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Interleukin 1 (IL1) and Tumour Necrosis Factor (TNF) Signal Transduction [and Discussion]

J. Saklatvala, W. Davis, F. Guesdon, M. Karin and C. J. Marshall

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Interleukin 1 (IL1) and tumour necrosis factor (TNF) signal transduction

J. SAKLATVALA, W. DAVIS AND F. GUESDON

Cytokine Laboratory, Department of Development and Signalling, The Babraham Institute, Cambridge CB2 4AT, U.K.

SUMMARY

The inflammatory cytokines interleukin 1 (IL1) and tumour necrosis factor (TNF) have a broad range of physiological effects. Whereas their immediate post-receptor events are not well understood, both have the potential to activate a range of protein kinases. These include the three types of mitogen activated protein (MAP) kinase (ERK, JNK/p54 and p38) and a β-casein kinase. The mechanisms by which these kinases are activated is discussed and the significance of their activation for particular biological responses is assessed.

1. INTRODUCTION: BIOLOGICAL ACTIONS OF THE CYTOKINES

Interleukin 1 (IL1) and tumour necrosis factor (TNF)α are primary inflammatory cytokines that mediate many of the local and systemic features of inflammation (Dinarello 1991, 1994; Beutler 1992). They are made mainly by monocytes and macrophages in response to a range of stimuli including various microbial products, viruses, immune complexes, activated T cells and the combined action of other cytokines such as interleukin 2 and interferon γ.

They have a similarly broad range of physiological effects. The main difference between them being that TNFα is cytotoxic to some transformed cell lines, whereas IL1 is generally not. They have a number of local inflammatory actions. They cause leucocytes to move out of capillaries and accumulate at sites of injury or infection. This is because of their stimulating production of chemotactic factors (e.g. IL8 and platelet activating factor) and inducing adhesion molecules for leucocytes on vascular endothelium. The up-regulation of surface molecules such as E-selectin and ICAM-1 causes leucocytes to attach to the endothelium, and they then move out into the extravascular space in response to chemotactic stimuli. TNF may then be involved in the further activation of polymorphonuclear leucocytes and monocytes, and both cytokines enhance lymphocyte responses. They stimulate synthesis of prostaglandins which cause other local effects by increasing blood flow, capillary permeability and enhancing perception of pain. IL1 and TNF also stimulate connective tissue cells to resorb their surrounding matrix. This results in destruction of tissues such as cartilage and bone.

If IL1 and TNF are made in large amounts they enter the circulation and act as hormones on distant target organs. They cause release of leucocytes from the bone marrow, they stimulate the liver to produce 'acute-phase' plasma proteins and they act on the thermoregulatory centre of the brain to cause fever. This last effect is mediated by increased prostaglandin production which somehow resets the central thermostat. There is also stimulation of the pituitary-adrenal axis which causes increased production of glucocorticoid hormones. Glucocorticoids tend to suppress cytokine production and some of the responses to IL1 and TNF (e.g. prostaglandin production).

Underlying the complex physiology of IL1 and TNF is their ability to induce expression of a number of genes (table 1), the identity of which depend upon the cell type undergoing the response. The promoters of many of these are controlled (in a complex manner) by known transcription factors such as NFκB, AP-1, ATF-2 and NFIL6. The activity of these factors is modulated by phosphorylation and the current hypothesis is that IL1 and TNF work by activating protein kinases which modulate the function of proteins that control transcription, as well as other cellular processes.

(*a*) *IL1 and TNF: ligands and receptors*

IL1 and TNF each occur in two forms, α and β, which are the products of related genes. TNFα and both forms of IL1 are made by activated monocytes and macrophages whereas TNFβ is made by activated T lymphocytes. The two forms of each cytokine interact with the same receptors.

IL1α and β signal by combining with the type I receptor, an 80 kDa member of the immunoglobulin super family with a single membrane-spanning region (Sims *et al.* 1988). Its 20 kDa cytoplasmic part is essential for signalling (Curtis *et al.* 1989), but its structure gives no clue as to its function. The type II IL1 receptor is a related protein but has only a short cytoplasmic extension of 29 amino acids (McMahan *et al.* 1991). It has not been shown to transmit any signal

Table 1. *Proteins induced by IL1 and TNF*

(Many of these changes are seen in cultured fibroblasts. Where a response occurs only in a specialized cell this indicated. Where a transcription factor is strongly implicated in regulation this is indicated: AP1*, NFKB^{+}, NFIL6^{o}, ATF2†.)

transcription factors	secreted proteins
c-Jun*†	IL2 (T cells)*
c-Fos	IL6^{o+}*
c-Myc	IL8^{o+}*
interferon response factor-1	interferon beta
NAK1	G-CSF, GM-CSF
NFIL6	gro
	NGF
	PDGF A chain
	HGF (Scatter factor)^{o}
	Procollagenase*
	Prostomelysin*
	Plasminogen activator
	acute phase proteins (hepatocytes)$^{o+}$*
intracellular metabolism	**surface membrane proteins**
glucose transporter	IL2 receptor (T cells)$^{+}$*
phospholipase A2 (group II)	E-selectin (ELAM) Vascular endothelium)$^{+}$†
cytosolic phospholipase A2	P-selectin
cyclooxgenases I and II	I-CAM-1^{+}
inducible nitric oxide synthase	
metallothionein	
Mn SOD	

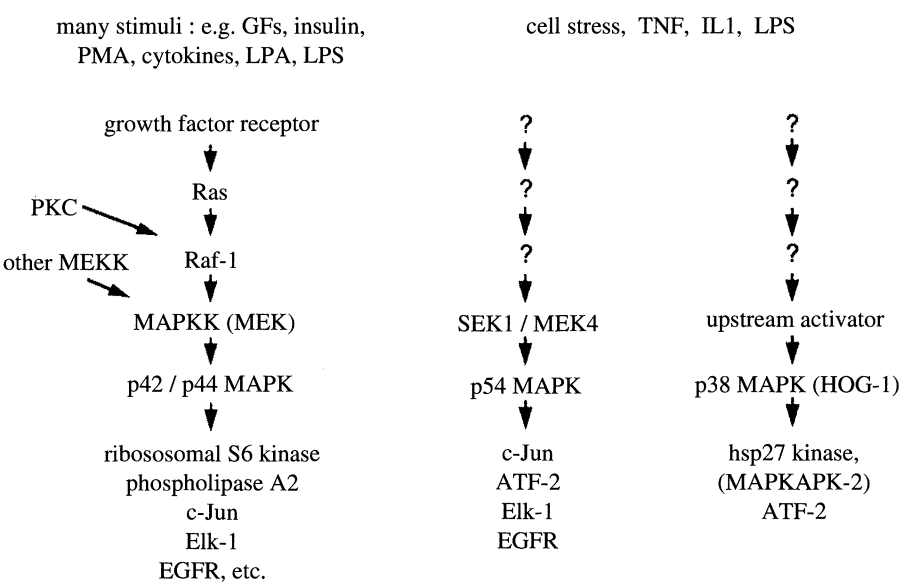

Figure 1. The MAP kinase pathways of mammalian cells.

(Stylianou *et al.* 1993; Sims *et al.* 1993) and seems rather to function as a cell-bound inhibitor of IL1 (Colotta *et al.* 1993).

There are also two TNF receptors, p55 (type 1) and p75 (type 2). Their extracellular parts are related to a family of cell-surface proteins that include a nerve growth factor receptor, Fas, CD40, 4-IBB and OX40 (Smith *et al.* 1994). They have single membrane-spanning regions and intracellular portions which are unrelated. The p55 receptor signals the inflammatory effects of the cytokine and apoptosis in appropriate target cells (Engelmann *et al.* 1990). The p75 may mediate the proliferative action of TNF on lymphocytes and may either augment or facilitate the action of p55 (Tartaglia 1993; Bigda *et al.* 1994).

The signalling mechanisms of the receptors are not yet understood, but over the last year or two it has been established that IL1 and TNF can activate a number of protein kinases. The best characterized of these are the different types of mitogen-activated protein (MAP) kinase (see figure 1).

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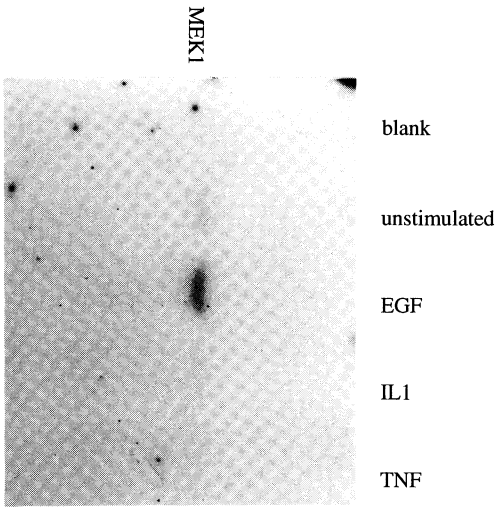

Figure 2. Effects of EGF, IL1 and TNF on activity of c-Raf in human gingival fibroblasts. Confluent monolayers of fibroblasts were stimulated with vehicle, EGF (10 ng/ml), IL1 (20 ng/ml) or TNF (40 ng/ml) for 6 min. Cells were then washed in ice-cold phosphate-buffered saline, then lysed rapidly in immunoprecipitation buffer. c-Raf was immunosorbed with antibody-coated protein A agarose. The rabbit antiserum (from Santa Cruz Biotechnology Inc.) was directed to the N-terminal sequence of MAPKK. Agarose beads were washed well then assayed for activity on recombinant MAPKK (inactive mutant) in the presence of [^{32}P]γATP. Reaction products were separated on SDS-PAGE and phosphorylation of MAPKK was detected by autoradiography.

(*b*) *p42 and p44 MAP kinases* (*ERK2 and 1*)

IL1 and TNF activate p42 and p44 MAP kinases (also called ERK for extracellular signal regulated kinases) in a variety of cultured connective tissue cells, such as fibroblasts, vascular endothelial cells and chondrocytes (Guy *et al.* 1991; Guesdon *et al.* 1993). In general they do not activate these kinases significantly in transformed cell lines such as Hela, KB, EL4 and Jurkat. Such transformed lines also respond to the cytokines in a limited way; they do not undergo many changes in gene expression when compared with primary cultures of fibroblasts or endothelial cells. The cytokines increase activity of MAPKK in fibroblasts and the enzyme concerned has been identified as MAPKK1 (Saklatvala *et al.* 1993). The best characterized regulated activator of MAPKK1 is c-Raf. So far we have been unable to establish that IL1 (or TNF) activate c-Raf. Figure 2 shows an experiment on human gingival fibroblasts. Following their stimulation they were lysed and c-Raf was immunosorbed to a antibody on agarose beads. The immunosorbed c-Raf was then tested for its ability to phosphorylate a kinase-inactive of mutant MAPKK1 of (MEK1). Whereas EGF clearly activated c-Raf, there was no evidence that either IL1 or TNF was doing so. There has been one report of TNF activating c-Raf in various cell lines (Belka *et al.* 1995) and another report of IL1 failing to do so (Bird *et al.* 1994); perhaps there are differences among cell lines. Regulation of c-Raf is complicated so all we can conclude for now is that the cytokines are not acting like EGF. The upstream mechanisms for activation of p42 MAP kinase by the cytokines is therefore unknown.

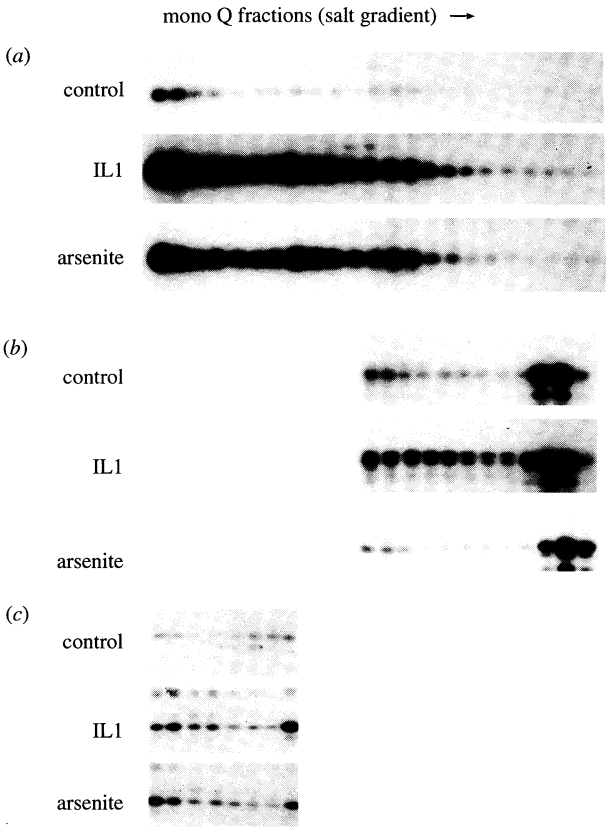

Figure 3. Effects of IL1 and sodium arsenite on β-casein kinase, Jun kinase and hsp27 kinase in human gingival fibroblasts. Confluent human gingival fibroblasts were treated with vehicle, IL1 (20 ng/ml) for 15 min, or sodium arsenite (200 μM) for 60 min. Cell layers were washed in ice-cold phosphate-buffered saline, then taken up in lysis buffer. Lysates were chromatographed at pH 8.5 on a MonoQ column in an FP liquid chromatography system. The column was eluted with a linear gradient from 0–0.5 ml NaCl. Fractions were assayed for their ability to phosphorylate GST-c-Jun (aa 1–135); hsp27 (purified from ME180 cells) or β-casein, in the presence of [^{32}P]γATP. Reaction products were electrophoresed on SDS-PAGE and phosphorylation of substrates was detected by autoradiography.

(*c*) *p54 MAP kinase* (*Jun kinase/stress activated protein kinase*)

IL1 and TNF activate p54 MAP kinase (Kyriakis *et al.* 1994; Kracht *et al.* 1994*a*, *b*; Bird *et al.* 1994). This enzyme is also strongly activated by many cell stresses and has been called stress activated protein kinase (SAPK) (Kyriakis *et al.* 1994). It phosphorylates Ser63 and Ser73 (Pulverer *et al.* 1991) in the transactivation domain of c-Jun and has also been named Jun kinase (JNK) (Dérijard *et al.* 1994). Its activation by IL1 and TNF has been shown not only in cultured cells, but also *in vivo* in liver (Kracht *et al.* 1994*b*). Figure 3 shows that IL1, like sodium arsenite activates c-jun kinases in fibroblasts. At least two main forms are separable on anion exchange chromatography of cell lysates. Two chromatographically similar major forms of kinase were activated by IL1 in rabbit liver, 8 min after

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intravenous injection of the cytokine. We purified these and obtained proteins of 55 kDa and 50 kDa on SDS electrophoresis. Amino acid sequencing of a number of tryptic peptides showed that both were forms of p54α MAP kinase: there was no evidence of β or γ forms of p54 (Kracht *et al.* 1994).

Various forms of p54 MAP kinase have been shown to be activated in cultured cells by the cytokines and stresses. Some of these are 54 kDa, others are smaller around 45 kDa (Derijard *et al.* 1994; Kracht *et al.* 1994*a*). It is not clear yet whether they are α, β or γ sequence. Shorter transcripts for the β and γ sequences have been found (Kyriakis *et al.* 1994). The different forms may turn out to have different substrate specificity, or regulatory mechanisms. Besides c-Jun, the enzyme also phosphorylates and activates transcription factors ATF-2 (on Thr69 and Thr71) (Gupta *et al.* 1995; Livingstone *et al.* 1995; Van Dam *et al.* 1995) and Elk1 (Whitmarsh *et al.* 1995). ATF-2 dimerizes with c-Jun and binds to non-typical AP1 sites found in the c-Jun promoter. Elk1 is a component of the serum response factor. p54 MAP kinase also probably phosphorylates the EGF receptor because the peptide of the sequence around Thr669 is a good substrate (Kracht *et al.* 1994*b*). It is not yet known exactly how the cytokines activate p54 MAP kinase. One activator (JNKK/SEK1/MEK4) which is a homologue of MAPKK, has been identified and implicated in both cytokine and stress induced activation of the pathway (Sanchez *et al.* 1994; Lin *et al.* 1995 and see accompanying articles by Woodgett & Karin).

(*d*) *p38 MAP kinase*

The p42/44 and p54 MAP kinases are activated by dual phosphorylation of the motifs Thr-Glu-Tyr and Thr-Pro-Tyr respectively. A third type of mammalian MAP kinase that was discovered more recently has the activation motif Thr-Gly-Tyr. This protein is a homologue of the yeast protein kinase HOG1 which lies on an osmosensing pathway (Han *et al.* 1994). Yeast respond to hyperosmolar conditions by synthesizing increased amounts of glycerol. The mammalian p38 MAP kinase can be activated by hyperosmolarity and other cell stresses in similar fashion to p54 (Rouse *et al.* 1994). It is also activated by IL1 and TNF (Freshney *et al.* 1994) and by bacterial endotoxin (Han *et al.* 1994). The enzyme phosphorylates and activates another protein kinase (hsp kinase or MAPKAP-K2) which phosphorylates the small heat shock protein, hsp27, in cells (Rouse *et al.* 1994). One other potential physiological substrate is the transcription factor ATF-2 (Dérijard *et al.* 1995).

IL1 and TNF activate p38 MAP kinase in various cell types in culture, but we have not so far been able to demonstrate such activation convincingly in animal tissues. The precise function of hsp27 and its phosphorylation is not established. Hsp27 can inhibit actin polymerization (Miron *et al.* 1991) and act as a molecular chaperone (Jakob *et al.* 1993). It is a constitutive cytosolic component and exists in very large aggregates of around M_r 0.5×10^6. Phosphorylation may cause disaggregation, allowing the hsp27 to then interact with other proteins (Kato *et al.* 1994). Overexpression of hsp27 is associated with increased thermo-tolerance, but there have been conflicting reports as to whether its phosphorylation is necessary for this (Knauf *et al.* 1994; Lavoie *et al.* 1995).

So far only one type of p38 MAP kinase protein has been identified, although a cDNA coding for a protein with 14 variant amino acids scattered between positions 230 and 255 was isolated from a human monocyte cDNA library (Lee *et al.* 1994). p38 MAP kinase can be activated *in vitro* by the p54 activator (SEK1/MEK4), and another activator, MEK3, has been discovered by cloning mammalian cDNAs homologous to the yeast activator PBS2 (Dérijard *et al.* 1995). To what extent MEKs 3 and 4 are involved in the physiological activation of p38 and p54 by cytokines or cell stress remains to be seen.

(*e*) *Inhibitors of MAP kinase pathways*

The MAP kinases have overlapping but different substrate specificities and are implicated in regulation of gene expression because they control activity of transcription factors through phosphorylation. One way of determining the physiological function of the pathways would be to selectively and specifically block one MAP kinase. This has recently become possible for the p38 MAP kinase. Certain bicyclic imidazoles can bind to p38 MAP kinase and inhibit its action in cells in the range 0.1–1 μM (Cuenda *et al.* 1995). These compounds were originally discovered as inhibitors of TNF production from endotoxin-stimulated monocytes (Lee *et al.* 1993) and later discovered to be inhibitors of p38 MAP kinase (Lee *et al.* 1994). As stated previously, p38 MAP kinase is activated by endotoxin. When TNF secretion is inhibited by the bicyclic imidazole, endotoxin still induces TNF mRNA, but production of the protein is prevented (Lee *et al.* 1993). It appears that p38 activation may remove a translational block. Production of IL1 is also affected.

Preliminary experiments with these bicyclic imidazoles show that they may block some, but by no means all, of the effects of IL1 and TNF. Early studies with vascular endothelial cells showed that they partly inhibited IL1-induced IL8 production. We have found they have little effect on IL6 production by gingival fibroblasts stimulated with IL1. They are, however, potent inhibitors of prostaglandin synthesis at concentrations (0.1–1 μM) where they do not appear to be inhibiting cyclooxygenase activity directly. Inhibition may be due to effects on phospholipase A2 activity or cyclooxygenase expression: these possibilities are being investigated. The inhibitors do not inhibit cytokine induction of E-selectin expression on vascular endothelial cells, or cartilage resorption. These initial results show that it may be possible to block particular cytokine actions by interfering selectively with one kinase pathway.

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(*f*) *Activation of NFκB*

IL1 and TNF are potent activators of the transcription factor nuclear factor (NF)KB. A number of the genes they induce are strongly regulated by this factor (see table 1). It is not possible yet to relate NFκB activation to the action of any of the protein kinases known to be induced by the cytokines. NFκB exists complexed to an inhibitor, IκB, in the cytoplasm. Upon cell activation the inhibitor becomes phosphorylated upon Ser32 and Ser36 and is rapidly degraded (Brown *et al.* 1995). This allows the nuclear factor to enter the nucleus where it binds to the appropriate nucleotide motifs. To what extent the subunits of NFκB (p50 and p65) are also regulated by phosphorylation is not yet clear. The kinase responsible for the IκB phosphorylation is activated over the same time period as all the other cytokine-activated kinases found so far: the peak of activity being 5–10 min after stimulation (Guesdon *et al.* 1995). The enzyme(s) responsible is probably novel, although it has been suggested that c-Raf can act as an IκB kinase (Li & Sedivy 1993).

IκB is likely to be degraded by the proteaseome, because inhibitors of this block its degradation and prevent NFκB activation (Read *et al.* 1995). A second IκB protein was cloned recently, this β form is homologous to the α but may be regulated differently (Thompson *et al.* 1995).

(*g*) *β casein kinase*

The MAP kinases are activated by a wide range of stimuli in addition to IL1 or TNF. In general p42/p44 MAP kinases (ERK2 and 1) are activated strongly by mitogens and phorbol esters, but only weakly by cell stresses. The p54 and p38 MAP kinases on the other hand are strongly activated by stresses and cytokines, but only weakly by mitogens or phorbol ester. One cytosolic kinase has been found which appears to be quite specifically activated by IL1 and TNF (Guesdon *et al.* 1993, 1994). This enzyme phosphorylates *in vitro* the milk protein, β-casein on Ser142 (F. Guesdon, unpublished data). It was first found in fibroblasts, but a highly similar enzyme is also activated by IL1 in Hela cells. It is not activated by a wide variety of stimuli including growth factors, phorbol ester, Ca^{2+} ionophore, interferons and other cytokines. Figure 3 shows an experiment in which fibroblasts were stressed with sodium arsenite or stimulated with IL1. Cell extracts were made and chromatographed on an anion exchange column. The fractions were assayed for protein kinase activity. Both arsenite and IL1 induced strong jun kinase activity. There are several chromatographic species which are probably different forms of p54 MAP kinase. Arsenite or IL1 also induced two peaks of hsp27 kinase activity. Thus both IL1 and arsenite induced the 'stress' kinases. However only IL1 induced the β-casein kinase activity. Similar experiments have also been done on fibroblasts exposed to heat-shock or hyperosmolar stress, and again the β-casein kinase was not activated.

The β casein kinase has not yet been purified to homogeneity. On gel filtration two forms are found: one of about 100 kDa and the other of about 400 kDa. The larger form is likely to be an aggregate or complex of some kind. The enzyme cannot be inactivated by treating it with phosphatases, so it is unlikely to be activated by direct phosphorylation (Guesdon *et al.* 1994). Phosphorylation might be involved indirectly, for example by removing the enzyme from an inhibitory subunit.

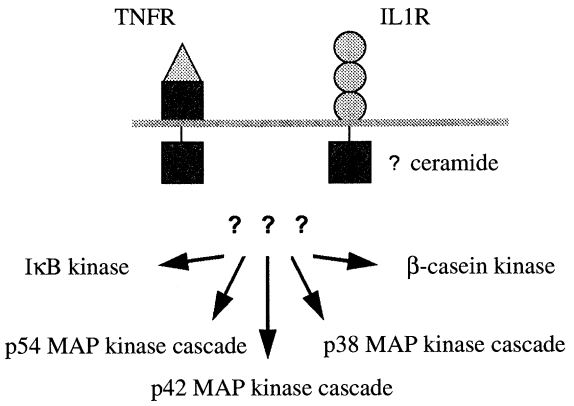

Figure 4. Protein kinase systems known to be activatable by IL1 and TNF.

The physiological substrates of the enzyme are unknown. The motif phosphorylated in β-casein (PLLQS*WMH) does not correspond to any so far known for other protein kinases (F. Guesdon, unpublished data). β-casein kinase could lie above the MAP kinase cascades. If it was downstream of any of them it should be inducible by stress or phorbol ester. Alternatively it may lie on a quite different pathway.

(*h*) *Proximal post-receptor events*

IL1 and TNF have been shown to activate at least five different protein kinase systems. These are summarized in figure 4. Activation of all these is detectable at 3–5 min after stimulation and not before. Thus there is a 2–3 min lag period before the cytosolic kinases are activated. There has been much controversy regarding possible second messengers for IL1 and TNF. One recently discovered potential mechanism is the generation of ceramide by activation of sphingomyelinases (Kolesnick & Golde 1994; Hannun 1994). During stimulation of cells by IL1 or TNF sphingomyelin breakdown is accelerated and ceramide levels rise. A ceramide-activated protein kinase has been detected in TNF-stimulated cells and could conceivably lie upstream of the kinases described here (Liu *et al.* 1994).

TNF is a trimer and cross-links receptors. Some TNF antibodies are agonists, so there is strong evidence that receptor oligomerization is involved in signalling. IL1α and β however are monomers and IL1 receptor oligomerization has not been proved to occur.

Exciting findings have recently been published about other proteins that interact with IL1 and TNF receptors. A 65 kDa transmembrane protein has been cloned that interacts with the type 1 IL1 receptor (Greenfeder *et al.* 1995). This protein forms a complex with IL1 and the receptor, but not with IL1 receptor antagonist protein and receptor. It could therefore be

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a signal transducer. Proteins that interact with the intracellular domains of the two TNF receptors have also been found. TNF receptor associated factors (TRAF) 1 and 2 interact with the cytoplasmic domains of the p80/type 2 receptor (Rothe *et al.* 1994), but their role in signalling is not known. Another protein, TNFR1-associated death domain protein (TRADD) interacts specifically with TNFR1. Overexpression of this 34 kDa protein led to NFκB activation and apoptosis (Hsu *et al.* 1995).

In addition to these receptor associated proteins there have been reports of receptor associated kinase activity for IL1 type 1 and both TNF receptors (Martin *et al.* 1994; Darnay *et al.* 1994*a*, *b*). These are serine–threonine kinases and may be associated with receptor activation.

Current challenges are to work out how the receptors are coupled to the downstream kinases and to understand which pathways are involved in which physiological responses to the cytokines. Detailed understanding of these processes will lead to the identification of new targets for anti-inflammatory therapy.

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Discussion

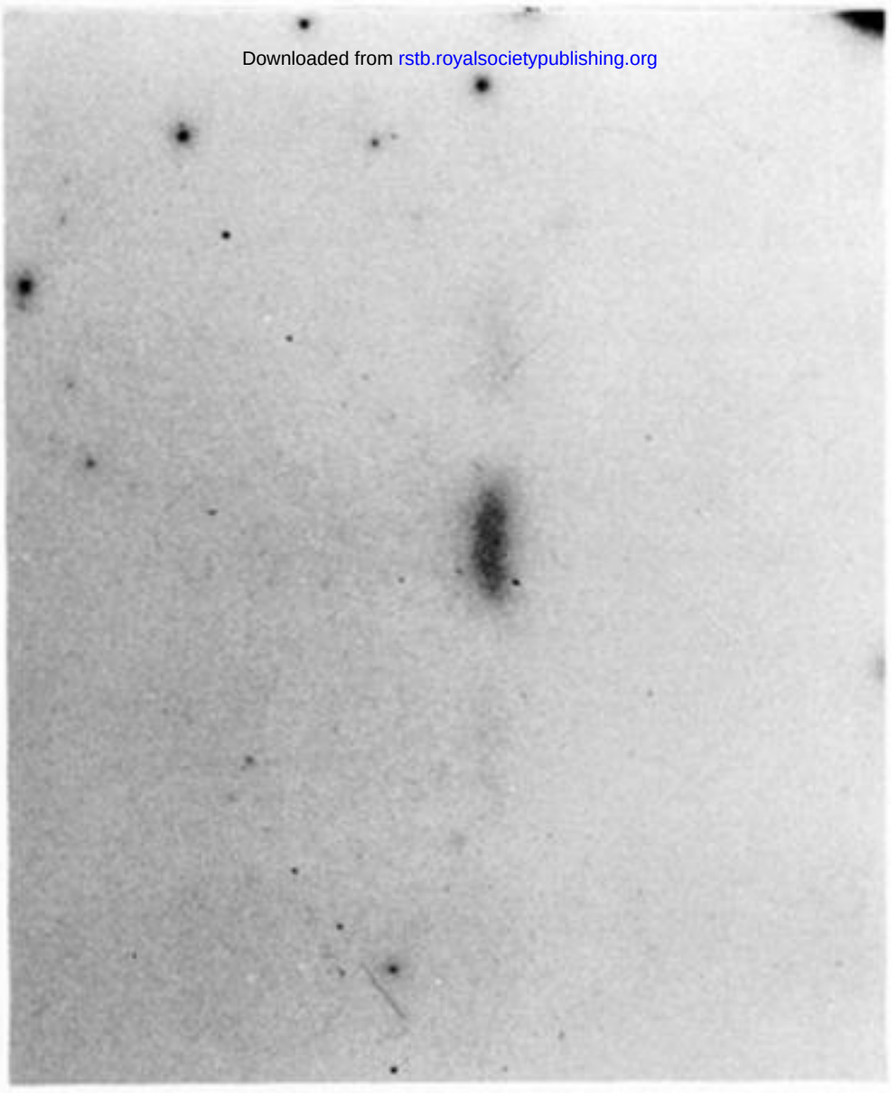
M. Karin (*Department of Pharmacology*, *University of California*, *San Diego*, *U.S.A.*). Is the target for inhibition of prostaglandin E_2 formation by the SKB inhibitor phospholipase A_2 or cyclooxygenase?

J. Saklatvala. These agents will inhibit cyclooxygenase at > 5–$10\ \mu$M, but our effects occur at 0.1–1 μM. Other than that, I do not yet have the necessary information to answer the question.

C. J. Marshall (*Chester Beatty Laboratory*, *Institute of Cancer Research*, *London*, *U.K.*). Why do these inhibitors prevent IL6 production in macrophages but not in fibroblasts?

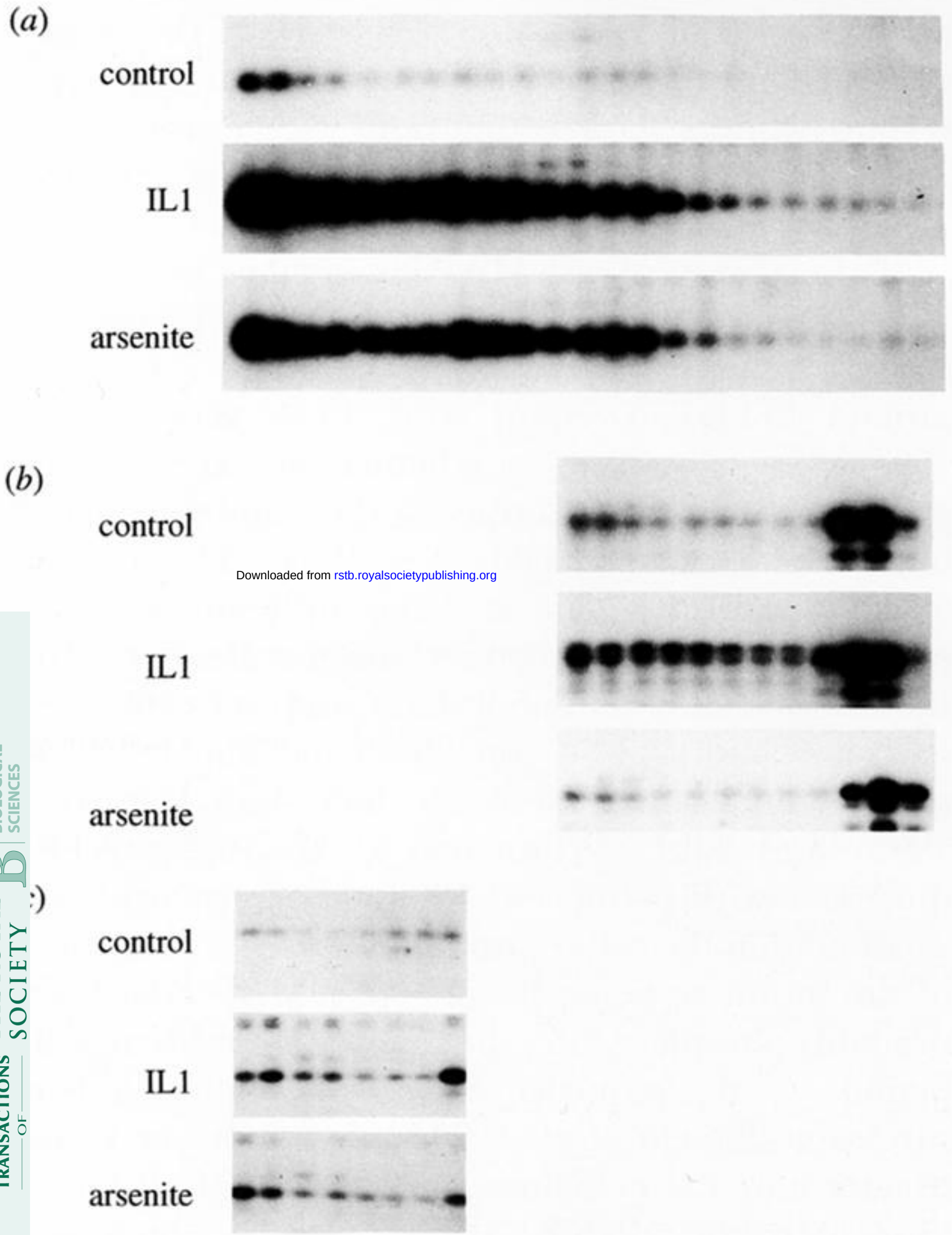
J. Saklatvala. This is not yet clear. However, the comparison is between TNF-stimulated macrophage lines and IL1-stimulated primary gingival fibroblasts, so it is not even clear whether the difference is between different cells or between cells stimulated by different agents.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES



gure 2. Effects of EGF, IL1 and TNF on activity of c-Raf human gingival fibroblasts. Confluent monolayers of broblasts were stimulated with vehicle, EGF (10 ng/ml), L1 (20 ng/ml) or TNF (40 ng/ml) for 6 min. Cells were en washed in ice-cold phosphate-buffered saline, then lysed pidly in immunoprecipitation buffer. c-Raf was immuno-rbed with antibody-coated protein A agarose. The rabbit ıtiserum (from Santa Cruz Biotechnology Inc.) was rected to the N-terminal sequence of MAPKK. Agarose ads were washed well then assayed for activity on combinant MAPKK (inactive mutant) in the presence of ^{32}P]γATP. Reaction products were separated on SDS-AGE and phosphorylation of MAPKK was detected by ıtoradiography.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES



gure 3. Effects of IL1 and sodium arsenite on β-casein nase, Jun kinase and hsp27 kinase in human gingival roblasts. Confluent human gingival fibroblasts were eated with vehicle, IL1 (20 ng/ml) for 15 min, or sodium senite (200 μM) for 60 min. Cell layers were washed in ice-ld phosphate-buffered saline, then taken up in lysis buffer. ysates were chromatographed at pH 8.5 on a MonoQ lumn in an FP liquid chromatography system. The column as eluted with a linear gradient from 0–0.5 ml NaCl. ractions were assayed for their ability to phosphorylate ST-c-Jun (aa 1–135); hsp27 (purified from ME180 cells) or casein, in the presence of [^{32}P]γATP. Reaction products ere electrophoresed on SDS-PAGE and phosphorylation of bstrates was detected by autoradiography.