   on   the thymocyte   assay.

74
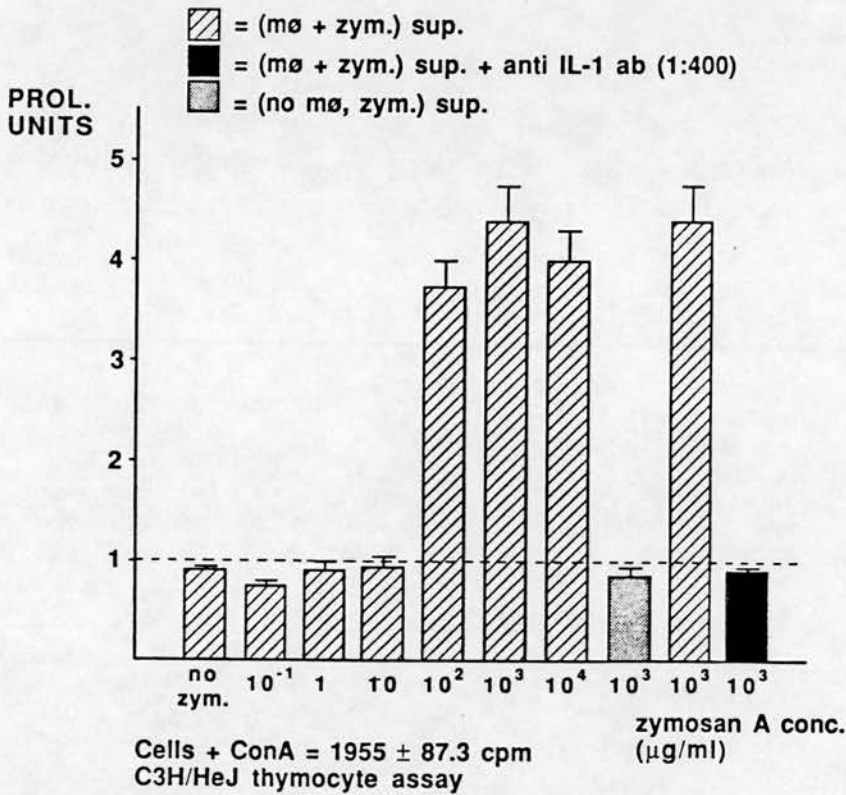
FIG 16 Induction of extracellular IL1 biological activity by Saccharomyces Cerevisiae

Zymosan A was tested for IL-1 inducing activity. Human monocytes (hatched bars, 10^6 monocytes/ml) were incubated in presence or absence of the stimulus for 20hrs (37°C, 5% CO_2). Supernatants were assayed in triplicate cultures for IL-1 activity in a C3H/HeJ thymocyte assay at 4 dilutions (1:20, 1:100, 1:500, 1:2500), and results from the 1:100 dilution are shown. A supernatant from the monocyte culture stimulated with 1mg/ml zymosan was assayed (1:100, solid bar) in the presence of 1:400 dilution of an antiserum to human purified IL-1 (kindly given by Dr C.A. Dinarello, Tufts University, Boston). Supernatant from Zymosan A (1mg/ml) incubated in absence of monocytes (dotted bar) failed to stimulate thymocyte proliferation.
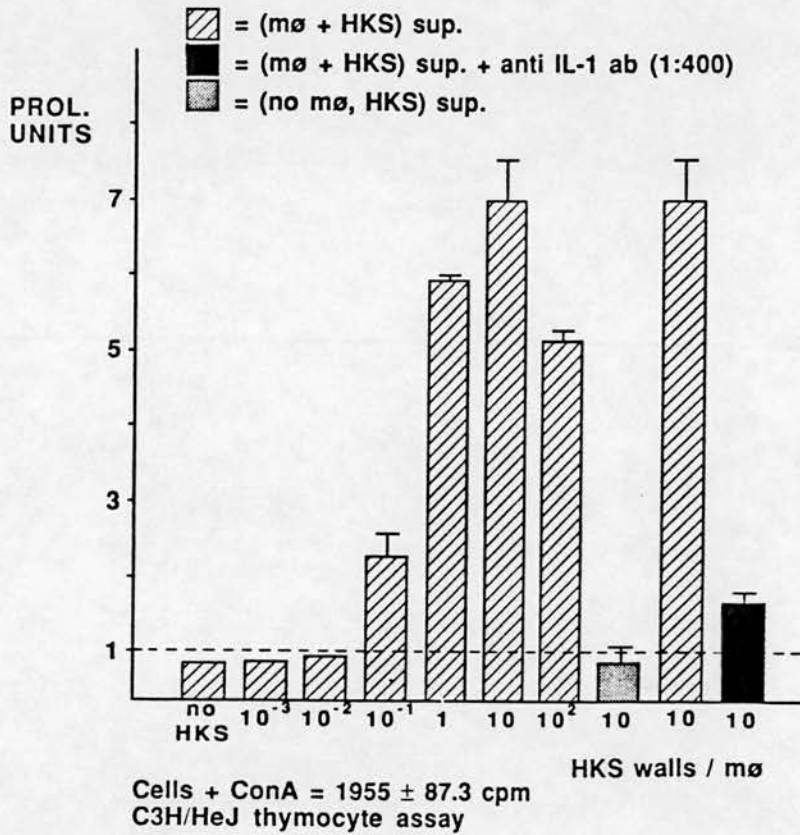
FIG. 17 Induction of extracellular IL1 biological activity by heat-killed staphylococci

HKS were used to stimulate 10^6/ml human monocytes for 20hrs (37°C, 5%), in concentrations ranging from 10^{-3} cell walls/monocyte to 10^2/monocyte. Control wells included monocytes cultured in absence of HKS and HKS incubated in absence of monocytes (dotted bar). One supernatant (from a culture of 10 HKS cell walls/monocyte) was tested in the absence (hatched bar) or presence (black bar) of anti IL-1 antiserum (1:400 dilution). Results are expressed in proliferation units of the 1:100 dilution. All supernatant assayed in triplicate and four dilutions (1:10, 1:25, 1:100, 1:1000).

76

PROL. UNITS

☒ = (mø + TSS) sup.
■ = (no mø, TSS) sup.
▨ = (mø + LPS 10 ng/ml) sup.

Cells + ConA = 679.89 ± 63.2 cpm
$D_{10}.G_4$. 1 assay
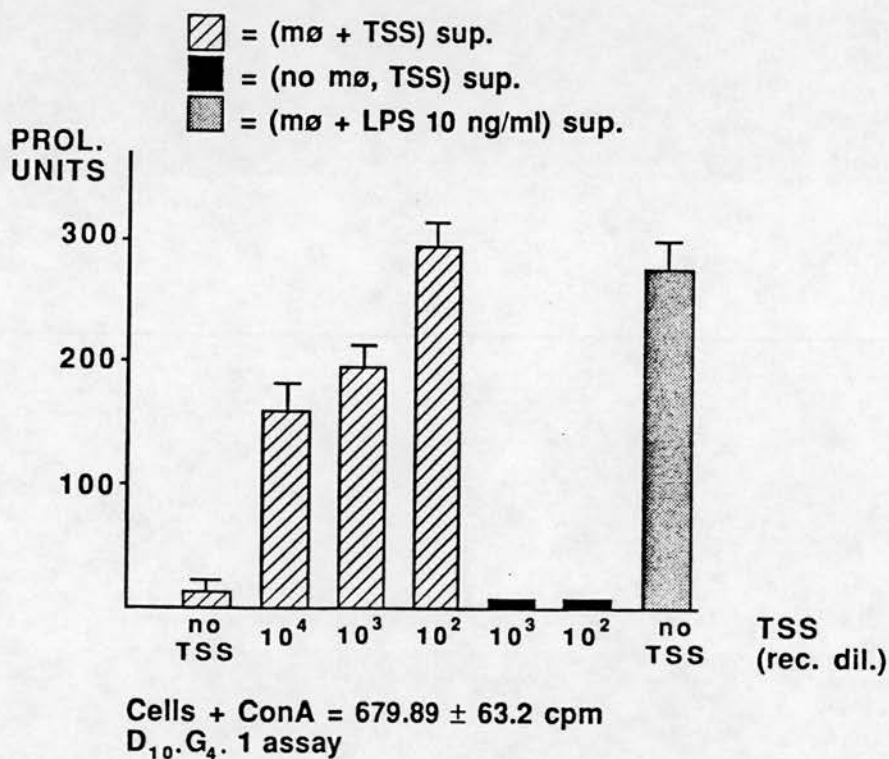
FIG. 18 Induction of IL1 extracellular activity from blood monocytes by Toxic Shock Syndrome exotoxin

Toxic shock syndrome exotoxin (TSS) in the dilution indicated (reciprocal of dilution) on the horizontal axis was added to human monocytes (5×10^5/ml, hatched bars) or in absence of cells (solid bars). Supernatants were harvested after 18 hours incubation and assayed in triplicate and three dilutions (1:1000, 1:100, 1:10) in the D10.G4.1 T cell proliferation assay for IL-1. Results are shown for 1:100 dilution of supernatant. In the same experiment, comparable IL1 bioactivity was present in cultures stimulated with TSS at 1:100 dilution and LPS at 10ng/ml (dotted bar).
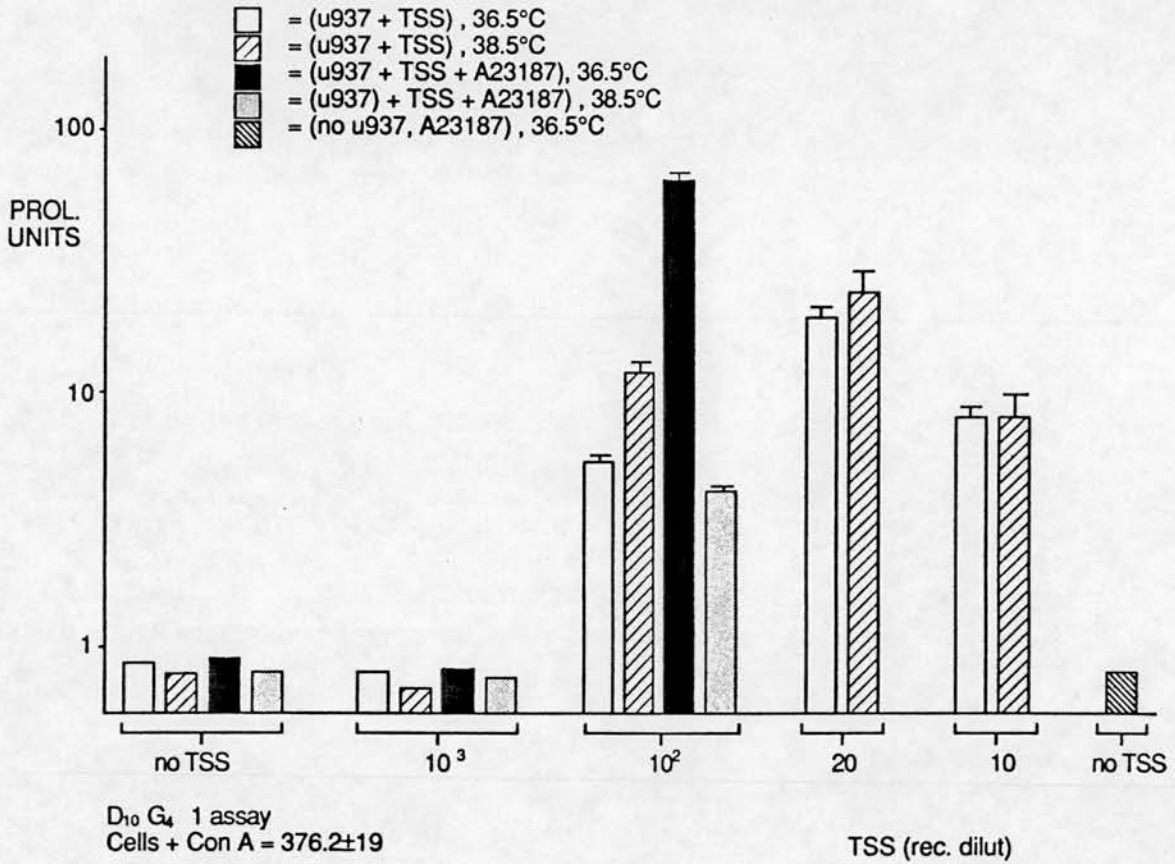
FIG. 19   IL1 bioactivity from U937 cells stimulated by toxic shock syndrome exotoxin

In this experiment, 2×10^6 U937 cells/ml were incubated with different dilutions of TSS (horizontal axis, reciprocal of dilution) in presence or absence of A23187 (10^{-6}M) and at 36.5°C or 38.5°C. Supernatants at 18hrs incubation were then tested in triplicate and four dilutions (1:100 shown) in the D10.G4.1 assay for IL-1 bioactivity. The hatched bar on the extreme right refers to A23187 10^{-6}M incubated in the absence of monocytes and then tested in the D10 assay.

78
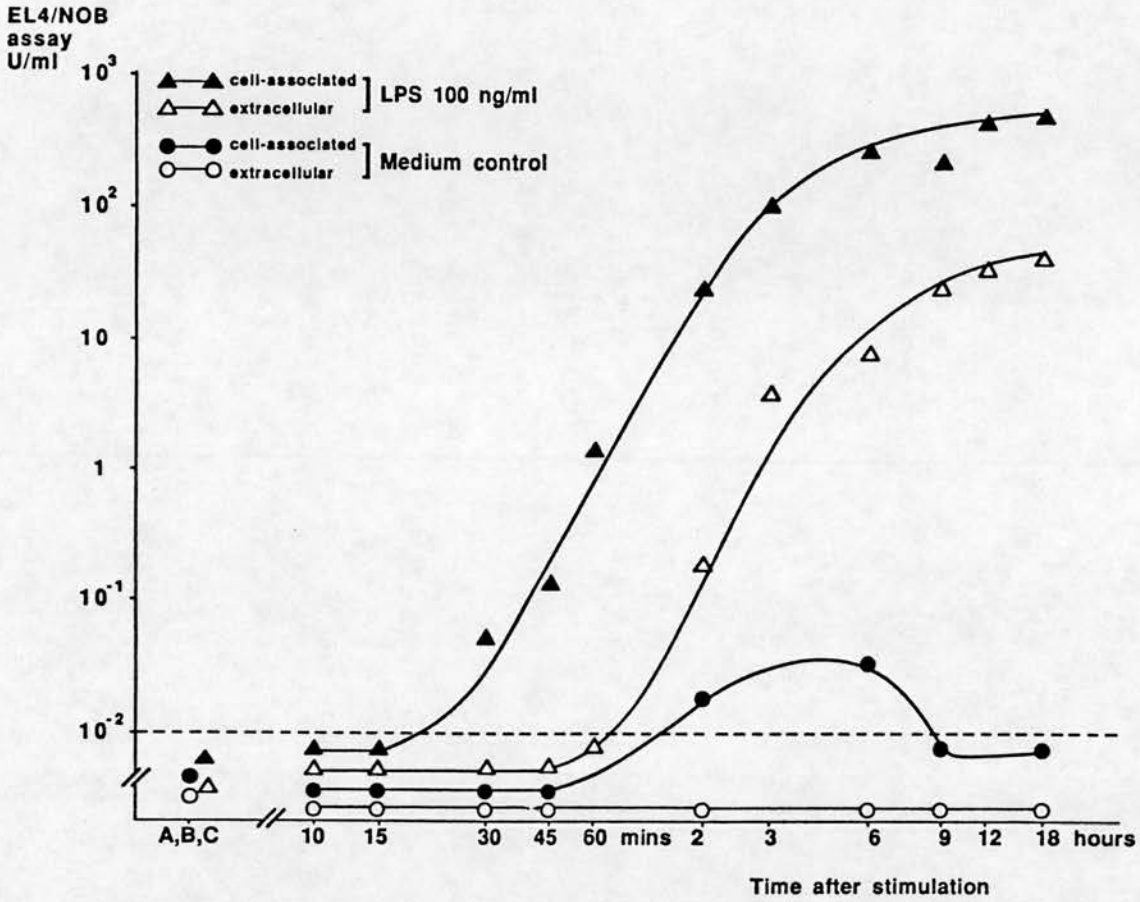
FIG 20   Time course of IL1 bioactivity accumulation and cellular distribution in human monocytes

Human adherent monocytes were cultured for up to 18 hours after stimulation (LPS, 100ng/ml) or simple addition of fresh medium. Supernatants were assayed for IL-1 in the EL4/CTL assay. On the vertical axis is indicated IL1 bioactivity expressed in EL4/CTL U/ml (1U = amount of IL-1-like activity comparable to 1ng/ml hr IL1 beta). Supernatants were tested in four dilutions and units calculated by best-fit in the standard curve.

(A)   cell-associated fraction from mononuclear cells after density separation.

(B)   Fractions from cells at the end of plastic adherence (40 mins).

(C)   Fractions from cells to which stimuli had just been added (time zero).

79
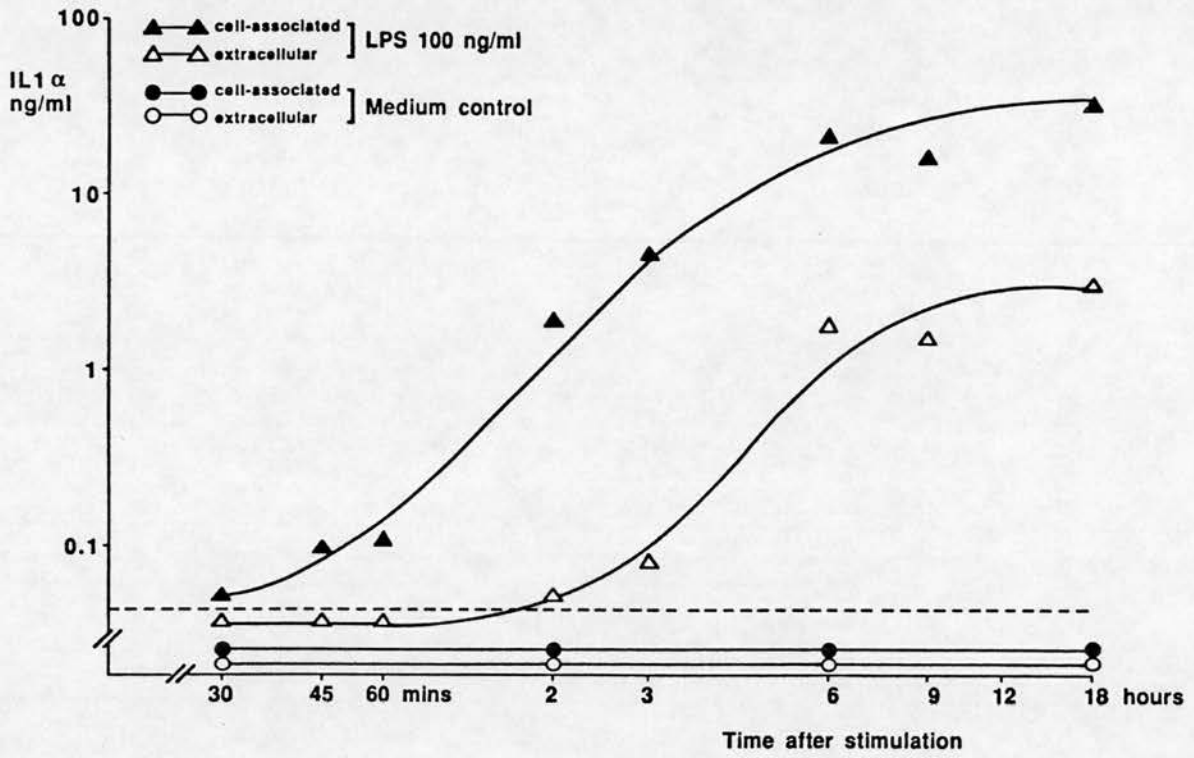
FIG 21   Time course of accumulation and cellular distribution of IL1 alpha in human blood monocytes

Extracellular and cell-associated fractions from cultures of human monocytes incubated in presence or absence of LPS (100ng/ml) were tested in an IL1 alpha-specific RIA (lower detection limit = 70pg/ml). Unstimulated cells failed to express immunoreactive IL1 alpha protein but LPS induced cells expressed detectable cell-associated IL1 alpha at 30 minutes, reaching a maximum at 18hrs (42ng/ml). Extracellular IL1 alpha was constantly expressed at lower levels, to a maximum of 3.5ng/ml at 18hrs after stimulation.

80
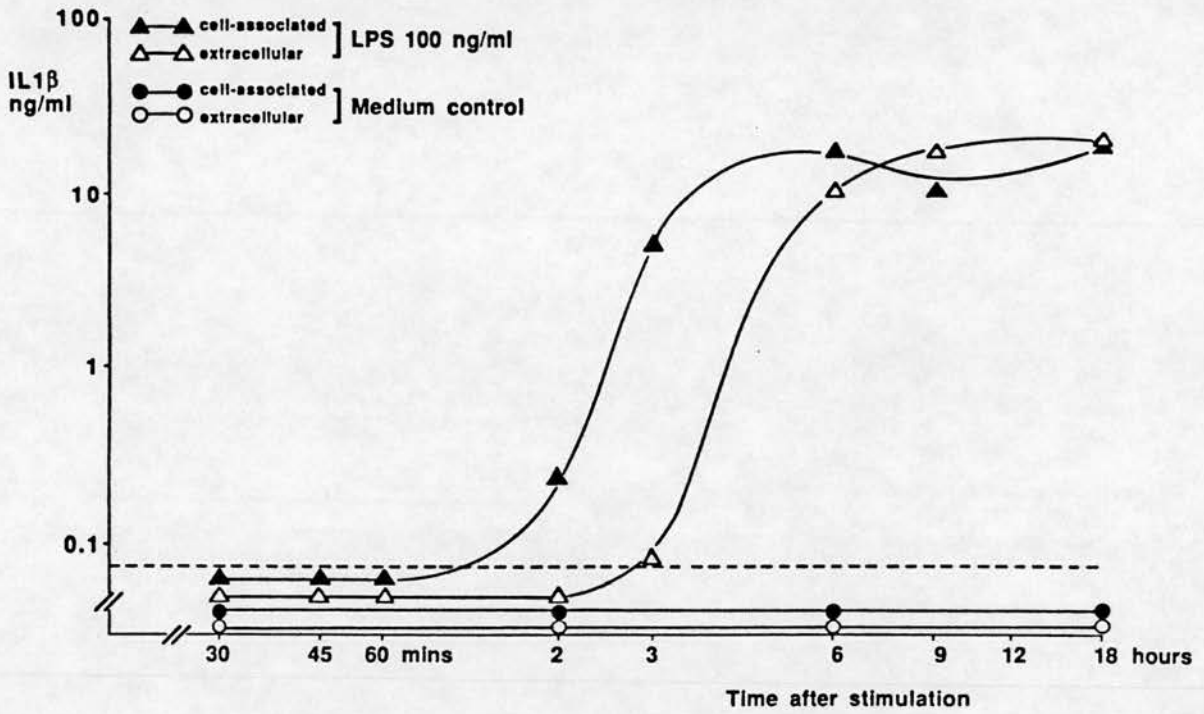
FIG 22   Time course of accumulation and cellular distribution of IL1 beta in human monocytes

Cellular and extracellular fractions indicated in Fig 21 legend were tested for immunoreactive IL1 beta using a sensitive IL1 beta ELISA (lower detection limit = 20pg/ml). Cell-associated IL1 beta was detectable by 2 hours: IL1 beta extracellular translocation started at 3 hrs, and levels comparable to the cell-associated fraction occurred by 6 to 18 hours.

2.c.3   DISCUSSION

In this study the production of IL1 from human monocytes in response to several microbial products (LPS, Toxic Shock Syndrome exotoxin and cell walls from Staph. Aureus and Saccharomyces Cerevisiae)was measured. Unstimulated cells failed to release IL1 bioactivity and each of the microbial components tested was a powerful, dose-dependent stimulator of IL1 bioactivity release.   This could not be attributed to carry-over of the stimulus in the bioassays used, nor to interference by parallel production of TNF, as human TNF fails to stimulate murine T-cell comitogenesis.   The IL1-like T cell co-mitogenic activity was inhibited by specific anti IL1 antiserum.

In the hope that a cell-line could substitute for human monocytes in studies requiring large cell populations such as time-courses of protein and mRNA accumulation, U937 cells were tested as IL1 producers.   Attempts to induce bioactive IL1 release from this line with LPS were unsuccessful. This could represent a failure of IL1 translocation or parallel production of agents such as PGE_2 that could inhibit IL1 production (Knudsen et al, 1986a) or interfere in the T cell assay (Stobo et al, 1979; Baker et al, 1981).   When TSS, reported to induce IL1 from U937 (Knudsen et al, 1986b), was used, IL1 bioactivity was obtained from U937 cells, with increased production in the presence of Ca Ionophore (10^{-6}M) at 36.5° C.   However, the bioactivity produced was 10 to 50 times less than that from human monocytes. This data and the report that U937 IL1 was different from normal human IL1's (Knudsen et al, 1986B) led to the conclusion that U937 cells are not reliable as a model of IL1 synthesis and release.   Adherent human mononuclear cells were used in further studies.

To study the kinetics of IL1 synthesis and release, IL1 bioactivity was tested in cell-associated and extracellular fractions from cultures of human monocytes.   These were incubated for different lengths of time in absence or in presence of a suboptimal concentration of LPS (100ng/ml). Unstimulated cells failed to accumulate IL1 bioactivity in the extracellular compartment, reaching a peak of 52pg/ml in the cell associated fraction at 6 hours.   Such amounts of IL1 (about 1000 times less than in the activated macrophages) agree with reports that demonstrate no activation of IL1 production in unstimulated human monocytes (Matsushima et al, 1986; Bakouche et al, 1987A).   It has been reported, however, that murine (Kurt-Jones et

82

al, 1985; Fuhlbrigge et al, 1987) and human (Kahn Bayne et al, 1986) macrophages could be activated by simple plastic adherence: this could be an effect of the type of plastic used or different experimental conditions.

In kinetic studies of IL1 bioactivity accumulation after stimulation (LPS 100ng/ml), IL1 activity in the cell-associated compartment always exceeded extracellular levels. This confirms previous studies (Gery, Lepe-Zuniga, 1983) which report IL1 as mainly a cell-associated biological activity and is in keeping with the lack of a leader sequence in either of the two peptides. The cell-associated fraction, as prepared in the present study, includes cytosol, intracellular organelles and cellular membranes. Biologically active IL1 has been demonstrated on the surface of fixed macrophages (Kurt Jones et al, 1985; Matsushima et al, 1986; Bakouche et al, 1987A; Weaver, Unanue, 1986), on B cells (Acres et al, 1987A) and in T cells (Tartakovski et al, 1986; Acres et al, 1987B). In human monocytes (Matsushima et al, 1986), membrane IL1 represented about half of the total cell associated bioactivity. Most of the remaining intracellular bioactivity seems to be associated with lysosomes (Bakouche et al, 1987A) and a major fraction of nonbioactive IL1 in the cytosolic compartment could be unmasked by trypsin treatment. From these studies, based on IL1 bioactivities, IL1 precursor seem to appear first in the cytosol, then in the lysosomal and membrane compartments, where processing to mature bioactive IL1 takes place. The mechanisms by which IL1 would gain access to the outer side of the membrane and behave as a membrane-bound protein are unknown. Two hypotheses have recently been advanced: IL1 would bind to 1,2, diacylglycerol (DAG) moieties of phosphatidyl inositol in the membrane (Bakouche et al, 1987A), stabilizing IL1 as an integral membrane protein; alternatively, a recent report (Brody, Durum, 1987) suggests the stabilization of IL1 on the mouse macrophage membrane *via* a lectin-like mechanism. Conjugation to DAG would explain the discrepancy in molecular weight between the membrane IL1, which is 22-23KDa (Matsushima et al, 1986) and the mw of 17.5KDa of the mature extracellular protein. In a recent experiment, (Baldari et al, 1987) it has been shown that nothing in the structure of the mature IL1 beta opposes extracellular translocation: when linked to a recognized leader sequence, IL1 beta is completely translocated and cleaved correctly to release mature peptide.

The interpretation of the data shown in the present study and in the previously mentioned work based on IL1 bioactivity ignores the possibility

that different patterns of biological inhibitors and different contributions from IL1 types might occur in different compartments.

To test this possibility the kinetics of specific IL1 alpha and beta distribution were measured by immunoassays. This is the first study to show that no immunoreactive IL1 alpha or beta (mature peptides or precursors) are detectable in cells in the absence of activation at any timepoint in an 18 hrs incubation. Both IL1 alpha and IL1 beta proteins in human monocytes are synthesized *de novo*, and not by processing from a preformed inactive protein pool.

Upon LPS stimulation, Interleukin 1 alpha protein was detectable earlier than IL1 beta in the cell-associated compartment (Fig. 23) and constantly exceeded IL1 beta levels within the cell. Extracellular levels of the cytokines were lower than in the cell-associated compartment and IL1 beta was the prevalent form in the supernatants. Considering the percentage of each cytokine which stays in the cellular fraction (Fig. 24), and assuming that the affinity of the antibodies in the immunoassays was comparable between the mature and unprocessed forms of IL1, it is evident that most (over 88%) of IL1 alpha, at each time point, remains cell associated. IL1 beta, by 6 hours, and further at 9 and 18 hours, achieved successful (over 50%) extracellular translocation. Recent reports (Conlon et al, 1987; Kurt-Jones et al, 1987) pointed out that the main membrane form on human cells is IL1 alpha. The present data show that about 70-80% of the total cell-associated IL1 is of the alpha type.

The mechanism of cellular release for IL1 beta, remains unknown but not unique, as FGF beta, also lacking a signal sequence, is efficiently translocated. Dissociation between IL1 synthesis and release has been reported by administration of LPS in liposomes (Bakouche et al, 1987B) or after anti Ia treatment (Durum et al, 1985). It is clear that in our conditions LPS induced synthesis of both IL1s but activated a translocation pathway only for IL1 beta. However, we obtained similar cellular distribution using different stimuli (data shown in Chapter 3) confirming that this is not simply an LPS-related phenomenon.

This difference in cellular distribution is one of very few reported biological differences between IL1 alpha and IL1 beta. Interleukin 1 alpha, the main form of cell-associated IL1 might be more important for the role of IL1 in the immune system, and cell-cell interactions, while Interleukin 1 beta, the "secretory" IL1, might mediate actions at a distance, such as fever and the induction of acute phase response.

84

IL1 (ng/ml)   IL1 (ng/ml)

60   40   20   0   0   20

Cell-associated
IL1

▨ IL1α

▨ IL1β

Extracellular
IL1

▨ IL1α

▨ IL1β

time after stimulation
(LPS 100 ng/ml)
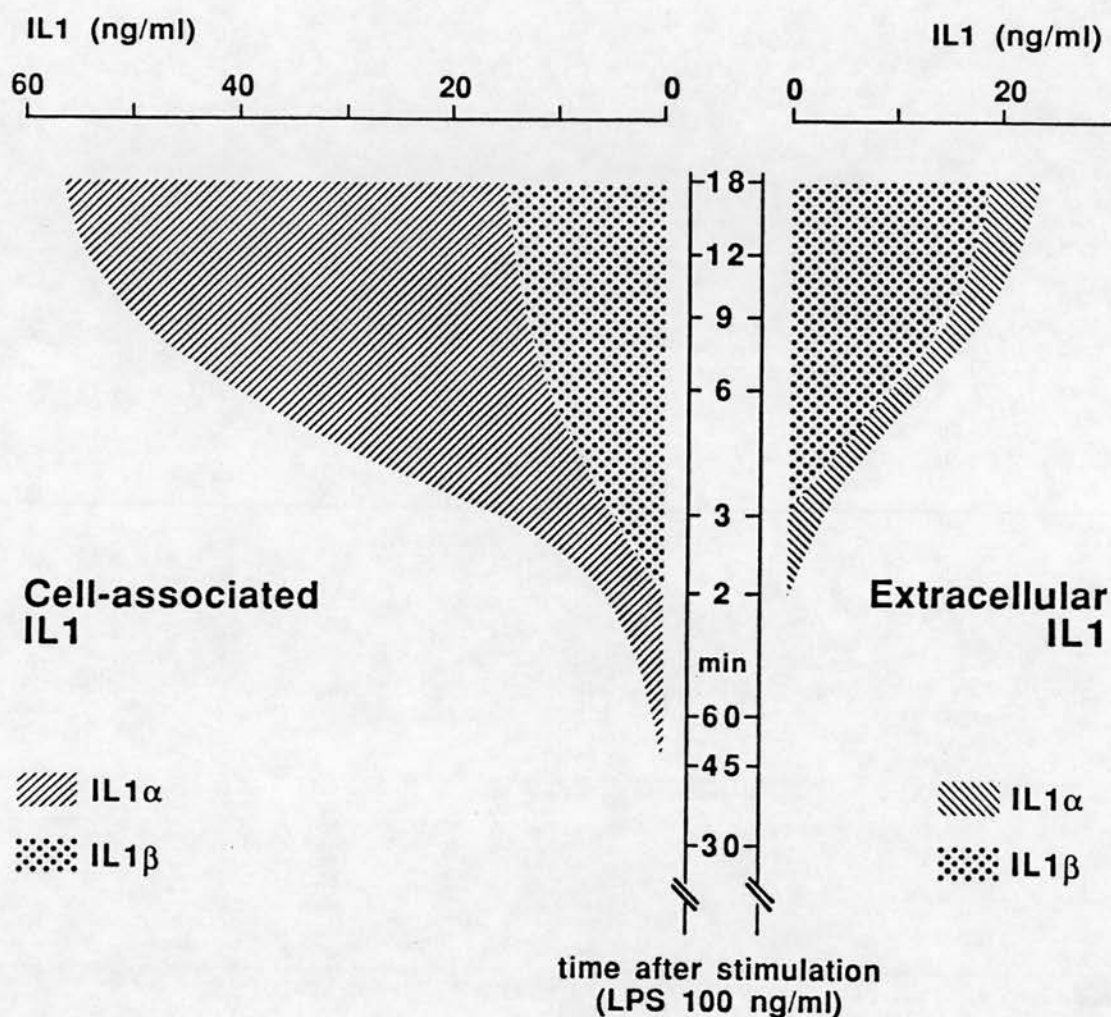
FIG 23   **Kinetics of IL1 alpha and IL1 beta protein accumulation in**
              stimulated human monocytes

In monocytes stimulated with LPS (100ng/ml) immunoreactive cell-associated IL1 alpha and IL1 beta were detectable, as expected, before extracellular protein. In this figure, which summarizes data in Figs. 21 and 22, the amounts at different time points have been expressed as shaded areas. Note that the origin for the single values is the lower limit of the relevant shaded area.

At 18hrs, cell-associated IL1 was mainly IL1 alpha (IL1 alpha = 42ng/ml; IL1 beta = 16ng/ml) while extracellular protein was mainly IL1 beta (IL1 beta = 18.5ng/ml; IL1 alpha = 3.5ng/ml). At the last time point studied, the total IL1 of each form was comparable (IL1 alpha = 45.5ng/ml; IL1 beta = 34.5ng/ml).
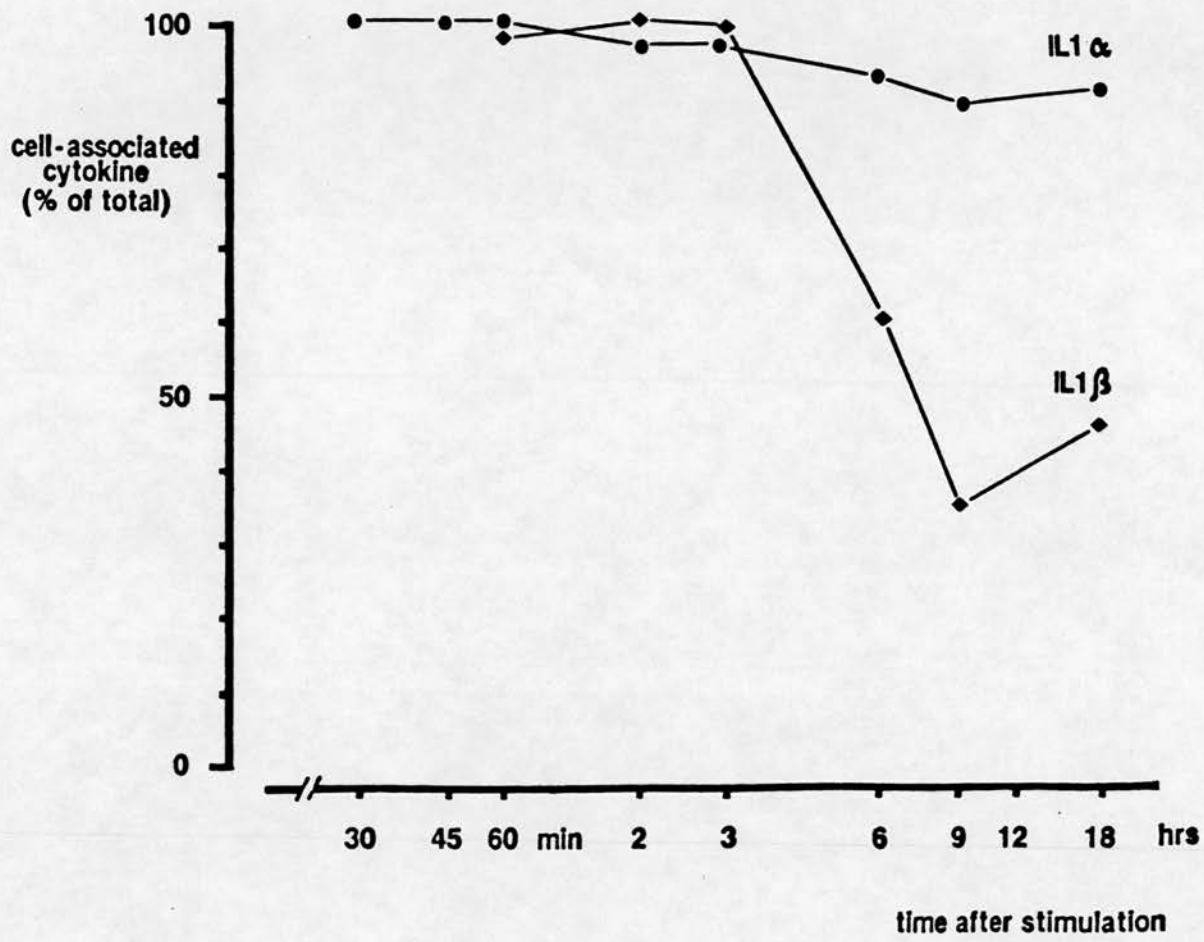
FIG 24   Kinetics of cellular distribution of IL1 alpha and IL1 beta

The total amount of each cytokine at different time points has been calculated by adding the cell-associated and extracellular fractions.   By 9 hours, 88% of IL1 alpha was still cell-associated, while only 35% of IL1 beta could be found within the cells.

2.d.   CHAPTER 2

Induction of Tumour Necrosis Factor by Microbial Products

2.d.1   INTRODUCTION

Infectious diseases often result in the depletion of the host's energy stores, vasculopathy and multiple organ failure. Tumour Necrosis Factor in particular, has been implicated in the progressive wasting diathesis (cachexia) of chronic infections and tumours (Rouzer, Cerami, 1980; Oliff et al 1987) and as an early mediator of lethal endotoxaemia (Beutler et al, 1985; Tracey et al, 1987).

The TNF gene has been conserved through mammalian evolution, suggesting that its expression confers survival advantage to the species. TNF has, infact,been shown to exert important anti-microbial effects (Wong, Goeddel, 1986; Paraut et al, 1980; Havell, 1987) and to act as a general immunepotentiator in vivo (Ghiara et al, 1987, Palladino et al, 1987).

The range of stimuli of TNF production is incompletely known. A variety of microbial agents are able to induce TNF synthesis in vitro (Table 2.4), and it seems that significant quantities of protein are produced in vivo under pathologic conditions: TNF has been detected in mice during listeriosis (Havell, 1987) and after experimental pneumonia by Legionella Pneumofila (Blanchard et al, 1987) and in humans during parasitic (Scuderi et al, 1986) and meningococcal (Waage et al, 1987) infections.

Lipopolysaccharide has been the best studied inducer of TNF: the active moiety appears to be Lipid A (Haranaka et al, 1984), but the mechanisms of TNF gene activation is unknown. It seems that LPS stimulation of TNF in vitro can be selectively blocked with inhibitors of calcium/Calmodulin kinase (Kovacs et al, 1988) and that the continuous presence of the stimulus on the outer membrane of the producing cells is required (Gifford, Lohmann-Matthes, 1986). Studies in vivo documented production of TNF alpha upon LPS stimulation in mice primed with "reticuloendothelial stimulators". These included Calmette-Guerin Bacillus (BCG) and Corynebacterium Parvum (now called Propionibacterium Acnes), which are facultative intracellular bacteria, described to enhance resistance to other infectious agents (Blanden et al, 1969; Adlam et al, 1972) or to transferrable tumours (Old et al, 1961; Halpern et al, 1966). The true nature of this "priming" and whether this is a necessary step leading to TNF production upon microbial recognition is unclear. It has been hypothesized (Gifford, Flick, 1987) that basal production of different cytokines after

88

"priming" infection could eventually synergize with LPS to induce TNF production: such a phenomenon has been documented *in vitro*, using human IFN gamma (Gifford, Lohmann-Matthes, 1987).

At a cellular level, the current understanding of TNF production and release (Beutler, Cerami, 1986) is that upon activation, TNF alpha synthesis starts with the synthesis of a 233aa long precursor polypeptide. The first 76aa represent an unusually long, highly conserved signal peptide, which is cleaved to smaller fragments during membrane translocation of TNF, the mature form of which (17KDa, 157aa) is released extracellularly. TNF would seem to be therefore a classical secretory protein.

This study tested the ability of human monocytes to produce TNF alpha upon stimulation with different bacterial and fungal constituents reported to induce IL1 synthesis. Kinetics of TNF alpha synthesis and release were also assessed in human monocytes stimulated by lipopolysaccharide.

TABLE 2.4   Microbial induction of Tumour Necrosis Factor

IN VIVO:

Agent	Phenomenon	Author(s)
LPS	induction of transferrable tumour necrosis activity	O'Malley et al, 1962 Carswell et al, 1975
LPS (LD100)	endotoxic shock; inhibitable by anti-TNF antibodies.	Beutler et al, 1985 Tracey et al, 1987
?LPS	injected in humans, cancer therapy	Coley, 1893 Shear, 1936
Streptococcal extract (OK-432)	cancer trials in patients, "endogenous TNF therapy"	Mizuno et al, 1987
Klebsiella pneumonia extract (RU-41740)	cancer trials in patients "endogenous TNF therapy"	Vacheron et al, 1987
Tripanosoma Brucei	inhibition of serum lipoproteinlipase activity	Rouzer, Cerami, 1980

IN VITRO:

Agent	Activity	Author(s)
Viruses Sendai	TNF cytotoxicity TNF "	Aderka et al, 1986 Berendt et al, 1986
Influenza	TNF "	Beutler et al, 1986
Bacteria LPS	TNF cytotoxicity LPLase inhibition	Kawakami et al, 1982 Rouzer, Cerami, 1980
muramyl-dipeptide	TNF cytotoxicity	Kildahl-Andersen, Nissen-Meyer, 1985
mycoplasma orale	TNF cytotoxicity	Lowenstein et al, 1987
Protozoa Tripanosoma Brucei	TNF cytotoxicity	Hotez et al, 1984

2.d.2. RESULTS

2.d.2.1 Induction of TNF alpha by LPS

Lipopolysaccharide (100pg to 10ug/ml) stimulated dose-dependent extracellular release of TNF from human peripheral blood mononuclear cells (Fig. 25). Such stimulating activity of LPS, as expected, could be completely neutralized by the addition of 250u/ml PMB; and supernatants from LPS induced cells, but not from unstimulated cells, contained immunoreactive TNF alpha. To check the specificity of the L929 bioactivity, three supernatants from this experiment were tested in presence or absence of specific monoclonal antibody to TNF alpha (Fig. 26). The bioactivity was completely neutralized, indicating that the L929 cytotoxicity was due to the presence of TNF alpha.

2.d.2.2. Induction of TNF alpha release from monocytes stimulated by different microbial products

Human peripheral blood adherent mononuclear cells were stimulated in different experiments with increasing concentrations of heat killed Staphylococcal cell walls (Fig. 27), toxic shock exotoxin (Fig. 28) and Saccharomyces Cerevisiae cell walls (Fig. 29). All agents were powerful, dose dependent stimuli. Some of the supernatants were also tested by specific TNF alpha RIA (Figs 27,28,29), and contained up to 6.3ng/ml immunoreactive TNF alpha. In parallel experiments, this TNF bioactivity was completely neutralized by anti-TNF alpha monoclonal antibody, and it was shown that carry-over of the agent used as stimulus could not be responsible for the L929 cytotoxicity measured.

2.d.2.3. Kinetics of production and cellular distribution of TNF alpha biological activity in human monocytes following LPS stimulation

Freshly isolated human adherent mononuclear cells (10^6 monocytes/ml) were cultured in presence or absence of a suboptimal dose of LPS (100ng/ml). At time-points ranging from immediately after density centrifugation to 18 hrs incubation, cell-associated and extracellular fractions were collected from stimulated and unstimulated cultures to be tested for L929 cytotoxicity (Fig. 30).

Cells which had been lysed immediately after density centrifugation or at the end of plastic adherence (40 mins) did not contain detectable TNF

activity (corresponding to less than 160pg/ml hrTNF). Unstimulated cells also failed to express detectable activity at any time-point, both in the cell-lysates and supernatants. TNF bioactivity accumulated in LPS-stimulated cells as early as 30mins, to reach a plateau between 9 to 18hrs. Starting from 45mins after induction, biological activity was mainly recovered in the extracellular fraction, and exceeded the cell-associated activity by 20 fold at 12 hrs.

To test the specificity of this biological activity, six of these fractions (indicated with [*] in Fig. 30) were tested in presence or absence of monoclonal antibodies to human recombinant TNF alpha (Table 2.5). Complete neutralization confirmed that the biological activity was entirely related to the presence of human TNF alpha (controls on the specificity of this mab have been previously shown).

2.d.2.4 Time course of TNF alpha production and release assessed by specific RIA

Cellular and extracellular fractions from the previously mentioned experiment were also tested in a TNF alpha RIA (Medgenix). The results confirmed (Fig. 31) that unstimulated cells did not express TNF alpha in either compartment, supporting the concept that no preformed protein pool is stored in cells prior to activation. The kinetics of cellular distribution reflected TNF biological activity, indicating that in these experimental conditions, the presence of immunoreactive protein in both compartments was proportional to TNF biological activity. At 18 hours, extracellular TNF alpha largely exceeded cell-associated (56.8ng/ml and 4.8ng/ml respectively).
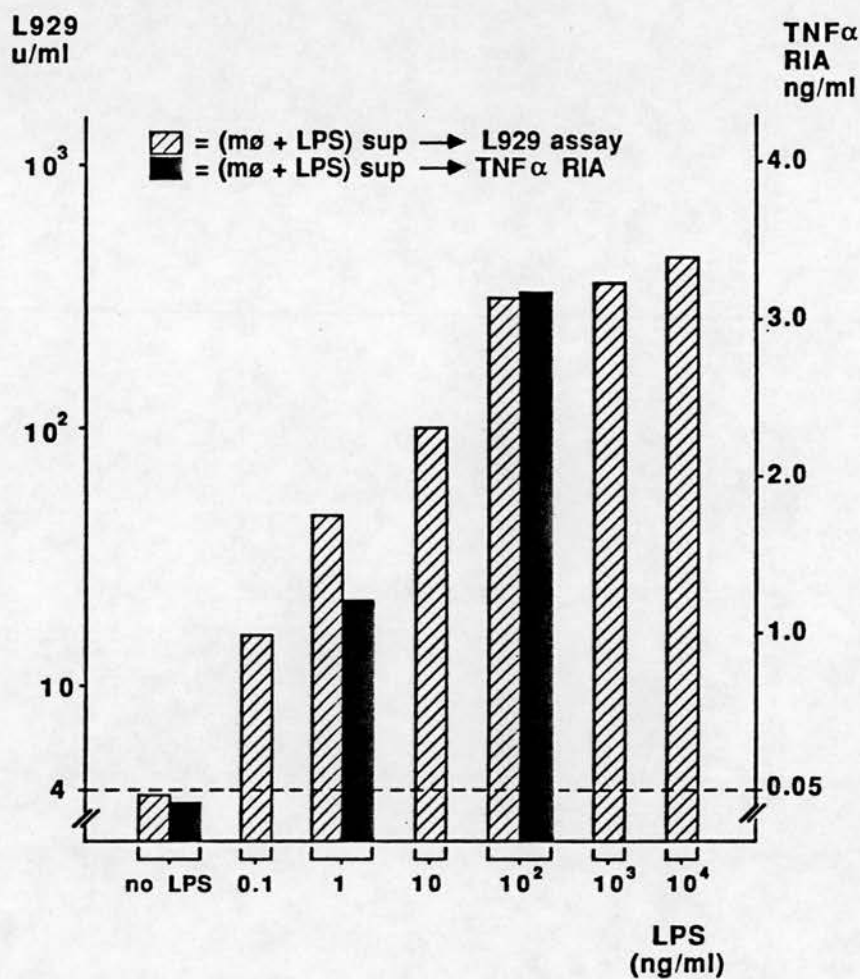
FIG 25 Induction of TNF release from human mononuclear cells stimulated by LPS

Human mononuclear cells (2×10^6/ml) were incubated for 12hrs in presence or absence of different concentrations of LPS (horizontal axis). Supernatants were assayed for TNF bioactivity in L929 cytotoxicity assay (hatched bars) and immunoreactive TNF alpha was assessed in a TNF alpha RIA (closed bars).
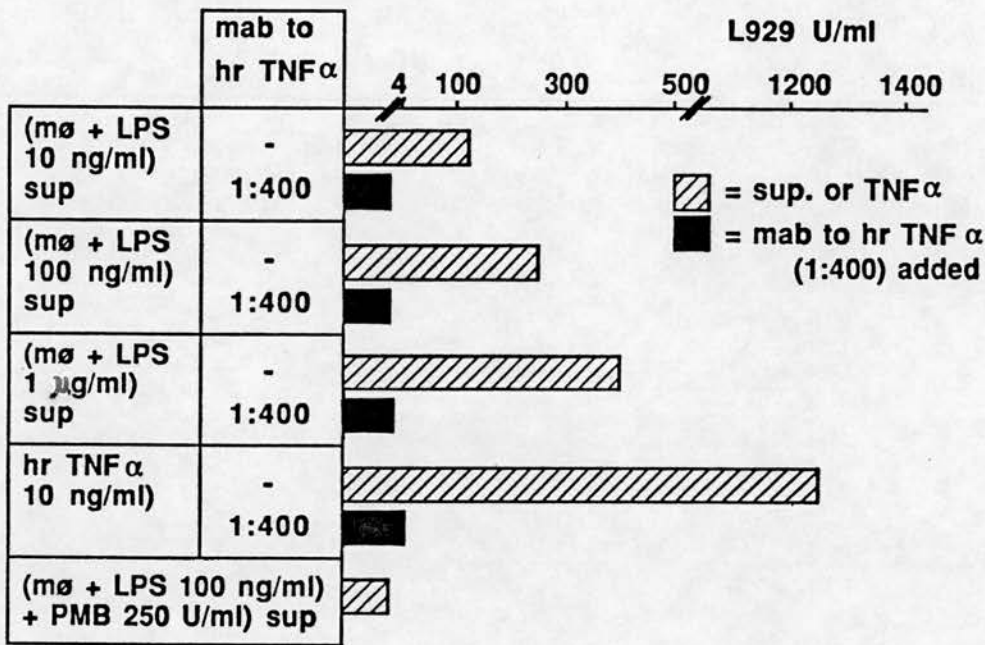
FIG 26   Antibody-neutralization of TNF-like bioactivity induced by LPS

To check that the previously tested bioactivity (Fig 25) was related to TNF alpha, supernatants from LPS stimulated mononuclear cells were tested in L929 assay in presence or absence of neutralizing mouse monoclonal antibody to human TNF alpha (1:400 dilution). Supernatant from mononuclear cells exposed to LPS (100ng/ml) in presence of 250U/ml Polymyxin B was also tested.
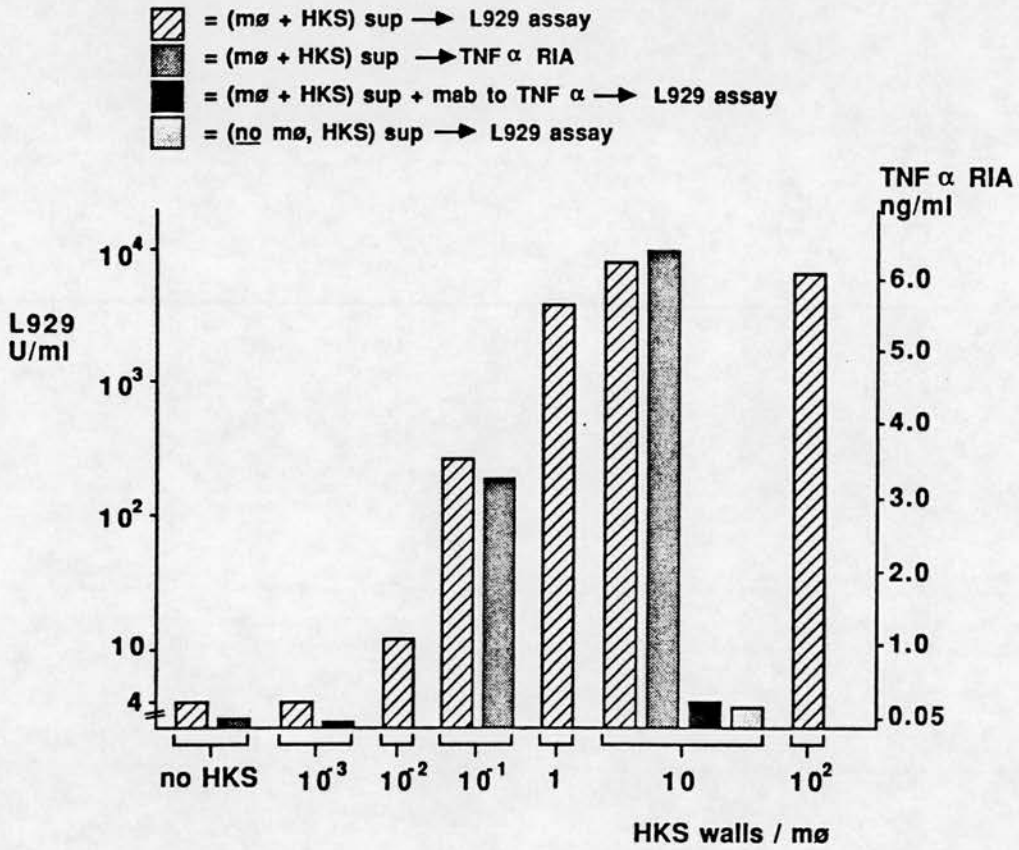
FIG. 27 Induction of TNF alpha release by heat-killed Staphylococci cell walls (HKS)

Cell cultures of human mononuclear cells (3.5×10^6/ml) were incubated with different concentrations of HKS (18hrs). Supernatants were tested for L929 cytotoxicity (hatched bars) or in TNF alpha RIA (dotted bars). The bioactivity in the supernatant from the culture stimulated with 10 bct/mononuclear cell, was completely inhibited by mab to human TNF alpha (1:800 dilution). Heat killed staphylococci, in absence of mononuclear cells, failed to induce L929 cytotoxicity.

95
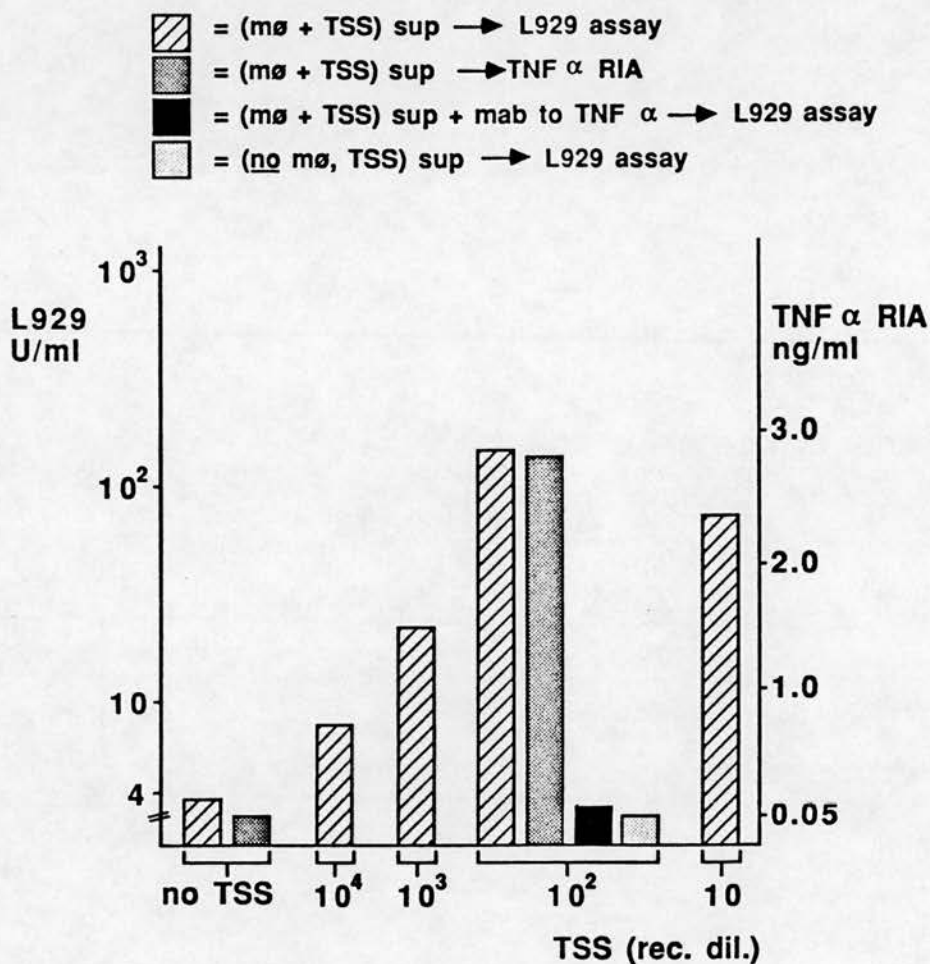
FIG. 28 Toxic shock syndrome exotoxin (TSS) induction of TNF alpha from human monocytes

TNF bioactivity and immunoreactivity was tested in supernatants from cultures of adherent mononuclear cells (10^6 monocytes/ml) incubated for 18 hrs with different dilutions of TSS (horizontal axis, reciprocal of dilution). TSS was a powerful, dose-dependent stimulator of TNF alpha release, and, when incubated in absence of monocytes, failed to elicit L929 cytotoxicity. L929 bioactivity was inhibited by preincubation of the supernatant with anti TNF alpha mab(1:400 dilution).
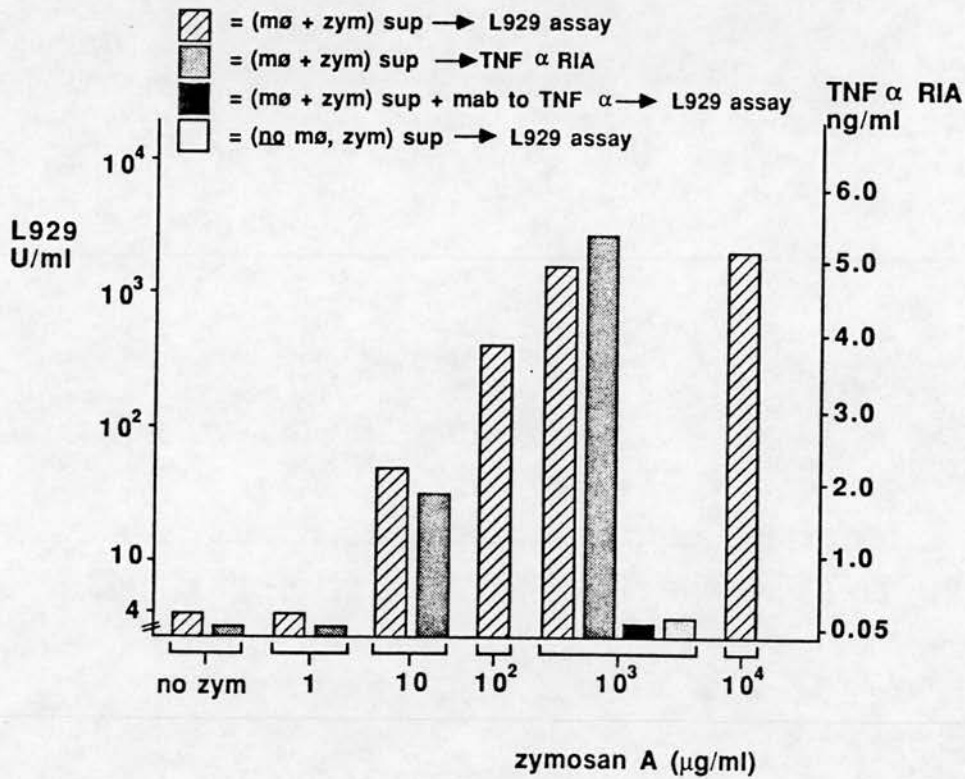
FIG. 29 Release of TNF alpha from human adherent mononuclear cells stimulated by Saccharomyces Cerevisiae cell walls (Zymosan A)

Supernatants from human adherent mononuclear cells (10^6 monocytes/ml, 18hrs) incubated in presence of different concentrations of fungal cell walls, were assayed for L929 cytotoxicity and in TNF alpha RIA. TNF bioactivity was inhibited when supernatants were preincubated with mab to TNF alpha (1:400 dilution); supernatant from Zymosan (1mg/ml) incubated in absence of cells failed to induce L929 cytotoxicity.
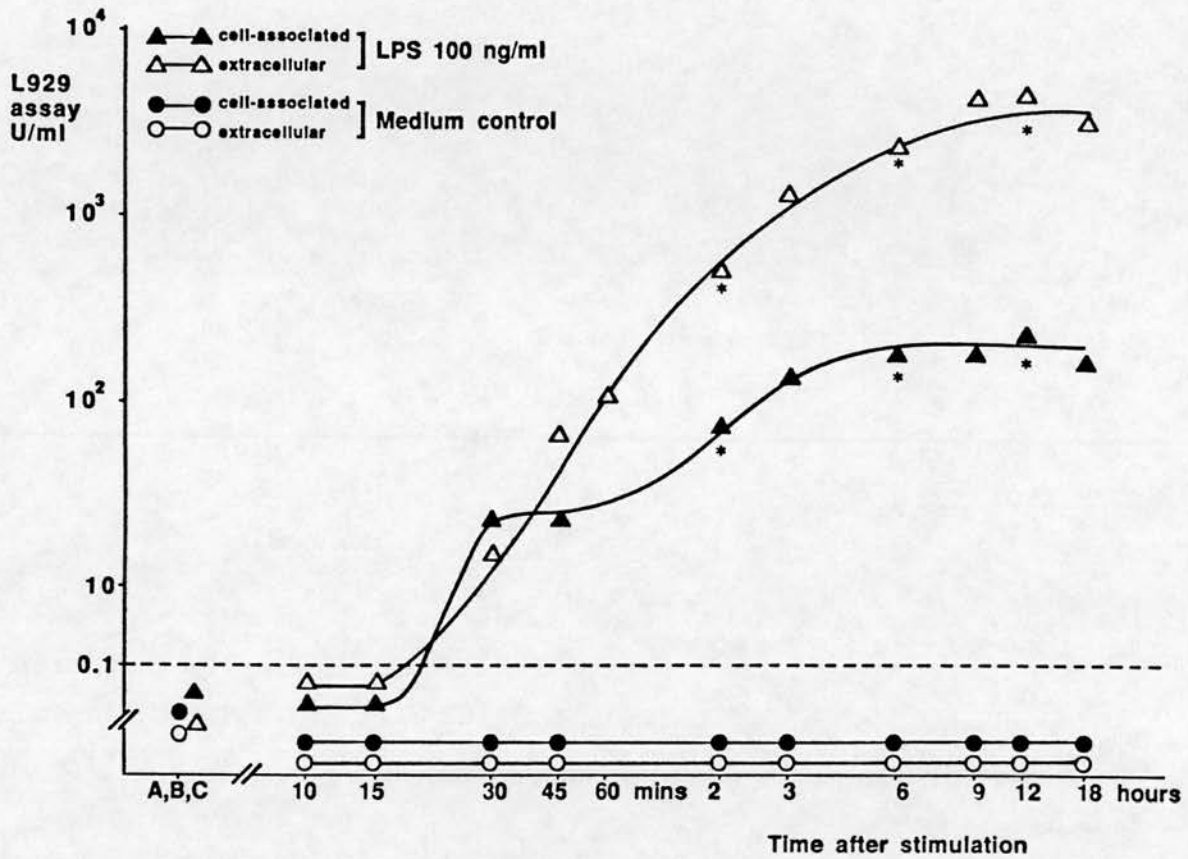
FIG. 30 **Cellular distribution and kinetics of TNF activity in LPS-stimulated human monocytes**

Human adherent mononuclear cells (1×10^6/ml) were cultured in absence or presence of LPS (100ng/ml). At different time points, supernatants and cell-lysates were prepared from the cultures, to represent extracellular or cell-associated fractions. These were tested in the L929 cytotoxicity assay for TNF activity.

Fractions indicated with (*) have also been tested in presence of neutralizing monoclonal antibody to hr TNF alpha (Table 2.5).

(A) cell-lysate of monocytes after density centrifugation
(B) supernatants and cell-lysate of cells after 40 mins plastic adherence
(C) supernatants and cell-lysate at the addition of stimulus (time point zero)

time after LPS stimulation	mab to TNF alpha	TNF bioactivity (L929 U/ml)	
		cell-associated	extracellular
2 hours	-	29.25	427.6
	1:400	< 4	< 4
6 hours	-	45.11	1544
	1:400	< 4	< 4
12 hours	-	65.33	1930
	1:400	< 4	< 4

TABLE 2.5   Effects of anti-TNF alpha antibodies on L929 cytotoxicity

To check if the biological activity on L929 cells of the fractions indicated in Fig. 30 was related to TNF alpha, they were tested in presence of monoclonal antibody to human TNF alpha (1:400 dilution).   Data on the specificity of this antibody have been presented (Fig. 13, p. 64).
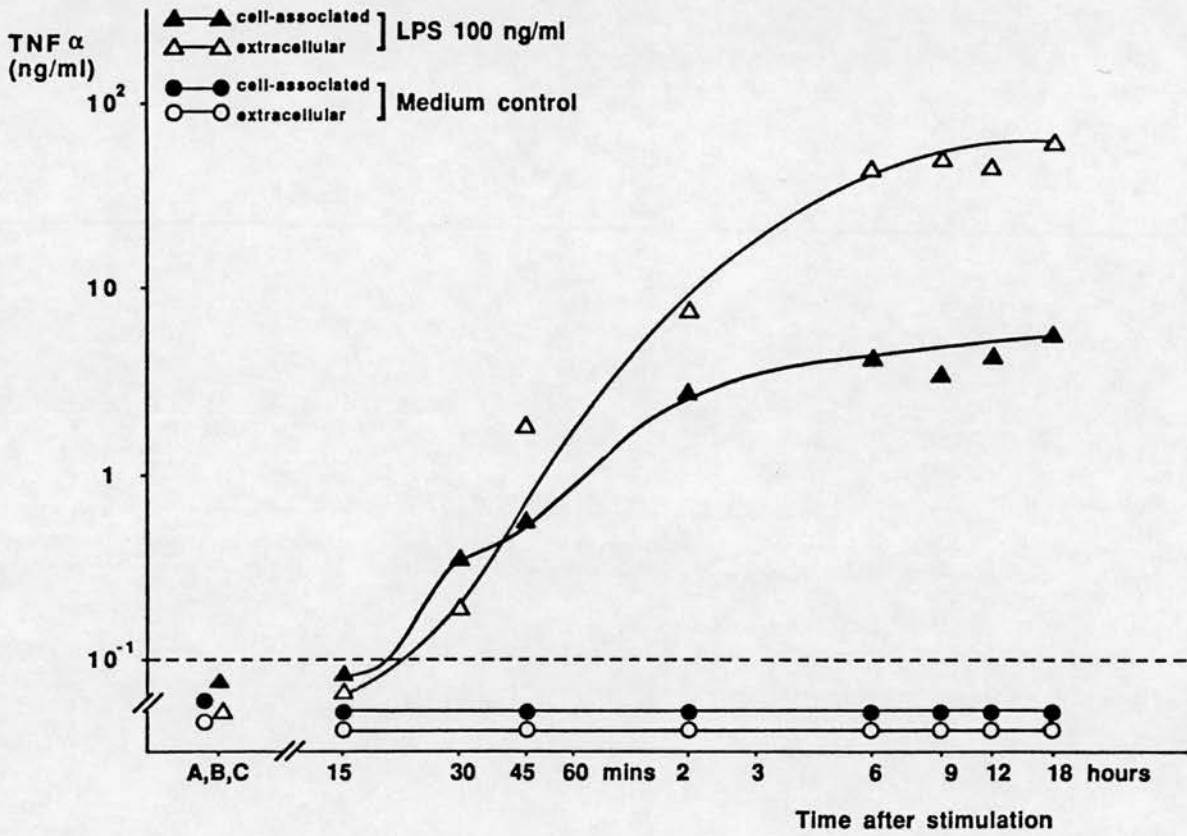
FIG. 31 Time course of TNF alpha production and release as assessed by radioimmunoassay

Kinetics of TNF alpha accumulation and cellular distribution in human monocytes were tested by TNF alpha RIA (Medgenix; assay detection limit: 100pg/ml TNF alpha)
(A) cell-lysate of monocytes after density centrifugation
(B) supernatants and cell-lysate of cells after 40 mins plastic adherence
(C) supernatants and cell-lysate at the addition of stimulus (time point zero)

100

2.d.3 DISCUSSION

The existence of a relationship between tumour necrosis factor production and infectious diseases was clearly indicated in the original observations (O'Malley et al, 1962; Carswell et al, 1975), when "Tumour Necrosis Serum" was described in BCG-primed mice injected with lipopolysaccharide (LPS).

The relevance of this association has been demonstrated in two extreme conditions: low levels of TNF production during infection with *Lysteria Monocytogenes* prevent microbial expansion and death (Havell, 1987) and seem therefore to have beneficial effects for the host; however, high and sustained levels of TNF mediate lethal endotoxaemia (Beutler et al, 1985; Tracey et al, 1987) leading to multiple organ failure and death. It could be argued that conditions leading to massive TNF synthesis (such as overwhelming infections) are going to be lethal anyway, and TNF could be beneficial in an evolutionary sense by rapidly removing a source of infection.

The production of TNF alpha by human monocytes in response to microbial agents, such as *E. Coli* LPS, toxic shock exotoxin, *Saccharomyces Cerevisiae* and *Staph. Aureus* cell walls has been tested in the absence of "priming" events. All of these products were powerful, dose dependent inducers of TNF activity which could be completely neutralized by specific anti human TNF alpha antibody. This observation is consistent with a recent analysis of the "priming" phenomenon (Gifford, Flick, 1987) which showed that single microbial agents, such as LPS can elicit TNF production *in vivo* and *in vitro* and the use of "priming protocols" (such as facultative intracellular bacteria *in vivo* or the presence of IFN gamma *in vitro*), only increase and/or extend the production of TNF.

In the experiments described here, unstimulated adherent mononuclear cells failed to release TNF alpha indicating a requirement for cellular activation. This was confirmed when we examined the kinetics of production and cellular distribution of TNF alpha: no TNF biological activity or immunoreactive protein could be demonstrated at any time-point in unstimulated cells or in their supernatants.

Upon LPS stimulation, TNF biological activity accumulated in the cell-associated fraction from 30 mins after induction, to reach a maximum by 9-12 hours. However, most of TNF activity (neutralized by specific antibody to TNF alpha) was found in the supernatants. These findings were confirmed when the presence of TNF alpha was tested by specific radioimmunoassay: TNF alpha mainly accumulated in the extracellular compartment (56.8ng/ml at 18hrs), exceeding immunoreactive TNF which remained cell associated (4.8ng/ml at 18hrs). It is noteworthy that, even if TNF alpha translocation appears to be efficient from the first minutes of protein accumulation, a small but significant proportion of TNF alpha biological activity and TNF alpha protein could be found cell-associated. This phenomenon, particularly evident in the first hour, is compatible with recent reports of cell-associated forms of TNF alpha (Kriegler et al, 1988; Decker et al, 1987) and could be of importance in cellular interactions.

When compared to the kinetics of cellular distribution of IL1 alpha and IL1 beta (Fig. 32), TNF alpha translocation mechanisms appear to be activated simultaneously with protein accumulation (44% translocated at 30mins), to achieve 90.5% release of TNF alpha at 18 hours: extracellular translocation of IL1 alpha and IL1 beta, on the contrary, is delayed of about 6-9 hours for IL1 alpha (amounting to 5-10% of total protein accumulated) and of 1 to 4 hours for IL1 beta (achieving a maximum of 65% at 9 hours after stimulation).

These differences probably reflect the different structures of these proteins (IL1 alpha and IL1 beta, unlike TNF alpha, lack a leader sequence). Given their broad spectrum of similar properties (Dinarello, 1987), the observation that the kinetics of secretion of TNF alpha, IL1 beta and IL1 alpha differ so dramatically could be surprising. However, the regulation of the activity of these three cytokines *in vivo* could be regulated in different situations by the level of their release from the cell or the availability of bioactive cytokine on the membrane during cell-cell interaction. These differences, and the possibility that structurally different proteins would have differential access to recognition structures *in vivo* could be the reason why three separate molecules should have evolved independently and yet remain functionally similar.

The kinetics of TNF alpha protein accumulation observed, and its absence in unstimulated cells, identify TNF alpha as a typical secretory protein, with short-lasting expression dependent on cellular activation.
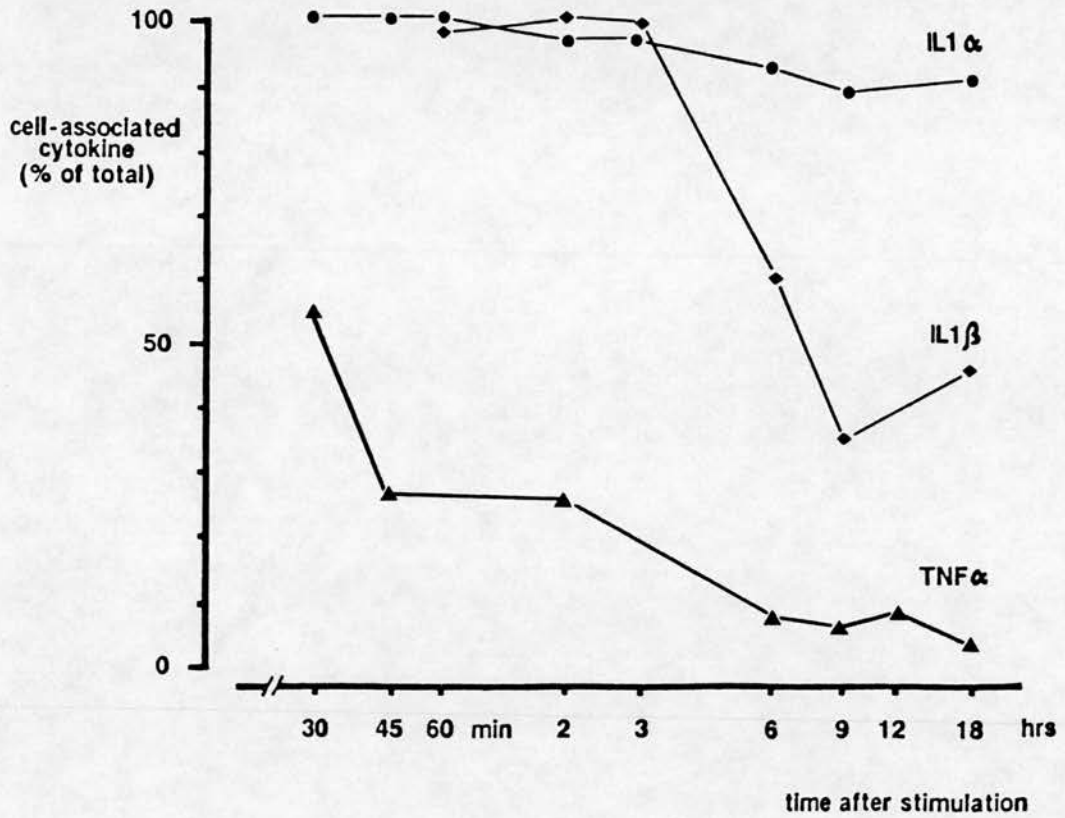
Fig. 32 Comparison of TNF alpha, IL1 beta and IL1 alpha kinetics of cellular distribution in human monocytes upon LPS stimulation

Time-related extracellular translocation of immunoreactive cytokines in human adherent mononuclear cells upon LPS stimulation (100ng/ml).

On the vertical axis is expressed the relative amount of TNF alpha, IL1 beta or IL1 alpha which accumulates in the cell-associated compartment.

103

2.e.   **CHAPTER 3**

Induction of IL-1 by pro-inflammatory crystals

2.e.1. INTRODUCTION

The role of IL1 in the host response to microbial pathogens has been discussed. Interleukin 1 is produced both by direct interaction of microbial products with competent cells, or within antigen-mediated immune responses, in which IL1 acts and is produced at different stages. Apparently unrelated to the concept of host defense against infections, a "miscellaneous" group of agents have been reported to induce LAF/EP activity (Table 2.6). It has to be stressed that before accepting a novel stimulus of IL-1 production, it must be extensively demonstrated that LPS, a ubiquitous contaminant in most laboratories was not responsible and that the stimulus itself, if carried over in the IL-1 bioassay could not mimick IL-1 effects. There are also reports of "spontaneous" production of IL-1 by mesangial cells (Lovett et al, 1983) glioma cell lines (Fontana et al, 1982), T cell leukemia lines (Okada et al, 1986) and in monocytes from a variety of diseases. Before accepting the concept of "spontaneous" production as reflection of *in vivo* activation, mRNA or protein data should be provided from samples that have been metabolically arrested seconds after excision or venepuncture.

Crystals of Monosodium Urate have previously been described as potent stimulators of EP activity from monocytes (Duff et al, 1983). This was a dose-dependent effect, did not require serum factors or mediation by other blood leukocytes, and was independent from phagocytosis of the crystals, and LPS contamination could not be implicated in this induction. Pyrogenicity however is not unique to IL-1, since recombinant TNF (Dinarello et al, 1986), IFN alpha (Dinarello et al, 1984) and IL6 (Dinarello et al, 1988) are also pyrogenic.

In the present study IL-1 stimulation by inflammatory crystals, was examined measuring IL-1 in a T-cell comitogenic assay in which TNF and alpha IFN are not stimulatory. Kinetics of cellular distribution were tested for IL1 biological activity and specific IL1 proteins, to study the mechanism of release involved.

The crystals tested, MSU, HA and CPPD are all associated with human arthritis: MSU is the necessary aetiologic agent of gout (Malawista, 1977), HA is involved in a complex inflammatory arthritis and CPPD crystals are

agents of chondrocalcinosis, and an inflammatory condition resembling gout - called "Pseudogout" (reviewed in Ryan and McCarty, 1985).

Establishing IL1 as a pathogenic link between endogenous crystal formation and local and systemic pathology could provide human models for the inflammatory consequences of acute and chronic overproduction of IL1 in the absence of infection.

TABLE 2.6

AGENT	PRODUCING CELL	ACTIVITY INDUCED	AUTHOR(S)
A. Natural Exogenous			
U.V. irradiation	Epithelial cells	LAF	Luger et al, 1983; Gahring et al 1984
	Macrophages	LAF	Matsushima and Oppenheim, 1985
Silica Crystals	Macrophages	LAF	Gery et al, 1981
	Macrophages	EP	Duff et al, 1983
	THP-1 cells	LAF	Krakauer et al, 1983
Colchicine	Macrophages	EP	Bodel, 1976
	Macrophages	LAF	Stosic-Grujicic, Simic, 1982
Aluminium hydroxide	Neutrophils	LAF	Yoshinaga et al, 1985
Tobacco glycoprotein	Macrophages	LAF	Francus et al, 1987
B. Synthetic Exogenous			
Muramyl-dipeptide	Macrophages	EP	Dinarello et al, 1978
	Macrophages	LAF	Oppenheim et al, 1980, Gery, Lope-Zuniga, 1983
Phorbol-Myristic	Monocytes	LAF	Mizel et al, 1978; Gery, Lope-Zuniga, 1983
Acetate	Epithelial cells	LAF	Luger et al, 1983
Dyalisis membrane	Monocytes	LAF	Lonnemann et al, 1987
Hydroxyurea	Monocytes	LAF	Matsushima, Oppenheim, 1985
Ca Ionophore A23187	Monocytes	LAF	Matsushima, Oppenheim, 1985
Poly I: Poly C	Rabbit leukocytes	EP	Nordlund et al, 1970B
C. Endogenous			
Urate crystals	Macrophages	EP	Duff et al, 1983
Etiocolanolone	Leukocytes	EP	Bodel, Dillard, 1968
Glucocerebrosides	Macrophages	LAF	Gery et al, 1981
Lithocolic Acid	Leukocytes	EP	Bondy, Bodel, 1971

2.e.2. RESULTS

2.e.2.1. Crystal induced IL-1 activity from blood monocytes

Crystals of monosodium urate in doses from 0.1 to 1.6mg/ml were potent inducers of monocyte extracellular IL-1 activity (Fig. 33). LPS-free crystal of MSU (8-40um), HA (3-15um) and CPPD (15-50um) were added at different concentrations to human monocytes ($1x10^6$/ml). After 20hr incubation, supernatants were assayed in triplicate for LAF activity. In Fig. 33 are shown cumulative data from different experiments. Individual induction experiments (Fig. 34) give much clearer dose/response relations. This is in keeping with the well known heterogeneity of IL1 production (Endres et al, 1987) in different individuals.

These supernatants were tested for IL2 bioactivity on CTLL-2 cells or T lymphoblasts and showed no detectable TCGF activity (data not shown). The T cell assay used for LAF activity was not influenced by the presence of human TNF alpha or IFN alpha (2.b.3.2, pp 46,47). When tested with rabbit antiserum to IL-1 (Table 2.7), MSU-induced IL-1 activity was completely neutralized. This antiserum did not neutralize T cell responses to IL2, and we never observed nonspecific suppressive effects of this antiserum on D10 cells, thymocytes or CTLL cells (data in Figs. 6-7, p. 58).

HA and CPPD crystals did not stimulate significant extracellular activity in any of the concentrations tested. Pretreatment of the crystals with serum (Fig. 35) did not consistently influence the ability of the crystals to induce IL1 activity, with exception perhaps of the highest concentration tested.

2.e.2.2. IL1 adsorption by crystals

It has been reported (Kozin, McCarthy, 1976; Hasselbacher, 1982) that proteins can be readily adsorbed on to the surface of crystals. It was possible that CPPD and HA crystals were stimulating extracellular IL1 release and adsorbing IL1 on their surface, so that IL1 could not be detected in a centrifuged supernatant.

To test this different concentrations of the three crystals were incubated with a human monocyte-derived IL1 crude preparation. After 20hrs ($37^{\circ}C$, 5% CO_2), the crystals were precipitated by centrifugation (1800G,

4°C, 30mins) and supernatants tested for mitogenic effects on D10 cells, compared with the same crude IL1 preparation that had been incubated in absence of crystals. None of the crystals (Fig. 36) reduced the biodetectable IL1 in the medium after coincubation. Significant IL1 adsorption cannot thereafter be responsible for the failure to detect IL1 in supernatants from monocytes induced with HA or CPPD crystals. Similar results were obtained when IL1 was exposed to crystals in an agitated incubation (data not shown).

2.e.2.3.   Co-stimulation with LPS and HA or CPPD crystals

HA and CPPD crystals could be stimulating intracellular IL1 production but failing to release it extracellularly, or non-specifically inhibiting IL1 processing, release or detection.

To test this, HA or CPPD crystals were used in conjunction with bacterial LPS. A dose of 1ng/ml LPS was used (in the suboptimal range for IL1 induction) sufficient to stimulate IL1 synthesis and its release in a bioactive form in the medium.

The presence of HA and CPPD with LPS in co-culture did not alter the amount of detectable IL1 activity stimulated by LPS alone (Fig. 37). This means that HA and CPPD crystals did not interfere with mechanisms of IL1 production, release, or the detection of IL1 biological activity.

2.e.2.4.   Fibroblast proliferation

Crude MSU-induced supernatants that had LAF activity on D10 cells were tested for induction of proliferation of human rheumatoid synovial fibroblasts (Fig. 38). Dose-dependent fibroblast growth was obtained with the monocyte supernatant, as well as with a highly purified human monocyte IL1 ("22KDa factor").

2.e.2.5.   IL1 activity from crystal-induced synovial fluid cells

Human rheumatoid synovial exudate adherent mononuclear cells $(1 \times 10^6/\text{ml})$ were incubated in presence or absence of MSU or HA crystals. Supernatants at 20hrs showed that "unstimulated" cells released significant amounts of IL1 activity, reflecting *in vivo* activation or inadvertent stimulation during the separation procedure. These could be further stimulated (Fig. 39) by MSU crystals but not by HA crystals. This IL1-like

activity was also completely neutralized by specific antiserum to human IL1 (Table 2.7).

2.e.2.6. Time course of production and cellular distribution of IL1 bioactivity and proteins by human monocytes stimulated by crystals

Supernatants and cell-lystates prepared by incubation of human peripheral blood monocytes with different crystals (MSU, HA, CPPD) for variable lengths of time (15mins to 18hrs) were tested for IL1-like activity in the EL4/CTLL conversion assay. In MSU-stimulated cells, the cell-associated fractions (lysates) (Fig. 40) yielded more bioactive IL1, exceeding the extracellular bioactivity at each time point. IL1 was detectable in the cell-lysates by 45 minutes, and in the extracellular compartment by 2 hours.

Cells cultured in presence of HA and CPPD crystals failed to release any detectable bioactivity, but expressed low levels of bioactivity (equivalent to 50-70pg of human recombinant IL1) in the cell lysates at 2-6 hours.

To test the relative amounts of IL1 alpha and IL1 beta in the different compartments after MSU stimulation, the fractions from the previous experiments were tested with specific immunoassays for IL1 alpha and IL1 beta.

IL1 alpha was detectable after 3 hours in the cell-lysates, to reach 6.25ng/ml at 18hrs. Cell-lysate levels constantly exceeded IL1 alpha content in the supernatants, where IL1 alpha was found at 6 hours after stimulation, to reach 0.75ng/ml at 18hrs (Fig. 41).

The pattern of IL1 beta cellular distribution (Fig. 42) appeared to be different, as immunoreactive IL1 beta in the supernatants exceeded cell-associated protein by 6 hours and at 18 hours 95% of total IL1 beta (6.3ng/ml) could be found in the extracellular compartment.

110
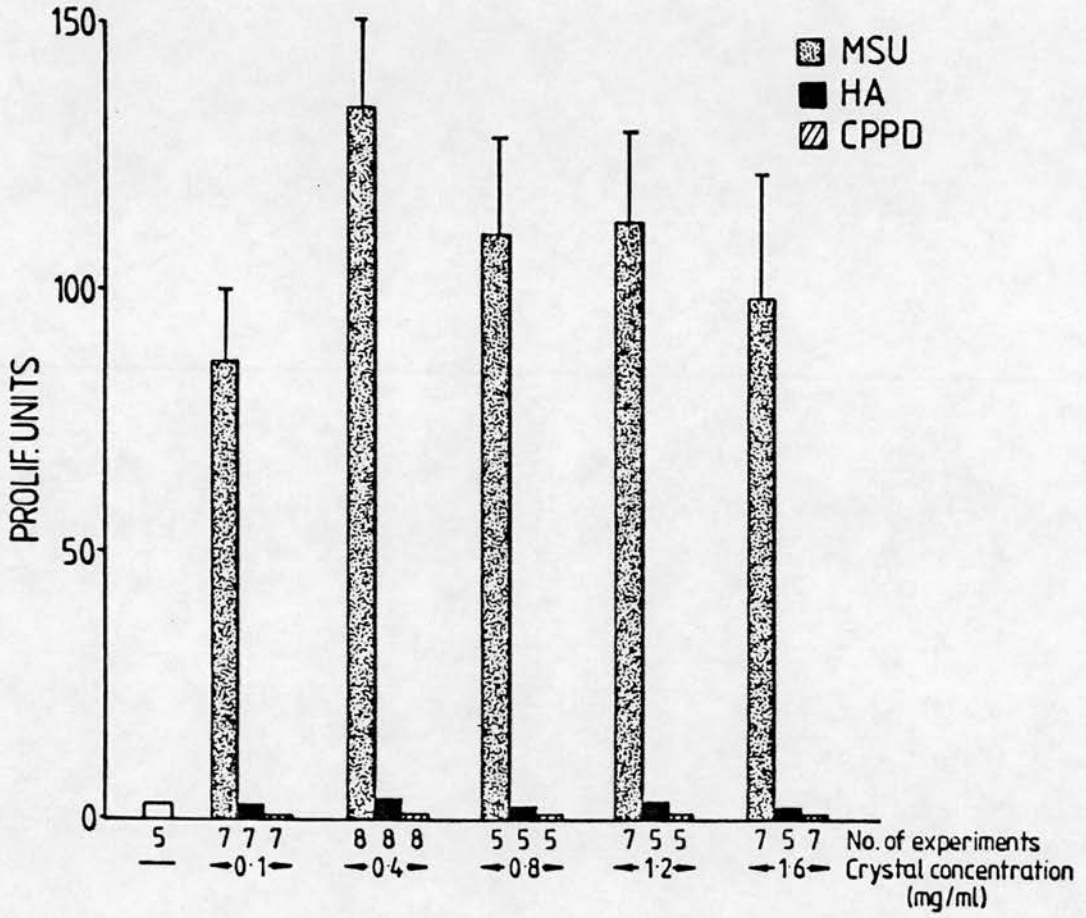
FIG 33   Release of IL1 bioactivity from human monocytes stimulated with crystals

Supernatants from crystal-stimulated mononuclear cells were assayed in three dilutions (1:1000, 1:100, 1:10) for LAF/IL1 activity in the D10.G4.1 assay. In the figure are shown results for 1:100 dilution (in complete medium) of supernatants. Data are expressed as mean (+SEM) for the number of experiments indicated.
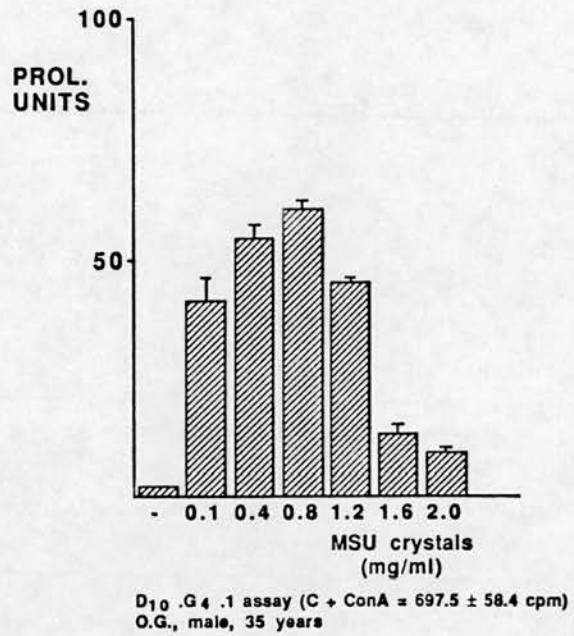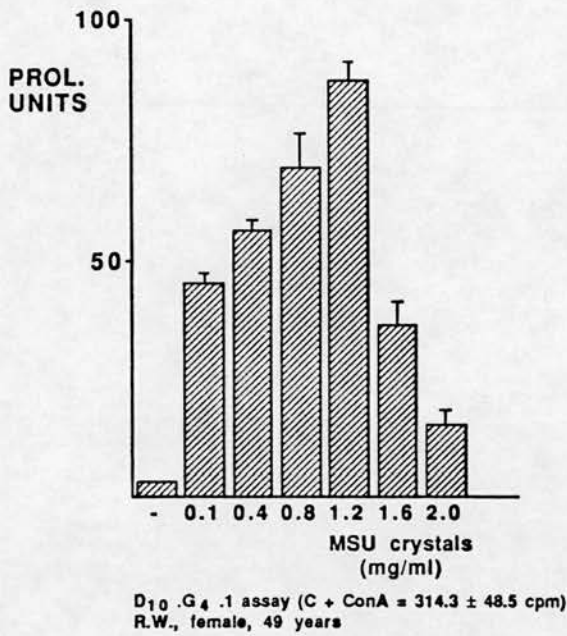
FIG. 34 Release of IL1 activity from monocytes stimulated with MSU crystals is dose-related.

Production of IL1 from monocytes of two normal volunteers after MSU crystal stimulation. Supernatants were assayed in three different concentrations (1:100 shown). Data are expressed as mean +SEM of triplicates in proliferation units.

	anti IL 1 antiserum				
	0	1:200	1:400	1:800	1:1600
IL 1	169±13	1.09±0.1	1.34±0.2	1.06±0.03	1.33±0.22
MSU-PBMC	62±5	1.14±0.2	3.92±0.34	12.43±0.82	29.66±1.48
MSU-SC	43±0.4	1.03±0.1	0.81±0.1	1.04±0.17	1.06±0.18

Table 2.7 Antiserum to IL1 inhibits LAF activity from cells stimulated with MSU crystals

Mitogenic responses of D10 cells (in proliferation units). Mean ±SEM of triplicate responses to IL1 and mononuclear cell supernatants are shown. Abbreviations: IL1, human recombinant IL1 beta (100pg/ml): MSU-PBMC, 1:100 supernatant of 10^6 adherent blood mononuclear cells cultured with 0.4mg/ml MSU crystals: MSU-SC, 1:100 supernatant of 10^6 adherent mononuclear synovial cells cultured for 20hrs with 0.4mg/ml MSU crystals (cpm cells + Con A = 300.67±12.6)
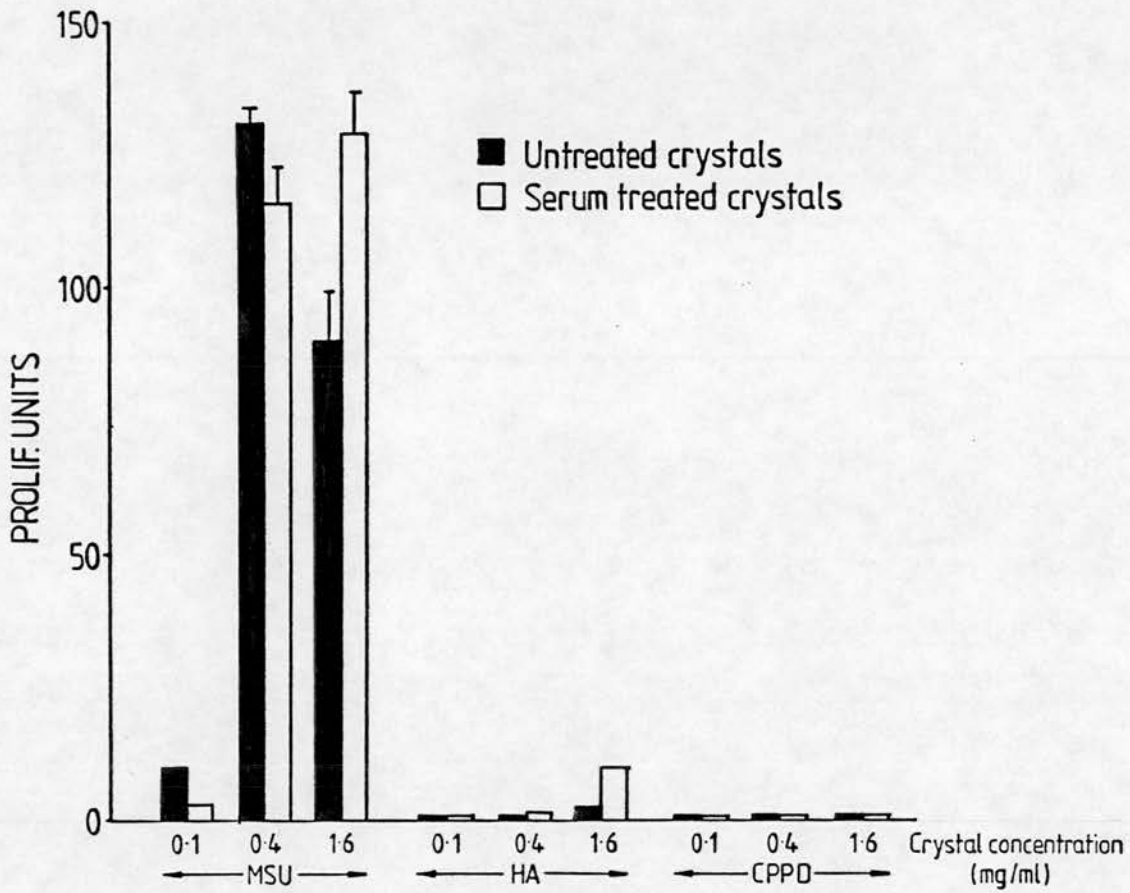
FIG 35   Serum-pretreatment of crystals does not consistently influence induction of IL1 activity

Untreated crystal and serum pretreated crystals were tested for their ability to induce IL1 activity from human peripheral blood monocytes (10^6/ml, 20hrs). Supernatants were tested in 3 dilutions (1:20, 1:100, 1:500) in D10.G4.1 assay. Results are expressed as mean (±SEM) of triplicate measurement of supernatants diluted in 1:100 in medium (cells + Con A = 265 ± 16cpm).
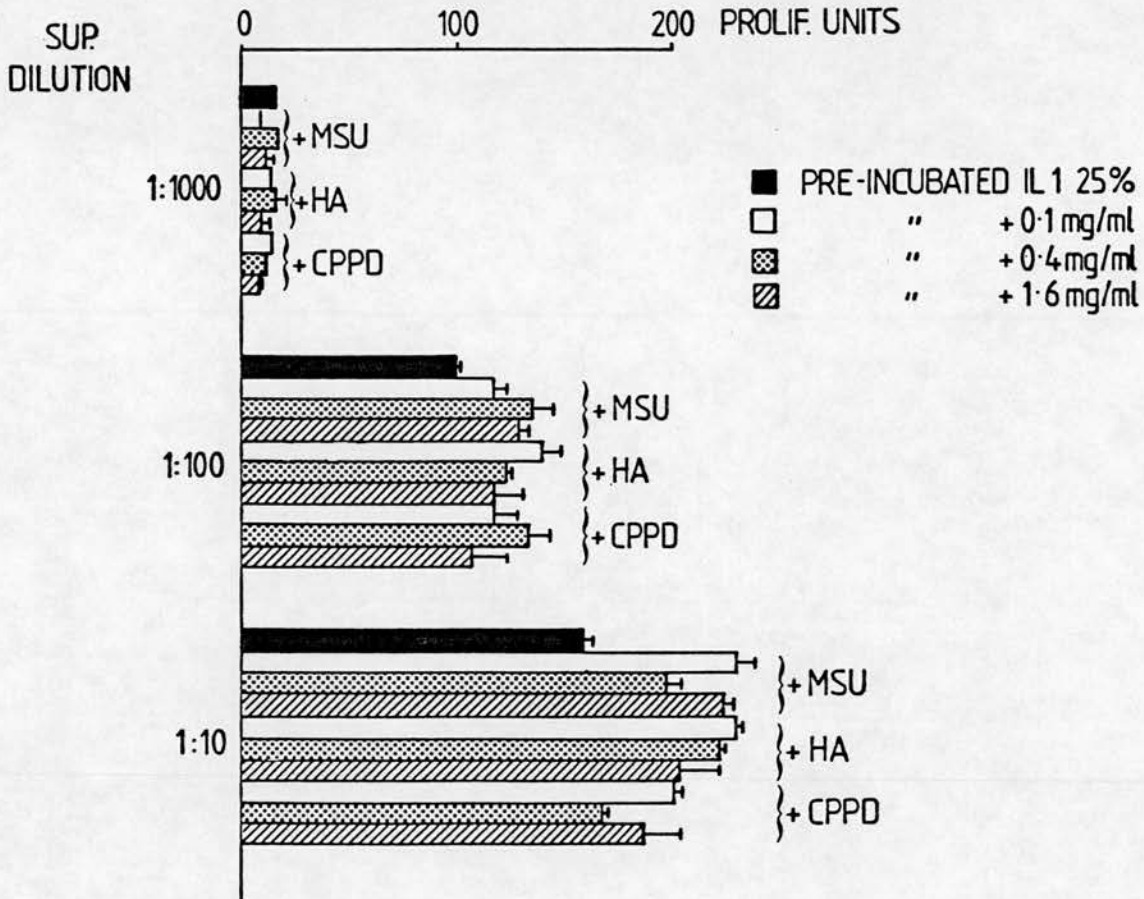
FIG 36 IL1 adsorption by crystals

To test whether surface adsorption of IL1 by any of the three crystals might remove significant quantities of IL1 from the solution, crude IL1 preparation was added to each of the crystals at 37° C for 20hrs.

Centrifuged supernatants were then tested for LAF activity in the D10.G4.1 assay and compared with the same IL1-containing supernatants incubated in absence of crystals. Means (±SEM) of proliferation units are given (cells + Con A = 179 ± 31cpm).

MONOCYTES WITH

NO STIMULUS
HA CRYSTALS
CPPD CRYSTALS
LPS
HA + LPS
CPPD + LPS

Crystals 0.5mg/ml
LPS 1.0 ng/ml
Sups dilution 1:100

100   200
PROLIF. UNITS
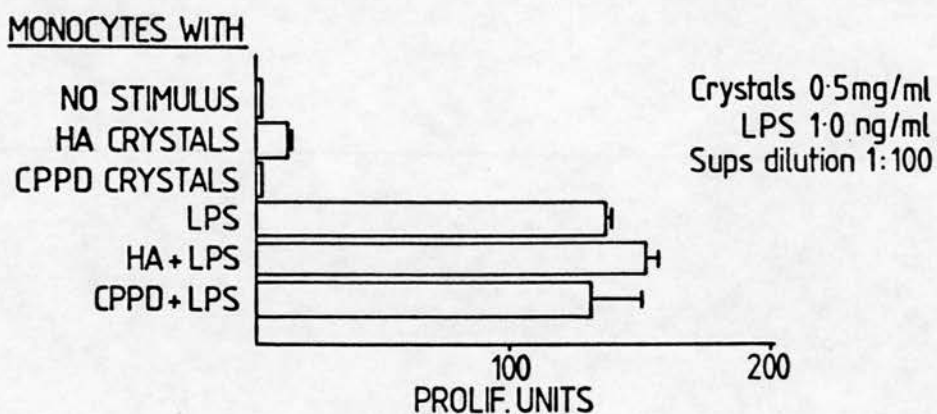
FIG 37   Co-incubation of crystals with lipopolysaccharide

To test if HA and CPPD were inducing IL1 synthesis but interfering with release or biodetection, crystals and bacterial LPS, an independent stimulus for both IL1 synthesis and release, were used.

As LPS was used in suboptimal concentration, significant augmentation or depression of IL1 release would have been detectable. Three dilutions of supernatant tested, 1:100 triplicate shown (D10.G4.1 assay; Cells + Con A = 334 ± 19cpm).

116
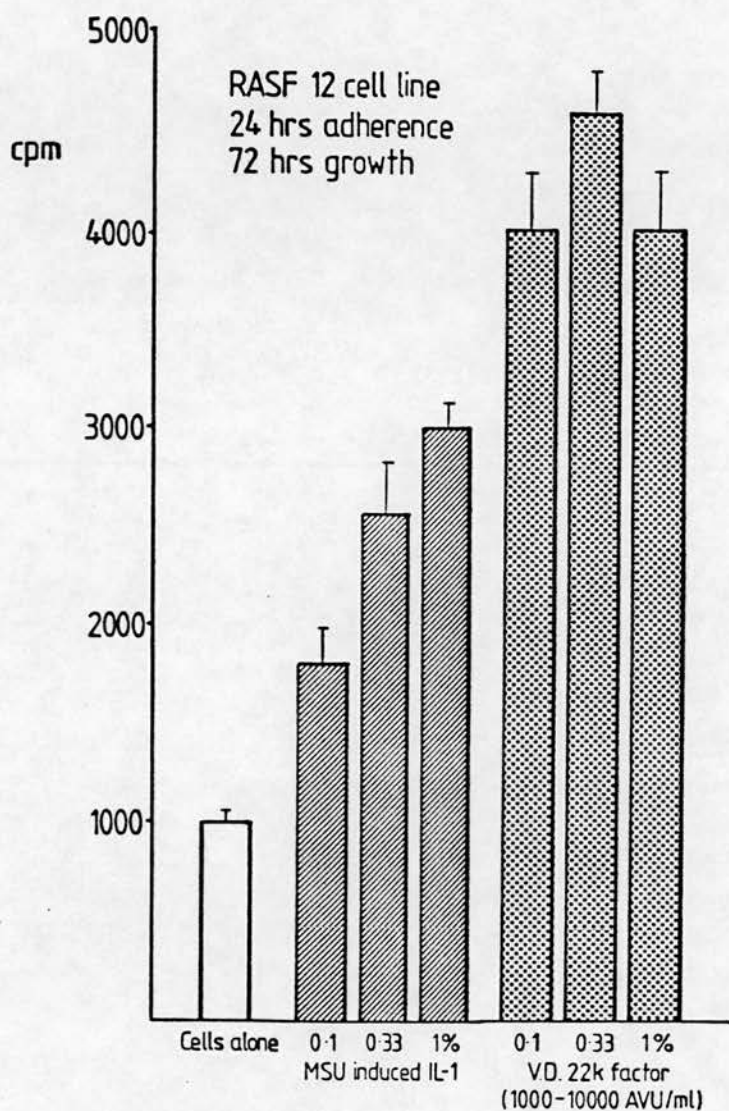
FIG 38   Fibroblast proliferation

MSU induced supernatants, with LAF activity on D10.G4.1 were tested for stimulation of human articular fibroblast proliferation.

^{3}H-thymidine incorporation was assessed after 3 days incubation. For comparison, response to highly purified IL1 beta (22Kd factor) in the same experiment is shown. Results represent mean ±SEM of triplicate wells.

117
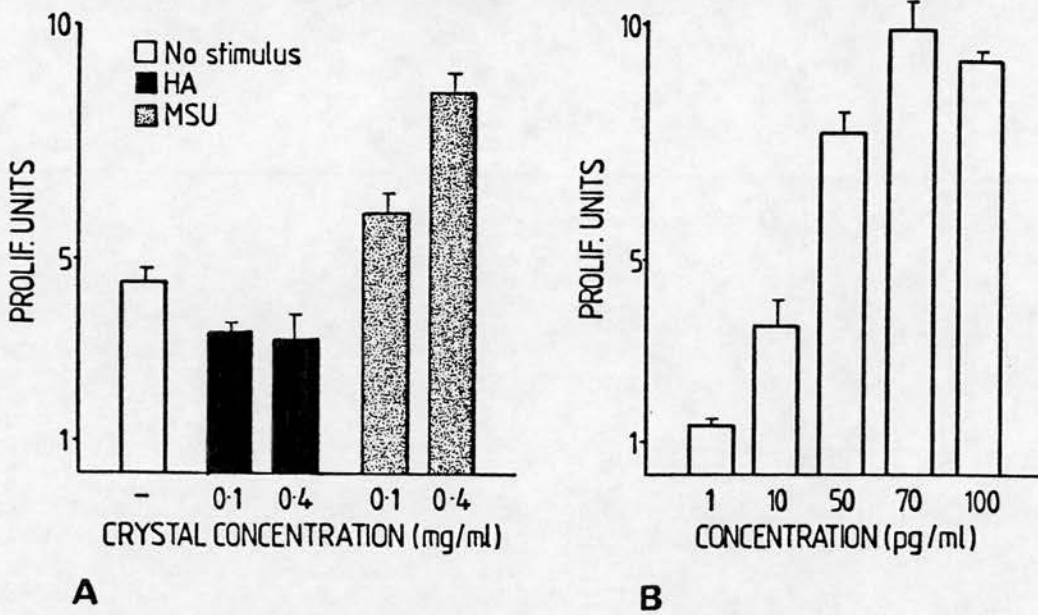
FIG 39   Crystal-induced IL1 activity from synovial fluid cells

A   Adherent synovial fluid mononuclear cells $(1 \times 10^6 \text{cells/ml})$ were incubated in absence or presence of crystals (HA, MSU) at different concentrations.   Supernatants of 20hrs incubation were tested in triplicate in a murine thymocyte assay (CBA/Ca; cells + Con A = 2493 ± 68cpm).   IL1 activity in the supernatant was completely neutralized by anti IL1 antiserum (Table 2.7).   Results are from supernatants diluted 1:100 and are means ±SEM of proliferation units in triplicate wells.

B   Activity of human recombinant IL1 beta in the same murine thymocyte assay.
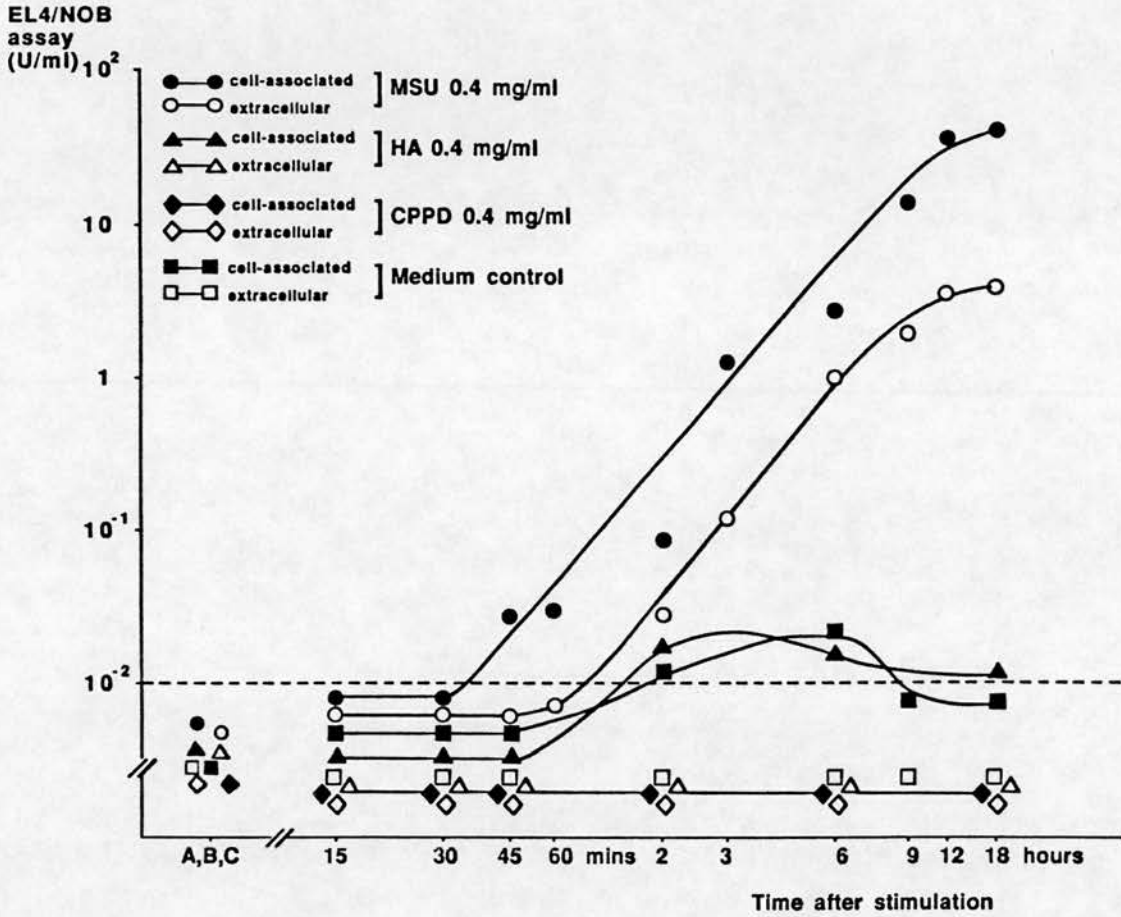
FIG 40   Kinetics of IL1 activity accumulation by crystal-stimulated adherent mononuclear cells

Extracellular and cell-associated fractions from human adherent mononuclear cells stimulated by crystals were tested for IL1 bioactivity in an EL4/CTLL2 assay. Results are expressed in U/ml from triplicate wells (1U = IL1-like activity equivalent to 1ng/ml human recombinant IL1 beta).
A = cell-lysate after density centrifugation
B = cell-lysate and supernatants after 40 mins plastic adherence
C = cell-lysate and supernatants at the addition of stimulus (time zero)

119
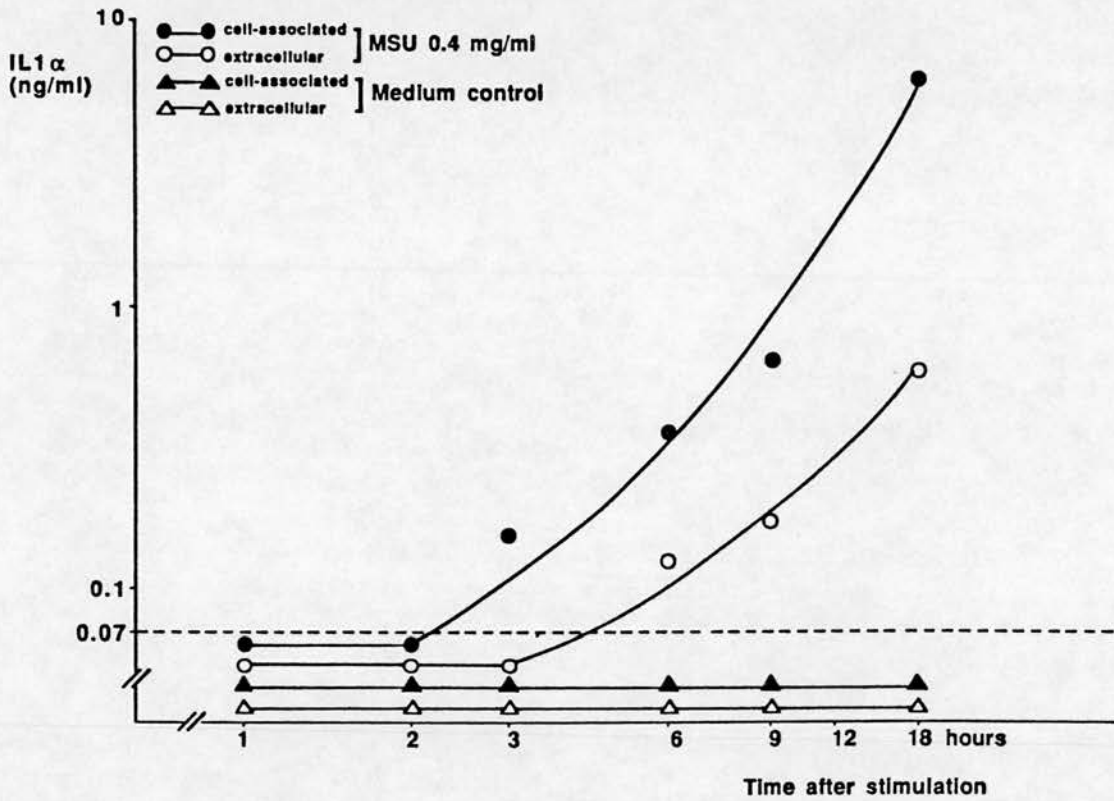
FIG 41   Kinetics of immunoreactive IL1 alpha accumulation in MSU stimulated monocytes

Interleukin 1 alpha was tested in the different fractions using an IL1 alpha-specific RIA (Amersham, UK). Results are means of duplicate tubes. The lower detection limit of this assay is 70pg/ml hr IL1 alpha.
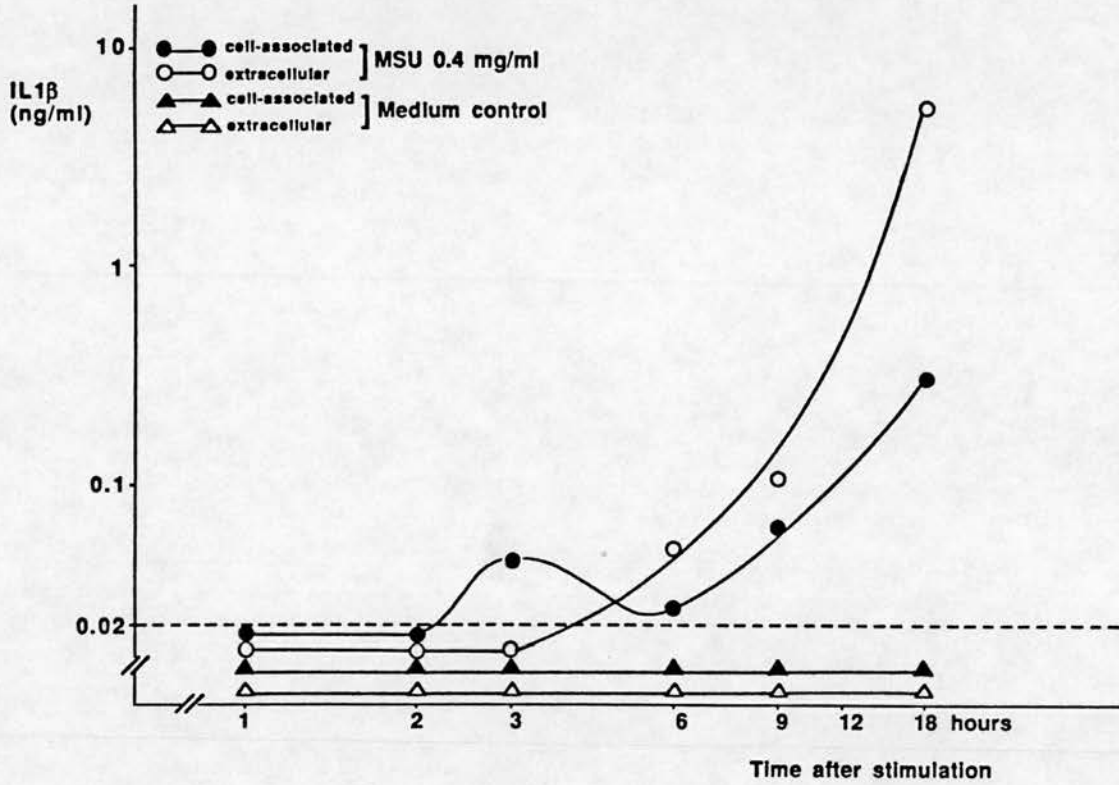
FIG 42 **Accumulation of immunoreactive IL1 beta in human monocytes with MSU stimulation**

Levels of IL1 beta in the cell lysates and supernatants from 10^6/ml human adherent mononuclear cells were tested using an IL1 beta-specific ELISA (Cistron Biotech./Lab. Impex; det. limit 20pg/ml). Results are mean of triplicate wells.

121

2.e.3.   DISCUSSION

The circumstantial evidence for IL1 involvement in human arthritis and the existence of a sterile rheumatic condition (gout) in which the arthritogenic stimulus (precipitation of MSU crystals) is known lead us to test whether MSU crystals were a sufficient stimulus for IL1 induction *in vitro*.

It had been reported that MSU crystals were potent stimulators of endogenous pyrogen (EP) activity from human monocytes and rabbit macrophages (Duff et al, 1983). In the present study, MSU and other pro-inflammatory crystals (HA, CPPD) were tested for their ability to release IL1 from human monocytes. Interleukin 1 bioactivity was tested in a LAF assay (D10.G4.1 T cell comitogenesis assay) insensitive to the actions of human TNF alpha and IFN alpha, two cytokines reported to have EP activity (Dinarello et al, 1986B, Dinarello et al, 1984). These cytokines did not induce proliferation in the T-cell assay nor modify the response to human IL1.

Monosodium urate, but not the other crystal-types tested, were potent stimulators of extracellular IL1 activity that was neutralized by specific anti IL1 antibodies. This property of MSU crystals was not related to the presence of LPS, a ubiquitous contaminant able to induce IL1 synthesis: cells that had not been deliberately stimulated failed to produce detectable IL1, indicating the absence of bioactive LPS in the media and plasticware used for the experiments. The crystals were LPS-free, as assessed by different criteria: MSU crystals were negative in the Limulus Gelation Test for LPS and did not inhibit the test; the inducing properties of MSU were not inhibited by the presence of Polymyxin B, a known neutralizer of LPS activities (Duff, Atkins, 1982B); and soluble products from the   crystals failed to induce any detectable IL1 activity from human monocytes.

In this study, it appeared that stimulating activity of MSU was not related to the presence of serum factors and previous work also showed that internalization of crystals was not necessary (Duff et al, 1983).

The widespread belief that phagocytosis is a sufficient stimulus for IL1 production is not supported by experimental data: phagocytosis of LPS-free latex beads *in vitro* and *in vivo* does not induce IL1 activity (Berlin, Wood 1964; Koyabashi, Friedman, 1964; Oken et al, 1981; Duff et al, 1983), nor do living intracellular parasites (such as *Leishmania Tropica*) or phagocytosis of their killed and opsonized amastigotes (Crawford et al,

1983). Conversely, several particulate agents were not prevented from inducing EP/LAF by preincubation of the monocytes with agents inhibiting internalization: colchicine did not inhibit EP induction by *Staph. Epidermidis* (Bodel, 1976) and cytochalasin B did not stop stimulation by MSU and silica crystals (Duff <u>et al</u>, 1983) or *Borrelia* spirochetae (Butler <u>et al</u>, 1982).

The two other crystal-types tested, HA and CPPD, did not induce extracellular IL1 activity at the concentrations tested. This failure appeared to be unrelated to interference with IL1 release or detection, since the presence of neither crystal altered the amount of extracellular IL1 produced by monocytes in response to a suboptimal concentration of an independent stimulus, LPS. It is also unlikely that the failure to detect IL1 was due to direct crystal adsorption of IL1 since monocyte supernatants did not show any loss of IL1 activity after incubation in presence of crystals. In time-course experiments, low levels of intracellular IL1 bioactivity were detected in HA-stimulated cells but also in unstimulated monocytes, suggesting that this was not related to the presence of the crystals. The different inducing properties of MSU, CPPD and HA crystals also seem to be unrelated to different crystal sizes (Malawista <u>et al</u>, 1985). The same HA and CPPD crystal preparations used in these experiments were, like MSU crystals, potent stimulators of neutrophil superoxide anion production, demonstrating that they were capable of pro-inflammatory leukocyte activation (Palit <u>et al</u>, 1986).

Regarding the mechanisms of IL1 induction by crystals, it has been suggested (Gery, Lope-Zuniga, 1983) that IL1 release was related to the membranolytic activity of the crystals. This would require the presence of an intracellular pool of IL1 in absence of stimulation.

In time-course experiments, we were unable to demonstrate significant bioactive or immunoreactive IL1 in unstimulated cells: IL1 accumulation only occurred following MSU-stimulation of monocytes. Bioactive IL1 was demonstrable in cell-associated fraction by 45' after stimulation, compatible with a direct induction of IL1 by MSU crystals; bioactivity in the cell-lysates at all time points exceeded extracellular bioactivity by a factor of 3 to 5. These data are comparable to those obtained in previous experiments using LPS stimulation.

Use of specific immunoassay showed that IL1 alpha was the predominant form of cell-associated IL1 (90% of the total, 6.25ng/ml) while in the

supernatant IL1 beta was more abundant (95% of total, 6.0ng/ml). Comparing kinetics of cellular distribution after stimulation with MSU or LPS (Fig 45), MSU crystals showed a trend towards extracellular release for IL1 alpha (at 6-9 hours) and mainly for IL1 beta at 6-18 hours.

We have not studied the occurrence of membrane damage, but release of unprocessed IL1 beta precursor from cells stimulated with MSU, could account for the different cellular distribution of immunoreactive IL1 beta in these cells (95% released) compared to IL1 biological activity.

Induction of IL1 by MSU could account for a large part of the previously reported EP activity (Duff et al, 1983; Malawista et al, 1985). It is, of course, possible that MSU stimulates the production of TNF or other factors in addition to IL1 from mononuclear phagocytes and that the multiple catabolic effects of TNF may contribute independently or synergystically with IL1 to the pathogenesis of gout or other inflammatory diseases.

The potential relevance of MSU crystal-induced IL1 to the pathogenesis of gouty arthritis is certainly increased by the present findings that human synovial adherent mononuclear cells, as well as blood monocytes, released IL1 activity following exposure to MSU crystals. Further, the proliferation of synovial fluid fibroblast-like cells was stimulated by urate crystal-induced monocyte supernatants with known IL1 content. Thus, cells derived from human joints both released IL1 and responded to IL1-containing supernatants induced by MSU crystals. Crystals of HA and CPPD, also related to inflammatory arthritides (Ryan, McCarthy, 1985; Nuki, 1984; Dieppe et al, 1984) failed to provide a direct stimulus for IL1 production in these experiments. This does not exclude, however, a role for IL1 in the pathogenesis of inflammation induced by these crystals, since IL1 release may be activated by mechanisms other than direct crystal stimulation of IL1 producing cells (Malawista et al, 1985).

As a model for the inflammatory consequences of overproduction of IL1, gout is the only sterile inflammatory disease where the local and systemic pathology is compatible with such overproduction; raised IL1 levels have been found at the site of inflammation (Wood et al, 1983) and a necessary etiologic agent, crystalline urate (Seegmiller et al, 1962; McCarthy, 1962; Malawista, 1977) has been shown unequivocally to be a direct activator of mononuclear cell IL1 release.
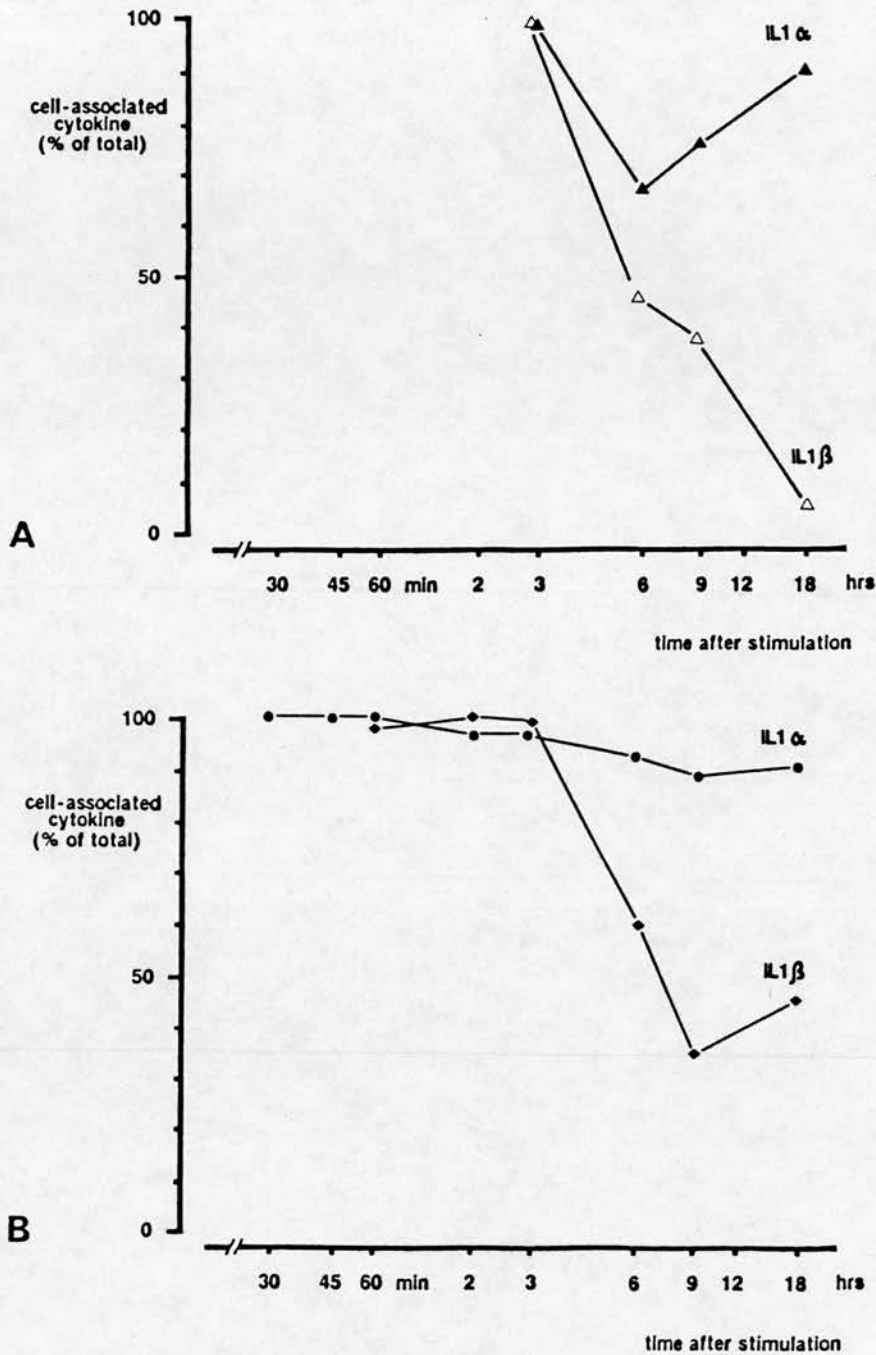
124

FIG 43   Comparison of kinetics of cellular distribution of IL1 alpha and IL1 beta in LPS or MSU stimulated monocytes

Derived from data shown in Fig 41 and Fig 42, in (a) is indicated (vertical axis) the percentage for each IL1 species which remains cell-associated, at different times after MSU stimulation.   At 2 hrs both cytokines were below the detection limit of the assay.   In (b) are presented, for comparison, data from time courses of induction previously shown (Fig 24), of IL1 alpha and IL1 beta in LPS-stimulated human monocytes.

125

2.f. **CHAPTER 4**

Induction of Tumour Necrosis Factor by Pro-inflammatory Crystals

126

2.f.1. INTRODUCTION

With the molecular cloning of TNF and the availability of pure protein, studies have flourished in the areas of TNF receptor/post receptor effects, metabolic and therapeutic effects *in vivo* and effects on cell-mediated immunity *in vitro*. The study of TNF alpha induction has, however, mainly involved infectious agents and little is known about the activation of TNF production by non-microbial agents.

In this study, we investigated the effects of several synthetic pro-inflammatory crystals on TNF alpha production in adherent human mononuclear cells.

The crystals tested, monosodium urate (MSU), hydroxyapatite (HA) and calcium pyrophophosphate dihydrate (CPPD), are associated with different forms of arthritis and MSU induces IL1 as described previously. Induction of TNF release by crystals might synergize with IL1 in determining the inflammatory consequences of crystal deposition.

2.f.2. RESULTS

2.f.2.1. Induction of TNF in blood monocytes by crystals

Human blood monocytes were incubated in presence or absence of LPS-free crystals of MSU, HA or CPPD for 18 hrs, when supernatants were collected and assayed in the L929 cytotoxicity assay (Fig. 44) for TNF activity. MSU-stimulated cells, and to a lesser extent, cells that had been treated with HA crystals, released significant amounts of TNF biological activity, while cells incubated in absence of stimulus or with CPPD crystals failed to produce any significant extracellular activity. TNF induction by MSU crystals was dose-dependent, and maximal stimulation occurred at 1.2mg crystals/ml.

To test the specificity of these bioassay results, supernatants from cells stimulated with MSU crystals were tested for L929 cytotoxicity in presence of monoclonal anti human TNF alpha antibody (kindly provided by Dr G Adolf, Boehringer, Wien) or irrelevant monoclonal antibody (mouse anti human DR). Results shown in Table 2.8 demonstrate selective inhibition of L929 cytotoxicity, indicating extracellular production of bioactive TNF

alpha by human monocytes stimulated by MSU crystals.

2.f.2.2.   Crystal-induced TNF alpha from synovial fluid cells

Freshly isolated human RA synovial fluid adherent mononuclear cells were found to release immunoreactive TNF alpha without being stimulated. This could reflect activation *in vivo* of the cells to produce TNF alpha or inadvertent activation during separation procedures (Fig. 45). The synovial cells could be further stimulated to release TNF by incubation with 0.4mg/ml MSU crystals but HA failed, at the concentration tested, to induce further significant release of TNF alpha.

2.f.2.3.   Kinetics of production and cellular distribution of TNF alpha in crystal-stimulated cells

Human adherent mononuclear cells (10^6/well/ml) were prepared from peripheral blood by density centrifugation. Cells were incubated with or without different concentrations of crystals of MSU, HA, or CPPD. At different time points (0 to 18hrs), supernatants were collected (extracellular fraction), and adherent cells reconstituted with equal volume of medium and lysed by rapid freeze-thawing (cell-associated fraction). Fractions were stored at -80° C until tested in the L929 cytotoxicity assay for TNF activity.

In Fig. 46 are shown the results of such experiments: kinetics of MSU induction of TNF activity follow the same pattern previously shown for LPS induction of TNF (Fig 30, p. 98). After an initial peak of cell-associated TNF at 3 hrs, extracellular TNF at 6 hours clearly exceeds cell associated to reach a maximum at 12-18 hrs.

To confirm on a quantitative basis the kinetics of cellular distribution, immunoreactive TNF alpha levels were tested in the same cellular fractions (Fig. 47). The results show comparable kinetics to those obtained by TNF biological activity (90.5% of TNF alpha translocated at 18 hrs), suggesting that in these conditions, no major accumulation of inactive TNF alpha precursor takes place.

Interestingly, MSU induced TNF activity is detectable at 3 hours, while upon LPS stimulation it occurred after minutes of induction; however, the peak of accumulation seems to be at the same time point, suggesting similar kinetics but on a smaller scale.

128

In cells stimulated with HA and CPPD crystals, TNF biological activity could not be detected in the supernatants nor in the cell-lysates. Unstimulated cells similarly failed to express any TNF activity or TNF alpha protein, suggesting the absence of a preformed TNF protein pool in resting cells.
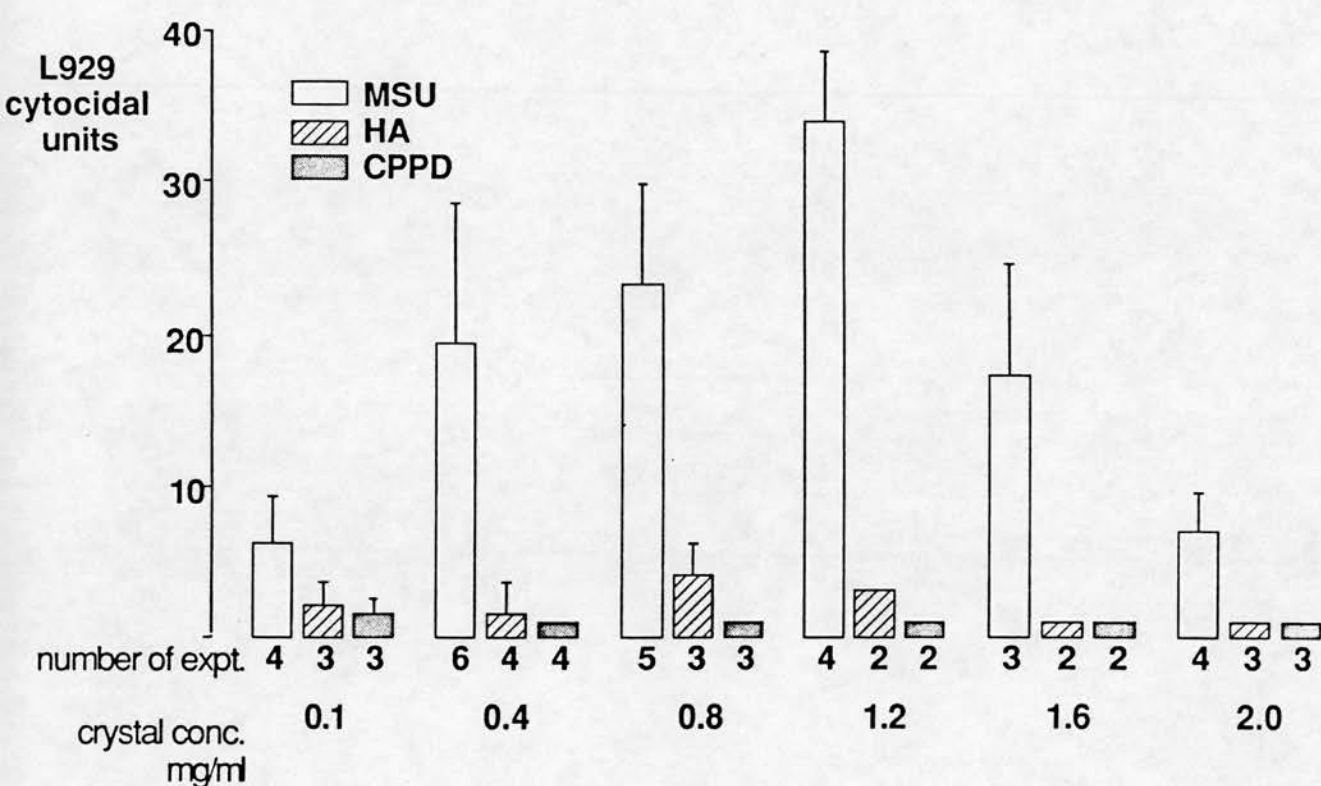
FIG. 44   TNF activity in supernatants of crystal-stimulated human adherent mononuclear cells

Different doses of crystals of MSU, HA and CPPD were tested in 18 hour incubation with human adherent mononuclear cells (10^6 monocytes/ml) for their ability to induce extracellular TNF release. Supernatants were tested in L929 cytotoxicity assay (in which 1U = 40pg/ml hr TNF alpha). Data are expressed as mean (±SEM) for the number of experiments indicated. In the same experiments, TNF bioactivity was not detectable in supernatants from cells incubated with medium only (data not shown).

	Sample alone	Sample with mab anti-hr TNF 1:800	Sample with control mab 1:800
MSU induced sup. (0.4 mg/ml)	17.61	< 4	24.16
MSU induced sup. (0.8 mg/ml)	36.92	< 4	42.77
MSU induced sup. (1.2 mg/ml)	27.36	< 4	28.75
LPS induced sup. (100 ng/ml)	1469.9	< 4	822.40
hr TNF beta (1 ng/ml)	24.08±3.34	21.68±2.85	29.52
hr TNF alpha (1 ng/ml)	22.13±2.45	< 4	20.91

TABLE 2.8   **Effect of anti TNF alpha monoclonal antibody on L929 cytotoxicity**

L929 cell cytotoxicity by supernatants of mononuclear cell populations exposed for 18 hours to different stimuli. Supernatants were tested for TNF biological activity in presence or absence of specific anti human TNF alpha monoclonal antibody, or irrelevant mouse anti human monoclonal (anti-human DR). Results are expressed in half-maximal units/ml.

131
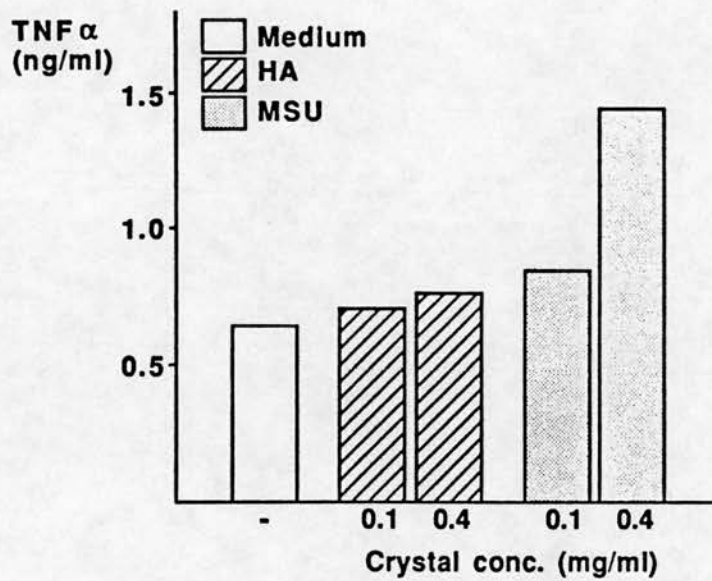
FIG. 45   Synovial fluid mononuclear cells produce TNF alpha

Synovial fluid mononuclear cells were separated by density centrifugation from freshly aspirated RA synovial exudate fluids, followed by adherence in plastic Petri-dishes (40 mins). Crystals of MSU or HA at the concentrations shown in the horizontal axis were added, and after 20 hr incubation supernatants were collected to be tested in a monospecific TNF alpha/RIA (Medgenix). Results are the mean of two determinations.
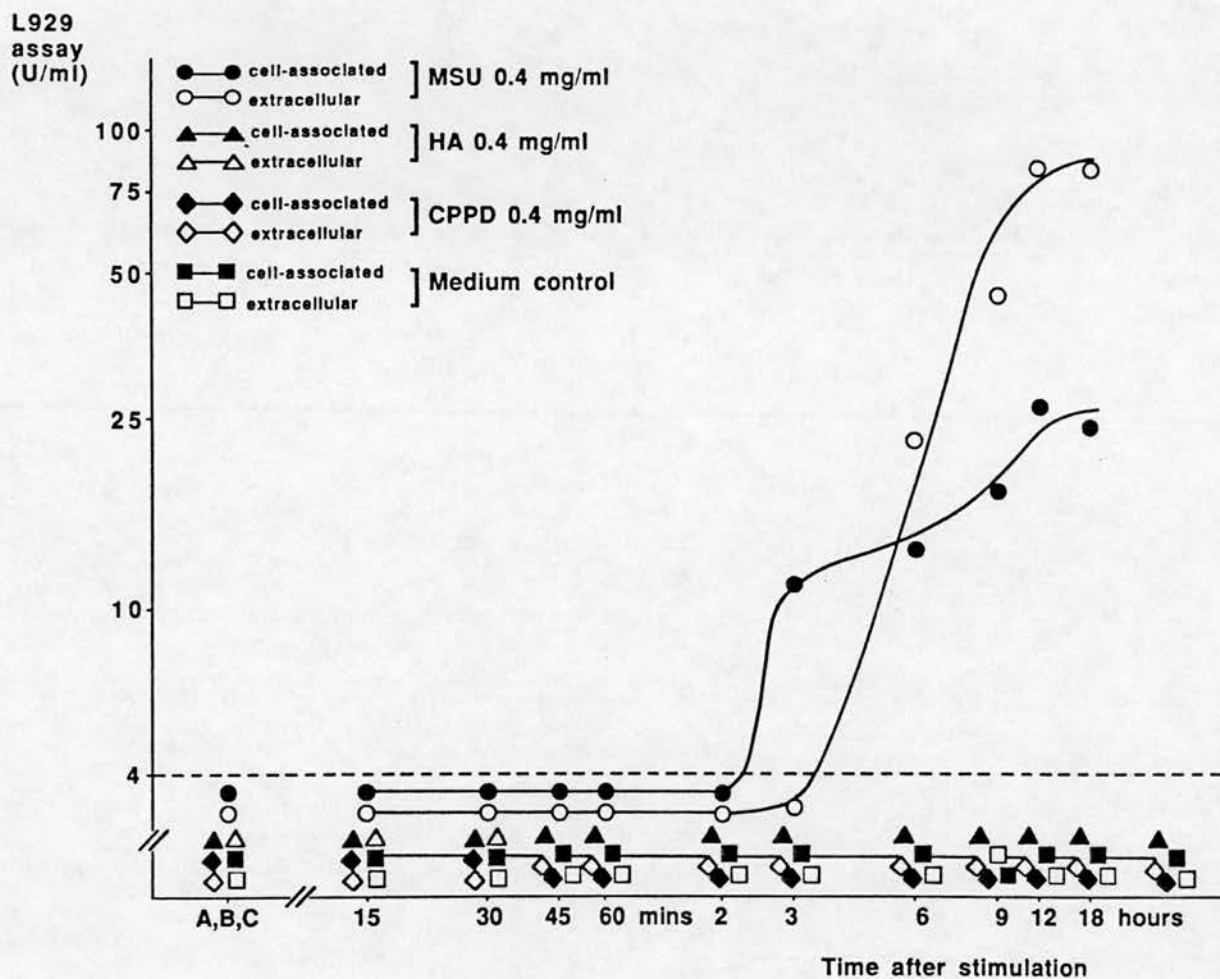
FIG. 46   Time course of TNF production and cellular distribution upon crystal stimulation

TNF biological activity in extracellular and cell-associated fractions from cells incubated for different times (horizontal axis) in presence or absence of different crystals. Fractions represent individual cultures of adherent mononuclear cells.

*A: cell associated TNF activity in 3×10^6 human mononuclear cells immediately after density centrifugation.

*B: cell-associated and extracellular supernatants of cells at the end of plastic adherence (40 mins).

*C: cell-associated and extracellular activities in cells (10^6 monocytes/ml) at the addition of stimuli (time-point zero).

133
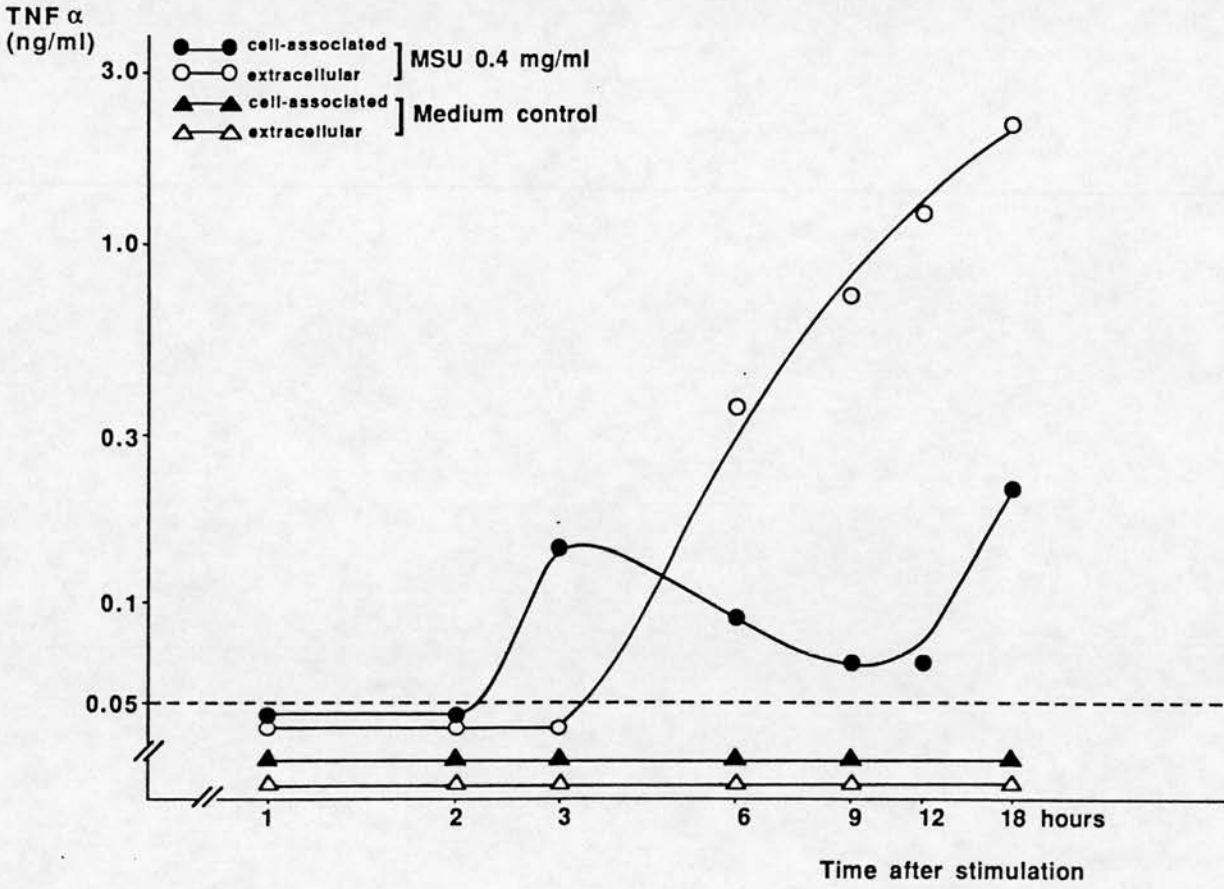
FIG. 47   Kinetics of TNF alpha cellular distribution

Fractions from the previous experiment (Fig. 46) were tested in a specific TNF alpha RIA (detection limit 50pg/ml). Results are mean of duplicate tubes.

2.f.3. DISCUSSION

A previous report showed that crystals of monosodium urate were potent stimulators of monocyte endogenous pyrogen activity (Duff et al, 1983). In further studies, the same authors (Malawista et al, 1985) demonstrated that such induction was not a universal property of arthritis-associated crystals, as HA and CPPD crystals, in similar conditions, failed to induce EP activity release from human monocytes.

Pyrogenicity is one of the diverse biological activities of IL1, but it is not unique to IL1 since recombinant TNF (Dinarello et al, 1986), alpha interferon (Dinarello et al, 1984) and IL6 (Dinarello et al, 1988) are also pyrogenic, even if with lower specific activity.

Using a biological assay insensitive to TNF and specific radioimmunoassays, we have shown (Chapter 1, pp 66-68) that IL1 production certainly occurred upon MSU crystal stimulation of human monocytes and MSU itself was a potent stimulus for both synthesis and release of IL1 peptides.

We report here that MSU crystals, but not CPPD, are dose-dependent, powerful inducers of TNF activity from human monocytes. HA in some experiments induced release of low level of TNF bioactivity but this could not always be reproduced and in subsequent time-course experiments was not confirmed.

The biological assay used (the standard L929 cytotoxicity assay) is not sensitive to human IL1 (2.b.6.1, p. 52), but will respond equally to TNF alpha and the lymphocytic product TNF beta (lymphotoxin). However, the biological activity induced by MSU crystals was completely neutralized by monoclonal antibody to human TNF alpha.

In these studies, we also show production of TNF alpha from rheumatoid arthritis synovial fluid adherent mononuclear cells. These released "spontaneously" significant amounts of TNF alpha, and such production could be further stimulated by addition of MSU, while HA crystals had no effect.

The failure of HA and CPPD to induce high levels of TNF synthesis (rather than a failure to stimulate release) was confirmed in time-course experiments where both crystals failed to induce cell-associated TNF biological activity, and in parallel experiments (2.g.3.3., p. 150) TNF alpha mRNA did not accumulate in HA or CPPD treated cells.

135

In contrast, LPS-free crystals of MSU induced time-dependent accumulation of TNF alpha protein and biological activity in both cell-associated and extracellular compartments, with detectable levels at 3 hours in cell-lysates, followed by steady extracellular translocation of biologically active TNF alpha, found mainly in the supernatants at 9-18 hours (extracellular TNF alpha = 2.24ng/ml - 90.5% of total). These kinetics of accumulation of TNF alpha upon stimulation of monocytes with MSU crystals seem thereafter to be comparable to those obtained with LPS stimulation, i.e. those of a typical secretory protein.

In different studies we have demonstrated that in similar conditions LPS-free crystals of MSU induce dose and time-related synthesis and release of biologically active IL1 alpha, IL1 beta and TNF alpha peptides. Such production could be triggered from both normal human blood adherent mononuclear cells and synovial adherent mononuclear cells from a patient with rheumatoid arthritis.

All of these cytokines have independent pro-inflammatory, catabolic and immunopotentiating activities and each can thereafter contribute independently to the inflammatory consequences of MSU crystal deposition. In view of the reported synergy of TNF and IL1 in several human systems (Elias et al, 1987; Stashenko et al, 1987; Ruggiero, Baglioni, 1987), and the possibility of mutual activation via cytokine-mediated amplification circuits (Oppenheim et al, 1987), overproduction of both IL1 and TNF could provide a pathogenetic link between MSU crystal deposition and the inflammation and tissue damage of gouty arthritis.

2.g. **CHAPTER 5**

Interleukin 1 beta and tumour necrosis factor alpha mRNA
accumulation in human blood mononuclear cells

2.g.1.   INTRODUCTION

The work reported in the previous chapters indicated that IL1 and TNF alpha are "transiently" induced proteins, i.e. cell products which are not released by resting cells and whose translocation only follows new protein synthesis, as no cell-associated protein pool could be demonstrated in absence of cell activation.   New IL1 protein synthesis is believed to be related to rapid translation of newly formed mRNA (Oppenheim et al, 1986). In maximally stimulated cells, IL-1 beta mRNA represents, according to different authors, from 0.1% (March et al, 1985) to 5% (Webb et al, 1985) of total cellular mRNA, exceeding by 40 (E. Kovacs, unpublished) to 300 (Fenton et al, 1987) times the low levels found in unstimulated monocytic cells. The levels of IL-1 beta mRNA appear to exceed, after stimulation, those of IL-1 alpha in a ratio of 10:1 (March et al, 1985) to 500:1 (Dinarello, 1986). The variability of these data can be related to the type and quality of stimulus used, duration of stimulation, conditions of cultures and cell-type used.

In the present study we tested IL-1 beta mRNA accumulation and protein synthesis in a 36 hours time-course in serum-free conditions and using stimuli at a concentration previously shown to be the most effective for the production *in vitro* of IL-1 bioactivity.   In particular, LPS was used at a concentration of 100ng/ml, that is 10-100 times lower than commonly used.

Most studies regarding induction of TNF synthesis have been performed *in vivo*, as previously indicated.   After analysis of protein and mRNA accumulation Beutler et al, (1986) reported the presence of an untranslated TNF alpha mRNA pool in "resting" cells (elicited murine peritoneal macrophages) and the ability of IFN gamma to synergize with LPS in inducing translation of this mRNA.   Lipopolysaccharide was able greatly to enhance mRNA accumulation in these cells representing up to 0.5% of the total cellular mRNA.

When the present study was started no description of TNF alpha mRNA accumulation kinetics in human monocytes was available.   To investigate kinetics of TNF alpha mRNA accumulation, we used a concentration of LPS (100ng/ml) lower than those used by other authors (1-10ug/ml).   High levels of LPS have, in fact, been reported to be down-regulators of IL-1 and TNF production, possibly *via* induction of PGE_2 (Kunkel et al, 1986; Wallach and Hahn, 1983).

In these experiments we also tested the inducing properties of the same range of pro-inflammatory crystals previously tested at a protein level. This was useful to complete the characterization of MSU as stimulator of IL1 and TNF gene expression as well as providing grounds for comparison with LPS-induced cellular activation.

2.g.2. MATERIALS AND METHODS

2.g.2.1 Cellular activation

Human peripheral blood mononuclear cells were separated on density gradient (Lymphoprep, pyrogen-tested, 1.077 density, Nyegaard, Oslo). After careful washes in LPS-free phosphate buffered saline, cells were incubated in RPMI 1640 medium (2mM glutamine, penicillin 100U/ml, streptomycin 100ug/ml) in absence or presence of different stimuli, such as LPS (100ng/ml) or MSU, HA, CPPD crystals (0.4mg/ml). In previous experiments, these concentrations had been demonstrated optimal or suboptimal for IL1 and TNF release from human monocytes.

For kinetic studies, cells were cultured in 1ml aliquots (4×10^6 mononuclear cells/ml; 50% monocytes; 1.5ml sterile tubes, Sarstedt, UK), for times ranging from 1hr to 36 hrs. After incubation, tubes were spun at 800G, 15 mins and supernatants stored for extracellular cytokine assay. The cell pellets were stored at -70° C until the experiment reached completion and then used for mRNA analysis.

In cultures that were going to be used for Northern analysis, cells were selected for plastic adherence (40 mins) before addition of stimuli (1×10^6 monocytes/ml; 20 to 30ml cultures in Petri dishes from Nunc, Denmark). After 6 hours incubation, supernatants were removed and cells lysed by direct addition of 3ml 4M Guanidinium and immediately processed for mRNA analysis.

2.g.2.2. cDNA probes

Human TNF alpha cDNA (1606 bp, inserted in the unique Pst I site of pAT 153) was kindly donated by Dr W. Fiers (Univ. Ghent, Belgium). To detect TNF alpha mRNA, we used the 1180 bp Pst I fragment, which contains the complete coding region (699 bp) for the human TNF alpha precursor (Marmenout et al, 1985).

Interleukin 1 beta mRNA was detected using a Pst I insert in pBr 322 (from Dr D. Carter, The Upjohn Company, Kalamazoo, MI) of a cDNA coding for aa 5 to 269 of the human IL1 beta propeptide (D. Carter, unpublished data).

The control probe was a 708 bp Pst I-Dra I fragment from p7B6 insert in pBr 322 (from Dr U Torelli, Univ. Modena, Italy), which codes for a species of mRNA whose levels are constant throughout the cell cycle. This was identified from a cDNA library prepared from a patient with chronic

lymphocytic leukemia (Kaczmarek et al, 1985).

All fragments were ^{32}P-labelled by random oligo-priming (Feinberg, Vogelstein, 1984). After separation on Sephadex G50 from unincorporated label, cDNA probes labelled, as assessed by Cerenkov radiation, at a specific activity of $4\text{-}10\text{x}10^8$cpm/ug DNA were obtained.

2.g.2.3. mRNA analysis

In kinetic studies, total cytoplasmic RNA was prepared from cells according to the method by White & Bancroft (1982). Cells were harvested by centrifugation (600G, 5 mins), resuspended in ice-cold 10mM Tris, 1mM EDTA (pH 7.0) and after detergent lysis (Nonidet P-40, Sigma) the nuclei were pelleted by spinning at 15000G (3 mins). Whole cytoplasmic extracts were transferred to tubes containing standard saline citrate and formaldehyde (final 0.9M NaCl/90mM Sodium Citrate, 7.4% formaldehyde) and incubated at 60° C for 15mins to denature RNA.

Cytoplasmic extracts were serially diluted 1:5 in a microtiter plate (in 2.25M NaCl/0.22M Sodium Citrate; starting from $4\text{x}10^5$cells/100ul/slot), and transferred on to nylon membranes (Hybond N, Amersham, UK) using a Minifold II slot blot apparatus (Schleicher & Schuell, FRG). The membrane was exposed to UV (5 mins) to fix cytoplasmic macromolecules and prehybridized for 8-20 hrs at 37° C in prehybridization solution (50% Formamide, 240mM NaCl, 30mM Sodium Citrate, 40mM Sodium Phosphate, 0.02% Ficoll, 0.02% polyvinyl pyrrolidine, 0.02% bovine serum albumin). Hybridization was carried out in fresh prehybridization solution to which calf thymus DNA (50ug/ml) and the ^{32}P-labelled probe had been added. After 18-24 hours at 37° C, membranes were washed four times (15 mins) in 0.1% sodium dodecyl sulphate and decreasing salt concentrations (from 0.3M NaCl, 30mM Sodium Citrate to 15mM NaCl, 1.5mM Sodium Citrate) and exposed to Agfa X-ray films at -80° C with intensifying screens for 12 hours to 4 days.

Autoradiographs were scanned at OD_{550} in a Shimadzu CS9000 densitometer using a calibration of 16x (Fig. 48). The integral of the area of the peaks was used as measure of the presence of specific mRNA within each particular series of cultures (Scanning Densitometry Units = SD units).

To ensure that these methods were producing consistent RNA extraction, loading and transfer, and to monitor variations in cell numbers during the kinetic studies, control experiments were performed (Fig. 49), probing with 7B6 cDNA which recognizes a cell-cycle independent form of human mRNA

(Kaczmarek et al, 1985).

For Northern analysis, total cellular RNA was isolated using a modified version of the guanidinium/hot phenol method (Feramisco et al, 1982). Briefly, cellular protein structures were disintegrated in 4M guanidine isothiocyanate and the preparations were treated with hot phenol (BRL, 60° C, 5 mins), followed by addition of ice-cold buffer (0.1M Sodium Acetate, pH 5.2; 10mM Tris, pH7.4; 1mM EDTA) and chloroform/isoamyl alcohol mixture. The extracted upper phase was transferred and underwent a second cycle of treatment. Total RNA in the final aqueous phase was precipitated in 70% ethanol. Final RNA pellets were resuspended in water and their concentration was determined by UV spectrophotometry. Samples were size-fractionated in agarose/formaldehyde gels (1.3% Seakem agarose, 6.7% BRL formaldehyde, 20mM MOPS, 5mM Sodium Acetate, 1mM EDTA), using as markers Lambda/Hind III digests, and transferred to nylon membranes (Hybond N, Amersham, UK) by capillary blotting. UV treatment, prehybridization, hybridization and all following procedures were as previously indicated for total cytoplasmic RNA preparations.

2.g.2.4.   Immunoassays of IL1 beta and TNF alpha

IL1 beta and TNF alpha concentrations in the supernatants of cultures used for mRNA analysis were assessed by IL1 beta RIA (Cistron Technology) and TNF alpha RIA (Medgenix) as previously indicated (2.b.3.4, p. 48 and 2.b.6.2, p. 52). Results reflect specific translocated cytokine accumulation at different time points from cultures which were assessed in parallel for kinetics of cytokine-specific mRNA accumulation.
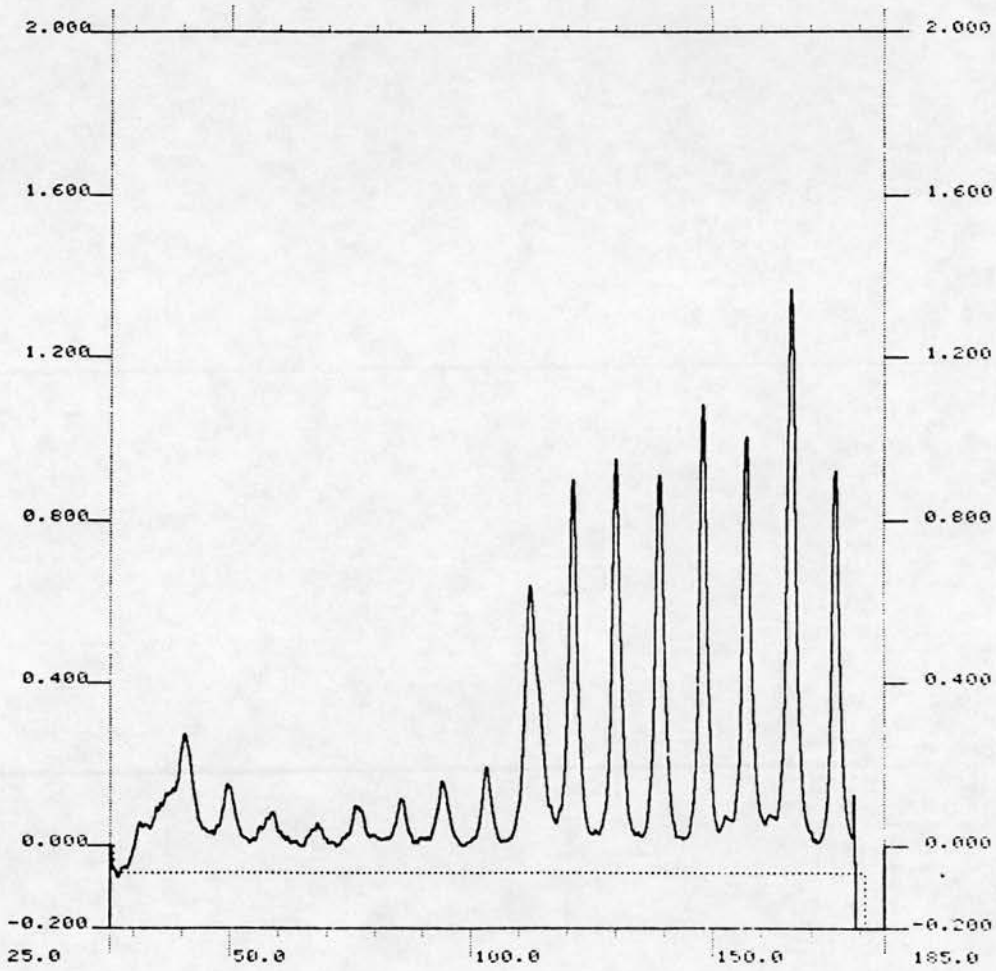
Fig. 48   Scanning Densitometry

Scanning densitometry of all autoradiographs was performed with a Shimadzu CS9000 densitometer, 16x calibration, OD_{550}. The area of the peaks, proportional to the amount of hybridization of the ^{32}P-labelled probe to the membrane, was measured in "Scanning Densitometry Units" (SD Unit). Semi-quantitative data obtained in such a way were used for kinetic analysis. The scan shown refers to unstimulated cells and LPS-induced mononuclear cells IL1 beta mRNA accumulation at 8 different time points.
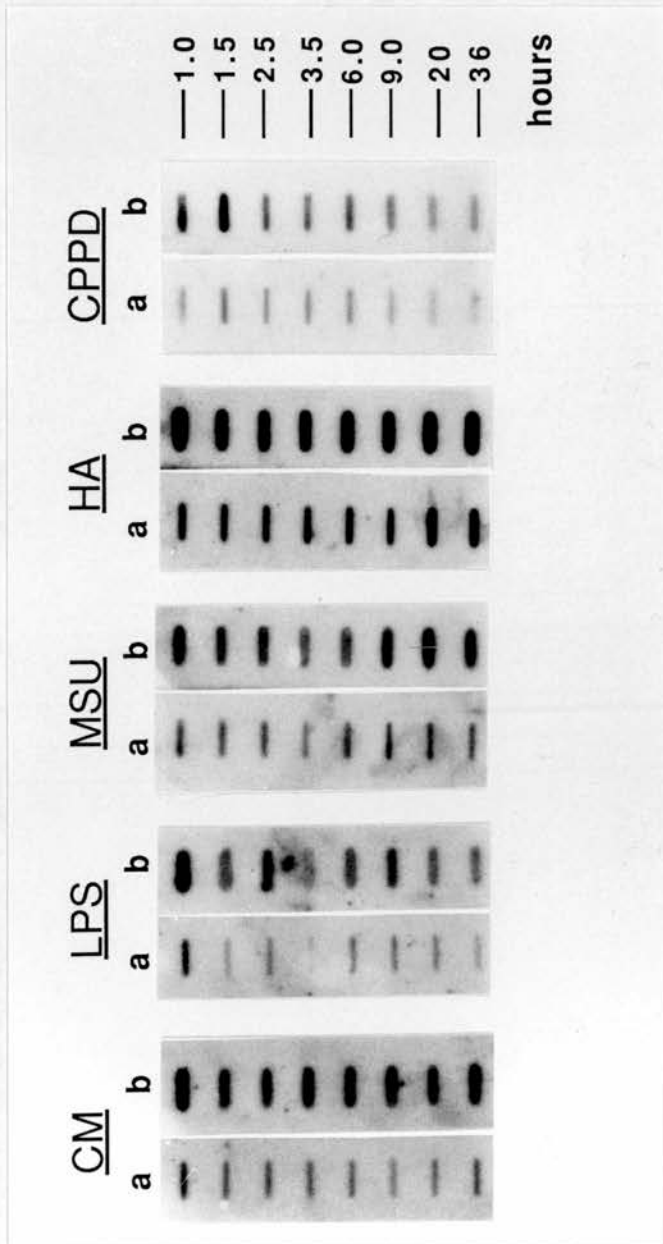
Fig. 49 **Hybridization for a cell-cycle independent mRNA species**

To control for variations in RNA extractions, loading and transfer, membranes were hybridized with radiolabelled p7B6 cDNA, recognizing a species of mRNA whose levels are constant though the cell-cycle. Total cytoplasmic RNA had been extracted from cells incubated in presence or absence of stimuli (LPS, 100ng/ml; MSU, HA, CPPD crystals at 0.4mg/ml) and transferred to nylon membranes.

a - dilution representing total cytoplasmic RNA from $8x10^4$ mononuclear cells

b - representing $4x10^5$ mononuclear cells.

2.g.3.   RESULTS

2.g.3.1.   Time related accumulation of IL1 beta mRNA and extracellular IL1 beta protein

Human peripheral blood mononuclear cells (4×10^6/ml, 50% monocytes) were incubated for times ranging from 1 hour to 36 hrs in presence of LPS (100ng/ml) or MSU (0.4mg/ml), HA (0.4mg/ml), CPPD (0.4mg/ml) crystals or medium control (serum-free RPMI). At different time points, cells were lysed for analysis of mRNA accumulation, and supernatants from MSU and LPS stimulated cultures, as well as from control culture, were tested for immunoreactive IL1 beta. Autoradiographs of cytoplasmic slot blot hybridized with IL1 beta cDNA probe are shown in Fig. 50, and quantitative data obtained by scanning densitometry are in Fig. 51-54.

Unstimulated cells (Fig. 51) and cells treated with CPPD (Fig. 52) failed to accumulate IL-1 beta mRNA, while HA induced low levels of IL-1 beta mRNA (Fig. 52). Lypopolysaccharide induced an early accumulation of IL-1 beta mRNA (probably from the first minutes after stimulation), with a first peak at 2.5 hours and a second at 9-20 hours (Fig. 53). MSU induced a first peak of accumulation at 6 hours and a second at 20 hours (Fig. 54).

As expected, unstimulated cells, which did not accumulate specific mRNA also failed to express any IL1 beta release; in MSU or LPS-stimulated cells, mRNA accumulation was followed concordantly by detection of IL1 beta protein in the supernatants, possibly with an earlier translocation in the MSU-treated cultures.

2.g.3.2.   Northern analysis (IL1 beta)

Total cellular RNA extracted by the guanidinium/hot phenol method from adherent mononuclear cells was examined by Northern Analysis. In Fig. 55, are shown results from a 6 hour induction experiment in which crystals of MSU, HA or CPPD had been used. The cells incubated with medium alone or HA or CPPD crystals failed to induce accumulation of IL-1 beta mRNA, while MSU-induced cells show at 6 hours an mRNA species that migrates as 1.6Kb and hybridizes to IL1 beta cDNA.
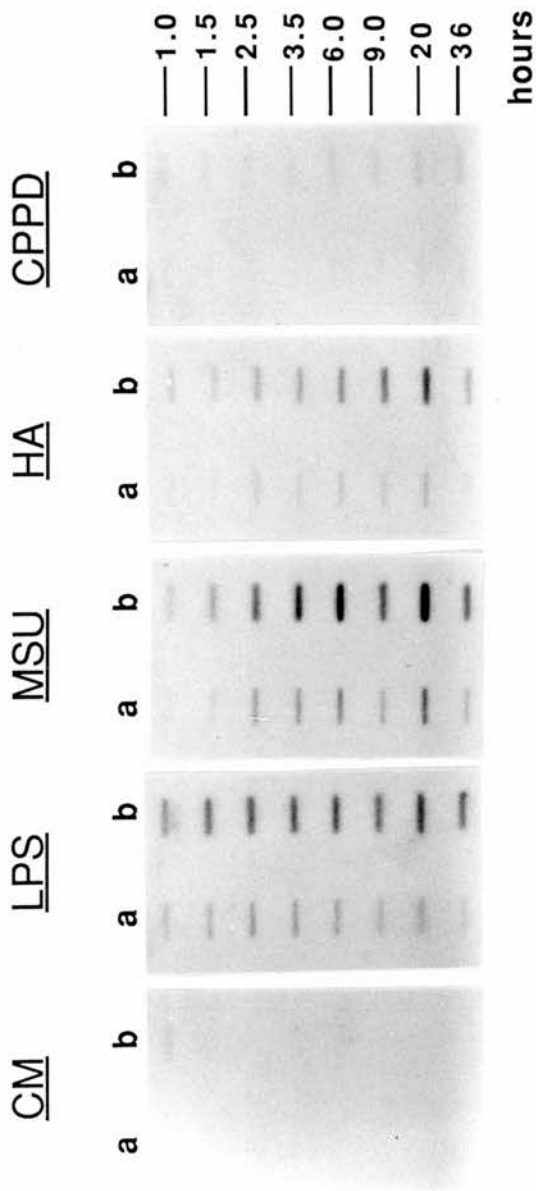
145

Fig. 50 Total cytoplasmic RNA slot blot analysis (IL1 beta cDNA)

Human mononuclear cells (4×10^6/ml, 50% monocytes) were incubated in presence or absence of different stimuli and harvested at different time-points (indicated on the right). Total cytoplasmic RNA was extracted and 100μls aliquots of $1/10$ dilution (b) or $1/50$ dilutions (a) transferred to the membrane. Slots in (b) represent total RNA from 4×10^5 mononuclear cells, and (a) from 8×10^4 cells. Autoradiography of the membrane, probed with human IL1 beta radiolabelled cDNA, is shown. Quantitative data, obtained by scanning densitometry, are shown in Fig. 51-54.
(**CM**) = unstimulated cells; (**LPS**) = LPS, 100ng/ml; (**MSU**), (**HA**), (**CPPD**) = different crystals, 0.4mg/ml.

146
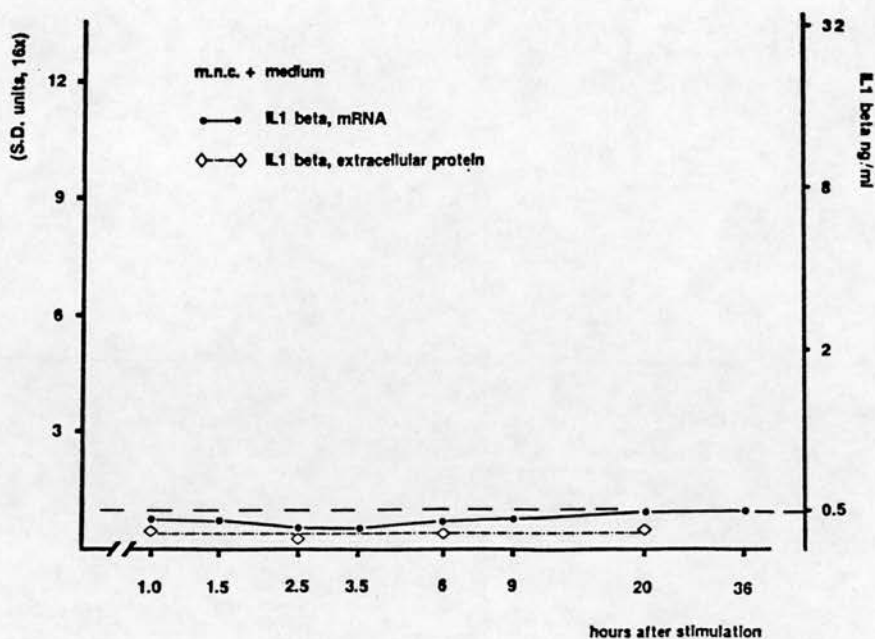
Fig. 51   IL1 beta mRNA and released protein accumulation in unstimulated mononuclear cells

Results from cultures of $4x10^5$ mononuclear cells/ml (50% monocytes) incubated and harvested at different time points. Shown are mRNA levels expressed in S.D. (scanning densitometry) units = integrated area $(x10^{-3})$ of the peaks above background, obtained by scanning of autoradiographs (Fig. 50) and levels of immunoreactive protein as assessed by specific R.I.A..
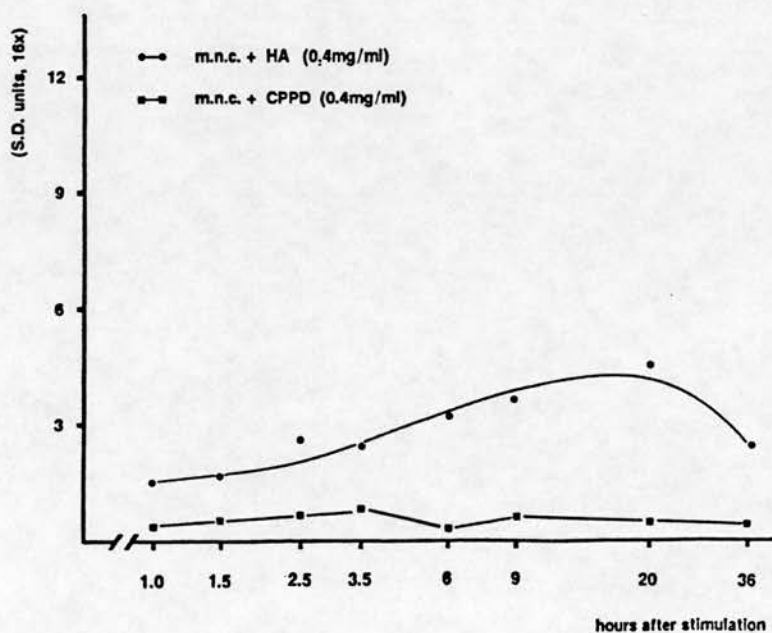

Fig. 52   IL1 beta mRNA accumulation in HA and CPPD crystal-stimulated mononuclear cells

Culture conditions and expression of results as in Fig. 51 legend. Original autoradiograph in Fig. 50.

147
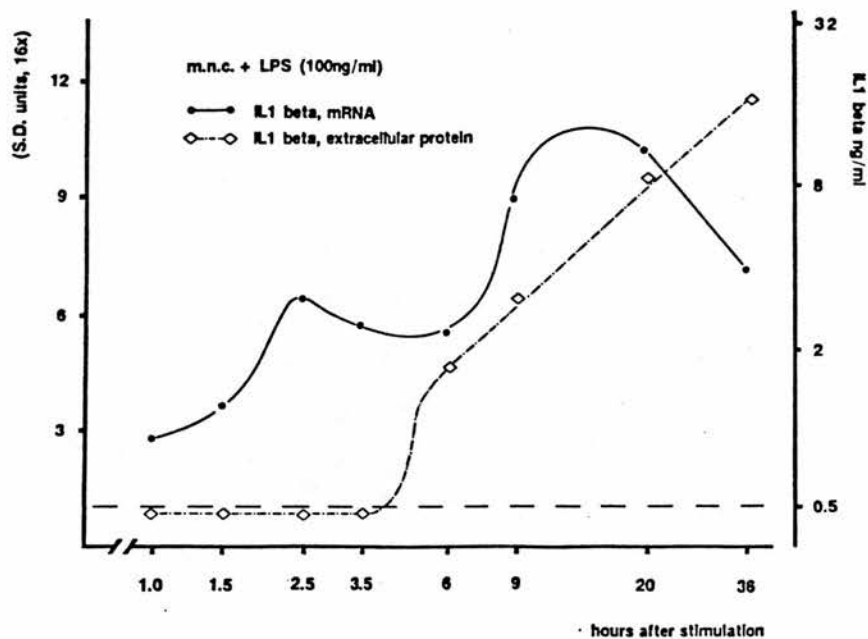
Fig. 53 IL1 beta mRNA and extracellular IL1 beta protein in cells stimulated with LPS

Cultures condition and expression of results as in Fig. 51 legend. Original autoradiography shown in Fig. 50, original densitometry in Fig. 48.
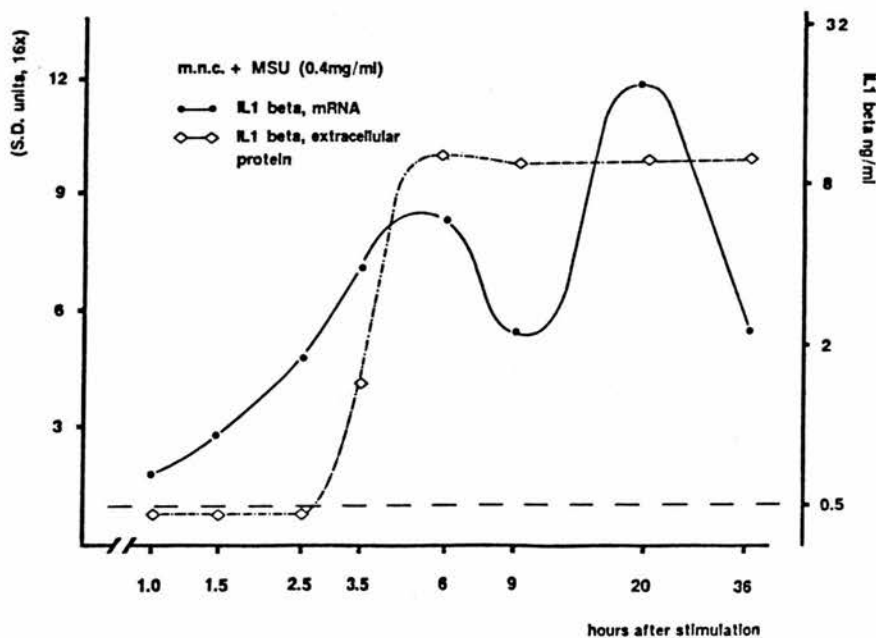

Fig. 54 IL1 beta mRNA and released protein in MSU crystal-stimulated cells

Expression of results and culture conditions as in Fig. 51 legend. Original autoradiography shown in Fig. 50.
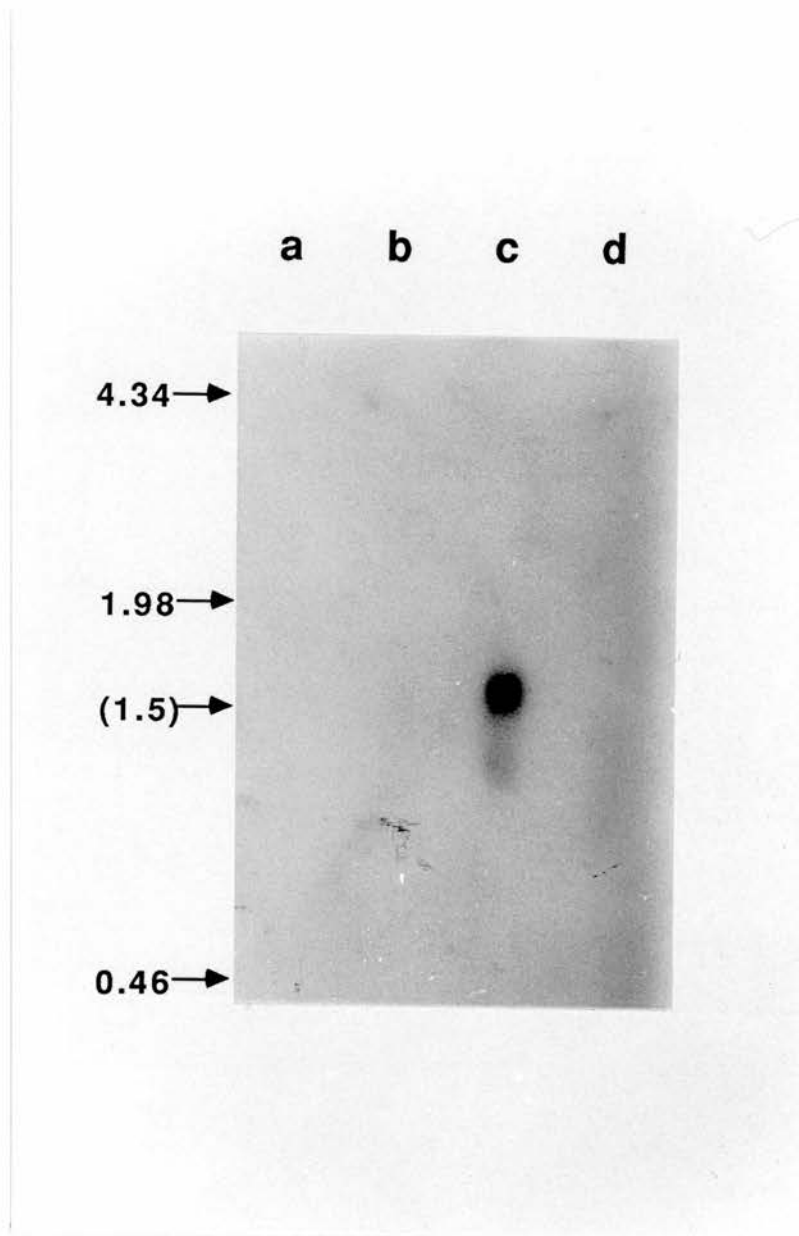
148

Fig. 55 Northern analysis of crystal-induced adherent mononuclear cells
IL1 beta mRNA

Northern analysis of human adherent mononuclear cells (mnc) RNA.
Total hot phenol / guanidine isothiocyanate-purified RNA (5ug/lane) from
$1x10^6$/ml adherent m.n.c. culture (a-d) was fractionated on
agarose/formaldehyde gel and transferred to nylon membrane for hybridization
to the IL1 beta probe. (a) stimulated with CPPD crystals, 0.4mg/ml; (b) HA
crystals, 0.4mg/ml; (c) MSU crystals, 0.4mg/ml; (d) incubated in absence of
stimulus. Molecular sizes are shown on the left.

149

2.g.3.3. Time course of TNF alpha mRNA accumulation

Cytoplasmic RNA preparations described in (2.g.3.1.) were transferred to nylon membranes and hybridized with TNF alpha cDNA. Autoradiographs are shown in Fig. 56 and quantitative data obtained by scanning densitometry are reported in Fig. 57-60. Unstimulated cells (Fig. 57) appear to accumulate low but significant levels of TNF mRNA at 6hrs and at 20-36 hours. Surprisingly, this was not followed by TNF alpha protein detection in the cellular supernatants. HA and CPPD-induced cells (Fig. 58) failed to accumulate TNF alpha mRNA, but LPS and MSU crystals (Figs. 59 and 60) stimulated early accumulation of TNF alpha mRNA. Such mRNA accumulation in LPS and MSU-treated mononuclear cells was followed by protein synthesis, and immunoreactive TNF alpha accumulated concordantly in the extracellular compartments.

2.g.3.4. Northern analysis (TNF alpha)

Specific TNF alpha mRNA accumulation was examined by Northern analysis on adherent mononuclear cells stimulated with LPS (100ng/ml), or MSU, HA or CPPD crystals (0.4mg/ml). Cells were harvested and total RNA extracted at 6hrs time point. As shown in Fig. 61, bands are visible in the 1.5Kb region in the MSU and LPS-stimulated cells but not in cells incubated in presence of control medium or crystals of HA and CPPD.
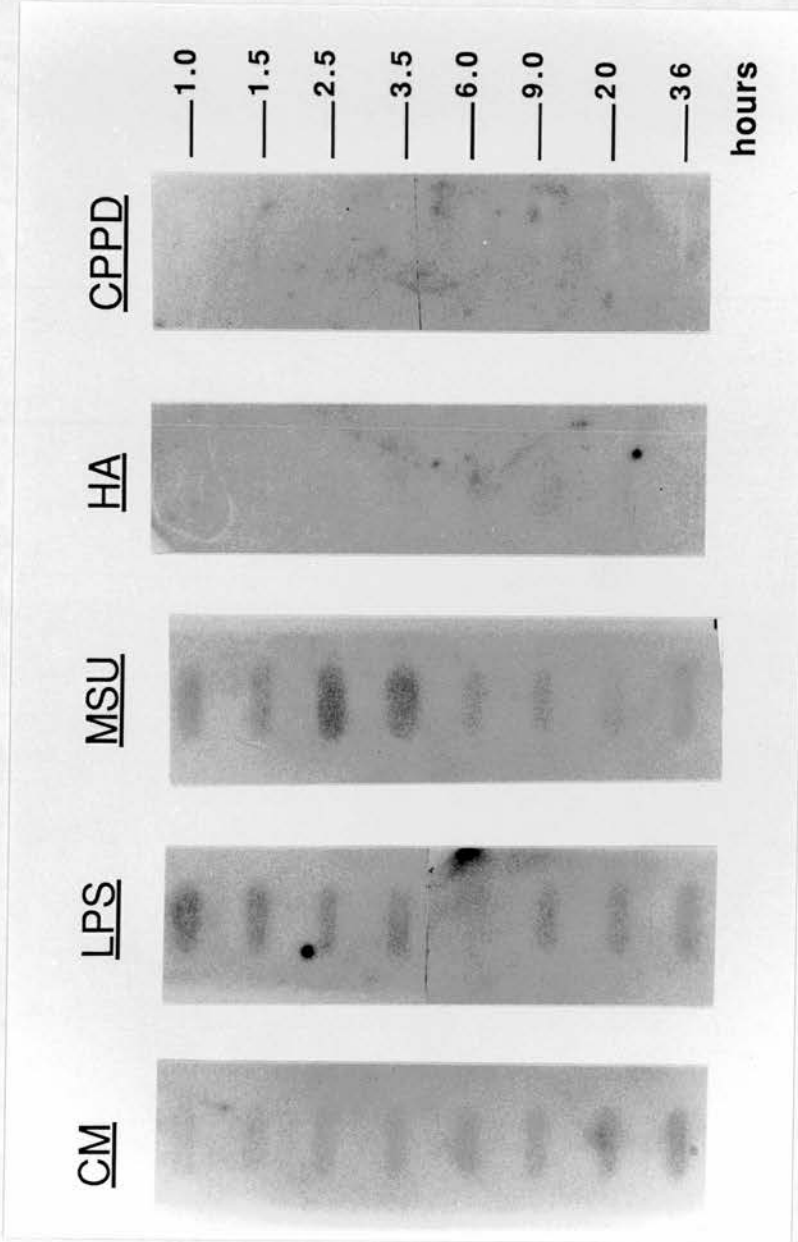
Fig. 56   **Total cytoplasmic RNA slot-blot analysis (TNF alpha cDNA)**

Total cytoplasmic RNA extractions, as from Fig. 50 legend, were transferred to nylon membranes and hybridized to ^{32}P-labelled human TNF alpha probe. Shown are results from 1/10 dilution (4×10^5 mononuclear cell total RNA/slot). Quantitative data by scanning densitometry are shown in Fig. 57-60. (**CM**) = unstimulated cells; (**LPS**) = LPS, 100ng/ml; (**MSU**), (**HA**), (**CPPD**) = different crystals, 0.4mg/ml.
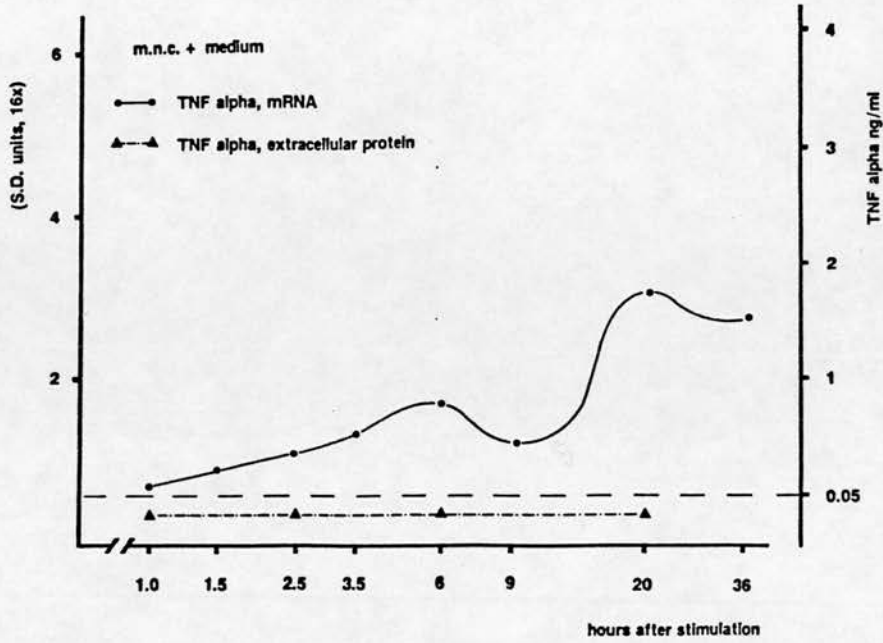
151

Fig. 57   TNF alpha mRNA and released protein in unstimulated cells

Scanning densitometry of autoradiograph shown in Fig. 56. Culture conditions and expression of results as in legend to Fig. 51.
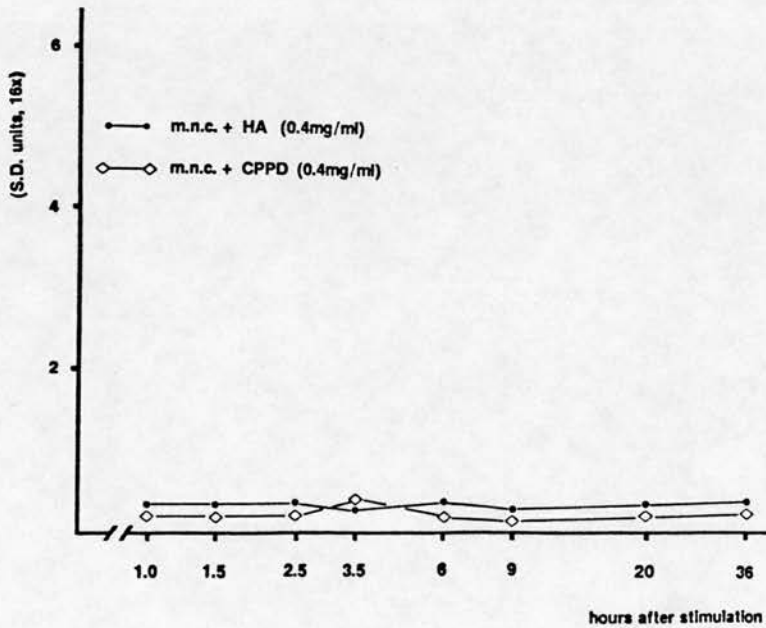

Fig. 58   TNF alpha mRNA accumulation in HA and CPPD-stimulated human mononuclear cells

Expression of results and culture conditions as previously described (legend Fig. 51). Autoradiograph scanned is in Fig. 56.
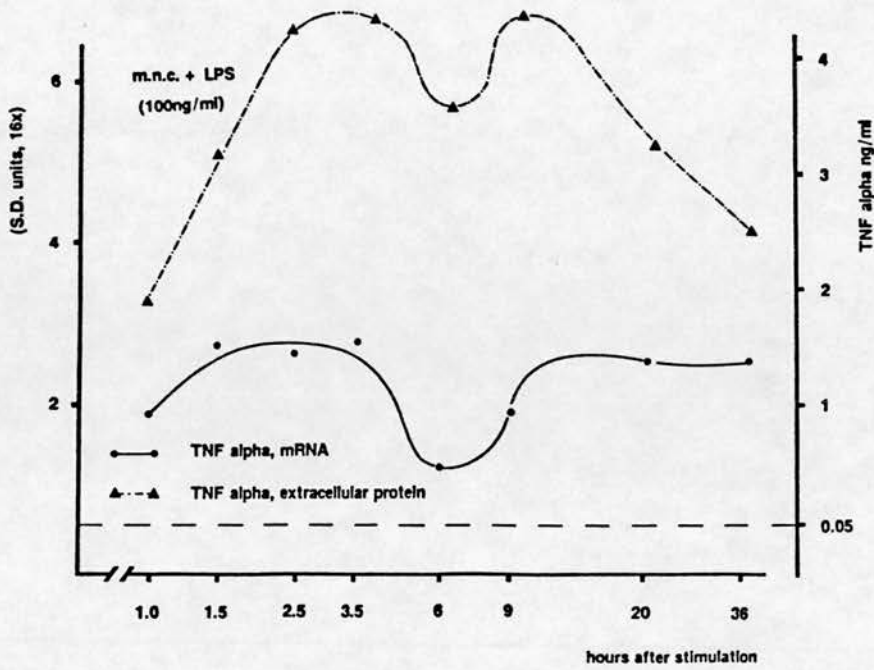
152

Fig. 59 **TNF alpha mRNA accumulation and TNF alpha protein release in cell stimulated with LPS**

Details of cultures and scanning densitometry as in legend to Fig. 51. Original autoradiograph in Fig. 56.

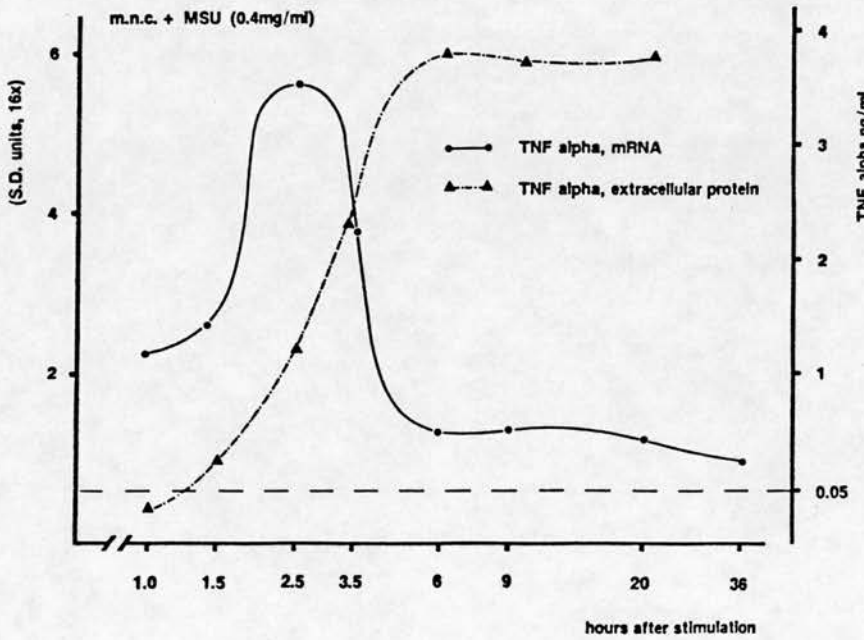
Fig. 60 **Kinetics of accumulation of TNF alpha mRNA and extracellular protein in MSU-stimulated cells**

Culture conditions and expression of results as in legend to Fig. 51. Densitometry has been performed on autoradiograph in Fig. 56.
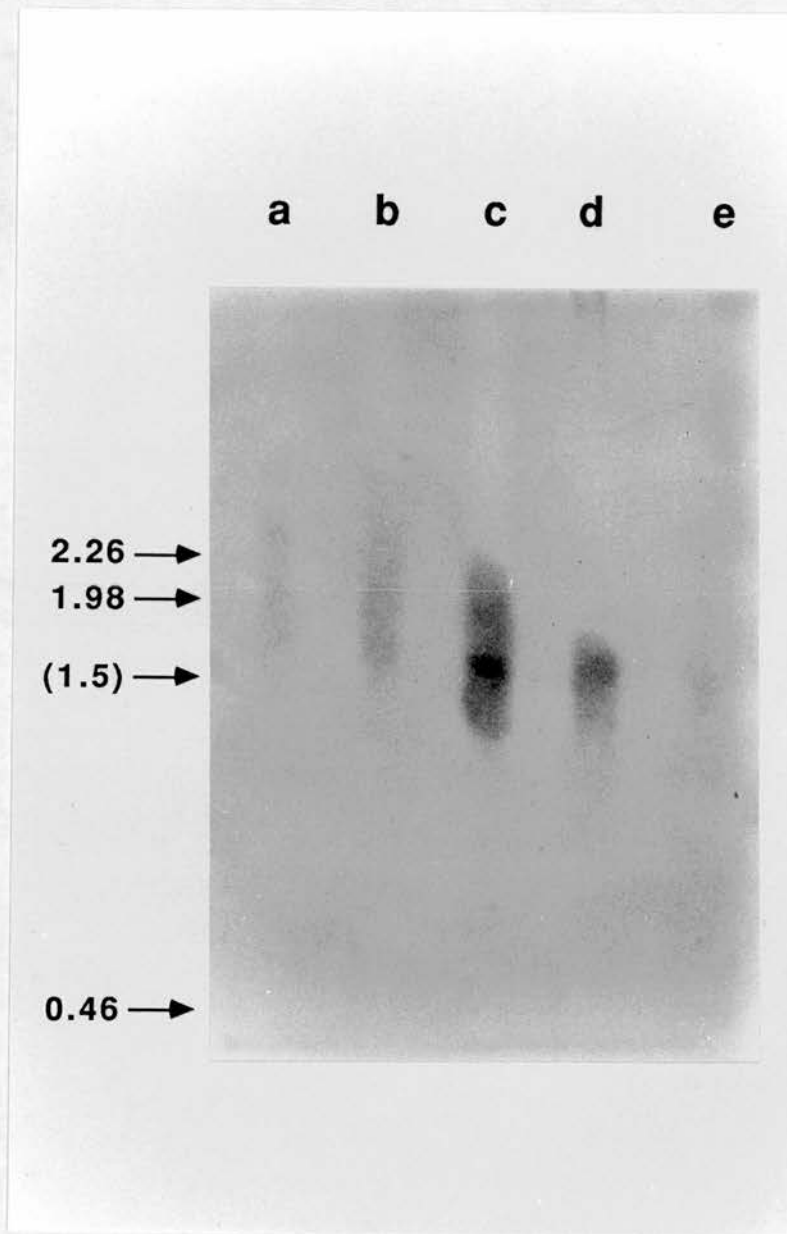
153

Fig. 61   Northern analysis of adherent mononuclear cells TNF alpha mRNA

Total extracted RNA (as described in 2.g.2.1) from 1×10^6 adherent mononuclear cells/ml cultures (6 hours stimulation) was fractionated (7.5µg RNA/lane) and transferred to Hybond N membranes. Hybridization to ^{32}P labelled human TNF alpha cDNA was monitored by autoradiography.
(a) CPPD crystals stimulation, 0.4mg/ml; **(b)** HA crystals, 0.4mg/ml; **(c)** MSU crystals, 0.4mg/ml; **(d)** LPS, 100ng/ml; **(e)** unstimulated cells.

2.g.4.   DISCUSSION

We previously showed that TNF alpha and IL1 beta were released from human monocytes only upon activation by competent stimuli, such as different microbial products or MSU crystals. As preformed proteins could not be demonstrated in unstimulated cells, this was not a mere activation of extracellular release, but induction of new protein synthesis. This view was also supported by very typical kinetics of protein accumulation in different compartments upon stimulation.

To investigate the control of production of these cytokines, in this study we tested the kinetics of IL1 beta and TNF alpha mRNA accumulation in human mononuclear cells upon activation.

The data demonstrate that IL1 beta and TNF alpha cytoplasmic mRNA rapidly accumulated in cells stimulated with lipopolysaccharide or MSU crystals. IL1 beta and TNF alpha proteins were subsequently synthesized and can be readily detected in the extracellular supernatants.

Interleukin 1 beta message accumulated only in cells that had been stimulated: unstimulated cells or cells stimulated with calcium pyrophosphate dihydrate failed to accumulate IL1 beta mRNA over a period of 36 hours. The absence of specific transcripts in unstimulated cells supports the view (Oppenheim et al, 1986) that IL1 beta is an inducible, transiently expressed cytokine and that, in contrast with previous reports (Fuhlbrigge et al, 1987), IL1 gene expression in human monocytes requires specific stimulation.

In our experiments, we studied IL1 beta mRNA accumulation in peripheral blood mononuclear cells, using a low dose of LPS (100ng/ml) or a separate stimulation, MSU crystals, that we previously reported to be a powerful inducer of IL1 synthesis and release. In these conditions, IL1 beta mRNA accumulates following biphasic kinetics, with a first peak at 2.5-6 hours (depending on the type of stimulus used) and a second at 9-20 hours. A recent report (Fenton et al, 1987) in which THP-1 cells, (a human monocytic leukemia cell line) was used, shows a single peak of mRNA accumulation which corresponds to the first peak that we observe. It is possible that the second peak of induction in our experiments is the result

155

of the use of a total mononuclear cell population, susceptible to interactions between the cellular subsets and possibly new IL1 induction through a cytokine network. However, a different study in which pure human monocyte populations had been used, confirmed high levels of IL1 beta mRNA still being produced at 16 hours (Matsushima et al, 1986B).

In the kinetics of TNF alpha mRNA accumulation, an early peak of mRNA (1-3.5 hours) occurred upon stimulation by LPS or MSU and extracellular protein could be detected in association with mRNA indicating efficient protein synthesis, processing and translocation.

Unstimulated cells accumulated low levels of TNF alpha mRNA in the second day of incubation which was not followed by translocation of immunoreactive protein. In view of the previous work on the secretory nature of TNF alpha, this could indicate the existence of an untranslated mRNA pool accumulating in monocytes at late stages of incubation. Such a phenomenon had been previously reported in elicited macrophages from mice (Beutler et al, 1986B) but has never been tested before on human mononuclear cells beyond the initial few hours of incubation.

Recent work has elucidated further the nature of TNF alpha and IL1 beta gene regulation. Nuclear transcription experiments have shown that stimulation determines the appearance of mRNA by direct activation of TNF alpha (Sariban et al, 1988) and IL1 beta (Fenton et al, 1987) transcription. In the same experiments, no transcription was active in unstimulated cells, demonstrating that TNF alpha and IL1 beta regulation differs from that of c-myc in activated T cells (Thompson et al, 1986) and of interferon beta in human fibroblast (Raj, Pitha, 1983). In these cases, the accumulation of short-lived mRNA was not regulated at a transcriptional level but by temporary inactivation of cytoplasmic nuclease, which was degrading a steady state pool of nuclear RNA transcribed at constant rate.

Both IL1 beta and TNF alpha mRNA's appear to have relatively short half lives, as in our experiment levels of accumulated mRNA decreased fairly rapidly during kinetic studies. The half-life of TNF alpha mRNA has been quantified as 20 mins (Sariban et al, 1988). Such mRNA instability has been associated in most-cytokines with the presence in the 3' flanking region of a repeating and overlapping octameric sequence, thought to confer message

156

instability (Caput et al, 1986; Shaw, Kamen, 1986): presumably the sequence facilitates nuclease degradation of mRNA. The observation that the protein synthesis inhibitor cycloheximide (CHX), when appropriately administered, paradoxically increases total cytokine accumulation (Billiau et al, 1973; Mizel, Mizel 1981), provided further insight into cytokine gene regulation. CHX augments IL1 beta (Collart et al, 1986; Fenton et al, 1987) and TNF alpha (Sariban et al, 1988; Collart et al, 1986) transcription, and prolongs the half-lives of their mRNA's. This latter could be related to inhibition of specific RNAse synthesis or inhibition of cytokine synthesis below the level where its intracellular concentration could provide a negative feedback on its own synthesis.

The increase in transcription might be related to the existence of a transcriptional repressor protein (Fenton et al, 1987) whose synthesis is stimulated by high-dose LPS and inhibited by CHX. This work has been followed, and regulatory sequences in the IL1 beta gene have been identified (Clark et al, 1988), suggesting a specific site of action for a transcriptional activator and a transcriptional repressor.

The work presented in this chapter analyzes quantitative changes in cytokine-specific mRNA under different conditions of cellular stimulation. These data, along with similar reports in this rapidly evolving field, represent a necessary first approach to the study of cytokine gene expression.

2.h.   CONCLUSIONS

Human immunity is based on a system of transiently-expressed genes encoding proteins ("cytokines") with powerful receptor-mediated effects on the different cellular elements of the immune system. The "adaptive" response is triggered by the binding of antigen to the lymphocyte antigen receptor (on B or T cells) selecting cells for clonal expansion and making them responsive to cytokine stimulation.

The work presented here stresses a different aspect of host defence, based on the induction of cytokines by microbial agents *via* a non-antigenic mechanism. In the case of IL1 and TNF, this response is likely to precede and augment antigen-activation of lymphocytes, and represents transient gene transcription with activation of protein synthesis, processing and release. We report evidence that, in this process, IL1 beta and TNF alpha are mainly released to the extracellular space (for autocrine, paracrine or endocrine actions) and IL1 alpha mainly accumulates within the producing cells, possibly for action during cell-cell interactions, as a membrane-associated form.

To test a potential role for cytokines in the pathogenesis of human arthritis, we studied the ability of sterile, LPS-free pro-inflammatory crystals to induce synthesis and release of IL1 and TNF. MSU crystals, whose deposition has been associated with gout (Seegmiller et al, 1962) were a powerful, dose-dependent stimulus for the production of these cytokines in normal human monocytes. In the same experiments, HA and CPPD, two other crystal-types associated with human arthritis, failed to induce cytokine production, suggesting that this is not a non-specific monocyte response to perturbation by particulate agents.

These results, and the arthritogenic, pro-inflammatory and catabolic properties of these cytokines *in vivo* (Pettipher et al, 1986) and *in vitro* (Dayer et al, 1976, 1985; Saklatvala, 1986) and the observation of immunoreactive IL1 and TNF in gouty synovial effusions (Wood et al, 1983; data presented in Section II, p. 161), provide evidence for a role of IL1 and TNF in urate crystal arthropathy.

Gene expression of IL1 beta or TNF alpha in human monocytes appears regulated at transcriptional level and the presence of specific mRNA is related to cellular activation with subsequent production of biologically

159

active protein. Abnormally high levels of these cytokines could result by dysregulation of gene expression, persisting stimulatory conditions, absence of negative feedback mechanism or reduced elimination rates.

To propose a role for these cytokines in the pathogenesis of human arthritis, evidence must be provided first for their presence *in vivo* at the site of inflammation. This was the aim of the work reported in the next section (Section II: "The presence of IL1 and TNF alpha in joint effusions from patients with rheumatic diseases").

3.   SECTION II :

The presence of IL1 and TNF in joint effusions from patients with rheumatic diseases

3.a.   INTRODUCTION

The concept of soluble leukocyte products as mediators of joint tissue destruction in arthritis (Bodel, Hollingsworth, 1967; Dayer et al, 1977; Dingle et al, 1979) is supported by the known pro-inflammatory, immunopotentiating and catabolic activities of cytokines, such as IL1 and TNF.

Further evidence might include:

1. Identification of such mediators at the site of inflammation.
2. Demonstration of a significant correlation between the presence of the cytokines and disease activity.
3. Reproduction of disease by administered cytokines.
4. Interruption of the pathogenic process by antagonists of cytokine production or effects.

An example where these criteria have been satisfied is the role of TNF alpha in lethal endotoxic shock. Circulating TNF is present in patients with endotoxic shock (Waage et al, 1986), lethal outcome in humans is related to TNF levels (Waage et al, 1987; Girardin et al, 1988), injection of TNF reproduces a toxic syndrome (Tracey et al, 1986B) and pretreatment of primates with anti TNF mab protects them from the otherwise lethal effects of LPS injection (Tracey et al, 1987). Some pathological conditions in which the presence of IL1 or TNF has been documented are summarized in Table 3.1.

The presence of IL1-like activity in joint fluids has been reported by several authors. The pioneering studies of Bodel ad Hollingsworth (1967), who detected EP activity in most synovial fluids tested were followed by the detection of IL1 bioactivity in unfractionated (Fontana et al, 1982B) or affinity purified (Wood et al, 1983: Bendtzen et al, 1985) synovial fluids. Because of the presence of powerful biological inhibitors in these effusions, purification was required, which made these studies non-quantitative and resulting in only a small number of patients being assessed.

As well as the many effects *in vivo* of IL1 and TNF on the immune system, liver and bone marrow (previously indicated in Table 1.9, p. 19 and Table 1.12, p. 28), IL1 also has potent arthritogenic properties *in vivo*. A

significant inflammatory exudate was obtained by injection of LPS or EP in rabbit joints (Hollingsworth, Atkins, 1965), and injection of recombinant IL1 (Pettipher et al, 1986) induced polymorphonuclear cell accumulation in the joint space and proteoglycan loss from the cartilage.

In the studies reported here the presence of IL1 or TNF was tested in synovial effusions from a large population of patients with rheumatic diseases. Biological assays and specific immunoassays were used and cytokine levels were compared with a full range of laboratory and clinical markers of disease activity within the population.

These experiments were performed for three reasons: Tumour necrosis factor, despite its pro-inflammatory actions in vitro, had not been previously studied at this site of inflammation; differential measurement of IL1 beta and IL1 alpha had not been performed in synovial fluids; the use of specific immunoassays for these cytokines allows quantitative comparison with clinical data.

Biologically significant levels of IL1 alpha, IL1 beta and TNF alpha were found in most synovial fluids from patients with different rheumatic conditions and their relationship to markers of disease activity is discussed.

Table 3.1   Detection of IL1 or TNF proteins or biological activity EX VIVO*

Study   Author(s)

IL1

   Circulating levels during human fever   (Bendtzen et al., 1984)

   LAF activity in normal urine   (Kimball et al., 1984)

   IL1 activity in fractionated human plasma
   after exercise   (Cannon, Kluger, 1983)

   IL1 activity in fractionated human plasma
   after ovulation   (Cannon, Dinarello, 1985)

   IL1 alpha activity in human amniotic fluid
   during normal pregnancies   (Brody et al, 1987B)

   LAF activity in cerebrospinal fluid in a
   model of multiple sclerosis (CREAE, guinea
   pigs) correlates with relapse   (Symons et al, 1987)

   LAF activity in extracts of human psoriatic
   skin lesions   (Camp et al, 1986)

   Serum IL1 beta (RIA) correlates with
   severity of meningococcaemia in children   (Girardin et al, 1988)

   Plasma IL1 beta (ELISA) correlates with disease
   activity in rheumatoid arthritis   (Eastgate et al, 1988)

TNF

   Lethal circulating levels in rabbits with
   endotoxic shock   (Abe et al, 1985)

   Raised serum TNF levels in human parasitic
   infections   (Scuderi et al, 1986)

   Association of TNF serum levels and fatal
   outcome in meningococcal disease   (Waage et al, 1987)

   Serum TNF levels in severly burned patients
   are raised and correlate with complications
   and death rate   (Reuter et al, 1988)

   Raised TNF levels in renal allograft rejection   (Maury, Teppo, 1987)

   Serum TNF levels correlate with severity of
   purpura fulminans in children   (Girardin et al, 1988)

*N.B. Studies on synovial effusions are discussed in the text.

3.b   MATERIALS AND METHODS

3.b.1.   Patients and clinical specimens

Synovial exudate fluids (SF) were obtained from patients undergoing aspiration of symptomatic knee joints. Clinical diagnoses covered a wide range of rheumatic diseases as indicated in the results section. All of the patients were receiving non-steroidal anti-inflammatory drugs, some of them were on second-line drugs (such as gold salts, chloroquine or penicillamine) and a small number were receiving low dose oral corticosteroids. The SF tested were mainly from patients with chronic rheumatic diseases but there were a few cases of septic arthritis or acute gout. Cytokine levels in synovial fluids were tested in different studies (over a period of 2 years) and compared with diagnosis, presence of radiological bone erosions, sex, age, duration of disease, and with drug therapy. Clinical laboratory tests included: haemoglobin (Hb), white cell count (WCC), platelet count (Plt) and erythrocyte sedimentation rate (ESR).

Synovial fluids were collected in EDTA (5mM) under sterile conditions. After centrifugation at 1800G for 30mins at 4° C, supernatants were aliquoted and stored at -70° C until analysis. Some aliquots of SF underwent an additional hyaluronidase digestion (37° C, 30 mins, 150U/ml bovine testicular hyaluronidase, Sigma, UK) followed by a second centrifugation. These supernatants were stored for subsequent parallel testing with the non-treated samples. A total of 228 SF were collected and used in these studies. Of these, 15 were tested for IL1 biological activity, 137 for TNF biological activity, 32 for immunoreactive IFN gamma, 119 for IL1 beta by RIA, 130 for IL1 alpha (IRMA) and 134 for TNF alpha (IRMA). Thirty two patients also had blood taken at the same time as joint aspiration with plasmas or sera being stored at -20° C.

3.b.2.   Reagents

Human recombinant (hr) IL1 alpha and IL1 beta were provided by Biogen (Geneva, CH), Immunex (Seattle, USA) and Dainippon (Osaka, Japan). Human recombinant TNF alpha, hr TNF beta, monoclonal antibody ("TNF-E") specific for hr TNF alpha and rabbit antiserum to hr TNF beta were all kindly donated by Dr G Adolf (Boehringer Inst, Wien, Austria). Mouse mab to human MHC class II was kindly provided by Dr M Steele (MRC Cytogenetics Unit,

Edinburgh). Human recombinant IFN gamma was kindly donated by Dr C A Dinarello (Tufts Univ., Boston, USA).

3.b.3. Assays for biological activity

The D10.G4.1 T cell proliferation and L929 cytotoxicity assays (described in 2.b.3.2, p. 45 and 2.b.6.1, p. 50) were used to test for IL1 and TNF biological activity in synovial fluids. Both of these biological assays have been previously tested for specificity and synergistic interactions against a panel of human and murine cytokines. In particular, D10.G4.1 T cell proliferation was not influenced by the presence of hr TNF alpha and L929 cytotoxicity could not be obtained by a wide range of IL1 alpha or hr IL1 beta concentrations (experiments shown in Fig. 5, p. 57 and Fig. 12, p. 63).

To confirm that L929 cytotoxicity by SF was due to TNF, some positive samples were assayed in the presence or absence of mab to hr TNF alpha or a control mab. These quantitative experiments indicated that some cytotoxic samples could not be neutralised by anti human TNF alpha mab. In order to determine the proportion of fluids with TNF alpha biological activity, a further 44 fluids were subsequently tested for L929 cytotoxicity in triplicate at one dilution only (1:8), after 1 hour preincubation with either culture medium, anti hr TNF alpha mab, anti-human DR (control ab) or rabbit antiserum to hr TNF beta.

3.b.4. Assays for biological inhibition

Eight SF were tested for inhibitory activity of L929 cytotoxicity by hr TNF alpha. Dilution of each synovial fluid were tested in the presence or absence of hr TNF alpha (40pg/ml). Each plate included wells in which only hr TNF alpha was added (40pg/ml, in one case 1ng/ml). Calculation of the TNF alpha-inhibitory activity of each SF dilution was possible after 24 hours culture. This was quantified by plotting SF dilutions against percentage of biological inhibition (Fig. 62). One inhibitory unit was defined as the dilution of SF able to neutralize 75% of the cytotoxicity induced by 40pg/ml hr TNF alpha. This concentration was chosen as it is easily detectable in L929 cytotoxicity but it is still in the suboptimal range of the assay. Details on biological inhibition assay and calculations are in Appendix (p. 211). Inhibition of IL1 biological activity was tested

in 4 synovial fluids using the D10.G4.1 assay, by comparing the proliferative response (expressed in proliferation units) to a suboptimal concentration of hr IL1 beta (60pg/ml) in the presence or absence of SF dilutions (Fig. 63).

3.b.5. Cytokine immunoassays

Interleukin 1 beta

Immunoreactive human IL1 beta was assessed using a commercially available radioimmunoassay (Cistron Biotech/Lab. Impex) according to a published protocol (Lisi et al, 1987). This assay is based on the competition for specific anti-human IL1 beta rabbit antiserum between ^{125}I-labelled IL1 beta and IL1 beta in the sample or standard tubes. The detection limits of this assay are 0.25-5ng/ml hr IL1 beta. Determinations were in duplicate tubes and experiments were performed to control for specificity (Fig. 64), recovery of exogenously added hr IL1 beta (78±5%; Table 3.2) linearity of dilutions (Fig. 65) and interassay variability (less than 10% in 5 samples tested in different assays).

Interleukin 1 alpha

Levels of human IL1 alpha in aliquots of synovial fluids were assessed by Dr S. Poole (N.I.B.S.C., Potters Bar, UK) using a sensitive two-site immunoradiometric assay (Thorpe et al, 1988). Briefly, wells of a flexible 96 well microtitre plate were coated with monoclonal ab to IL1 alpha. After washes and blocking of remaining protein binding sites, samples or standard hr IL1 alpha were added followed by overnight incubation. After further washes, ^{125}I-labelled "developing" antibody (sheep antiserum to hr IL1 alpha) was added and after final vigorous washes residual radioactivity was estimated in a gamma counter. This assay was demonstrated to be suitable for SF determinations by studying interassay variability, recovery of exogenously added hr IL1 alpha and linearity of dilutions (Thorpe et al, 1988). Detection limits were 0.02-10ng/ml hr IL1 alpha.

Interferon gamma

A commercially available IRMA (Sucrosep-Gamma, Boots-Celltech, UK) was used to test human IFN gamma levels in 32 synovial fluids. The test samples were incubated with ^{125}I-labelled monoclonal antibody to IFN gamma, followed

by capture of the ^{125}I-labelled complex with a sheep-antihuman IFN gamma antibody coupled to a solid phase. Bound labelled mab was isolated by sucrose layering separation, with radioactivity in the lower, denser phase being proportional to the IFN gamma content in the sample or standards. To detect the presence of interfering substances, different dilutions of IFN gamma were tested in presence of two synovial fluids (Fig. 66). Detection limits for this assay were 1-1024U/ml.

Tumour necrosis factor alpha

The presence of immunoreactive human TNF alpha in SF samples was determined by Dr A Meager (N.I.B.S.C., Potters Bar, UK) using a solid phase, two-site "sandwich immunoassay" method, with less than 10% inter-assay or intra-assay variation over the range 25pg to 25ug/ml hr TNF alpha (A. Meager, Personal Communication). This IRMA is based on the capture of ligand in the samples or standards on etched polystyrene beads which have been coated with hr TNF alpha monoclonal antibody. After extensive washings, ^{125}I-labelled anti-human TNF alpha is used to assess the amount of ligand trapped by the first antibody. Under these conditions, the IRMA has a lower detection limit of 12.5pg/ml hr TNF alpha. Although hyaluronidase treatment dramatically reduced SF viscosity, when paired samples (n=9) were tested in blind experiments (Fig. 67) no significant difference was observed (signed rank test, p=>0.5) in TNF immunoreactivity. Other blind controls included testing the recovery of exogenously added hr TNF alpha (104.32±8.35%; Table 3.3) and linearity of dilutions (Fig. 68).

3.b.6.   Statistical analysis

This was performed with an "EPISTAT" statistical package for IBM PC. Analysis included:

1. Assessment of correlation between different cytokines in SF samples. Qualitative agreement was studied by Kappa statistic (Everitt, 1968), quantitative correlation by Spearman's rank correlation, before and after selection for samples which were "positive" for both cytokines.

2. Comparison between levels of one cytokine in SF samples, and clinical data from patients:
    a. continuous variables (Hb,ESR, Plt, WCC, age, duration of disease)

were tested by Spearman's rank coefficient, and by one-way analysis of variance (after division of patients in 3 groups, according to the cytokine levels in their SF)

b. dichotomous variables (x-ray erosions, sex) were tested by unpaired T-test, on two groups divided according to sex or the presence of erosions

c. non-continuous variables (diagnosis, drug treatment) were analysed by one-way analysis of variance, and by chi-square test (dividing the patients in 3 groups, according to the cytokine levels in their SF).

3. Differences in cytokine levels between paired samples. Signed rank-test was used to compare samples that underwent different treatment (hyaluronidase digestion).
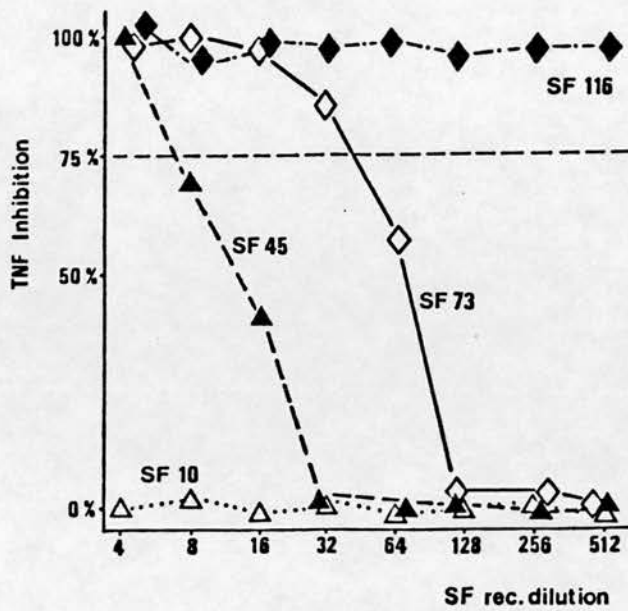
Fig. 62   Inhibition of TNF alpha biological activity by SF dilutions

Typical dilution curves of TNF alpha-inhibiting activities in some of the SF tested; SF 10 was tested against hr TNF alpha at 1ng/ml, while SF 73, 45 and 116 against 40pg/ml hr TNF alpha. The four fluids have respectively no inhibition of 1ng/ml TNF alpha and about 8, 58 and >512 inhibition U/ml on 40pg/ml hr TNF alpha.
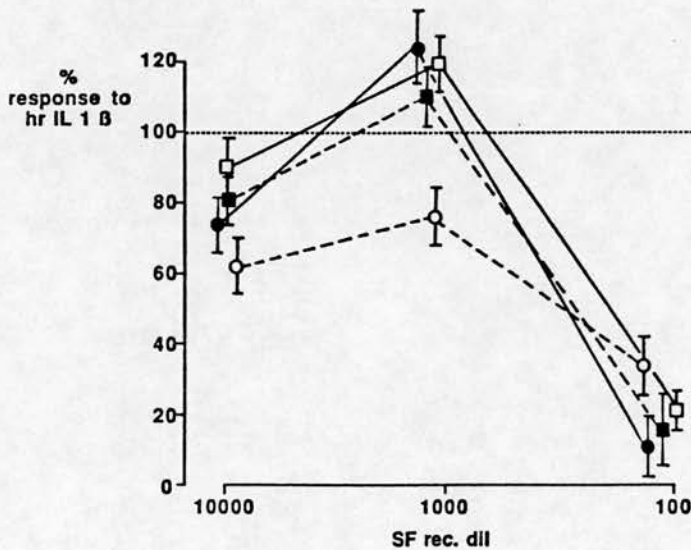

Fig. 63   Inhibition of IL1 biological activity by SF dilutions

Dilutions of SF were tested for their ability to inhibit IL1-driven D10.G4.1 proliferation. T-cell mitogenesis, expressed in proliferation units (cpm sample + cells + Con A/cpm cells + Con A) were compared in triplicate cultures incubated with hr IL1 beta (60pg/ml) and in the presence or absence of SF dilutions.
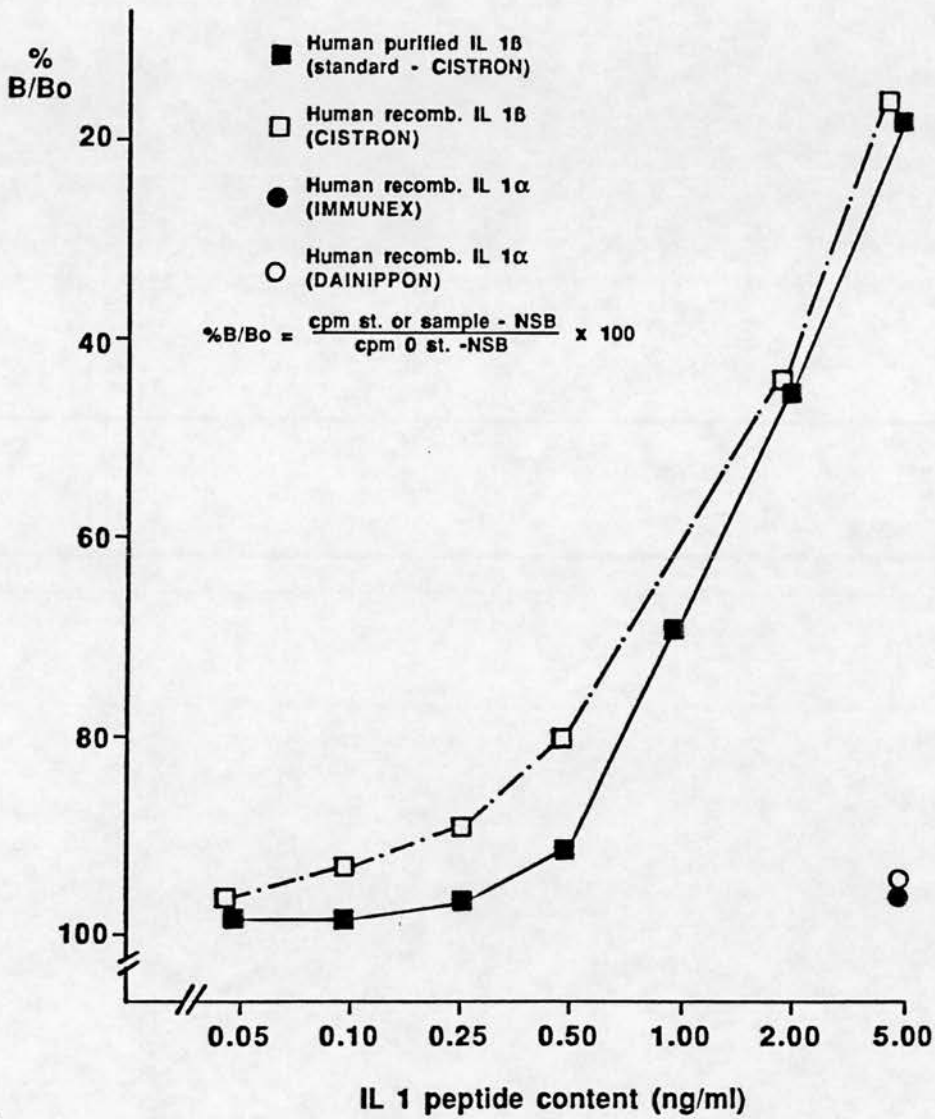
170

Fig. 64   Interleukin 1 beta specificity control

Different IL1 beta and IL1 alpha proteins were tested in the IL1 beta radioimmunoassay. IL1 alpha was not detected in the assay, indicating no antibody-crossreactivity. Other cytokine preparations tested and similarly undetected by the RIA included hr IFN gamma (1000U/ml) and hr TNF alpha (5ng/ml).

171

		hr IL 1 beta added (ng/ml)			
		–	0.5	1.0	2.0
SF 120	expected		0.5	1.0	2.0
	determined	< 0.125	0.304	0.731	1.632
	% recovered		60.8 %	73.1 %	81.6 %
SF 116	expected		1.321	1.821	2.821
	determined	0.821	0.972	1.582	2.687
	% recovered		73.6 %	84.1 %	95.25 %

Table 3.2   "Spiking" of fluids with hr IL1 beta

Human recombinant IL1 beta was tested in the presence or absence of SF. hr IL1 beta solutions or BSA buffer were mixed with four volumes of SF and the final dilutions(80% SF) tested in IL1 beta RIA.   Overall recovery was 78.07±4.78%.
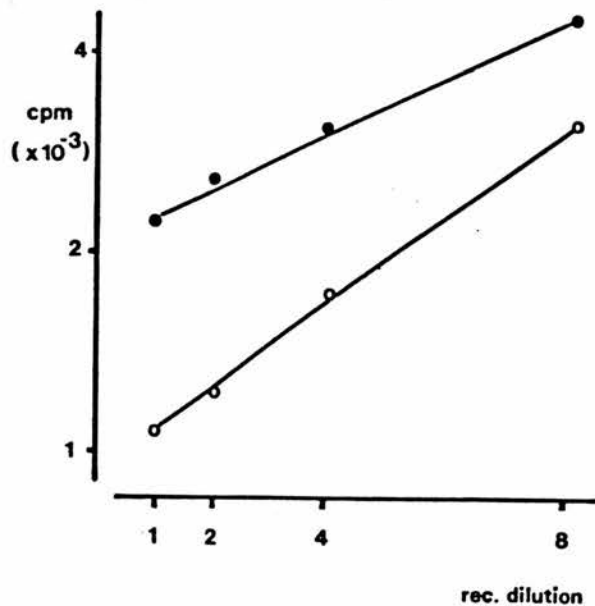

Fig. 65   Immunoreactive IL1 beta in SF dilutions

Two synovial fluids were tested in different dilutions to test for linearity.   The two fluids tested had 4.41ng/ml (closed circles) and 18.05ng/ml (open circles) IL1 beta.
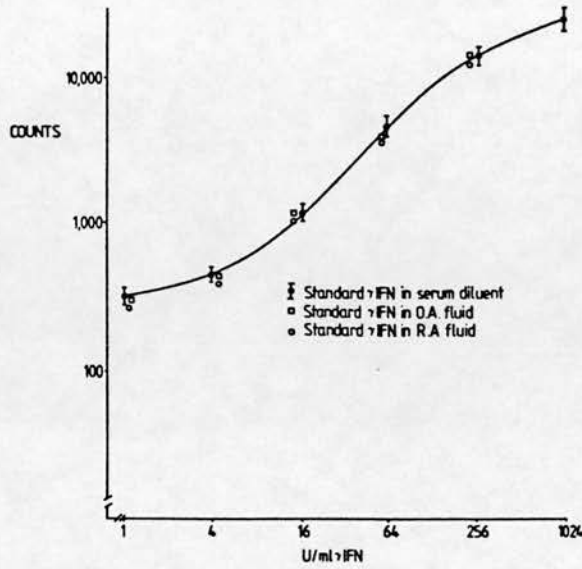
Fig. 66 "Spiking" of fluids with human IFN gamma

Different concentrations of human IFN gamma were tested in presence or absence of two different synovial fluids. These did not influence IFN gamma detection in the IRMA used.
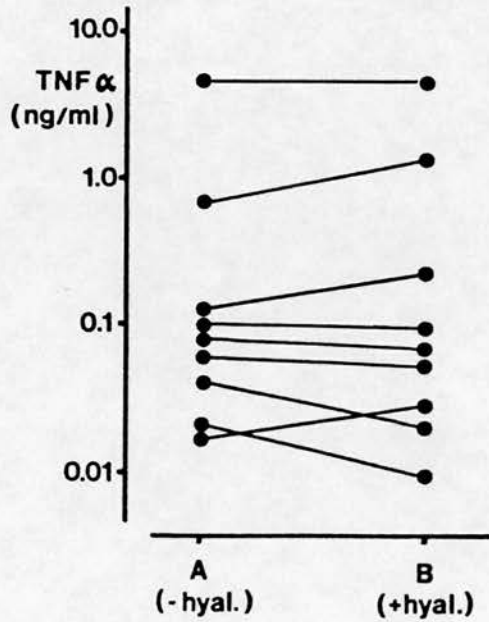

Fig. 67 Hyaluronidase treatment of synovial fluids

In each of nine SF, supernatants from one aliquot (A) were stored at -80° C after centrifugation (1800G, 30 mins); a second aliquot underwent hyaluronidase treatment (150U/ml, 37° C, 30 mins) prior to a second centrifugation. These aliquots (B) were stored until testing in parallel with (A) in a TNF alpha IRMA. Signed-rank test confirmed that hyaluronidase treatment does not influence yield of TNF alpha from synovial fluids.

173

		hr TNF alpha added (pg/ml)			
		–	50	500	5000
SF 73	expected		4250	4700	9700
	determined	4200	4500	5500	9000
	% recovered		105.9 %	117 %	92.8 %
SF 159	expected		50	500	5000
	determined	< 12.5	70	340	4500
	% recovered		140 %	68%	90 %
SF 133	expected		73	523	5023
	determined	23.0	105	480	4500
	% recovered		143.8%	91.8 %	89.6 %

Table 3.3   "Spiking" of synovial fluids with TNF alpha

Recovery of hr TNF alpha added to different SF was studied in these experiments.   Overall   recovery   was   104.3±8.35%.   Dilutions   performed   as   in Table 3.2 legend.
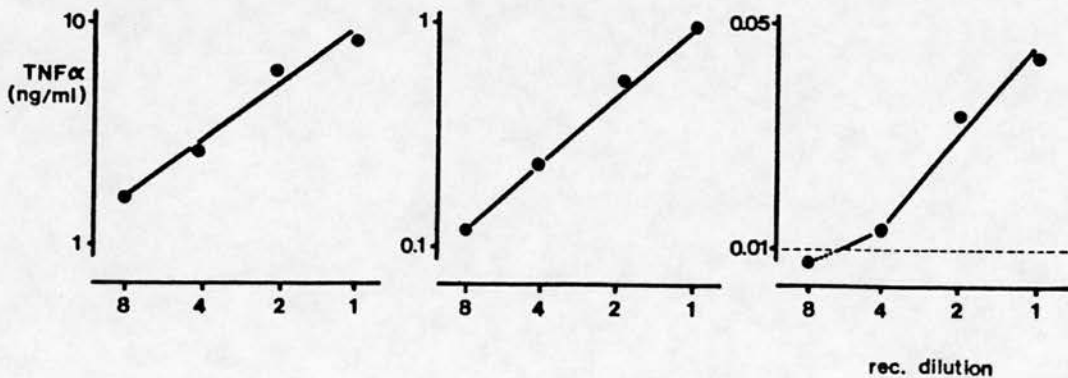

Fig. 68   Dilutions of immunoreactive TNF alpha in synovial fluids

Three   different   synovial   fluids   were   tested   for   immunoreactive   TNF alpha in a blind experiment, in different dilutions.   The three SF had been originally   chosen   from   a   wide   range   of   TNF   alpha   concentrations   (40pg/ml   to 7ng/ml).

3.c. RESULTS

3.c.1. Interleukin 1 biological activity in synovial fluids

Our preliminary attempts to demonstrate IL1 bioactivity in unfractionated joint effusions revealed strong inhibitory activity of the fluids (at 1:100 or higher concentrations) on D10.G4.1 T-cell proliferation in tissue culture (Fig. 63). When tested in the presence of a suboptimal concentration of hr IL1 beta (60pg/ml) which gave 67.4 ± 2.7 p.u., synovial fluids were powerful inhibitors of T-cell mitogenesis. However, at higher dilutions of SF an increased response of 86.9 ± 5.05 p.u. (3 SF in triplicate) was observed, suggesting the unmasking of IL1 biological activity by dilution of the inhibitory action of synovial fluids.

3.c.2. Interleukin 1 beta RIA

A total of 119 joint fluids were tested for the presence of immunoreactive IL1 beta (Table 3.4). High levels of IL1 beta (up to 18ng/ml) were present in most of the fluids tested (76.5%) without association with any particular rheumatic condition. The "miscellaneous" group included two samples from a patient with septic arthritis (before and after two weeks antibiotic therapy), one palindromic rheumatism, one gout (three days after the acute attack: 2.44ng/ml IL1 beta) one ankylosing spondylitis, one recurrent monoarthritis and one psoriatic arthritis.

Interleukin 1 beta was not related to TNF alpha (n=55), IL1 alpha (n=54) or IFN gamma (n=7) levels in fluids which had been tested for these cytokines. No correlation with clinical and laboratory parameters could be demonstrated.

Circulating levels of IL1 beta (in 23 sera and 9 plasmas) were below the assay detection limit (250pg/ml), while parallel synovial fluids had high levels of immunoreactive cytokine (up to 4.85ng/ml IL1 beta in 31 out of 32 samples).

3.c.3 Interleukin 1 alpha IRMA

Levels of immunoreactive IL1 alpha were determined in 130 synovial fluids (Table 3.5). Of these, fifty (38.5%) had detectable levels of IL1 alpha (up to 3.5ng/ml). The "miscellaneous" group included one patient with Behcet's syndrome and four with ankylosing spondylitis (all negative), five psoriatic arthritis (2 being positive), three seronegative monoarthritis

(one was positive), one gout (62pg/ml IL1 alpha), three septic arthritis fluids (all positive, two from the same patient after two weeks therapy), one hairy-cell leukemia synovitis (82pg/ml), two palindromic rheumatisms (both positive). No correlation was demonstrated with other cytokines in fluids which had been tested for several proteins (TNF alpha : n=130; IL1 beta; n=54; IFN gamma; n=25). Clinical and laboratory tests of disease activity did not correlate with IL1 alpha levels in this cross-sectional study.

3.c.4. Interferon gamma IRMA

Interferon gamma levels were studied in 32 synovial fluids (27 rheumatoid arthritis, 5 osteoarthritis). All fluids read below the detection limit of the IRMA used (1U/ml). This could not be attributed to the presence of interfering substances in the fluids as demonstrated by "spiking" experiments.

3.c.5. Tumour Necrosis Factor biological activity in joint fluids

Of the initial 93 SF tested, 35 had TNF-like activity of 4U/ml or greater. Table 3.6 summarizes the clinical diagnoses and levels of TNF-like activity. The two positive fluids in the miscellaneous group were from patients with recurrent monoarthritis. Several SF with the highest levels had been assayed fresh, suggesting that activity may be lost after freeze-thawing or storage at -70° C.

To test for specificity of these readings, eight of the 35 fluids with TNF-like activity were tested in full titrations alone or with anti-TNF alpha or control mab (Table 3.7). Six were completely neutralized by anti-TNF alpha but two were not, suggesting that other cytotoxic factors were present in these samples.

To determine the proportion of SF that contained cytotoxic activity attributable to TNF alpha, we tested a second group of 44 SF in triplicate at one dilution only (1:8) following pre-incubation with medium control or antibodies (1:1600, 30 mins, room temp.). Of these 44 fluids 13 (30%) contained TNF activity that was unaffected by control antibody but completely neutralized with anti TNF ab. These included SF from patients with rheumatoid arthritis, septic arthritis, ankylosing spondylitis, osteoarthritis, psoriatic arthritis, reactive arthritis and gout. Twenty three samples (52%) had no TNF cytotoxicity, and eight (18%) were cytotoxic

176

for L929 cells but were either not neutralized with anti-TNF ab or were affected by the irrelevant mab. Interestingly, of the 21 SF cytotoxic for L929 cells, none was neutralized by antiserum to lymphotoxin.

No association was found between levels of TNF activity in SF and clinical or laboratory parameters. No correlation was demonstrated between levels of IL1 beta (n=53) or IL1 alpha (n=127) and TNF biological activity.

3.c.6.   Inhibitors of TNF biological activity in SF

Of the nine fluids tested, one fluid did not inhibit 1ng/ml hr TNF alpha cytotoxicity at any of the concentrations tested (1:4 to 1:512). Five SF contained TNF inhibitory activity quantifiable at between 8 to 54U/ml (respectively 8,12,12,48,54 U/ml, representing inhibitory activity of 0.24 to 1.62ng/ml TNF alpha. Three other SF showed very high inhibitory activity against 40pg/ml hr TNF alpha that did not dilute even at 1:512 dilution.

3.c.7.   Tumour Necrosis Factor alpha IRMA

Because of the presence of such powerful biological inhibitory activity in SF, TNF alpha was tested by TNF IRMA on a total of 134 fluids. Of these, 64 (47.8%) had levels of TNF exceeding 12.5pg/ml (range: 12.5pg/ml to 4.9ng/ml) (Table 3.8). The miscellaneous group included patients with a variety of rheumatic diseases. The eleven positive SF in this group were from one juvenile chronic arthritis, one palindromic rheumatism, one gout, two ankylosing spondylitis, one psoriatic arthritis, one polymyalgia rheumatica and three septic arthritis (two from the same patient at different times).

No significant correlation was noted with clinical parameters in the whole population, nor within each disease group. There was no significant difference in TNF titres between disease groups and no relation to drug therapy. No quantitative correlation existed with IL1 beta (n=55), IL1 alpha (n=130) and IFN gamma (n=26) levels, nor with TNF biological activity in the forty samples which were positive in both assays. This last observation was not surprising in view of the presence of powerful inhibitors of biological activity differentially distributed in SF.

When qualitative comparison was performed on 130 SF tested for TNF alpha IRMA and L929 cytotoxicity (Table 3.9) however, 61 fluids were negative in both assays and 40 positive in the two different tests (77% agreement). Seven fluids out of 130 (5.4%) were negative in the IRMA and

177

positive in the L929 assay, possibly indicating degradation of TNF alpha in the fluids between the experiments (12 to 6 months) or cytotoxic activity not mediated by TNF alpha. Of the 22 fluids positive in the IRMA and negative in the L929 assay, 20 had a level of TNF alpha that was below the sensitivity of the L929 assay. When these 20 fluids are excluded on the basis of different assay sensitivities, agreement increases to 91.8% (101/110). K value for these comparisons were 0.506 (n=130) and 0.830 after exclusion (n=110) (ideal agreement: K= +1; complete disagreement : K= -1)

IL1 beta (ng/ml)	RA+	RA-	OA	RT	MISC	TOTAL
< 0.25	9 *	8	4	7		28
0.25-1.0	11	9	1	2		23
1.0-2.5	21	7	1	3	2	34
2.5-4.0	11	2	1	3		17
4.0-5.5	4	1		1	2	8
5.5-10.0	2	2	1		3	9
> 10.0		1				1
TOTAL	58	30	8	16	7	119
% "positive" **	84.5	73	50	56.2	100	76.5

Table 3.4. Immunoreactive IL1 beta in joint exudate fluids

RA+ = seropositive rheumatoid arthritis; RA- = seronegative rheumatoid arthritis; OA = osteoarthritis: RT = reactive arthritis; Misc = miscellaneous group. Each fluid was tested in duplicate tubes.
* Number of patients in this category.
** Percentage of patients with IL1 beta levels >0.25ng/ml (detection limit)

IL1 alpha (ng/ml)	RA+	RA-	OA	RT	MISC	TOTAL
< 0.025	32 *	21	10	6	11	80
0.025-0.1	7	6	3	2	5	23
0.1-0.5	11	4	2	3	2	22
0.5-1.0	1					1
> 1.0	1		1		2	4
TOTAL	.52	31	16	11	20	130
% "positive"**	38.5	32.2	37.5	45.5	45.0	38.5

Table 3.5 Immunoreactive IL1 alpha in synovial fluids

Abbreviations used are as in Table 3.4 legend. Each sample tested in triplicate.
* Number of patients in this category.
** Percentage of patients with IL1 alpha levels >0.025ng/ml (detection limit)

179

TNF activity (U/ml)	RA+	RA-	OA	RT	PA	AS	Misc	Total
< 4	29 *	11	6	2	3	1	6	58
4-16	9	7	3	4	2	1	1	27
16-28	2		1	1		1		5
28-40			1					1
40-52	1							1
> 52							1	1
TOTAL	41	18	11	7	5	3	8	93
% "positive"**	29	39	46	71	40	67	25	38

Table 3.6   TNF biological activity in SF from patients with different rheumatic diseases

RA+ = seropositive rheumatoid arthritis; RA- = seronegative rheumatoid arthritis; OA = osteoarthritis; RT = reactive arthritis; PA = psoriatic arthritis; AS = ankylosing spondylitis; Misc = miscellaneous group. Each fluid was measured at least three times in 11 dilutions in different assays.
*   Number of patients in this category.
**   Percentage of patients with TNF levels >4U/ml (detection limit)

	Sample only	Sample with anti-TNF *	Sample with anti-DR **
SF 73 (OA)	39	< 4	34
SF 69 (AS)	42	< 4	41
SF 78 (OA)	14	< 4	ND
SF 158 (OA)	11	< 4	11
SF 161 (RA+)	50	< 4	49
SF 91 (RA+)	5	< 4	ND
SF 116 (RA+)	19	5	10
SF 112 (RT)	7	9	ND
hr LT (1ng/ml) ***	24	22	30
hr TNF (1 ng/ml)	22	< 4	21

Table 3.7   Antibody neutralization of synovial fluid TNF-like activity

All values are expressed in L929 cytotoxicity units/ml.
*   mab to human recombinant TNF alpha (1:1600)
**   mab to human MHC class II product as control antibody (1:800)
***   hr LT = human recombinant lymphotoxin (TNF beta); ND = not done; other abbreviations as in Table 3.6.

TNF alpha (ng/ml)	RA+	RA-	OA	RT	Misc	Total
< 0.0125	29	18	11	3	9	70
0.0125-0.025	6	2		1	5	14
0.025-0.25	16	9	1	4	3	33
0.25-2.5	3	1	5	3	3	15
> 2.5	1		1			2
Total	55	30	18	11	20	134
% "positive"	47.3	40.0	38.9	72.7	55.0	47.8

Table 3.8   Immunoreactive TNF alpha in SF

Fluids were tested in triplicate tubes. "Positive" samples are those with >12.5pg/ml TNF alpha (detection limit). Other abbreviations as in legend for Table 3.4.

		TNF alpha IRMA (det. limit = 12.5 pg/ml)		
		"positives" (n)	"negatives" (n)	
L929 bioassay (det.limit = 160 pg/ml)	"positives" (n)	40	7	Total = 47
	"negatives" (n)	22 *	61	Total = 83
		Total = 62	Total = 68	Total = 130

Table 3.9   Qualitative comparison between bioactive and immunoreactive TNF in SF

Of the 22 fluids labelled with [*], 20 had TNF alpha IRMA levels between 12.5 to 160pg/ml, below the detection limit of the L929 bioassay ("negatives" for L929 cytotoxicity) but detectable in the immunoassay ("positives" in the TNF alpha IRMA).

181

3.d.   DISCUSSION

The aim of this study was to test whether unfractionated synovial fluids from arthritic patients contained biologically active or immunodetectable levels of IL1 alpha, IL1 beta, IFN gamma or TNF alpha.

Different proportions of the fluids tested contained immunoreactive IL1 alpha (38.5%), IL1 beta (76.5%), TNF alpha (47.8%) in biologically significant levels. These cytokines, but not IFN gamma, were present in synovial effusions from different rheumatic diseases, arguing against any of these cytokines being a specific factor in the pathogenesis of any particular disease. There have been previous reports (Fontana etal, 1982B) of detected bioactive IL1 in unfractionated fluids from patients with rheumatoid arthritis but not with osteoarthritis. In our studies, osteoarthritis patients had comparable levels of IL1 alpha and IL1 beta to the rheumatoid patients and we failed to detect any IL1 biological activity in unfractionated fluids. This is in contrast with the high levels of immunoreactive IL1 beta protein that we detected in most of the samples. It is possible that IL1 beta is present in the fluids only in its inactive precursor form. This seems unlikely given the presence in SF of proteolytic enzymes and particularly of elastase (Pryce-Jones, Wood, 1975), which cleaves the IL1 beta propeptide to its mature form (Webb et al, 1987). Furthermore, we have previously shown that RA synovial cells release bioactive IL1 (chapter 3), and in other studies this has been reported to be mainly of the pI7 form (Wood et al, 1985).

An explanation for this lack of IL1 biological activity is provided by the observations that SF from RA and osteoarthritis contain powerful biological inhibitor(s) of murine T cell co-mitogenesis. Their presence has been documented previously (Wood et al, 1983; Bendtzen et al, 1985) and could be of potential importance, but specific inhibitors have not been characterized and their actions in a human system are only hypothetical.

Tumour necrosis factor biological activity could be measured in 35 (38%) of 93 unfractionated synovial fluids. In six of eight positive SF tested, this activity was neutralized by monoclonal anti-TNF alpha. Of a further 44 fluids which were tested in a single dilution assay, 13 (30%)

contained TNF activity which was neutralized only by mab for TNF alpha. None of the 21 cytotoxic samples in this series was neutralized by antiserum to LT. When compared with TNF measurements by immunoassay, they showed excellent qualitative agreement (92%) when the difference in assay sensitivity was taken into account. Among the samples in which TNF alpha was tested by a specific IRMA, a large proportion (47.8%) were "positive", the difference being related to the higher sensitivity of the assay (12.5pg/ml was detectable against 160pg/ml TNF alpha in the bioassay). On a quantitative basis, however, no correlation could be demonstrated between TNF alpha bioactivity and immunoreactive protein in SF. In analogous experiments to those previously discussed for IL1, we could demonstrate the presence in SF of inhibitor(s) of TNF biological activity, whose pattern of distribution may account for this lack of correlation.

Aside from the issue of biological inhibition, immunoreactivity and bioactivity of a peptide ligand measured ex vivo, will be different at any time, immune recognition not being restricted to receptor binding regions. Inert molecules, both larger and smaller than the mature peptide could react with antibody raised to the mature form. However, immunoreactive levels reflect cytokine production over time (rather than what happens to be biologically active at any one moment) and may well be a better indicator of chronic disease activity.

Nonetheless, when in our study synovial fluid levels of immunoreactive TNF alpha, IL1 beta or IL1 alpha were compared with a variety of clinical or laboratory parameters of disease activity, no significant association could be demonstrated. The conclusions are that systemic markers of inflammation are more likely to be related to levels of cytokines in the circulation rather than in the synovial space. In addition, the well known inter-individual variability in cytokine production (Endres et al, 1987) suggests that it may be more appropriate to correlate disease activity with cytokine levels in a longitudinal study, which would require the study of blood levels.

In preliminary experiments we could not detect immunoreactive IL1 beta in sera and plasma from 32 patients, probably related to the low sensitivity of the RIA used (0.25ng/ml IL1 beta lower detection limit) or the presence

of carrier protein(s) for IL1, masking binding sites.

As a development of these studies, and using a sensitive IL1 beta ELISA (detection limit : 20pg/ml) in combination with a recently developed extraction method (Cannon et al, 1988), a study from our group (Eastgate et al, 1988) has, for the first time, correlated IL1 beta levels in plasma with several laboratory and clinical parameters of disease activity in patients with rheumatoid arthritis. This longitudinal study also demonstrates that IL1 levels correlate with patient clinical score both during clinical improvement and exacerbation.

The only "longitudinal" data in the present study are from a patient with septic arthritis, where diagnostic joint aspirations could be performed at the clinical onset, and repeated after two weeks of antibiotic therapy. Clinical improvement was accompanied by a dramatic fall of the cytokine levels in SF (TNF alpha: from 1.22 to 0.43ng/ml; IL1 alpha: from 0.110 to undetectable levels; IL1 beta: from 8.09 to 4.02ng/ml).

Since cytokine genes are not constitutively expressed, the findings of IL1 beta, IL1 alpha and TNF alpha in rheumatic patients *ex vivo* strongly suggests that these genes are activated *in vivo* in the course of the disease. We have previously shown that synovial cells of rheumatoid patients released IL1 and TNF without deliberate induction. This has been more appropriately confirmed by demonstrating cytokine-specific mRNA in snap frozen surgical specimens, by using in situ hybridization in this laboratory (Duff et al, 1988).

A more complete understanding of the interactions between these cytokines in the immunoregulatory network and their roles in the pathogenesis of arthritic diseases is now accessible with the reagents available and should allow for the development of novel treatments.

4.   CONCLUDING DISCUSSION

The data presented in the first section of this work support the idea that IL1 and TNF protein are produced in human cells only following specific stimulation and that their synthesis is mainly regulated at the level of transcription. Further studies, in which we reported the presence of these cytokines at the site of inflammation in patients with rheumatic diseases, indicate that IL1 and TNF genes are activated in these patients during the course of disease.

Interleukin 1 has been shown to be arthritogenic *in vivo* (Pettipher et al, 1986) and plasma IL1 beta levels correlate with disease activity in patients with rheumatoid arthritis (Eastgate et al, 1988). IL1 and TNF mRNA have been detected in synovium from RA patients (Buchan et al, 1988; Duff et al, 1988) and both cytokines are capable of bone and cartilage resorption in organ culture.

Several unanswered questions remain and will provide scope for further investigations in this area:

1.   Can we identify a common pro-inflammatory event in the cytokine network? Several cytokines other than IL1 and TNF have been identified at the site of inflammation in rheumatic diseases. These include IL2, IL6 (Houssiau et al, 1988) and TGF beta (M. Sporn, unpublished), each of which is able to play a role both as effector molecule or in feed-back networks. The regulation of cellular receptors certainly plays a role in determining the final biological function of the system, and soluble receptors that retain their binding capacities, must also be regarded as immunoregulatory entities. Among the latter, soluble CD8 and soluble IL2R have been recently demonstrated to correlate with clinical status in rheumatic patients (Symons et al, 1988A; Wood et al, 1988A).

A more complete understanding of the physiology of immunoregulation might facilitate the identification of the "lesion" in chronic inflammatory diseases such as RA. More direct approaches, i.e. testing a monoclonal antibody to an effector or regulatory molecule, have been frustrated partly by the lack of adequate reagents, and mainly by the absence of a suitable animal model of rheumatoid arthritis.

2.   Which cell populations produce these pro-inflammatory cytokines in the course of disease? Methods involving cell separation procedures are probably not suitable for such studies, because of the high risk of

inadvertent cell activation. Furthermore, the availability of human synovium is mostly from patients undergoing joint replacement surgery, and therefore at a late stage of disease. The study of cytokine gene expression in earlier disease will require the use of needle-biopsy specimens, with obvious problems related to the quantity of tissues available. Combined in situ-hybridization-immunophenotyping has, so far, demonstrated IL1 beta to be predominantly produced in the stromal areas of the synovium, while the IL6 gene is expressed mainly in perivascular cells and lymphoid follicles. In contrast to IL1, the predominant cell-type expressing IL6 appears not to be the macrophage (Wood et al, 1988B). To study cytokine gene abnormalities in such small numbers of cells will probably require amplification of DNA sequences using polymerase chain reaction (PCR) to overcome the extremely low yield of cells; combination with *in vitro* transcription could provide material for qualitative mRNA studies. Quantitative mRNA analysis could be achieved by reverse transcription followed by automated PCR.

Assessment of immune functions in synovium at different stages of disease and identification of cell-types and their activities, is clearly still a basic goal in the understanding of rheumatic inflammation.

3. **Why and how does a pro-inflammatory cytokine become pathogenic?** Sustained cytokine production could be related to abnormal transcriptional activation (due to persistence of the stimulus - i.e. chronic infection - or dysregulation of transcriptionally-active regulatory elements) or to lack of active repressor mechanisms. Our data, showing induction of IL1 and TNF by MSU crystals and the detection of these cytokines in gouty patients, suggest that gout could be a model of arthritis in which IL1/TNF over-production is the key mechanism of the acute attack with fever, swelling and all the typical inflammatory signs.

For the establishment of chronic inflammation, self-sustaining mechanisms are needed. The reduction of T cell sensitivity to potentiating factors such as IL2 is an early observation in several chronic inflammatory diseases. Such reduced susceptibility could lead to failure of appropriate immune responses (such as eradication of foreign antigen). In autoimmune diseases, failure to respond to IL2 stimulation has been correlated with increased levels of sIL2R (Symons et al, 1988B). As soluble IL2R could

mediate deficient T cell activation in RA, it could also determine the lack of T-cell derived inhibition of IL1 synthesis, which is thought to be mediated by IFN gamma (Ghezzi et al, 1988). This view of the pathogenic cycle is supported by the absence of IFN gamma in SF, which we have reported, by the correlation between sIL2R and IL1 beta levels in synovial fluids and the correlation of both indexes in plasma with clinical signs of disease activity (Wood et al, 1988A; Eastgate et al, 1988).

A possible positive contribution to the net action of these pro-inflammatory cytokines could be decreased production of agents that inhibit their biological actions. We have demonstrated the presence of inhibitory activities for IL1 and TNF in SF, and several inhibitory moieties have been described (Lotz et al, 1986; Seckinger et al, 1987; di Giovine et al, 1987; Seckinger et al, 1988). Dysregulation of such inhibition might underline an imbalance of the normal cytokine-homeostatic mechanisms.

Current biotechnology and the huge recent interest in this area of research make these objectives within reach in the years to come. It should be possible soon, with the development of therapeutic agents able to block specific pathogenic mechanisms, to take a new approach to the treatment of chronic rheumatic conditions and, at least, bring them into the same clinical category as hypertension or diabetes in which therapeutic intervention can effectively control the development of tissue damage and disease progression. With the recognition and characterization of the different cytokine systems we can now give a molecular substrate to the associations between fever, inflammation and host defence that were familiar to ancient peoples some 6000 years ago.

5.   REFERENCES

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196

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6.   APPENDIX

Preparation of crude TCGF

1. Remove spleen from Wistar rats under aseptic conditions.
2. Dissociate into a single cell suspension in PBS, between two sterile ground glass slides.
3. Wash the cells by spinning at 100g (10 mins) and resuspend at 1×10^7 cells/ml in serum-free RPMI 1640.
4. Add Concanavalin A (5ug/ml) and incubate 20-24 hours (37° C, 5% CO_2).
5. Collect supernatants, spinning first at 300G, then at 1800G (10 mins each).
6. Cell-free supernatants (crude rat TCGF) are stored in aliquots at -30° C and sterile-filtered before use.

Inactivated feeder/antigen presenting cells

1. Dissociate H-2K spleen (i.e. from C3H mice) as in 6.1.
2. Lyse the red blood cells by hypotonic shock.
3. Wash the cells by centrifugation at 100G (10 mins) and resuspend at 2×10^7 cells/ml in PBS containing mytomycin C (50ug/ml; Sigma) and incubate at 37° C for 60 mins.
4. Wash the cells by centrifugation 4 times in PBS to remove the mitomycin C and add to D10.G4.1 cultures at a final concentration of $5-10 \times 10^5$ cells/ml.

Limulus gelation test (LAL assay)

1. Ensure all materials and reagents at LPS-free.
2. Reconstitute lyophylized LAL (limulus amebocyte lysate) on ice, according to manufacturer's instructions.
3. Add 100uls of LAL solution and 100uls of standard LPS or test solution to the bottom of LPS-free tubes (Falcon, 10x75mm stoppered plastic tubes) and mix gently. Include as negative control pyrogen-free PBS.
4. Transfer the tubes to a motionless water bath at 37° C. Leave undisturbed for 1 hour.
5. The presence of an adherent gel (which remains adherent on gentle inversion of the tube) indicates a positive tube.

Several dilutions of standard LPS solution should be assayed to check the sensitivity of the lysate. If the sample is tested at different dilutions, quantitative data can be produced. It is also necessary to check that negative samples do not contain inhibitors of LAL gelation (by addition of a known amount of LPS to the sample).

False negatives have been reported such as when testing trypsin inhibitors or calcium chelants like EDTA (Levin and Bang, 1968), high molarity salt concentrations (>2M; Nandan and Brown, 1977), semisynthetic penicillins (Rhodes et al, 1974), tetracyclines and dexamethazone (van Noordwijk and de Jong, 1977). False positives can take place with trypsin-like enzymes (Sullivan and Watson, 1975), thromboplastin, poly I:C (Elin and Wolff, 1973), ribonuclease (Jorgensen and Smith, 1973), high concentration of calcium (Sullivan and Watson, 1974), muramyl dipeptide (Dinarello et al, 1978), peptidoglycan (Kotani et al, 1977) and exotoxins A, B and C from streptococci (Bruson and Watson, 1976).

Polymyxin B co-incubation

In this experiment, LPS (100ng/ml) and MSU (0.5mg/ml) were tested for their ability to induce IL1 activity from blood monocytes (1×10^6/ml) in presence or absence of polymyxin B (250U/ml), a well known inhibitor of LPS biological activities.

		IL1 bioactivity produced (p.u.)
monocytes incubated with:	medium	0.922 ± 0.115
	LPS	3.592 ± 0.271
	LPS + PMB	0.666 ± 0.421
	MSU	3.051 ± 0.245
	MSU + PMB	3.181 ± 0.112

The IL1 biological activity in supernatants is expressed as mean (±SEM) of proliferation units of triplicate measurements of supernatants (1:25 dilution indicated) assayed in a C3H/HeJ proliferation assay (cell + Con A = 10164 ± 906.4 cpm).

The presence of PMB did not modify the ability of MSU crystal to induce IL1 release, while completely neutralized the IL1-inducing property of LPS.

MSU-crystals do not release soluble activators of IL1 production

To test the presence of soluble activators of IL1 production in association with the crystal preparation, aliquots of crystals (10mg/ml) were resuspended in PBS. After incubation (30 mins) and centrifugation, supernatants (crystal "wash") were added to human monocyte cultures (10% vol/vol). Monocyte supernatants (20 hrs) were then assayed in D10.G4.1 assay for IL1 biological activity.

		IL1 bioactivity produced (p.u.)
monocytes incubated with:	medium	0.824 ± 0.175
	LPS (100ng/ml)	24.680 ± 3.26
	HA (1mg/ml)	1.246 ± 0.182
	HA wash (10%)	1.462 ± 0.330
	MSU (1mg/ml)	61.140 ± 12.21
	MSU wash (10%)	0.485 ± 0.04

Shown are results from the 0.1% dilution of supernatant. These indicate that no soluble activator of IL1 production was released by the crystals (cells + Con A = 311.6 ± 39cpm).

Effects of hrIL1 on L929 cytotoxicity

In this experiment, different amounts of hrIL1 alpha and hrIL1 beta, previously demonstrated to be active on T cells, were tested for L929 cytotoxicity in presence or absence of different concentrations of hr TNF alpha.

		hrIL1 beta (ng/ml)				hrIL1 alpha (ng/ml)			
		0	0.1	1	10	0	0.1	1	10
hr TNF alpha (ng/ml)	0	<4*	<4	<4	<4	<4	<4	<4	<4
	0.1	<4	<4	<4	<4	<4	<4	<4	<4
	1	11.32	11.73	9.94	10.33	11.32	10.57	10.38	10.34
	10	121.96	105.17	132.80	103.40	121.96	128.37	118.24	116.48

IL1 preparations failed to induce L929 cytotoxicity or to synergize with hrTNF alpha in its activity.
* L929 cytotoxicity expressed in half-maximal U/ml.

TNF alpha inhibition studies

1. Seed L929 cells in the inner 60 wells of a microtiter plate ($3x10^4$ cells/100ul EMEM - 10% FCS/well).
2. After 20hrs incubation (5% CO_2, 36.5° C), remove spent medium and replace with 50ul Actinomycin D in EMEM (final conc. = 1ug/ml).
3. Dispense double dilutions of the sample to be tested for inhibition (synovial fluids 1:4 to 1:512) in each row, in absence or presence of hrTNF alpha (final conc : 40pg/ml). Five wells receive only hrTNF alpha (40pg/ml) and five EMEM 10% FCS ("total cells" control). Incubate the plate for 18-20hrs at 38.5° C.
4. Remove the medium, add 0.5% crystal violet in 20% methanol in all wells. After vigorous washing, O.D. is assessed by microELISA spectophotometry at 470mm.
5. For each single well, percentage cytotoxicity (%CTX) is calculated with the formula

$$\% \; CTX = \frac{O.D. \; "total \; cells" \; - \; O.D. \; sample}{O.D. \; "total \; cells"} \; x \; 100$$

6. The ability to inhibit the cytotoxic action of 40pg/ml hrTNF alpha is calculated for each SF dilution with the formula

$$\% \; inhibition = \frac{(\%CTX \; by \; SF)+(\%CTX \; by \; TNF)-(\%CTX \; by \; TNF \; with \; SF)}{(\%CTX \; by \; SF)+(\%CTX \; by \; TNF)} \; x \; 100$$

7. Dilutions of SF are plotted against % inhibition. One inhibitory unit is defined as the dilution of SF able to neutralise 75% of the cytotoxicity induced by 40pg/ml hrTNF alpha.

INTERLEUKIN 1 (IL 1) AS A MEDIATOR OF CRYSTAL ARTHRITIS

Stimulation of T Cell and Synovial Fibroblast Mitogenesis by Urate Crystal-Induced IL 1[1,2]

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We reported before that monosodium urate (MSU) crystals were potent stimulators of endogenous pyrogen (EP) production from human and rabbit mononuclear phagocytes, and proposed that this property of MSU crystals may be important in the pathogenesis of gout. EP activity is now attributed to interleukin 1 (IL 1) peptides but IL 1 is not the only pyrogenic monocyte-derived cytokine, since both interferon-α (α-IFN) and tumor necrosis factor (TNF) are also pyrogenic in rabbits. Using a T cell comitogenic assay based on a murine helper T cell clone that does not respond to IFN or TNF, we now report the release of IL 1 activity from human blood monocytes and synovial fluid mononuclear cells (MNC), following stimulation with MSU crystals. MSU-induced supernatants with IL 1 activity were neutralized with rabbit antiserum to human IL 1 and also stimulated the growth ([^{3}H]thymidine incorporation) of long-term fibroblast-like cell lines derived from human synovial rheumatoid exudate. Two other crystals associated with articular inflammation were tested: hydroxyapatite was a much less potent stimulus compared with MSU crystals, and calcium pyrophosphate dihydrate did not stimulate IL 1 release from human monocytes or synovial fluid MNC. As a model for the inflammatory consequences of acute and chronic overproduction of IL 1, gout is the only sterile inflammatory disease where the local and systemic pathology is compatible with such overproduction; raised IL 1 levels have been found at the site of inflammation, and a necessary etiologic agent, crystalline urate, has been shown unequivocally to be a direct activator of mononuclear IL 1 release.

We have previously found that crystals of monosodium urate (MSU) were potent stimulators of monocyte IL 1[4] release measured in vivo as endogenous pyrogen (EP) activity. This property of MSU crystals was dose-dependent, did not require phagocytosis of the crystals, was independent of serum factors and mediation by other blood leukocytes, and could not be attributed to contamination of the crystals with Gram-negative bacterial endotoxin (lipopolysaccharide: LPS) (1).

In further studies we reported that EP/IL 1 induction was not a universal property of arthritis-associated crystals and specifically that crystalline hydroxyapatite (HA) and calcium pyrophosphate dihydrate (CPPD), when tested over a range of concentrations and particle sizes, failed to stimulate significant EP/IL 1 release from monocytes (2).

Pyrogenicity is only one of a wide range of biological activities of recombinant IL 1 peptides (3) and is not unique to IL 1 since both recombinant tumor necrosis factor (TNF) (4), and α-interferon (α-IFN) (5) are also pyrogenic. We have, therefore, in the present study reexamined IL 1 stimulation by inflammatory crystals measuring IL 1 activity in a T cell comitogenic assay in which neither TNF nor α-IFN is stimulatory (6).

We found that MSU crystals stimulated both blood monocytes and adherent synovial exudate mononuclear cells (MNC) to release IL 1 activity but CPPD crystals had no activity and HA crystals were only minimally stimulatory at the highest concentration of crystals tested. Urate crystal-induced monocyte supernatants that contained IL 1 activity in the T cell assay also stimulated the growth of human synovial long-term fibroblast lines. Since we have now shown that urate crystals stimulate the release of IL 1 activity from synovial exudate cells and that urate-induced, IL 1-containing cellular supernatants are active on target cells derived from human synovium, we believe these present findings support our earlier hypothesis that IL 1 may be an important mediator of the inflammatory reactions that occur in gouty arthritis.

MATERIALS AND METHODS

Media and reagents. All media and reagents used were demonstrably free from significant endotoxin contamination as assessed by the high sensitivity limulus lysate gelation assay (Sigma Chemical Co., St. Louis, MO) (7). In all experiments we used RPMI 1640 medium (GIBCO Ltd, Paisley, Scotland) supplemented with penicillin 100 U/ ml, streptomycin 100 μg/ml, glutamine 2×10^{-3} M, 2-mercaptoethanol (6×10^{-5} M) (Sigma), and MOPS buffer (1.25×10^{-2} M) (Sigma). In some experiments culture medium contained 10% fetal calf serum (GIBCO) ("complete medium"). For positive controls in IL 1 production experiments, LPS was used (*Escherichia coli* 0127:B8,TCA extracted, Sigma). Polymyxin B (Aerosporin-Wellcome Foundation Ltd., London) was used to check for LPS activity in stimulatory crystals (8). A rabbit antiserum to highly purified human IL 1 (kindly donated by Dr. C. A. Dinarello, Tufts University, Boston) was used to check the specificity of responses in lymphocyte activating assays for IL 1.

Crystals. The three crystal types studied here, MSU, HA, and CPPD, were chosen for their associations with arthritic diseases. Details of crystal manufacture and sizing have been previously published (2). Additional crystals of MSU, HA, and triclinic CPPD were prepared and generously donated by Dr. Paul Dieppe (Bristol University, UK). All crystal types were sterile and supernatant fluid from crystal suspensions did not stimulate monocytes to release IL 1 activity in 20-hr cultures indicating that no soluble activators of IL 1 production were associated with the crystal preparations. The crystals were also LPS-free since all three types at 1 mg/ml scored negative in the Limulus assay but did not inhibit Limulus lysate gelation in response to 500 pg/ml *E. coli* LPS. Further evidence against significant LPS contamination of crystals was obtained by using polymyxin B (200 U/ml) which completely prevented the induction of monocyte IL 1 activity in response to 100 ng/ml LPS (3.59 to 0.66 thymocyte P.U.) but had no effect on MSU-induced IL 1 activity (3.05 to 3.18 thymocyte P.U.). In most experiments, crystals were used in concentrations of between 0.1 and 1.6 mg/ml. MSU crystals were between 8 and 40 μm in length, HA 3 to 15 μm, and CPPD 15 to 50 μm. Crystals were suspended at 10 mg/ml in pyrogen-free phosphate-buffered saline (PBS) and stored at -30°C in 500 μl aliquots until used. Before use, some crystal aliquots (see figure legends) were pretreated with fresh normal human serum (10 mg crystal/ml serum) at 37°C for 1 hr followed by two washes in PBS.

Cells. In most experiments human blood from normal volunteers was taken into preservative-free heparin (10 U/ml) (Unihep—Leo Labs Ltd, Aylesbury, Bucks, UK) and mononuclear cells were obtained by density centrifugation (Lymphoprep, density 1.077, Nyegaard, Oslo) in pyrogen-free conditions. MNCs were allowed to adhere to multiwell plates (24 flat-bottomed well plates, Linbro, Flow Labs, Herts, UK) at 37°C for 2 hr and after removal of the nonadherent population, culture medium was added to achieve a final concentration of 1×10^6 adherent MNCs/ml. In some experiments adherent, synovial exudate mononuclear cells are used. These were obtained from fresh synovial fluid aspirated from acutely inflamed knee joints of patients with rheumatoid arthritis (RA). The fluids were taken into EDTA (5×10^{-3} M) and treated with bovine testicular hyaluronidase type VIII (Sigma, 100 U/ml synovial fluid) for 45 min at 37°C. Cells were removed by centrifugation at $400 \times G$ for 20 min and washed in serum-free medium before density centrifugation and culture in tissue culture flasks (25 cm², Corning, Corning, NY) at 1×10^6 adherent MNCs/ml in complete medium. In IL 1 production cultures these cells were used immediately but similar cells were kept in long-term culture in the presence of 0.1% highly purified human monocyte 22,000-dalton peptide with potent IL 1 activity (from Dr. J. van Damme, Leuven, Belgium) (9). After 2 wk in culture the remaining viable cells were entirely of fibroblast morphology and these cells continued in culture for up to 18 wk with cultures divided at approximately three weekly intervals following treatment with trypsin-EDTA solution (1×) (GIBCO) to loosen the cells. These cells were used in assays for growth factor activity in later experiments.

Exposure of cells to crystals. Adherent blood or synovial exudate MNCs at 1×10^6/ml in complete medium were incubated for 20 hr either alone or with different concentrations of crystals as indicated in the figure legends. Crystals were either untreated or pretreated with fresh human serum for one hour at 37°C. In early experiments culture medium was serum-free but 10% fetal calf serum was added in later experiments since the presence of serum did not significantly influence supernatant IL 1 content induced by any of the three crystal types except at the highest concentrations tested. After 20 hr of incubation, supernatants were aspirated and centrifuged at $1800 \times G$, 4°C for 30 min. Supernatants were either stored at -80°C or tested immediately for IL 1 content.

Assay of IL 1 activity. 1. T cell assay. IL 1 activity in most experiments was tested in a co-mitogenic assay using the murine,

antigen-specific (conalbumin) helper T cell clone D10.G4.1 (D10) from Drs. Charles Janeway and Jonathan Kaye of Yale University (10). Briefly, 2×10^4 D10 cells per well were added to microtiter plates (96-well, flat bottom—Titertek, Flow Labs) with concanavalin A (Con A) (Sigma) 2.5 μg/ml, and different dilutions of supernatants to be tested for IL 1 content as indicated in the results section. Total volume per well was 200 μl. After 70 hr (37°C, 5% CO_2) 1 μCi of [³H] thymidine (5 mCi/ml, TRK296, Amersham, Little Chalfont, Bucks, UK) was added to each well. After 5 hr, cells were lysed on glass fiber filters (PHD cell harvester—Cambridge Technology Inc.) and incorporated [³H]thymidine was measured by beta scintillation counting. The assay was calibrated using human recombinant IL 1β (pI7) (11) and IL 1α (pI5) (12) (gifts from Dr. C. A. Dinarello, Tufts University, Boston, and Dr. S. Gillis, Immunex Corporation, Seattle, WA) and highly purified pig IL 1 (from Dr. J. Saklatvala, Cambridge, UK). All of these IL 1 peptides gave optimal responses at 70 to 100 pg/ml. We also tested recombinant human tumor necrosis factor (13) (Genetech Inc., San Francisco, CA) which, in the range 0.1 pg/ ml to 1 μg/ml, had no activity alone in the D10 assay and did not modify the response to purified or recombinant IL 1α and IL 1β (e.g., 64 ± 2.8 D10 P.U. with purified pI5 IL 1 vs 65.4 ± 1.9 P.U. with pI5 plus 10 ng/ml TNF). This TNF-α preparation was active in the L929 cytotoxicity assay at 256 half-maximal bioassay units for 10 ng/ml recombinant peptide.[5] Highly purified human leukocyte IFN-α (Boots-Celltech, Slough, UK) tested over the range 20 to 2000 U/ml, suppressed the D10 background (293 ± 19 cpm to 182 ± 28 cpm with 200 U/ml) and reduced the D10 responses to recombinant IL 1β by 50% at 200 U/ml.

2. Thymocyte assay. As indicated in *Results*, in some experiments the T cell co-mitogenic assay was performed using fresh thymocytes from 6- to 8 wk-old CBA/Ca mice. Details of the murine thymocyte assay for lymphocyte activating properties of IL 1 have been previously published (14). Both of these assays (D10 clone and thymocytes) are based on the induction of T cell growth factors such as interleukin 2 (IL 2) by Il 1. Both assays are sensitive to exogenous IL 2. Our supernatants were also tested for IL 2 content using IL 2-dependent cells (3-day-old Con A-induced lymphoblasts (15) or the cytotoxic T cell line CTLL-2 (16). Urate crystal-induced supernatants that stimulated D10 or murine thymocytes had no detectable IL 2 content in these assays (<2BRMP U/ml). The concentrations of recombinant human IL 1 that gave optimal responses (70 to 100 pg/ ml) were similar in both the thymocyte and D10 assays. A rabbit antiserum raised against purified human monocyte IL 1 completely inhibited the response to optimal concentrations of recombinant human IL 1 pI7 (100 pg/ml) at a dilution of 1:1600 in both thymocyte and D10 assays (Table I). At 1:400 this antiserum did not affect the CTLL-2 response to IL 2 (1 to 125 BRMP U/ml) and did not inhibit the response of D10 cells to IL 2: 125 BRMP U/ml gave 15 ± 4.5 D10 P.U. alone and 45 ± 6.4 P.U. in the presence of anti-IL 1 antiserum. This augmentation by IL 1 antiserum of the D10 response to IL 2 also occurred with IL 1 α-stimulated D10 cells and is being investigated. We have never observed nonspecific suppressive effects of this antiserum on D10, thymocyte, or CTLL cells.

3. Fibroblast assay. Urate crystal-induced supernatants were also tested for stimulation of fibroblast growth (17). These experiments employed 8- to 14-wk-old fibroblast-like cell lines obtained by outgrowth from human RA adherent synovial exudate cells in the presence of highly purified IL 1 (as described above). These fibroblasts were allowed to readhere for 24 hr to the bottoms of flat microtiter wells (Titertek, Flow Labs) at 1×10^4 cells/well (nonconfluent). Urate crystal-induced monocyte supernatants or 22,000-dalton factor (highly purified monocyte-derived peptide with IL 1 activity) (9) were then added to the wells. After 70 hr, [³H]thymidine (0.5 μCi/well) was added. After 6 hr the medium was replaced with 100 μl trypsin-EDTA solution (1×) (GIBCO) (37°C, 30 min) to loosen the cells before harvesting. Incorporated [³H]thymidine was measured by scintillation counting.

RESULTS

Crystal-induced IL 1 activity from blood monocytes. Crystals of MSU at 0.1 to 1.6 mg/ml were potent inducers of monocyte supernatant IL 1 activity (Fig. 1). MSU crystal-induced IL 1 activity was completely neutralized by rabbit antiserum to highly purified human IL 1 (Table I). In the same experiments, CPPD and HA crystals did not stimulate any significant extracellular IL 1 activity from human blood monocytes. At higher concentration (2.0

TABLE I

Effect of IL 1 antiserum on D10 mitogenic responses[a]

	Anti-IL 1 Antiserum				
	0	1:200	1:400	1:800	1:1600
IL 1	169 ± 13	1.09 ± 0.1	1.34 ± 0.2	1.06 ± 0.03	1.33 ± 0.22
MSU-PBMC	62 ± 5	1.14 ± 0.2	3.92 ± 0.34	12.43 ± 0.82	29.66 ± 1.48
MSU-SC	43 ± 0.4	1.03 ± 0.1	0.81 ± 0.1	1.04 ± 0.17	1.06 ± 0.18

[a] Mitogenic responses of D10 cells (in proliferation units = cpm Con A + sample divided by cpm with Con A alone). Mean $\pm$ SEM of triplicate responses to IL 1 and mononuclear cell supernatants are shown. Abbreviations: IL 1, human recombinant IL 1β (100 pg/ml); MSU-PBMC, 1% supernatant of 1×10^6 adherent blood mononuclear cells cultured with 0.4 mg/ml MSU crystals; MSU-SC, 1% supernatant of 1×10^6 adherent mononuclear synovial cells cultured for 20 hr with 0.4 mg/ml MSU crystals (cpm cells + Con A = 300.67 ± 12.6).
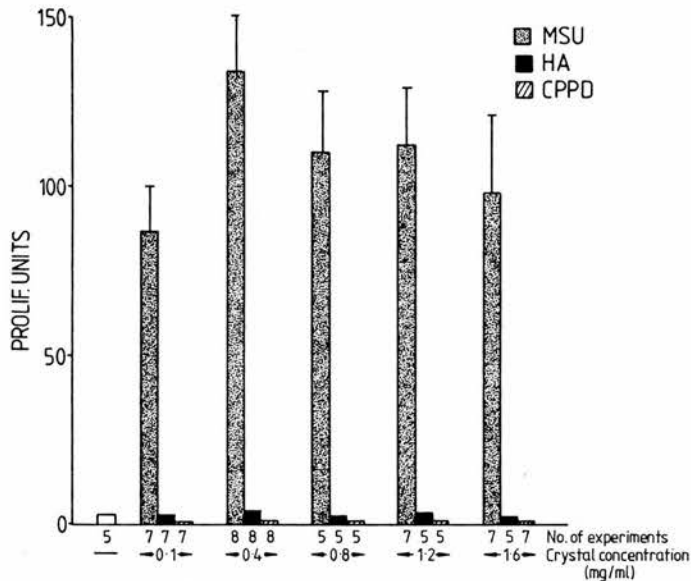

Figure 1. Pyrogen-free crystals of MSU (size 8 to 40 μm), HA (3 to 15 μm), and CPPD (15 to 50 μm) were added at different concentrations to human monocytes (1×10^6/ml) and incubated for 20 hr (37°C, 5% CO$_2$) in complete medium. Supernatants were than assayed in triplicate, in three dilutions (1:1000, 1:100, 1:10) in the D10.G4.1 assay for LAF/IL 1 activity. The figure shows the results (in proliferation units = cpm with sample plus Con A divided by cpm with Con A alone) for 1:100 dilution (in complete medium) of supernatants. Data are expressed as mean (+SEM) for the number of experiments shown. The same supernatants were tested for IL 2 content and all contained less than 2BRMP μ/ml compared with NCI standard IL 2.
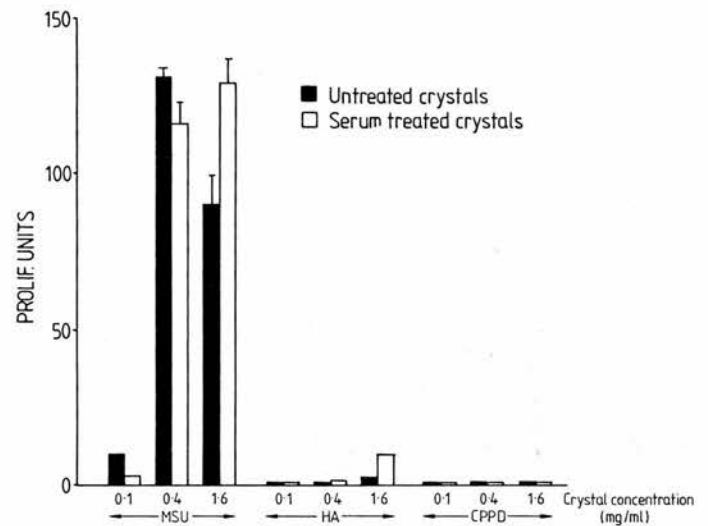

Figure 2. Untreated crystals and serum pretreated crystals were tested for their ability to induce IL 1 activity from human peripheral blood monocytes. Serum treatment of aliquots of crystals was performed by incubation with fresh human serum (10 mg crystals/ml of serum) at 37°C for 1 hr on a rotating agitator. At the end of the incubation the crystals were washed twice in pyrogen-free PBS. The "untreated" crystals received the same treatment in PBS instead of serum. Crystals were then incubated at the concentrations shown with human monocytes (1×10^6/ml) and the 20-hr supernatants were tested at different dilutions for IL 1 content in the D10 assay. Results are expressed as (+SEM) of proliferation units (see Fig. 1 legend) in triplicate measurement of supernatants diluted 1:100 in complete medium.

mg/ml) CPPD crystals also consistently failed to stimulate but HA crystals occasionally stimulated a very small release of IL 1 calculated to be at least 100- to 200-fold less potent than urate crystals. When cell lysates obtained by rapid freeze-thawing were assayed CPPD-stimulated cells contained no co-mitogenic activity. Both MSU and to a lesser extent HA-stimulated cells contained intracellular co-mitogenic activity but only the intracellular co-mitogenic activity induced by MSU crystals could be neutralized by the IL 1 antiserum (data not shown). The behavior of MSU, CPPD, and HA crystals in IL 1 induction experiments was not consistently influenced by serum pretreatment of the crystals (Fig. 2).

IL 1 adsorption by crystals. Inflammatory crystals are known to adsorb proteins to their surfaces (18, 19) and it was possible that HA and CPPD might be stimulating extracellular IL 1 release and adsorbing the IL 1 which would not then be detectable in a centrifuged supernatant. To test this we added each of the three crystals (made and stored in protein-free conditions) to monocyte supernatants with known IL 1 content. Following 20 hr of incubation at 37°C the crystals were precipitated by centrifugation (1800 × G, 4°C, 30 min) and the superna-

tant IL 1 content tested on D10 cells and compared with the same IL 1-containing supernatant that had been incubated in parallel in the absence of crystals. Neither HA, CPPD, nor urate (Fig. 3) reduced the amount of biodetectable soluble IL 1 in serum-free culture medium after 20 hr co-incubation. We take this as an indication that the failure of HA and CPPD to stimulate detectable IL 1 in our earlier experiments cannot be attributable to significant IL 1 adsorption to the surface of the crystals in the culture conditions that we employed. The same results were obtained when crystals were exposed to IL 1 in an agitated incubation (data not shown).

Co-culture of HA and CPPD crystals with LPS. It was also possible that HA and CPPD crystals stimulated intracellular IL 1 production but had nonspecific inhibitory effects on IL 1 processing, release or detection. We tested this directly by co-culturing HA or CPPD crystals with bacterial LPS, an independent stimulus of IL 1 synthesis and release. The LPS concentration used (1 ng/ml) was found in preliminary experiments to be in the suboptimal range for IL 1 induction from human monocytes. Figure 4 shows that alone, HA and CPPD did not stimulate significant IL 1 release, and the presence of HA or CPPD in co-culture with LPS did not alter the amount of bio-
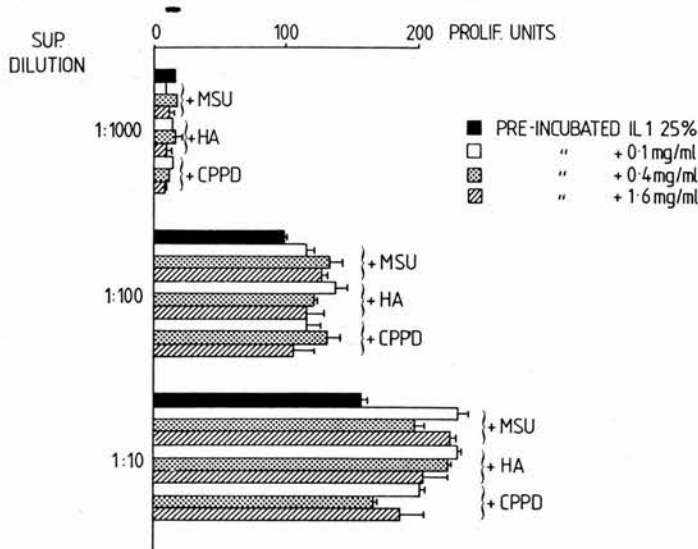
Figure 3. To test whether surface adsorption of IL 1 peptides by any of the three crystal types might remove significant quantities of IL 1 from solution, we added human monocyte supernatants with known IL 1 content to each of the crystal types at 37°C for 20 hr. The centrifuged supernatants were then tested for IL 1 content in the D10 assay and compared with the same IL 1 containing supernatants that had been incubated for 20 hr in the absence of crystals. Supernatants were tested in triplicate at dilutions of 1:10, 1:100, 1:1000. Means (+SEM) of proliferation units (see legend Fig. 1) are given.
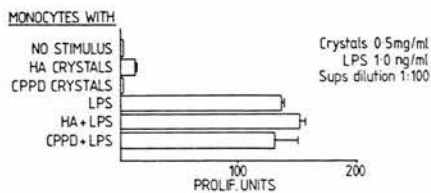

Figure 4. To test whether HA and CPPD crystals might be inducing IL 1 synthesis but interfering with release or biodetection in an unknown way, we co-incubated both crystals and bacterial LPS, an independent stimulus for both IL 1 synthesis and release. The LPS was used at a suboptimal concentration (1 ng/ml) so subsequent augmentation or depression of IL 1 release in the presence of crystals would have been detectable. Data are expressed in mean (+SEM) of proliferation units (see legend in Fig. 1) in triplicate. Results shown were obtained with supernatants diluted 1:100 in complete medium.

detectable IL 1 activity stimulated by LPS alone.

Fibroblast proliferation. MSU crystal-induced monocyte supernatants with IL 1 activity in the D10 assay were also tested for their growth-stimulation of human synovial fibroblasts. Figure 5 shows dose-dependent fibroblast growth stimulation by MSU crystal-induced supernatants. For comparison the response of the same synovial fibroblast cells to a highly purified monocyte IL 1-like peptide (9) is also shown. Human recombinant IL 1β (pI 7) also stimulated [³H]thymidine incorporation to a comparable extent in these synovial fibroblasts (data not shown).

Crystal-induced IL 1 activity from synovial fluid cells. In these experiments freshly obtained human RA synovial exudate mononuclear cells were found to release significant amounts of IL-1 activity measured in the murine thymocyte assay without the need for extrinsic stimulation. This might indicate that the cells were activated for IL 1 release in vivo or that they were inadvertently activated during our separation procedures. Although these cells were already releasing IL 1 activity, they could be further stimulated by MSU crystals but again, as with normal peripheral blood monocytes, HA crystals failed to stimulate IL 1 release (Fig. 6A). For comparison, the
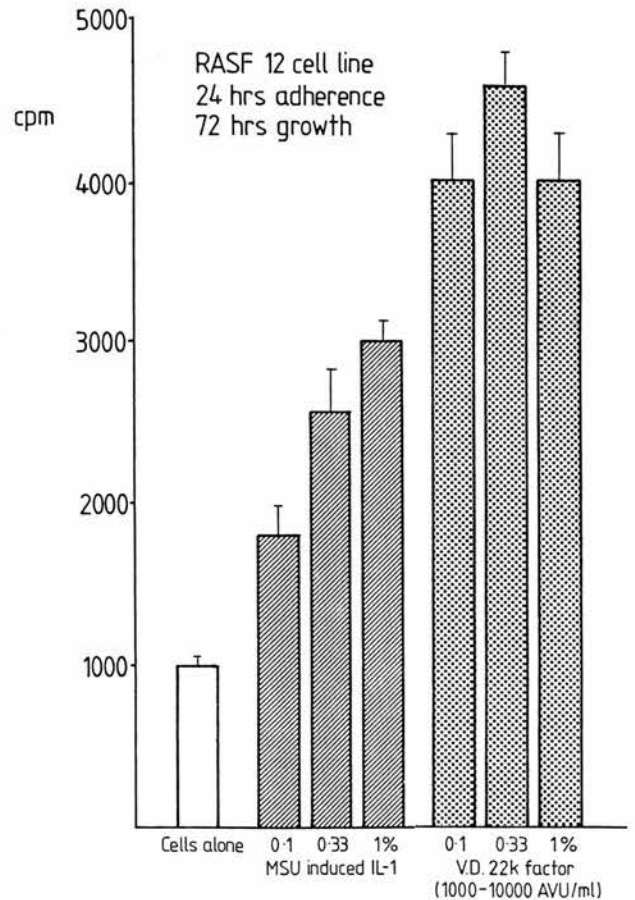

Figure 5. MSU-induced monocyte supernatants with IL 1 content by D10 assay were tested for their ability to stimulate human articular fibroblast proliferation. The fibroblast-like cells were obtained by outgrowth from RA synovial effusion MNC in 8 to 14 wk cultures in the presence of 22,000 dalton factor (a highly pure human IL 1-like peptide). Cells were loosened with trypsin-EDTA and allowed to re-adhere in non-confluent conditions at 1 × 10⁴/well for 24 hr before addition of 22,000-dalton factor or dilutions of urate-induced monocyte supernatants. After 70 hr in culture [³H]thymidine incorporation was measured following a 6-hr pulse. The results are expressed as means (+SEM) (cpm) of triplicate wells.
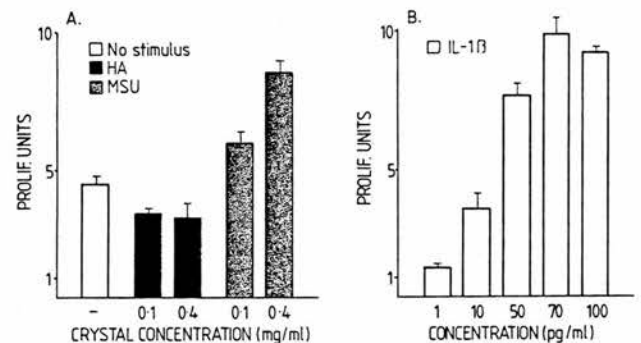

Figure 6. *A.* Synovial fluid MNC were tested for IL 1 release in response to MSU crystals. The cells were separated by density centrifugation from freshly-aspirated RA synovial exudate fluids and adhered to plastic for 1 hr. Final cell concentration was 1 × 10⁶ adherent MNC/ml of complete medium. Crystals of MSU or HA at the concentrations shown were added, and after 20 hr of incubation supernatants were tested at different dilutions for IL 1 content in a murine thymocyte assay (CBA). The IL 1 activity in the supernatants was completely neutralized by IL 1 antiserum at a dilution of 1:800. Results are from supernatants diluted 1:100 with complete medium and are means (+SEM) of triplicate cultures (proliferation units). *B.* Activity of human recombinant IL 1β is seen in the same murine thymocyte assay as shown in *A.* Optimal responses were obtained with IL 1 concentrations of 70 pg/ml. Thymidine incorporation by thymocytes stimulated with Con A alone (without added IL 1) was 2493 ± 68 cpm.

response of the same murine thymocytes to human recombinant IL 1β is shown (Fig. 6B). MSU-induced synovial cell IL 1-like activity was also neutralized by antiserum to purified human monocyte IL 1 (Table I).

DISCUSSION

The wide-ranging biological activities of IL 1 that have been reported from studies with highly purified natural IL 1 (20) and recombinant human and mouse IL 1 (21) suggest that IL 1 may play an important role as a mediator of the acute phase response, as a regulator of cellular and matrix components in skeletal and connective tissue, and as a powerful immunopotentiator with activities on both T and B cells (22). Inappropriately high or sustained release of IL 1 has been suggested as a possible mechanism contributing to the pathogenesis of inflammatory joint diseases. In support of this idea, raised IL 1 levels have been detected in joint effusion fluids in several arthritic conditions including RA (23, 24) and gout (25) and synovial cells have been found to release IL 1 in vitro (26–28).

We have previously reported that crystals of MSU but not HA or CPPD were potent stimulators of endogenous pyrogen (EP) activity from human monocytes and rabbit peritoneal macrophages (2). EP activity has been associated with IL 1 (29, 30) but IL 1 peptides are not the only "endogenous pyrogens" since both recombinant human IFN-α (5) and TNF (4) are pyrogenic when injected into animals. Indeed TNF and IL 1 seem to share a large number of biological effects which can cause confusion in several bioassay systems (31). We have, therefore, in the present study used the in vitro lymphocyte activating factor assay for IL 1 activity (32) with both an antigen-specific murine helper T cell clone (10) and murine thymocytes. First we established that the proliferation of these cells was not stimulated by recombinant TNF (or IFN-α) which makes the LAF assay an important differentiator of IL 1 and TNF bioactivities. It is, of course, possible that urate crystals stimulate the production of TNF in addition to IL 1 from mononuclear phagocytes and that the multiple catabolic effects of TNF may contribute independently or synergystically with IL 1 to the pathogenesis of gout and other inflammatory rheumatic diseases. As in our earlier studies, we found that MSU crystals but not HA or CPPD were potent stimulators of IL 1 activity from human blood monocytes. This property of MSU crystals was independent of serum factors and the failure of HA and CPPD crystals to stimulate biodetectable IL 1 release is unlikely to be due to IL 1 adsorption by these crystals since a direct test of crystal-adsorption of IL 1 from monocyte supernatants failed to demonstrate any loss of IL 1 activity from the supernatants.

It is also unlikely that HA or CPPD crystals could have induced IL 1 synthesis but then failed to stimulate or subsequently inhibited intracellular IL 1 processing and release since the presence of neither crystal altered the amount of extracellular IL 1 produced by monocytes in response to a suboptimal concentration of an independent stimulus—bacterial LPS. This conclusion is also supported by the absence of significant levels of IL 1 in lysates of monocytes following exposure to CPPD crystals for 18 hr.

These results indicate that IL 1 release is not a nonspecific monocyte response to perturbation by particulate agents. The same HA and CPPD crystals that failed to stimulate monocyte IL 1 in these experiments were, like MSU crystals, potent stimulators of human polymorphonuclear cell superoxide anion production demonstrating that they were capable of proinflammatory leukocyte activation (33). Stimulation of IL 1 by MSU crystals apparently does not require serum factors and the precise nature of the MSU stimulus for IL 1 production is unclear. Our previous work showed that internalization of crystals was not necessary (1) and the different properties of MSU, CPPD, and HA crystals were unlikely to be related to different crystal sizes (2).

The potential relevance of MSU crystal-induced IL 1 to the pathogenesis of gouty arthritis is increased by the present findings that human synovial adherent mononuclear cells, like blood monocytes, released IL 1 activity following exposure to MSU crystals. Further, the proliferation of synovial fluid fibroblast-like cells was stimulated by urate crystal-induced monocyte supernatants with known IL 1 content. Thus, cells derived from human synovial fluid both released IL 1 and responded to IL 1 containing supernatants induced by MSU crystals.

Although HA and CPPD, the other crystal types tested here, are also associated with inflammatory arthritic syndromes (34–37) neither appeared to provide a direct stimulus for IL 1 production in our experiments. This does not, however, exclude a role for IL 1 in the pathogenesis of inflammation induced by these crystals since IL 1 release may be activated by mechanisms other than direct crystal stimulation of IL 1 producing cells (2).

Gout is probably the only sterile inflammatory disease where: the local and systemic pathology is entirely compatible with overproduction of IL 1 (2); raised IL 1 levels have been found at the site of inflammation (25); and a necessary etiological agent, crystalline urate (38–40), has been shown to be a direct activator of mononuclear cell IL 1 release.

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IMMUNOREACTIVE TUMOUR NECROSIS FACTOR ALPHA AND BIOLOGICAL INHIBITOR(S) IN SYNOVIAL FLUIDS FROM RHEUMATIC PATIENTS

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The powerful proinflammatory and catabolic activities of tumour necrosis factor (TNF), the presence of its mRNA and biologically active TNF in inflamed joints, suggest a role for TNF in the pathogenesis of inflammation and joint destruction in rheumatic diseases. We have tested unfractionated joint exudate fluids for the presence of biologically active TNF alpha, and inhibitors of TNF activity. Different degrees of inhibition were observed in most samples. To avoid biological interference, 134 synovial fluids were tested by a specific and highly sensitive TNF alpha IRMA which detected significant TNF alpha levels in about 48% of the samples. Immunoreactive TNF alpha titres in synovial fluids were not clearly related to clinical parameters or type of disease.

Tumour necrosis factors (TNF) are inducible cytokines with multiple biological activities of which the first described was the necrosis of inducible tumours in animal models. Two members of this family have been biochemically characterized (1,2) and their cDNAs cloned and expressed (3,4).

TNF alpha (also known as cachectin) is a 17Kd protein resulting from cleavage of a larger precursor (5). It is mainly produced by activated macrophages and forms biologically active oligomers. TNF beta (or lymphotoxin) is predominantly a T cell product with similar biological actions but different structure, and its production is regulated independently of TNF alpha (6). TNF alpha and TNF beta compete with equal affinity for the same receptor on a variety of target cells (7).

Freshly isolated human mononuclear cells do not express TNF protein or mRNA (8), but soon after activation TNF alpha production and extracellular translocation occurs (9,8), often representing up to 1-5% of total protein synthesis in an activated macrophage (9).

The biological activities of TNF include a wide range of actions including fever (10), induction of acute phase proteins (11), actions on lipid and carbohydrate meta-

bolism (12) and membrane potential (13). Its different antimicrobial actions (14,15) and direct cytotoxicity for some tumour cells (16) point to a role in host defence against pathogens and transformed cells. Raised TNF alpha levels have been found in cancer (17), parasitic (18) and bacterial (19) infections.

Other activities of TNF are relevant to the pathogenesis of chronic inflammation and arthritis. These include induction of fibroblast proliferation (20), bone (21) and cartilage (22) resorption, polymorphonuclear cell activation (23) and adherence to activated endothelial cells (24), induction of GM-CSF (25), class I and II MHC genes (26,27), and induction of PGE_2 and collagenase from synoviocytes (28). These actions are very similar to those of IL-1 alpha and IL-1 beta which have been strongly implicated in the pathogenesis of several rheumatic diseases (29). TNF acts in synergy with IL-1 in a number of systems (30) and directly activates macrophages to produce IL-1 (10). IL-1 itself is also able to induce TNF alpha activity directly (31) or *via* IL-2 (32).

We previously demonstrated the presence of bioactive TNF alpha in synovial exudate fluids from patients with rheumatic diseases (33,34). When synovial fluids (SF) from a variety of rheumatic conditions were tested, about one third contained TNF-like bioactivity on L929 cells that was specifically neutralized by monoclonal antibody (mab) to hr TNF alpha.

In this study we present data describing the presence of inhibitor(s) of TNF alpha activity in synovial fluids. To bypass the interference of such inhibitors, a specific and highly sensitive TNF alpha IRMA was used to test immunoreactive TNF alpha levels in 134 joint exudate fluids and the results compared to the clinical parameters in the population tested.

MATERIALS AND METHODS

Synovial Fluids

Synovial fluids were aspirated from 134 consecutive patients attending a Rheumatology Out-Patient Clinic for therapeutic joint aspiration and/or injection. Fluids were collected in EDTA (5×10^{-3}M) in sterile conditions. After centrifugation at 1800G for 30 mins 4°C, supernatants were aliquoted and stored at -70°C. Several aliquots of SF underwent an additional hyaluronidase digestion (37°C, 30min, 150U/ml bovine testicular hyaluronidase (Sigma)) followed by a second centrifugation. These supernatants were stored for subsequent parallel testing with the non-treated samples. No evidence of significant cellular carry over and release of TNF was obtained (double centrifugation ± hyaluronidase treatment did not influence SF TNF content).

Patients

The SF assayed were mainly from patients with chronic rheumatic diseases and a few with acute syndromes such as gout or septic arthritis. Patients (female 57%) with a mean age of 56.4 ± 16.4 years (mean ± SD), were studied.

TNF alpha levels in synovial fluids were compared with diagnosis, presence of radiological bone erosions, sex, age, duration of disease, and drug therapy. Clinical laboratory tests included: haemoglobin (Hb), white cell count (WCC), platelets (Ptl), erythrocyte sedimentation rate (ESR).

Cell Cytotoxicity Assay and Inhibition Studies

L929 cells were used as targets for TNF cytotoxicity *in vitro* (35). Cells were grown in EMEM (Sigma) with Penicillin (100U/ml), Streptomycin (100µg/ml, Sigma), and 5% fetal calf serum (FCS) (heat inactived 56°C, 30mins (Gibco)). For maximum sensitivity to TNF activity, cells were grown for at least two weeks in non-confluent status. To assay TNF, cells were seeded at 3×10^4/well in the inner 60 wells of 96 well microtiter plates (Costar). After 20hrs incubation in a 5% CO_2, 36.5°C, 95% humidity atmosphere in antibiotic-free EMEM-10% FCS, the spent medium was removed and replaced with 50µl actinomycin D in EMEM (final conc. = 1µg/ml).

Double dilutions of synovial fluids (1:4 to 1:512) in the absence or presence of hr TNF alpha (final concentration: 40pg/ml) were dispensed in each row (final volume: 200µl/well). Each plate included wells in which only hr TNF alpha was added (40pg/ml, in one case 1ng/ml), or EMEM 10% FCS ("total cells" control). Plates were incubated for 18-20hrs at a higher temperature (38.5°C) to achieve increased cytotoxicity (36). Under these conditions half maximal killing was obtained with 40-50pg/ml hr TNF alpha. After removal of medium, the adherent, living cells were fixed and stained with 0.5% (v/v) crystal violet in 20% methanol). After vigorous washing, optical density (O.D.), which is proportional to cell survival, was assessed by microELISA spectrophotometry at 470nm. For each single well, percentage cytotoxicity (% CTX) was calculated with the formula

$$\% \text{ CTX} = \frac{\text{O.D. "total cells"-O.D. sample}}{\text{O.D. "total cells"}} \times 100$$

The ability to inhibit the cytotoxic action of 40pg/ml hr TNF alpha was calculated, for each SF dilution, with the formula

$$\% \text{ inhibition} = \frac{(\% \text{ CTX by SF}) + (\% \text{ CTX by TNF})-(\% \text{ CTX by TNF with SF})}{(\% \text{ CTX by SF}) + (\% \text{ CTX by TNF})} \times 100$$

To measure inhibition by synovial fluids, dilutions were plotted against % inhibition (Fig 1). One inhibitory unit was defined as the dilution of SF able to neutralize 75% of the cytotoxicity induced by 40pg/ml hr TNF alpha.

Using this assay, 8 samples were tested for inhibition of hr TNF alpha activity (40pg/ml), and one sample was tested against 1ng/ml hr TNF alpha.

Immunoradiometric Assay for TNF alpha (TNF alpha IRMA)

This immunoassay was based on a solid phase two-site "sandwich immunoassay" method (37). Etched polystyrene beads (Northumbria Biologicals, UK; 6mm diam.) were immersed in a solution of 3.101.23 anti hr TNF alpha monoclonal antibody (200µg Ig protein/ml PBS) (38) and stored overnight at 4°C. Before use, antibody-coated beads were washed four to five times with washing buffer, 0.5% BSA-PBS and then stored in this buffer at 4°C before use. LP4 tubes (Luckham, UK) were blocked with 5% BSA-PBS for at least one hour before use.

For the assay, LP4 tubes were emptied and synovial fluid samples and serial dilutions of hr TNF alpha interim standard (86/659) transferred to the tubes (0.225ml/tube). To lower the viscosity of synovial fluids, they were either pretreated with hyaluronidase (40 u/ml, 30 mins) or diluted 1:5 in PBS. Human recombinant TNF alpha standard was serially diluted in a synovial fluid known to be negative for TNF alpha. Monoclonal antibody-coated beads were dried by blotting on paper towels and then one bead was transferred to each tube. Incubation was for 18 hours at 4°C, following which samples were removed by aspiration and the beads washed three times with 3ml washing buffer.

Radio-iodinated anti-human TNF alpha Ig (2-33-G2) (38) was appropriately diluted in washing buffer and approximately 2×10^5 cpm in 0.225 ml added to each tube.
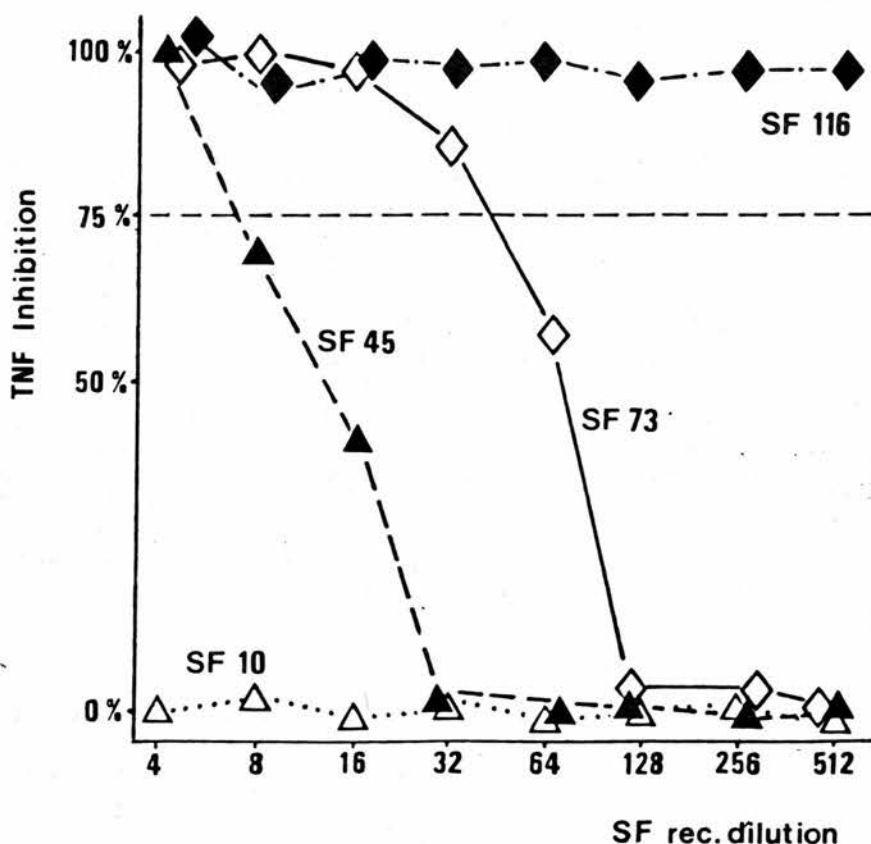
F. S. di GIOVINE ET AL.

Fig. 1. Typical dilution curves of TNF alpha-inhibiting activities in some of the synovial fluids (SF) te-
sted; SF 10 was tested against hr TNF alpha 1ng/ml, while SF 73, 45 and 116 against 40pg/ml hr TNF al-
pha. The four fluids have respectively no inhibition of 1ng/ml TNF alpha and about 8 and 58 inhibition
U/ml, and >512 inhibition U/ml of 40pg/ml TNF alpha.

Following further incubation at 4°C for 4-5hr, excess [125] I-2-33-G2 was removed and the
beads washed three times with washing buffer before counting in an LKB gamma counter.
The IRMA had in these counditions, a detection limit of 0.5U(12.5pg/ml) when calibrated
with the hr TNF alpha interim standard (86/659).

In several fluids, exogenous hr TNF alpha was added in concentrations ranging from
50pg/ml to 5ng/ml: percent recovery was 115.16 ± 14.86 (n = 9); dilutions of synovial fluids
were also tested (1/2, 1/4, 1/8), with a recovery from expected values of $112.02 \pm 10.96\%$
(n = 9). In both cases, differences between TNF alpha titres obtained and TNF alpha values
expected was not significant by signed rank test ($p > 0.5$) Interassay variability was less than
10% and intra-assay variation less than 10% over the range 25pg-25μg/ml TNF alpha.

Statistical Analysis

Qualitative agreement was assessed by Kappa statistic (39), quantitative differences bet-
ween groups by one-way analysis of variance, quantitative correlation by Spearman's rank
coefficient, analysis of dichotomous variables in different groups were tested by chi-square
test. For estimations on paired samples, differences were tested by signed rank test.

RESULTS

The presence of inhibitors of TNF bioactivity in SF.

Of the nine fluids tested, one fluid did not inhibit 1ng/ml hr TNF alpha cytotoxicity at any of the concentrations tested. Five synovial exudates contained TNF inhibitory activity quantifiable at between 8 to 54 u/ml, representing potential inhibitory bioactivity of 0.24 to 1.62ng/ml TNF alpha. Three other synovial fluids showed very high inhibitory activity against 40pg/ml TNF alfa that did not dilute out even at the 1:512 dilution.

TNF alpha IRMA

Tumour necrosis factor alpha was tested by IRMA on a total of 134 fluids. Of these, 64 (47.8%) had levels of TNF alpha exceeding 12.5pg/ml (range = 12.5pg/ml to 4.9ng/ml) (Table I). The miscellaneous group included patients with a variety of rheumatic diseases. The eleven positive exudates in this group were from two juvenile chronic arthritis, two ankylosing spondylitis, one psoriatic arthritis, one palindromic rheumatism, one gout, one polymyalgia rheumatica and three septic arthritis. Significant levels were well distributed in each disease category.

The possibility of mononuclear cell carryover and production of TNF within the assay was not an issue as the assay was carried out in the presence of 1ug/ml Actinomycin D (L929 assay) or 50ug/ml Azide (TNF IRMA). To determine the effect of hyaluronidase treatment, paired samples were assayed by IRMA. The treatment dramatically reduced SF viscosity. However, in paired samples (n = 9) no significant difference was observed (signed rank test, p = >0.5) in TNF immunoreactivity.

Qualitative comparison between bioactive and immunoreactive TNF alpha in synovial fluids

Of the fluids tested in the TNF alpha IRMA 130 had previously been tested for L929 cytotoxicity (34). Table II shows the qualitative comparision between these data. Sixty one fluids were negative in both assays and 40 positive in the two different assays (total = 77.7% agreement). Seven fluids out of 130 (5.4%) scored negative in the IRMA and positive in the previous L929 assay. Of the 22 fluids positive in the IRMA and negative in the L929 assay, 20 had a level of TNF alpha that was below the sensitivity of the L929 assay (between 12.5 and 160pg/ml). When these 20 fluids are excluded on the basis of different assay sensitivities, agreement increases to 91.8% (101/110). K value for these comparisions was 0.506 in the first case (n = 130) and 0.830 after eclusion (n = 110) (ideal agreement: K = +1; complete disagreement: K = -1).

Quantitative correlation between bioactivity and immunoactivity

The forty samples that were positive for L929 cytotoxicity and for TNF alpha immunoreactivity were analyzed for quantitative correlation. These values did not significantly correlate, as might be expected due to the presence of inhibitors of the bioassay.

Correlation with Clinical Parameters

No significant correlation was noted with clinical parameters in the whole population, nor within each disease group (synovial fluid TNF vs Hb, WCC, Ptl, ESR, disease duration, bone erosions). There was no significant difference in TNF titres between disease groups, and no relation to drug therapy.

DISCUSSION

In a previous study (34), we demonstrated the presence of TNF-like activity that was neutralizable by a specific anti-hr TNF mab in approximately 30% of synovial fluids from patients with rheumatic diseases.

The levels of bioactive SF TNF alpha did not correlate with routine laboratory markers of inflammation in patients or disease group. This could be due to several factors, the most likely of which seemed to be the presence of biological inhibitor(s) in the fluids tested. In this study we demonstrated the presence of such inhibitory activity in most of the exudates fluids tested. To measure reliably TNF alpha levels, a TNF alpha IRMA was used on 134 fluids, 130 of which had been previously tested for TNF bioactivity. Significant TNF alpha levels (range: 12.5pg/ml to 4.9ng/ml) were detected in 48% of the SF tested.

Qualitative agreement between bioactivity and TNF alpha immunoreactivity was excellent (about 92% for the fluids (n = 110) with titres exceeding 160pg/ml or below 12.5pg/ml) but, as expected, no quantitative relationship was evident when positive fluids were compared. Seven fluids out of 130 (5.4%) scored negative in the IRMA and positive in the L929, possibly indicating degradation of TNF in the fluids between the experiments (12 to 6 months) or cytotoxic activity not mediated by TNF alpha. Non-specific killing in a small proportion of samples was in fact previously recorded (34).

However, clinical laboratory measurements (ESR, platelets, Hb, WCC) related to systemic inflammation, radiological joint erosions, and disease category did not correlate with TNF alpha levels in synovial fluids.

The lack of correlation with markers of disease activity is unlikely to be explained by dissociation between immunoreactivity and bioactivity of TNF alpha as TNF propeptide is apparently fully processed before extracellular translocation (5). In human monocyte supernatants, L929 bioactivity significantly correlates with TNF alpha immunoreactivity as determined by RIA (8). It is possible however that different aggregation states of TNF in synovial fluid might give unequal exposure of receptor binding sites and immunoreactive epitopes.

It is also possible that TNF production varies between individuals similarly to IL-1 beta (40). This might suggest that comparision of TNF alpha levels between patients is inappropriate and that longitudinal studies in individuals could be more useful. This, of course, is not feasible for synovial fluids, but is possible with blood samples.

Interestingly, of two patients with septic arthritis in which repeated aspiration was performed, TNF alpha levels were initially raised and fell with treatment as joint inflammation resolved (e.g. 1.22 to 0.43ng/ml after 2 weeks'antibiotic therapy).

The estimation of TNF in plasma would probably be more helpful in studies on the role of TNF in the pathogenesis of rheumatic diseases because it would allow comparision with a normal population, who have low but detectable levels of plasma TNF

Table I. Immunoreactive TNF alpha in Synovial Fluids

Diagnoses		RA +	RA-	OA	RT	Misc	Total
No. of cases		55	30	18	11	20	134
<12.5	pg/ml	29	18	11	3	9	70
12.5-25	pg/ml	6	2	—	1	5	14
25-250	pg/ml	16	9	1	4	3	33
0.25-2.5	pg/ml	3	1	5	3	3	15
>2.5	pg/ml	1	—	1	—	—	2
Total "positives"		26	12	7	8	11	64
% "positives"		47.3%	40%	38.9%	72.7%	55%	47.8%

Table I. A total of 134 synovial fluids were assayed for TNF alpha with a specific and sensitive TNF alpha IRMA. Of these, 47.8% had a level of TNF alpha over the detection limit ("positive" = >12.5pg/ml). RA + = rheumatoid arthritis seropositive; RA- = rheumatoid arthritis seronegative; OA = osteoarthritis; RT = reactive arthritis; Misc = miscellaneous group of arthritis, including ankylosing spondylitis, psoriatic arthritis, septic arthritis, juvenile chronic arthritis, palindromic rheumatism and gout.

Table II. Qualitative Comparison Between Bioactive and Immunoreactive TNF in Synovial Fluids

		TNF alpha IRMA (det. limit = 12.5 pg/ml)		
		"positives" (n)	"negatives" (n)	
L 929 bioassay	"positives" (n)	40	7	Total 47
(det. limit = 160 pg/ml)	"negatives" (n)	22*	61	Total = 83
		Total = 62	Total = 68	Total = 130

Table II. Qualitative comparision between L929 cytotoxicity assay and TNF alpha IRMA on 130 joint exudate fluids tested by both methods. Of the 22 fluids labelled with (*), 20 had TNF alpha IRMA levels between 12.5 to 160pg/ml, below the detection limit of the L929 bioassay ("negative" for L929 cytotoxicity) but detectable in the immunoassay ("positive" in TNF alpha IRMA).

alpha (J. Eastgate, personal communication). It would also allow serial measurements on the same patient and testing of correlation with clinical disease activity in individuals.

It would be predicted that markers of systemic disease activity such as those measured in this study would be more likely to correlate with TNF levels in blood rather than levels in individual inflammed joints.

Synovial fluid TNF concentration though, is higher than autologous blood levels. TNF is produced in the synovium of rheumatoid patients (41) and synovial cells "spontaneously" release immunoreactive TNF alpha (42), indicating TNF is produced within the synovium during inflammation.

Clearly, the local and systemic biological effects of this TNF production will depend upon a complex balance between TNF and synergystic cytokines on one hand and inhibitory factors on the other.

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Tumour necrosis factor in synovial exudates

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SUMMARY   The actions of tumour necrosis factor (TNF) include resorption of bone and cartilage, suggesting a potential role in the pathogenesis of arthritis. TNF activity was looked for in synovial fluids from 137 patients with different rheumatic diseases. Unfractionated samples were tested in the L929 bioassay. Significant TNF activity that was neutralised by monoclonal antibody to TNF alpha occurred in 13 (30%) of 44 samples. Raised TNF levels were not associated with any particular disease type or routine laboratory markers of inflammation but were related to disease duration in osteoarthritis. The finding of biologically active TNF in symptomatic joints of arthritic patients supports the idea that it may contribute to the pathogenesis of joint damage in chronic rheumatic diseases.

Key word: arthritis.

Cytokines are inducible peptides with receptor mediated biological actions on many different cellular targets. Cytokine production has mainly been associated with leucocyte or fibroblastoid cells during responses to injury or infection.[1] [2] Tumour necrosis factors (TNFs) are cytokines that have recently been characterised at molecular level.[3] [4] TNF alpha (also called cachectin) is a 17 000 Mr peptide that forms biologically active oligomers.[5] Mononuclear phagocytes are potent sources of TNF alpha,[6] but T cells also produce a similar molecule[7] as well as the related product, lymphotoxin (also called TNF beta).[8] Resting cells release little or no TNF alpha, but after activation mRNA accumulates and TNF peptide appears soon after.[9] The biological activities of human TNF alpha are similar to those of interleukin 1 (IL1) alpha and beta except that human TNF does not activate murine T cells.[10]

The biological role of TNF has not yet been completely defined, but its properties in vitro and in vivo suggest it could be an important mediator of host defence against tumours and pathogens.[11] It is also likely that TNF may contribute to autoimmune and inflammatory pathology such as occurs in rheumatic diseases. The defined activities of TNF relevant to arthritis include resorption of cartilage and bone[12] [13]; endothelial adherence and activation

of granulocytes[14] [15]; stimulation of fibroblast growth[16]; stimulation of synovial cell prostaglandin and collagenase release[17]; and the systemic reactions of fever,[18] liver acute phase protein synthesis,[19] and catabolism of protein and fat leading to muscle loss and cachexia.[20] Whether resident cells in healthy skeletal tissues include populations that produce TNF is not known, but the mononuclear infiltrating cells that characterise synovial pathology in many arthritic diseases are likely sources of TNF. Cytokine studies in arthritis have previously concentrated on interleukins 1 and 2 and interferons,[21] but the connective tissue effects of TNF raise the possibility that it may mediate bone and cartilage injury in chronic joint disease.[20]

As there was no previous information on the occurrence of TNF in human arthritic diseases we tested unfractionated synovial exudate fluids from 137 patients with different rheumatic diseases and report here that biologically significant levels of TNF alpha were found in approximately 30% of the samples.

Materials and methods

SYNOVIAL FLUIDS

Synovial effusion fluids (SF) were obtained from patients referred for aspiration of symptomatic knee joints. Clinical diagnoses covered a wide range of rheumatic diseases as indicated in the results section. All of the patients were being treated with non-steroidal anti-inflammatory drugs and some

received slow acting anti-inflammatory drugs or low dose oral corticosteroids. Fluids were collected in sterile containers in the presence of 5×10^{-3} M edetic acid, centrifuged at $1800\,g$ for 30 minutes, and sample aliquots stored at $-70°C$ or tested immediately for TNF content. The fluids were collected over 16 months.

REAGENTS
Human recombinant (HR) TNF alpha, human recombinant lymphotoxin (TNF beta), mouse monoclonal antibody specific for human TNF alpha and rabbit antiserum against HR lymphotoxin were all kindly donated by Dr Gunther Adolf (Ernst-Boehringer-Institut fur Arzneimittel-Forschung, A-1121 Wien, Austria). HR IL1 alpha and IL1 beta were obtained from Biogen (Geneva). Reagents for immunoradiometric assay for human interferon gamma were from Boots-Celltech (Slough, Berkshire).

TNF ASSAY
The conventional bioassay for TNF activity based on cytotoxicity for the mouse connective tissue cell line L929 was used.[22] Briefly, L929 cells were cultured in Eagle's minimum essential medium (Sigma, St Louis, MO) with 5% fetal calf serum (Gibco, Paisley, UK) at 4×10^4 cells/well for 20 hours in flat bottomed, 96 well, microtitre plates. The medium above the non-confluent cell layer was replaced with fresh medium containing actinomycin D (1 µg/ml) and serial dilutions of standard HR TNF alpha or samples to be tested for TNF content. After a further 20 hour incubation the medium was removed and a 0·5% in 20% methanol solution of crystal violet was added to the wells. The number of viable (adherent) cells remaining in each well was assessed by scanning spectrophotometry (Dynatech MR 700) at 540 nm. The concentration of TNF giving 50% maximal cell killing (one bioassay unit) was equivalent approximately to 50 pg/ml of recombinant human TNF alpha. A range of cytokines, including recombinant and purified natural IL1 alpha and beta, purified interferon alpha and recombinant interferon gamma, and crude interleukins 2 and 3, gave no appreciable activity (less than 4 units/ml) in this assay but HR lymphotoxin was equipotent with TNF alpha.

Synergy between interferon gamma and TNF has been reported for human cell cytotoxicity[23] and in other human systems.[24] Therefore we measured interferon gamma levels in these synovial exudates. Of 27 SF tested for IFN gamma in a specific radioimmunometric assay, none reached the detection limit (1 U/ml). With this assay there was no loss of detection of HR interferon gamma added to

osteoarthritis or rheumatoid arthritis synovial fluids compared with culture medium.

Ninety three SF were tested at 11 serial dilutions in at least three separate assays, from which quantitative data (units of TNF activity) could be derived.

SPECIFICITY TESTING
To test that the L929 cytotoxicity of the SF was mediated by TNF alpha some positive samples were reassayed in the presence of monoclonal antibody (MAb) specific for HR TNF alpha or MAb specific for human major histocompatibility complex (MHC) class II product (control antibody). These quantitative experiments indicated that some cytotoxic samples were not neutralised by anti-TNF alpha. To determine the proportion of fluids with cytotoxic activity that were neutralised by anti-TNF alpha we tested a further 44 fresh fluids at one dilution only (1:8) after preincubation with culture medium, anti-TNF alpha, irrelevant MAb, or a rabbit antiserum against HR lymphotoxin.

It is known that both IL1 alpha (di Giovine, Poole, and Duff, unpublished observation) and beta[25] are detectable by radioimmunoassay in human synovial exudate fluids. IL1 is reported to act synergistically with TNF in a number of biological systems.[26-28] To test whether our bioassay estimate of TNF content might be influenced by synovial fluid IL1 we performed L929 bioassays of HR TNF in the presence of HR IL1 alpha and beta. Fig. 1 shows that IL1 did not affect the L929 response to TNF alpha.

Results

TNF-LIKE ACTIVITY IN SYNOVIAL FLUIDS
Of the first 93 SF tested, 35 had TNF-like activity of 4 U/ml or greater. Table 1 summarises the clinical diagnosis and level of TNF-like activity. The two positive fluids in the miscellaneous group were from patients with recurrent monarthritis. Several SF with the highest levels had been assayed fresh, suggesting that activity may be lost after freeze-thawing or storage at $-70°C$.

ANTIBODY NEUTRALISATION OF TNF-LIKE ACTIVITY
Eight of the 35 fluids with TNF-like activity were retested in full titrations alone or with anti-TNF alpha, or control MAb (Table 2). Six were completely neutralised by anti-TNF alpha but two were not, suggesting that other cytotoxins were present in these samples. To determine the proportion of SF that contained cytotoxic activity attributable to TNF alpha we tested a new group of 44 SF in triplicate at

one dilution only, either alone or with antibodies. Of these 44 fluids, 13 (30%) contained TNF activity that was unaffected by control antibody but completely neutralised with anti-TNF. They included SF from patients with rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, psoriatic arthritis, reactive arthritis, septic arthritis, and gout. SF that did not contain TNF activity were also found from patients in most of these disease groups. Twenty three samples (52%) contained no TNF-like activity at all and eight (18%) were cytotoxic for L929 cells but either were not neutralised with anti-TNF or were affected by the irrelevant MAb. Notably, of the 21 SF that were cytotoxic for L929, none was neutralised by antiserum to lymphotoxin. This antiserum at 1:800 dilution completely inhibited the activity of 10 ng/ml human recombinant lymphotoxin in the L929 assay.
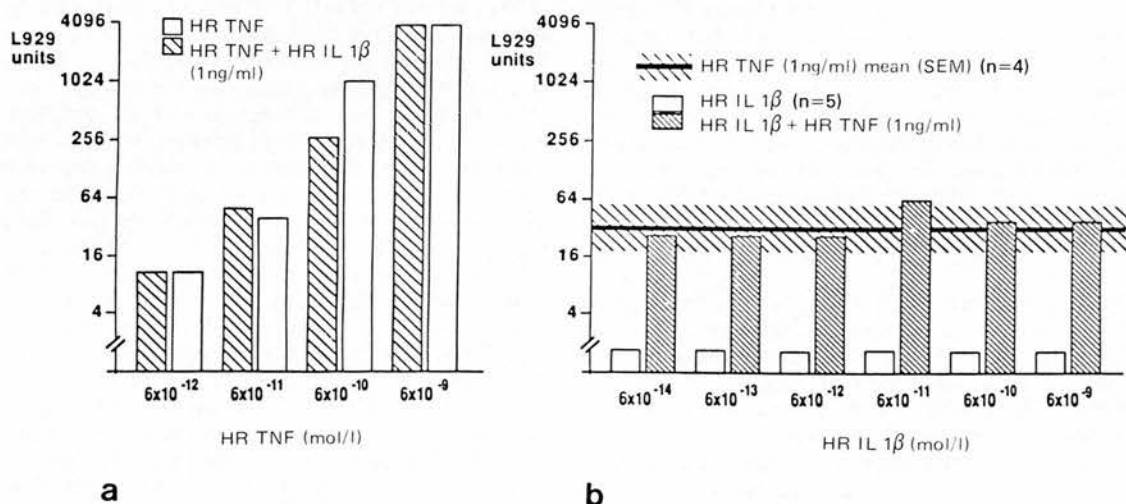

a

b

Fig. 1   *Human recombinant tumour necrosis factor (HR TNF) alpha and HR interleukin 1 beta (IL 1β) were tested for synergy in the L929 cytotoxicity assay. (a) Different concentrations of HR TNF alpha were tested in the presence or absence of HR IL1 beta (1 ng/ml). (b) HR TNF alpha (1 ng/ml) was tested in the presence or absence of different concentrations of HR IL1 beta. No synergy in L929 cytotoxicity was observed and HR IL1 beta alone in a wide concentration range was inactive in this assay. Identical results (data not shown) were obtained in separate experiments in which TNF was tested at higher concentrations (10 ng/ml) or HR IL1 alpha was used. Biological activity of both IL1 preparations was confirmed by EL4.NOB.1/CTLL conversion assay.*

Table 1   *TNF activity in SF from patients with different rheumatic diseases*

TNF activity* (U/ml)	RA+	RA−	OA	ReA	PsA	AS	Misc	Total
<4	29†	11	6	2	3	1	6	58
4–16	9	7	3	4	2	1	1	27
16–28	2		1	1		1		5
28–40			1					1
40–52	1							1
>52							1	1
Total	41	18	11	7	5	3	8	93
% Positive‡	29	39	46	71	40	67	25	38

RA+=seropositive rheumatoid arthritis; RA−=seronegative rheumatoid arthritis; OA=osteoarthritis; ReA=reactive arthritis; PsA=psoriatic arthritis; AS=ankylosing spondylitis; Misc=miscellaneous group.
*Each synovial fluid was measured at least three times at 11 dilutions in different assays.
†Number of patient SF in this category.
‡Percentage of patients in each category with TNF levels> 4 U/ml (detection limit).

Table 2 *Antibody neutralisation of synovial fluid TNF-like activity*

	Sample alone	Sample with anti-TNF†	Sample with anti-DR‡
SF 73 (OA)	39*	<4	34
SF 69 (AS)	42	<4	41
SF 78 (OA)	14	<4	ND§
SF 158 (OA)	11	<4	11
SF 161 (RA+)	50	<4	49
SF 91 (RA+)	5	<4	ND
SF 116 (RA+)	19	5	10
SF 112 (ReA)	7	9	ND
HR LT (1 ng/ml)§	24	22	30
HR TNF (1 ng/ml)	22	<4	21

*Values are TNF bioassay units/ml.
†Monoclonal antibody against human recombinant TNF alpha (1:1600).
‡Monoclonal antibody against human MHC class II product as control antibody (1:800).
§HR LT=human recombinant lymphotoxin; ND=not done; other abbreviations as in Table 1.

Discussion

The purpose of this study was to test whether unfractionated synovial exudate fluids from arthritic patients contained biologically active TNF. Using a bioassay for TNF based on cytotoxicity for the murine L929 connective tissue cell line we tested a total of 137 synovial exudate fluids. In quantitative assays for TNF activity 35 (38%) of 93 samples were positive at a concentration of at least 4 U/ml (the limit of detection). In six of eight positive SF tested, TNF-like activity was neutralised by monoclonal anti-TNF alpha. Of a further 44 fluids that were tested in a single dilution assay, 13 (30%) contained TNF activity neutralised only by MAb for TNF alpha. None of the 21 cytotoxic samples in this series was neutralised by antiserum to lymphotoxin.

TNF alpha was found in SF from patients with a variety of rheumatic diseases, but in this preliminary study we noted no association between synovial fluid TNF level and disease category, drug treatment, or laboratory data (haemoglobin, white cells, platelets, erythrocyte sedimentation rate). The only significant difference in clinical terms was in disease duration in osteoarthritis and reactive arthritis, where those with detectable TNF levels had a mean duration of disease of 9·3 years (n=10), while similar patients with no detectable TNF had mean disease duration of 3·3 years (n=14, p=0·0067). Presumably, systemic markers of inflammation are more likely to be related to levels of TNF in the circulation rather than in the synovial space.

There have been recent reports of TNF in samples from patients with meningococcal[29] or protozoal[30] infections and in cancer,[31] but no previous reports of TNF in sterile inflammatory diseases seem to be available. Our results indicate that about 30% of synovial exudates from different rheumatic diseases contain biologically detectable levels of TNF alpha (equivalent to nanomolar concentrations of HR TNF). The presence of TNF in synovial exudate from several rheumatic diseases argues against TNF production as a specific factor in the pathogenesis of any particular disease. It seems that TNF may be associated with the process of joint effusion that is common to a number of articular disorders with apparently different aetiologies. This is in keeping with the known variety of stimuli able to induce TNF production in mononuclear cell populations. For example, monosodium urate crystals stimulate monocyte TNF release in vitro,[32] which could account for TNF in gouty effusions. Presumably, in septic arthritis macrophage TNF production is directly stimulated by microbial products, and TNF release could be a direct or indirect result of lymphocyte activation in both acute and chronic rheumatic diseases. It is likely that these biodetection results underestimate the amount of TNF present as in some fluids we could detect only 50–60% of known quantities of added HR TNF alpha. It is also possible that TNF activity was lost on prolonged storage or during freeze-thawing as the highest levels we found were in fresh or recently collected samples. Finally, the assay itself is relatively insensitive and preliminary findings with more sensitive immunoassays indicate that TNF is detectable in a much larger proportion of samples.

As TNF is known to activate polymorphonuclear cells,[14 15] stimulate synovial prostaglandin production,[17] and increase bone[13 33] and cartilage[12] destruction in vitro, its presence in synovial effusion fluids may well be significant. Recently, TNF has also been shown to induce other cytokines, including IL1 and granulocyte-monocyte colony stimulating factor.[18 34] IL1 is, like TNF, proinflammatory and catabolic[21] and is found in human synovial exudates.[25 35 36] It is made by synovial cells[37 38] and was reported to be arthritogenic when injected into rabbit joints.[39] In addition to IL1 induction by TNF, IL1 itself has been shown to increase TNF activity,[40] suggesting a mechanism for the maintenance of inflammation.

This report now provides evidence that TNF alpha in a biologically active form occurs at the site of inflammation in several types of arthritis and supports the idea that mononuclear cytokines mediate joint tissue destruction.[41] An understanding of the cellular origins and activators of these cytokines should help elucidate the pathogenesis of several types of rheumatic disease.

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