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# A novel secretory pathway for interleukin-1β, a protein lacking a signal sequence

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**Interleukin 1 (IL-1) is a major soluble mediator of inflammation. Two human IL-1 genes, α and β, have been isolated, which encode polypeptides with only 20-30% amino acid sequence homology. Unlike most secreted proteins, the two cytokines do not have a signal sequence, an unexpected finding in view of their biological role. Here we show that IL-1β is actively secreted by activated human monocytes via a pathway of secretion different from the classical endoplasmic reticulum-Golgi route. Drugs which block the intracellular transport of IL-6, of tumour necrosis factor α and of other secretory proteins do not inhibit secretion of IL-1β. Secretion of IL-1β is blocked by methylamine, low temperature or serum free medium, and is increased by raising the culture temperature to 42°C or by the presence of calcium ionophores, brefeldin A, monensin, dinitrophenol or carbonyl cyanide chlorophenylhydrazone. IL-1β is contained in part within intracellular vesicles which protect it from protease digestion. In U937 cells large amounts of IL-1β are made but none is secreted. In these cells IL-1β is not found in the vesicular fraction, and all the protein is accessible to protease digestion. This suggests that intracellular vesicles that contain IL-1β are part of the protein secretory pathway. We conclude that IL-1β is released by activated monocytes via a novel mechanism of secretion which may involve translocation of intracellular membranes and is increased by stress conditions.**

Key words: interleukin-1/secretion/signal sequence/endoplasmic reticulum/translocation

## Introduction

Most of the pre-secretory proteins that have been so far analysed are extended at their N-terminus by a 'leader' or 'signal' sequence of 13-30 hydrophobic amino acids. This allows the co-translational, vectorial transfer of the protein across the membrane of the endoplasmic reticulum (ER), and is often cleaved before completion of the transmembrane transport of the protein (Milstein *et al.*, 1972; Blobel and Dobberstein, 1975a,b). The signal sequence may also be located up to 12 amino acid residues downstream from the N-terminus of the protein: in the case of ovalbumin, it is not proteolytically removed (Tabe *et al.*, 1984). Translocation usually occurs co-translationally. However, in yeast, completed polypeptide chains may translocate the ER membrane. This post-translational translocation requires heat shock protein 70 (hsp 70) (Chirico *et al.*, 1988; Deshaies *et al.*, 1988). Core glycosylation of proteins occurs cotranslationally at Asn residues followed by the sequence X-Ser/Thr (Kornfeld and Kornfeld, 1985). Secretory proteins are then transported to the Golgi apparatus where they undergo further glycosylation, and finally reach secretory vesicles, which upon fusion with the plasma membrane release their contents in the extracellular space (Palade, 1975). Drugs which block the transport of proteins at different steps along the secretory route have been instrumental in dissecting the process of secretion. For example, brefeldin A (BFA) seems to prevent the egress from the ER (Misumi *et al.*, 1986), while monensin blocks the transport of secretory proteins within the Golgi complex (Tartakoff, 1983).

IL-1α and IL-1β are two cytokines produced by activated monocytes which mediate a wide range of biological activities (Oppenheim *et al.*, 1986). IL-1α and IL-1β are synthesized as larger precursor molecules (pro-IL-1, $M_r$ 31-34 kd) which are processed to the mature form ($M_r$ 17-18 kd) upon secretion (Matsushima *et al.*, 1986; Auron *et al.*, 1987). Both forms of IL-1 lack a typical signal sequence, either N-terminal or internal (Auron *et al.*, 1984; March *et al.*, 1985). In agreement with this, immunoelectron microscopy (Singer *et al.*, 1988) and subcellular fractionation (Matsushima *et al.*, 1986; Bakouche *et al.*, 1987) studies failed to demonstrate IL-1 molecules in the ER and in the Golgi apparatus, raising the question of how they can be released by the producer cells. Here we show that IL-1β is selectively secreted by human activated monocytes via a novel pathway of secretion.

## Results

### *IL-1β is selectively released by activated monocytes*

Human monocytes activated with lipopolysaccharide (LPS) synthesize comparable amounts of IL-1α and IL-1β which are detected in the cell lysates as unprocessed pro-IL-1α and pro-IL-1β, migrating as major bands of ~33 kd (Figure 1a). The 29 kd band present in lane 1 is recognized by anti-IL-1β in Western blots, and probably represents a proteolytic fragment. By contrast, only IL-1β, in its mature form of 17 kd, but not IL-1α, is precipitated from culture supernatants (lanes 2 and 4, Figure 1a).

The absence in culture supernatants of pro-IL-1β and pro-IL-1α (Figure 1a) and other intracellular proteins (see Figures 2 and 4) as well as of cytosolic enzymes (such as lactate dehydrogenase, see Table I) indicated that the release of 17 kd IL-1β was not due to cell death. Cell lysis as a mechanism of release of IL-1β was also ruled out by the finding that when pulse-labelled monocytes were killed by freezing and thawing before chasing at 37°C, only the immature forms of IL-1β (33 and 29 kd) accumulated in the culture supernatant (Figure 1b). Thus, viable cells are required for secretion of processed 17 kd IL-1β. Although

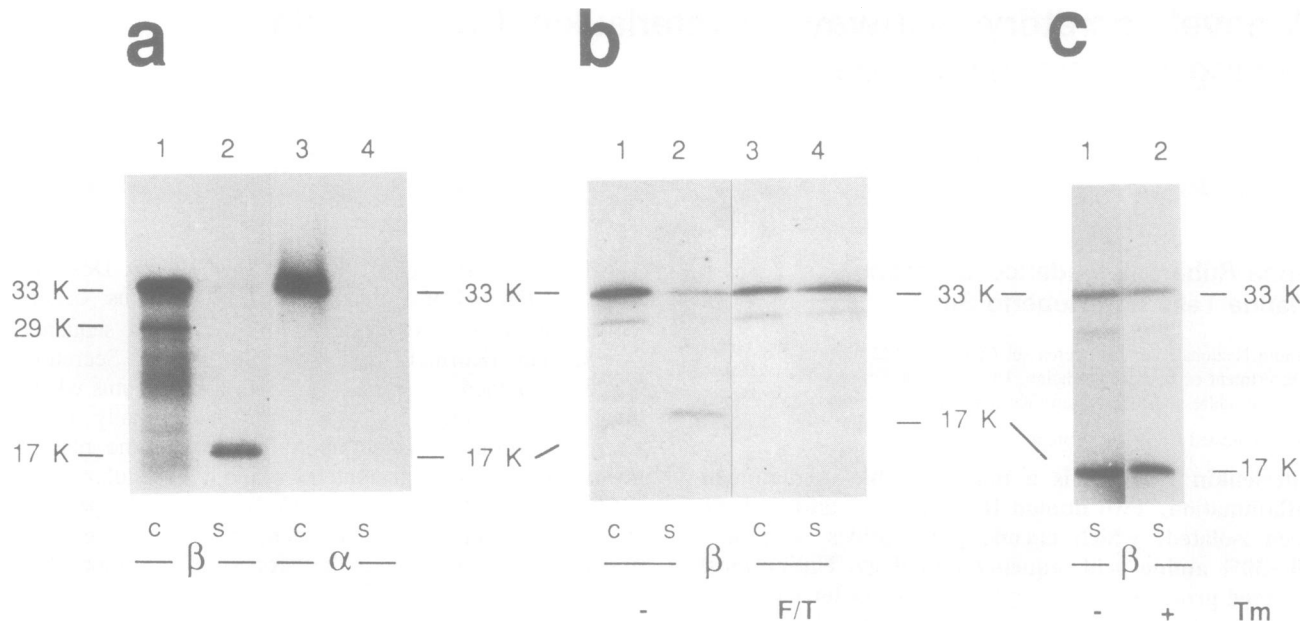


**Fig. 1.** IL-1$\beta$ is selectively secreted by activated monocytes and is not glycosylated. (**a**) IL-1$\beta$ (lanes 1 and 2) and IL-1$\alpha$ (lanes 3 and 4) immunoprecipitated from cell lysates (c, lanes 1 and 3) and supernatants (s, lanes 2 and 4) of LPS activated monocytes pulsed for 4 h with [$^{35}$S]-methionine. (**b**) IL-1$\beta$ immunopreciptated from cell lysates (lanes 1 and 3, c) or supernatants (lanes 2 and 4, s) of cells pulsed for 10 min with [$^{35}$S]-methionine and chased for 3 h at 37°C (lanes 1 and 2, −) or lysed by 2 cycles of freezing and thawing before the chase (lanes 3 and 4, F/T). (**c**) IL-1$\beta$ immunoprecipitated from supernatants of LPS activated monocytes labelled for 4 h with [$^{35}$S]methionine in the presence (lane 2) or absence (lane 1) of 5 $\mu$g/ml of Tm. Tm was added 30 min before [$^{35}$S]methionine. Autoradiograms of 12% SDS−PAGE run under reducing conditions are shown.

IL-1$\beta$ contains a potential site for *N*-linked glycosylation at Asn123 (Auron *et al.*, 1984; March *et al.*, 1985), treatment with tunicamycin (Tm) did not affect the electrophoretic mobility of the IL-1$\beta$ bands (Figure 1c). Since addition of a leader sequence to IL-1$\beta$ results in glycosylation of the molecule expressed in yeast (Baldari *et al.*, 1987) and in transfected mammalian cells (M.Melli *et al.*, Sclavo, Siena, Italy, personal communication), the finding that native IL-1$\beta$ is not glycosylated further supports the notion that it does not enter the ER.

### *Secretion of IL-1$\beta$ does not follow the ER−Golgi pathway*

To establish whether IL-1$\beta$ indeed follows an independent secretory pathway we employed BFA and monensin, known to block vesicular traffic at the ER and Golgi level, respectively. To eliminate any possible effect on protein synthesis, cells were pulsed for 10 min with [$^{35}$S]-methionine (Figure 2, panels a, c and e) or cysteine (panels b and d), and chased in the presence or absence of the drugs. As expected, most of the protein bands found in the supernatant of control cells decreased or disappeared upon treatment with the two drugs (panels a and b, compare lane 2 with lanes 4 and 6). Only two bands of 17 and 46 kd escaped the inhibitory effect of the two drugs: the former, the intensity of which was actually increased by both monensin and BFA, corresponds to mature IL-1$\beta$ (see below) while p46 remains to be identified. We studied in detail the effects of the drugs on two 'classical' secretory proteins, IL-6 and TNF$\alpha$, and compared them with IL-1$\beta$ (panels c, d and e, respectively). After 3 h of chase, all IL-6 and TNF$\alpha$ labelled in control cells were extracellular (lane 2). Treatment with BFA inhibited secretion of both IL-6 (panel c, lane 6) and TNF$\alpha$ (panel d, lane 4). Monensin also inhibited secretion of IL-6, but, unlike BFA, it caused intracellular degradation of IL-6. By contrast, both BFA and monensin increased secretion of IL-1$\beta$ several fold (panel e, lanes 4 and 6, and Table I).

The observations that BFA and monensin inhibited the secretion of IL-6, TNF$\alpha$ and other secretory products by the same cells in which secretion of IL-1$\beta$ was increased, demonstrate that the latter indeed follows an alternative secretory pathway.

### *Secretion of IL-1$\beta$ is inhibited by methylamine and increased by stress conditions*

To gain information on the secretory pathway of IL-1$\beta$, we investigated the effects of other drugs known to affect different intracellular compartments. The results are shown in Figure 3 and are quantitated in Table I. Chloroquine caused a slight enhancement of secretion of IL-1$\beta$, whereas carbonyl cyanide chlorophenylhydrazone (CCCP) and dinitrophenol (DNP) (two uncouplers of oxidative phosphorylation), and the $Ca^{2+}$ ionophore A23187 increased 17 kd IL-1$\beta$ secretion 3- to 15-fold as compared to controls. Protein synthesis during the chase period was not essential, as cycloheximide did not block, but actually enhanced, secretion of 17 kd IL-1$\beta$. A common feature of the drugs that increased secretion of IL-1$\beta$ is their capacity to induce synthesis of heat shock proteins (hsp) (Lindquist, 1986; our unpublished results). We thus studied the effect of heat shock on IL-1$\beta$ secretion by chasing monocytes at 42°C. Under these conditions, IL-1$\beta$ secretion was increased, 70% of the total labelled IL-1$\beta$ recovered after 3 h being in the supernatant (Figure 3, lanes 11 and 12). Interestingly, processing to 17 kd was inhibited: only 33 and 29 kd IL-1$\beta$ were detected in the supernatants of cells chased at 42°C.

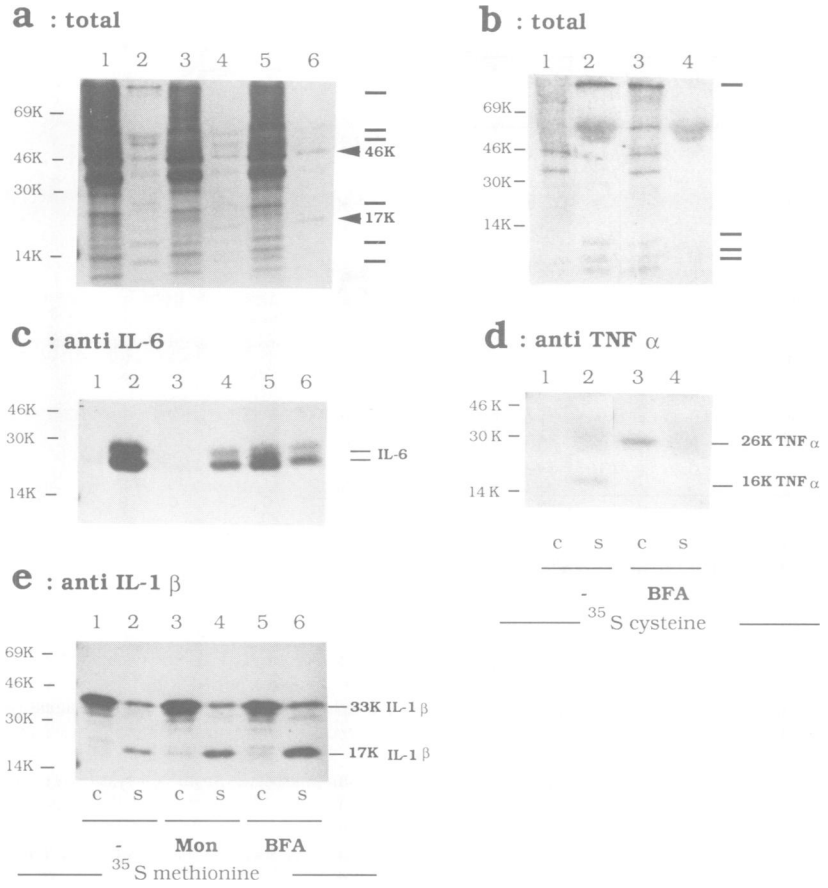


**Fig. 2.** IL-1β does not follow the ER−Golgi pathway. Activated monocytes were pulsed for 10 min at 37°C with either [$^{35}$S]methionine (panels a, c and e) or [$^{35}$S]cysteine (panels b and d) and chased for 3 h in the absence (lanes 1 and 2) or presence of 10 μM monensin (lanes 3 and 4 of panels a, c and e) or 0.5 μg/ml of BFA (lanes 5 and 6 of panels a, c and e; lanes 3 and 4 of panels b and d). Lanes 1, 3 and 5: cell lysates (c); lanes 2, 4 and 6: supernatants (s). Cell viability was controlled by trypan blue exclusion and determination of LDH activity in supernatants at the end of the chase (see Table I). Panels show electrophoretic analysis of an aliquot of total cell lysates and supernatants of cells labelled with [$^{35}$S]-methionine (**a**) or with [$^{35}$S]cysteine (**b**) before immunoprecipitation. Marks on the right hand margin indicate the secretory bands which decrease upon treatment with monensin or BFA, while in panel a arrows point at the two bands which increase under the same conditions (17 and 46 kd). As indicated by immune precipitation or blotting, the 200 kd band consists of at least three proteins, i.e. fibronectin, C3 and C4 (Whaley, 1980), which co-migrated on 12% SDS−PAGE; the broad band of ~25 kd was probably IL-6. The 200 kd band results stronger after labelling with [$^{35}$S]cysteine (b) than with [$^{35}$S]methionine (a). This is probably related to the high cysteine content of fibronectin (Kornblihtt *et al.*, 1985). (**c**) Anti-IL-6 immunoprecipitates. Note that an intracellular IL-6 band, absent in control and in monensin treated cells, accumulated in the lysates of BFA treated cells. This may suggest that BFA, but not monensin, inhibits intracellular degradation of IL-6. (**d**) Anti-TNFα immunoprecipitates. The 26 kd intracellular band accumulating in the lysates of BFA treated cells probably corresponds to the unprocessed, signal sequence-bearing, cell associated TNFα (Kriegler *et al.*, 1988). (**e**) Anti-IL-1β immunoprecipitates. Molecular weight markers are shown on the left of each panel.

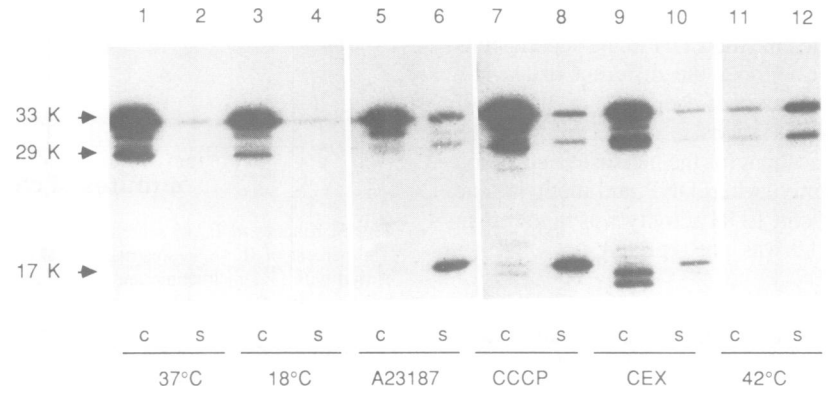


**Fig. 3.** Secretion of IL-1β is increased by stress conditions. Activated monocytes were pulsed for 10 min with [$^{35}$S]methionine and chased for 3 h at 37°C (**lanes 1** and **2**), 18°C (**lanes 3** and **4**), 37°C in the presence of 5 μM A23187 (**lanes 5** and **6**), 5 μM CCCP (**lanes 7** and **8**), 100 μM cycloheximide (CEX) (**lanes 9** and **10**) or at 42°C (**lanes 11** and **12**). Cell lysates (c, lanes 1, 3, 5, 7, 9, 11) and supernatants (s, lanes 2, 4, 6, 8, 10, 12) were immunoprecipitated with anti-IL-1β and electrophoresed.

**Table I.** Modulation of IL-1β secretion by different drugs

| Chase conditions[a] | Secretion of IL-1β[b] | LDH activity (mU/ml)[c] | % of cell lysis[d] |
|---|---|---|---|
| Complete medium alone | 100 | 0.32 ± 0.17 | 8 |
| Methylamine (10 mM)[e] | 10 | 0.36 ± 0.30 | 9 |
| Serum free medium | 0 | 0.30 | 8 |
| 18°C | 0 | ND[f] | ND |
| BFA | 500 | 0.40 ± 0.33 | 10 |
| Chloroquine | 180 | ND | ND |
| Monensin | 340 | 0.60 ± 0.41 | 14 |
| A23187 | 1300 | 1.65 | 38 |
| DNP | 1000 | 0.70 ± 0.14 | 16 |
| DNP + methylamine | 0 | 0.77 ± 0.53 | 18 |
| CCCP | 900 | 0.33 ± 0.01 | 8 |
| CEX | 420 | ND | ND |
| 42°C | 1700 | 0.47 ± 0.10 | 12 |

[a]LPS activated monocytes were pulsed for 10 min with [$^{35}$S]methionine and chased for 3 h at 37°C under different conditions.
[b]The figures were normalized to the amount of IL-1β secreted by control cells, as calculated by densitometric analyses of the autoradiograms (see Materials and methods for details). Mean of two or more different experiments.
[c]LDH activity was measured in supernatants at the end of the chase period. Background, represented by the LDH activity present in complete medium, was subtracted. Data express the mean of three different experiments ± SEM. The high SEM probably reflects the variability in cultures from different donors.
[d]The percentages of cell lysis were deduced from a curve of LDH activity versus cell mortality, as detailed in Materials and methods.
[e]Cells were preincubated with methylamine for 1 h.
[f]ND, not determined.

Methylamine (Figure 4), a drug known to inhibit endocytosis (Maxfield *et al.*, 1979), as well as performing the chase at 18°C or in serum free medium, inhibited secretion of IL-1β. When an inducer (DNP) and an inhibitor (methylamine) were added simultaneously, the inhibitory effect prevailed (Figure 4a, lane 6) indicating that, in cells induced with DNP, IL-1β follows the same pathway of secretion as in control cells. Methylamine also inhibited the secretion of p46 (Figure 4b, lane 4); in contrast, secretion of 17 kd IL-1β and of p46 was selectively induced by DNP (lane 8). The similar response of IL-1β and p46 to these various drugs suggests that both may well utilize the same secretory route.

To exclude the possibility that the effects on IL-1β secretion were due to reduced cell viability, we measured the activity of the cytosolic enzyme LDH in the supernatants of cells cultured with or without the different drugs and correlated it with the percentage of dead cells at the end of the chase (Table I). The variations in cell viability in the different samples cannot account for the increase in secretion of IL-1β. More importantly, when DNP and methylamine were simultaneously present, LDH activity was higher than in controls, and yet IL-1β was not secreted.

### *Kinetics of IL-1β secretion*

The kinetics of IL-1β secretion under control and stress conditions are shown in Figure 5. The 17 kd IL-1β became detectable in the supernatants of control cells after 90 min of chase and continued to increase even after 5 h, in agreement with the data of Hazuda *et al.* (1988). A23187 and high temperature induced a faster secretion, an IL-1β band becoming detectable after 45 and 20 min of chase,

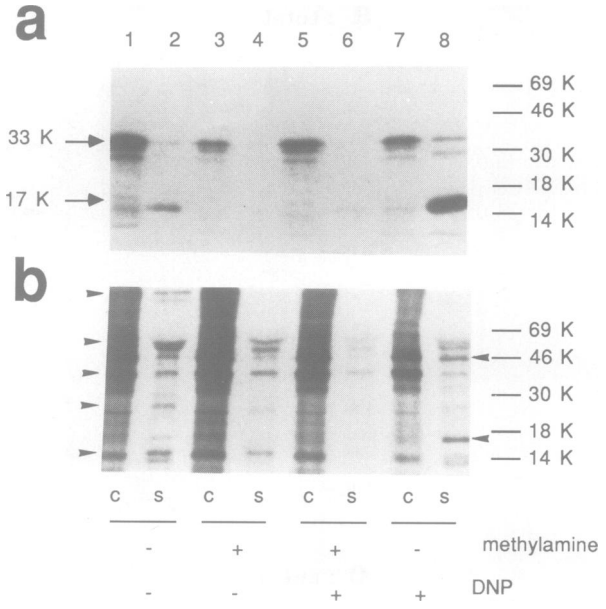


**Fig. 4.** Secretion of IL-1β is inhibited by methylamine. Activated monocytes were pulsed with [$^{35}$S]methionine for 10 min and then chased for 3 h in complete medium alone (lanes 1 and 2) or in the presence of 1 mM methylamine (lanes 3 and 4), 0.3 mM DNP (lanes 7 and 8) or both (lanes 5 and 6) as indicated. (**a**) Anti-IL-1β immunoprecipitates from cell lysates (c) and supernatants (s). (**b**) Aliquots (1/200) of total cell lysates (c) and supernatants (s) before immunoprecipitation. Arrows on the left hand margin indicate migration of secretory bands which decrease of intensity upon treatment with DNP. Migration of 17 kd IL-1β and p46 is indicated by arrows on the right hand margin.

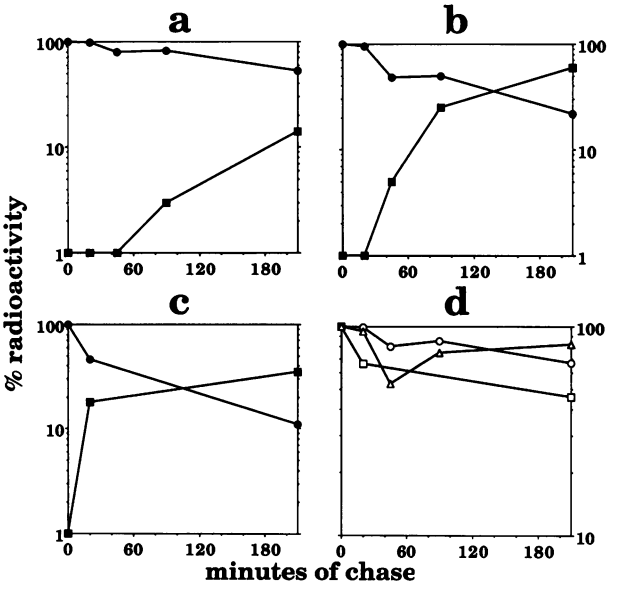


**Fig. 5.** Kinetics of IL-1β secretion under different chase conditions. The percent of IL-1β in supernatants (■) or lysates (●) of monocytes pulsed with [$^{35}$S]methionine and chased at 37°C in the absence (**a**) or presence (**b**) of 5 μM A23187 or at 42°C (**c**) were determined by densitometric analyses of SDS–PAGE autoradiograms, as described in Materials and methods. Panel (**d**) shows the total amount of IL-1β recovered (intracellular + secreted) at 37°C (○), in the presence of A23187 (△), or at 42°C (□).

respectively. Under stress conditions, IL-1β secretion reached a plateau within 3 h. Chasing at 42°C also resulted in increased degradation of IL-1β (Figures 3 and 5d).

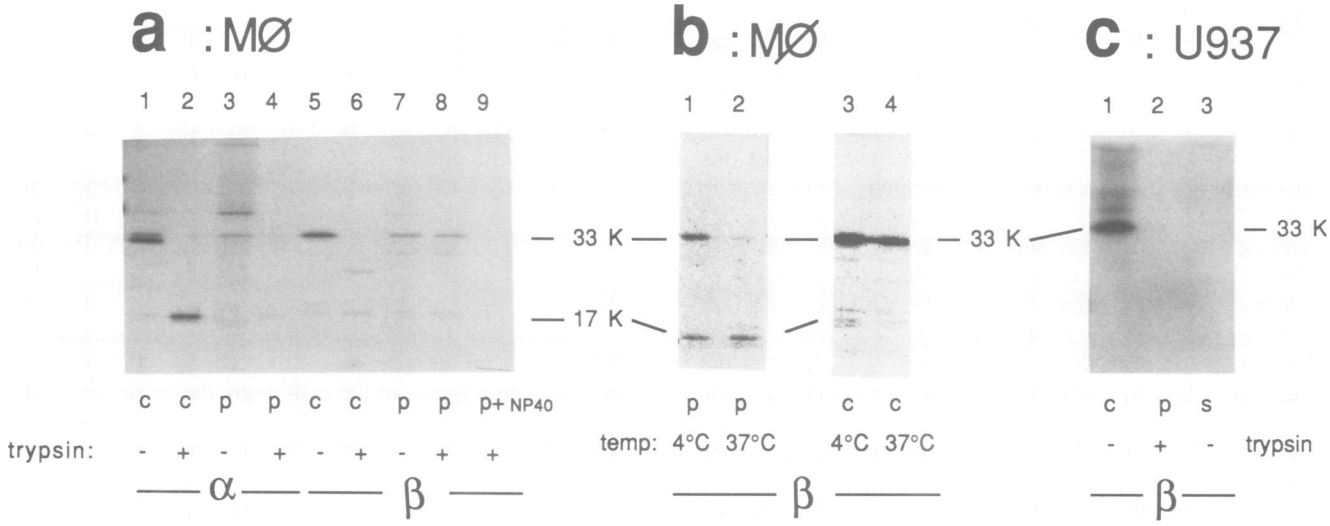


**Fig. 6.** In activated monocytes, but not in U937 cells, IL-1β is in part contained within vesicles probably involved in processing. (**a**) IL-1α (lanes 1–4) and IL-1β (lanes 5–9) were sequentially immunoprecipitated from cytosol (c, lanes 1, 2, 5 and 6) or particulate fractions (p, lanes 3, 4, 7–9) obtained from LPS activated monocytes, pulsed for 3 h with [$^{35}$S]methionine. Aliquots of the samples (lanes 2, 4, 6, 8 and 9) were digested with 100 μg/ml of trypsin for 3 h on ice before solubilization and immunoprecipitation. In one sample NP-40 was added before trypsin (lane 9). (**b** The particulate fraction (p, lanes 1 and 2) or cytosol (c, lanes 3 and 4) obtained from LPS activated monocytes pulsed for 110 min with [$^{35}$S]methionine and chased for 3 h were incubated for 1 h at 4°C (lanes 1 and 3) or at 37°C (lanes 2 and 4), and immunoprecipitated with anti-IL-1β after solubilization with NP-40. Data from lanes 3 and 4 are not quantitative. (**c**) U937 cells were activated with LPS for 12 h, pulsed for 3 h with [$^{35}$S]-methionine, and fractionated into cytosol and particulate fraction. IL-1β present in the two fractions and in the supernatants was immunoprecipitated and analysed on SDS–PAGE.

### *Part of intracellular IL-1β is contained in trypsin resistant vesicles*

Secretion of IL-1β implies its translocation across a cellular membrane, either the plasma membrane or an intracellular one. To distinguish between the two possibilities, we investigated the state, soluble or membrane bound, of IL-1α and IL-1β in monocytes. As shown in Figure 6a, the two cytokines were present both in the cytosol and in the particulate fraction, although in different amounts. Treatment of the two fractions with trypsin demonstrated that, whereas IL-1α and IL-1β contained in the cytosol were sensitive to digestion, most IL-1β in the particulate fraction was not digested, indicating that it was contained within membrane surrounded vesicles. Under the same conditions, IL-1α was protected only partially (Table II). Addition of detergent to the particulate fraction rendered all IL-1β accessible to proteolysis (lane 9a). The finding that IL-1β was selectively protected against proteolysis not only suggests that the vesicular form represent an intermediate in secretion, but also rules out the possibility that the vesicles were a fractionation artifact (note also that the slower band co-precipitating with anti-IL-1α in Figure 6a, lane 3, probably representing cytosolic actin, was fully accessible to digestion).

### *Are the intracellular vesicles containing IL-1β involved in processing and secretion?*

The absence of mature 17 kd IL-1β in NP-40 cell lysates (Figure 1a), and the failure to obtain processing by incubating soluble pro-IL-1β with activated monocytes or supernatants thereof (data not shown, see also Hazuda *et al.*, 1988) suggest that processing occurs while the molecule is being secreted. However, as shown in Figure 6a (lanes 7 and 8), a faint 17 kd IL-1β band could be detected in the particulate fraction after subcellular fractionation. When the two fractions were incubated at 37°C, the 17/33 kd ratio increased in the particulate fraction (from 1.33 to 4.34) but not in the cytosol (from 0.25 to 0.28) (see panel b). The intensity of the 17 kd band in lane 2b is lower than expected from the entire conversion of the 33 kd pro-IL-1β into 17 kd molecules. It is possible that non-specific proteolysis during incubation at 37°C of the particulate fraction yielded non-immunoprecipitable fragments. These data indicate that processing to 17 kd IL-1β may occur *in vitro* within isolated vesicles, and suggest that these vesicles represent a step in the secretory pathway of IL-1β. If this were indeed the case, one would not expect to detect trypsin protected IL-1β in U937 cells, which on induction produce, but do not process or secrete, IL-1β. As shown in Figure 6c, whereas a pro-IL-1β band was easily detectable in the cytosol of LPS-stimulated U937 cells, no IL-1β was found in the particulate fraction or in the supernatants, even after a long exposure of the autoradiogram.

**Table II.** Particulated IL-1β is protected from trypsin digestion[a]

| | Experiment | % protection[b] | |
|---|---|---|---|
| | | cytosol | particulated |
| IL-1α | 1 | 1 | 22 |
| | 2 | 2 | 50 |
| IL-1β | 1 | 0 | 74 |
| | 2 | 0 | 90 |

[a]IL-1α and IL-1β present in the cytosol and in the particulated fraction, before and after treatment with trypsin, were quantitated by densitometric analysis of the fluorograms. Results from two experiments are shown.
[b]The percent of protection was calculated according to the formula: area of 33 kd pro-IL-1α or β after trypsin treatment/control × 100.

## Discussion

In this study we show that IL-1β, a protein lacking a typical signal sequence, is secreted by activated monocytes via a novel pathway of secretion. The presence of a high cytosolic pool of pro-IL-1 raised the possibility that the cytokine is released by cell lysis. Several lines of evidence exclude this possibility: (i) IL-1β is selectively secreted, (ii) processing to the 17 kd form requires viable cells and (iii) certain drugs increase IL-1β secretion without affecting cell viability.

In addition, several drugs which block the transport of proteins along the classical secretory pathway cause an increase, rather than an inhibition, in IL-1β secretion. These findings, together with the observations that IL-1β is not found in the ER by morphological and biochemical criteria (Bakouche *et al.*, 1987; Singer *et al.*, 1988) and is not glycosylated at Asn123, demonstrate that IL-1β is not translocated into the ER.

Active secretion of a protein requires its translocation across a cellular membrane: the presence of a protease protected pro-IL-1β in cell homogenates indicates that translocation may involve some intracellular vesicular compartment. Since no particulate IL-1β is detectable in cells which produce but do not secrete IL-1β, these vesicular elements are probably part of the protein secretory pathway. The nature of these vesicular elements remains to be established: as secretion of IL-1β is inhibited by agents and conditions known to prevent endocytosis, such as methylamine, low temperature or serum free medium (Maxfield *et al.*, 1979; Steinman *et al.*, 1983), recycling endosomes might play a role in the export of IL-1β.

IL-1β may be sequestered into vesicles by either active translocation or autophagy. Neoformation of vesicles involved in the selective release of cellular proteins has been described in several systems. In cultured macrophages, cytosolic blebs or 'podosomes', containing selected cytosolic proteins, protrude and detach from the cell surface (Davies and Stossel, 1977). Similarly, loss of transferrin receptors from differentiating reticulocytes involves concentration of the receptor molecules to a pole of the cell, followed by vesiculation and selective loss of that patch of the membrane (Pan and Johnstone, 1983). The vesicular elements formed contain abundant hsp 70 (Davis *et al.*, 1986). The presence of some pro-IL-1β within intracellular vesicles does not obviously exclude the possibility that translocation also occurs directly at the plasma-membrane.

In any case, the question as to how IL-1β is specifically recognized among other cytosolic proteins remains to be answered. The alternative secretory pathway described here for IL-1β might be used by other proteins with a defined extracellular function but lacking a signal sequence, such as bFGF (Abraham *et al.*, 1986); PD-ECGF (Ishikawa *et al.*, 1989); ADF, a thioredoxin-like molecule released by leukaemic T cells (Tagaya *et al.*, 1989); blood coagulation factor XIIIa (Grundmann *et al.*, 1986) and the yeast mating pheromone a-factor (Kuchler *et al.*, 1989). Since a motif common to these proteins is not readily identifiable, it is possible that post-translational modifications, such as acylation, are responsible for their selective export. In yeast, secretion of a-factor requires the STE 6 gene product, a protein homologous to the mammalian multidrug resistance (MDR) glycoprotein (Kuchler *et al.*, 1989; McGrath and Varshavsky, 1989). The stimulatory effects of apparently unrelated drugs on IL-1β secretion might be related to MDR function. A possible role for heat shock proteins is also worth consideration; in either case, since the cycloheximide experiments indicate that protein synthesis is not required, a post-translational activation of pre-existing molecules should be postulated.

Post-translational translocation of certain yeast and mitochondrial proteins requires hsp70 and ATP (Chirico *et al.*, 1988; Deshaies *et al.*, 1988). However, ATP is not required if the protein is unfolded (Eilers and Schatz, 1986). There are at least two possible explanations for the observation that IL-1β secretion occurs in the presence of CCCP or DNP: either a store of energy sufficient for translocation remains in the cells even after treatment with the two drugs, or cytosolic pro IL-1β is in a loose conformation, which allows translocation in the absence of energy. Indeed, unlike pro-IL-1α, pro-IL-1β is biologically inactive (Mosley *et al.*, 1987; Hazuda *et al.*, 1989). A different intracellular folding of IL-1α and IL-1β, might explain the slower release of the former. Alternatively, this may reflect the existence of a membrane associated form of IL-1α (Fuhlbrigge *et al.*, 1988).

The observation that stress conditions increase IL-1β secretion may be relevant for the control of the local production of bioactive cytokines in response to tissue damage. Secretion of unprocessed IL-1β at 42°C indicates that secretion and processing are only temporally associated. In addition, since pro-λ IL-1β is inactive, it may also represent a feed-back mechanism to control the pyrogen function of the cytokine.

The question of where does IL-1β processing take place, and its relationships with secretion are intriguing. The mature form of the molecule is detectable in the particulate fraction but not in the cytosol: this suggests that, at least *in vitro*, IL-1β-containing vesicles represent the site of 33 to 17 kd processing. However, 17 kd IL-1β is not detected in NP-40 lysates, even when secretion is blocked by methylamine: thus, in intact cells, conversion to 17 kd may take place as the vesicles containing the pro-IL-1β release their content extracellularly.

The use of an alternative route of secretion may be finalized to avoid the oxidizing milieu of the ER lumen. We have recently obtained evidence that partially assembled IgM molecules are retained intracellularly because they expose a free sulphydryl group (Sitia *et al.*, 1990): as the bioactive form of IL-1β contains two exposed cysteine residues (Priestle *et al.*, 1988), passage into ER might be detrimental to its biological function. Free thiol groups are present also on bFGF, PD-ECGF, ADF and factor XIIIa (Takahashi *et al.*, 1986). These proteins may share with IL-1β the requirement of a reducing milieu and hence the alternative secretory pathway.

Alternatively, this novel pathway of secretion might allow intracellular compartmentalization of a ligand and its receptor. Co-localization of a growth factor and its receptor in the ER−Golgi pathway can result, in some circumstances, in internal activation and uncontrolled autocrine stimulation (Bejcek *et al.*, 1989; Dunbar *et al.*, 1989): in the case of proteins without a signal sequence, transfection of a leader sequence-containing bFGF gene into 3T3 cells (which do express bFGF receptors) indeed led to cell transformation (Rogelj *et al.*, 1988).

## Materials and methods

### Reagents

LPS from *Escherichia coli* (used at 10 μg/ml), NADH and sodium pyruvate, trypsin and trypsin inhibitor (used at 100 and 200 μg/ml, respectively) were purchased from Sigma (St Louis, MO). Tm (Sigma) was used at 5 μg/ml, BFA (kind gift of Sandoz, Milano, Italy) at 0.5 μg/ml, methylamine (Sigma) was used at 10 mM, A23187 (Sigma) at 10 μM (Copeland *et al.*, 1988), DNP (Sigma) at 1 mM (Copeland *et al.*, 1988), chloroquine (Sigma) at 150 μM (Poole and Okhuma, 1981), cycloheximide (CEX: Boehringer, Mannheim, FRG) at 100 μM, monensin (Sigma) at 10 μM.

### Preparation and activation of human monocytes

Peripheral blood mononuclear cells were isolated from buffy coats obtained from healthy donors by Ficoll–Hypaque gradients. Cells were washed twice, resuspended in RPMI 1640 medium (Gibco) supplemented with 10% foetal calf serum (FCS), 1 mM L-glutamine and 100 U/ml of penicillin–streptomycin (complete medium) and plated in 24-well plates (Costar Instruments) at $10^7$/ml. After 1 h at 37°C, non-adherent cells were removed and the remaining cells (>95% monocytes) were activated for 1.5 h with 10 μg/ml of LPS (Sigma).

U937 cells (Koren *et al.*, 1979) were maintained in complete medium and activated with 1 μg/ml of LPS for 12 h before endogenous labelling.

### Endogenous labelling

Adherent cells prepared as above or U937 cells ($10^6$) were washed 3 times with methionine- or cysteine-free RPMI, and incubated with the same medium supplemented with 1 mM glutamine, 5% dialysed FCS, 10 μg/ml of LPS (Sigma) and 100 μCi/ml of [$^{35}$S]methionine or [$^{35}$S]cysteine (sp. act. >800 Ci/mmol, Amersham, Milano, Italy) for 3 or 4 h at 37°C in 5% $CO_2$. For pulse–chase experiments, monocytes were pre-activated with 10 μg/ml of LPS for 1.5 h, washed in methionine-free RPMI containing 5% dialysed FCS and glutamine, and pulsed for 10 min at 37°C with 800 μCi/ml of [$^{35}$S]methionine. For detection of TNFα, which does not contain methionine residues, [$^{35}$S]cysteine was used. After removal of the labelling medium, cells were washed 3 times with RPMI 1640 medium and cultured for different periods of time at 37 or 42°C in complete medium in the presence or absence of different drugs. When methylamine or chloroquine were used, cells were pre-incubated for 1 h in the presence of the drug before the pulse.

Supernatants were then removed, and cells washed in cold PBS and lysed in 0.25% NP-40 and 1 mM PMSF, unless diversely specified. Nuclei and cell debris were removed by centrifugation for 10 min at 12 000 *g*, and supernatants and cell lysates were subjected to immunoprecipitation.

### Inhibition of N-linked glycosylation

Monocytes, prepared as above, were pre-incubated in complete medium at 37°C with 5 μg/ml of Tm (Kornfeld and Kornfeld, 1975) for 30 min before the addition of [$^{35}$S]methionine. Labelling was performed in the presence of 5 μg/ml of Tm.

### Subcellular fractionation and trypsin protection

Activated monocytes were labelled with [$^{35}$S]methionine for 3 h, scraped from the plate, washed twice in cold phosphate buffered saline (PBS) and once in a homogenizing buffer containing 20 mM HEPES, pH 7.4, 4 mM 2-mercaptoethanol, 5 mM EDTA. Cells were resuspended in 1 ml of the same buffer, kept for 10 min in ice, disrupted by 20 strokes with a glass pestel in a Dounce Homogenizer (Wheaton, Millville, NJ, USA) and supplemented with 1 ml of 0.66 M sucrose in the same buffer (Matsushima *et al.*, 1986). Nuclei and undisrupted cells were eliminated by centrifuging for 10 min at 200 *g*, and the post-nuclear supernatants layered onto 1.5 ml of a 40% sucrose solution in homogenizing buffer and centrifuged at 100 000 *g* or 1 h at 4°C. After centrifugation, supernatants were collected and kept as cell cytosol, and the pellet (particulate fraction) was resuspended in 0.1 ml of PBS. Cytosol and particulate fractions were incubated for 3 h in ice in the presence or absence of 100 μg/ml of trypsin (Blobel and Dobberstein, 1975b). At the end of the incubation periods, 200 μg/ml of trypsin inhibitor and 5% FCS were added to each sample before solubilization in 0.25% NP-40 and immunoprecipitation. As a control, an aliquot of the particulate fraction was solubilized with 0.25% NP-40 before incubation with trypsin.

### Immunoprecipitation and SDS–PAGE

All the immunoprecipitations were carried out as follows. Samples were precleared by rotating 1 h at 4°C with 50 μl of a 50% solution of protein A–Sepharose (Pharmacia, Uppsala, Sweden) and then immunoprecipitated with 1 μl of the following antisera: anti-IL-1α or anti-IL-1β (kind gift of Dr A.Shaw, Glaxo, Geneva, Switzerland), anti-IL-6 and anti-TNFα (kind gift of Dr S.Poole, National Institute for Biological Standards and Control, Potters Bar, UK), anti-C3 and -C4 complement components (Dakopatts, Glostrup, Denmark), anti-fibronectin (kind gift of Dr L.Zardi, Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy) or anti-hsp 70 (kind gift of Dr H.Pelham, MRC, Cambridge, UK) followed by protein A–Sepharose. The immunoprecipitates were washed 4 times in 0.5 M NaCl, 0.25% NP-40, 50 mM Tris–HCl, pH 7.6, 1 mM PMSF, once in 10 mM Tris–HCl, pH 7.6, eluted in SDS-2ME Laemmli buffer (Laemmli, 1970) and run on a 12% SDS–PAGE, which was treated with Amplify (Amersham), dried and exposed to Hyperfilm MP (Amersham) at −70°C. For analysis of total labelled proteins, an aliquot of cell lysates and supernatants was denatured in Laemmli buffer and run on SDS–PAGE before immunoprecipitation.

### Determination of cell viability

Cell viability at the end of the period of chase was evaluated by trypan blue exclusion as well as by measurement of LDH activity in culture supernatants of cells chased in the presence or absence of the different drugs. LDH activity was determined by spectrophotometric analysis as described by Bergermeyer *et al.* (1983). To correlate it with cell viability, macrophages were lysed by either three cycles of freezing and thawing or by sonication, and aliquots corresponding to 25, 50 or 100% of the cells used in the pulse–chase experiments were added to complete medium before LDH determination. 1.06 ± 0.17, 2.08 ± 0.69, 3.67 ± 0.64 mU/ml of LDH activity in the supernatants corresponded to 25, 50 and 100% of cell lysis, respectively (mean of three different experiments ± SEM).

### Densitometric analysis

Densitometric analyses were performed by scanning at least two exposures of the same fluorogram with a LKB Ultrascan XL densitometer. The data were corrected according to the number of methionine residues present in the different IL-1 molecules or of cysteine residues in the different TFNα molecules. The percent of secreted molecules was calculated according to the formula: % secreted molecules = area of secreted molecules/(area of intracellular molecules + area of secreted molecules) × 100.

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