Secreted proteins of human monocytes

Analysis by two-dimensional gel electrophoresis and effect of lipopolysaccharide

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A monocyte-rich preparation from the adherent cell fraction of human peripheral blood leukocytes was incubated for 1–8 h with [³⁵S]methionine or [³H]leucine in the presence and absence of bacterial lipopolysaccharide (LPS). The macromolecules released into the supernatant were analysed by two-dimensional gel electrophoresis and radioautography. A complex labelling pattern involving at least 20 easily demonstrable and apparently distinct products with a broad range of molecular masses and isoelectric points was observed. LPS or LPS plus actinomycin in combination markedly stimulated the labelling and release of at least twelve different macromolecules ranging in apparent M_r from 12 000 to 46 000. Studies with monocytes that had been additionally purified by centrifugal elutriation and with the monocyte-like human cell line U-937 indicated that monocytes rather than contaminating cells were the source of these products. The majority of the secreted products were unique and did not cross-react with antibodies to interleukin 1 or tumour necrosis factor. The high resolving capacity of two-dimensional gel electrophoresis may be useful to define further the diverse biological activities and potential monokines released from monocytes at various stages of their differentiation and activation.

INTRODUCTION

The mononuclear phagocyte's role in host defence includes the capacity for microbial and tumour cell killing, phagocytosis and secretion of biologically active products [1]. The products known to be secreted by mononuclear phagocytes include reactive metabolites of oxygen, biologically active lipids, numerous complement and coagulation proteins, other proteases, α_2 -macroglobulin and other antiproteases, lysozyme, phosphatases, fibronectin, and growth factors including interleukin 1 (IL-1), tumour necrosis factor (TNF), interferon and colony stimulating factor [1–3]. Many of these activities undergo change during cell differentiation and their secretory rates are modified by exogenous agents such as bacterial lipopolysaccharides (LPS) or lymphokines [4–11]. Secreted proteins capable of monokine function may require gene activation and new protein synthesis, as exemplified by IL-1 and TNF [12,13].

Functional assays of conditioned media from mononuclear phagocytes has demonstrated numerous protein factors capable of enhancing or suppressing the immune response [14–16]. Many of these biological activities have been only partially characterized in molecular terms. Other authors have analysed radiolabelled secreted proteins produced by mononuclear phagocytes by immunoprecipitation and/or one-dimensional gel electrophoresis; however, these methods may underestimate the secretory capacity of mononuclear phagocytes [17,18].

The analysis of complex protein mixtures by two-dimensional gel electrophoresis of biosynthetically label-

led proteins allows determination of changes in the levels of specific proteins in precise molecular terms and allows analysis of relationships between multiple products. A detailed analysis of the effects of LPS on protein synthesis by mouse peritoneal macrophages has been published [19]. Two-dimensional gel electrophoresis permitted these authors to define alterations in the accumulation of specific cellular proteins following LPS stimulation and revealed that the macrophage response to LPS was more complex than previously anticipated. The secreted proteins produced by human mononuclear phagocytes have not been similarly studied.

In this paper we define the newly biosynthesized secreted proteins produced by freshly isolated human mononuclear phagocytes and the U-937 human tumour cell line. A number of constitutively secreted products not previously defined have been characterized by two-dimensional gel electrophoresis. Additionally, the effects on specific protein accumulation produced by LPS, concanavalin A (Con A), and phorbol myristate acetate (PMA) have been defined. LPS or LPS plus actinomycin markedly stimulated the biosynthesis and secretion of several proteins which are different from IL-1 and TNF [20,21]. The spectrum of biosynthesized proteins secreted by monocytes before or after LPS stimulation and their temporal relationship to stimulation has the potential to identify new monokines of biological importance.

MATERIALS AND METHODS

Materials

Reagents and their sources were as follows. Human IL-1, specific activity (250–500) $\times 10^3$ half-maximal

Abbreviations used: FBS, fetal bovine serum; NP-40, Nonidet P-40; LPS, lipopolysaccharide; PBM, peripheral blood mononuclear cells; SRBC, sheep red blood cells; Con A, concanavalin A; IL-1, interleukin 1; (r)TNF, (recombinant) tumour necrosis factor; PMA, phorbol myristate acetate; sIg, surface immunoglobulin; ER+, E rosette-forming; M_r is used in this paper as apparent relative molecular mass based on behaviour in two-dimensional gels; similarly, pI is used to represent apparent isoelectric point based on behaviour in the isoelectric focusing dimension of two-dimensional gels with urea present; EBSS, Earle's balanced salt solution.

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Table 1. Approximate average M_r and pI values of the labelled bands present in 6 h monocyte supernatants

Band	Induction with LPS	$10^{-3} \times$ Apparent M_r	Average pI (uncorrected)
1-4	—	24	5.6, 5.8, 6.0, 6.4
5	—	64	5.6
6	—	70	5.6
7	—	16	5.5
8	—	12	6.4
9	—	22	6.0
10	—	36	6.7
11,12	—	37	6.6, 6.8
13	—	42	5.7
14	—	43	6.1-6.5
15	—	64	5.7, 5.8, 5.9
16	—	80	5.8
17	—	120	4.9
18	—	135	4.9
19	—	190	5.0
20	—	230	5.4
21	—	150	5.8
22	—	140	6.1
23	—	185	6.1
24	—	210	6.2
25	+	17	6.6
26	+	37	4.8
27	+	46	4.8
28	+	45	5.9
29	+	34	5.8
30	+	23	> 7.1
31	+	24	> 7.1
32	±	25	6.8
33	+	32	7.0
34	+	45	7.0
35	±	100	5.6
36	±	120	5.7
37	+	33	6.0
38	+	24	6.0
39	+	22	5.7

units/mg, Collaborative Research (Lexington, MA, U.S.A.); rabbit anti-(human IL-1), Genzyme Corporation (Boston, MA, U.S.A.); formalin-fixed *Staphylococcus* A cells, Bethesda Research Laboratories (Gaithersburg, MD, U.S.A.). rTNF, specific activity 14.5×10^6 units/mg (95 % pure by SDS/polyacrylamide-gel analysis) and rabbit anti-TNF were a kind gift from Dr. Leo Lin, Cetus Corp. (Emeryville, CA, U.S.A.). [35 S]Methionine (sp. radioactivity > 600 Ci/mmol), Amersham International; [3 H]leucine (> 140 Ci/mmol), NEN Products; ampholytes (pH 3.5-10 and pH 5-8), LKB; electrophoresis reagents and protein standards, BioRad; nylon wool, Du Pont; culture media and nutrient supplies, Grand Island Biological Co.; FBS, KC Biological Inc. (Lenexa, KS, U.S.A.); NP-40, Omega Corp. (Bensonville, IL, U.S.A.); human sera were obtained from normal AB positive donors; sheep red blood cells, Colorado Serum Co. (Denver, CO, U.S.A.); monoclonal antibodies OKT 3 and OKM 1, Ortho Pharmaceuticals (Raritan, NJ, U.S.A.); monoclonal antibody HNK-1, Becton Dickinson (Sunnyvale, CA, U.S.A.); fluorescein isothiocyanate-labelled Fab $_2$ goat anti-(human immunoglobulin) and rhodamine isothiocyanate-labelled Fab $_2$ rabbit anti-(mouse immunoglobulin), Cappel (Cochranville, PA, U.S.A.); neur-

aminidase, Calbiochem; LPS (*E. coli*), Difco; PMA, Con A, actinomycin D, cholesterol, L- α -phosphatidylcholine and bovine albumin (fatty acid free), Sigma.

Cell preparations

Ficoll-Hypaque-purified peripheral blood mononuclear cells (PBM) and plastic-adherent monocyte-rich mononuclear cells were prepared from human peripheral blood as described [22]. In preparation for biosynthetic labelling, monocytes were either harvested from petri dishes by scraping with a rubber policeman or harvested by centrifugal elutriation [23]. The adherent cells were > 90 % OKM 1 positive and < 3 % OKT 3 positive as determined by immunofluorescence and > 90 % ingested latex beads. The monocytes prepared by centrifugal elutriation and plastic adherence were greater than 99 % non-specific-esterase positive. Purified T cells (> 96 % OKT 3 positive) were obtained by lysis of ER+ cell fractions of the nonadherent cells [24]. Enriched T cells were also obtained by filtration of PBM through nylon wool columns [22]. The nylon wool nonadherent cells (92-96 % OKT 3 positive) contained < 1 % polymorphonuclear leukocytes and < 1 % cells which phagocytosed latex beads or had the morphological appearance of monocytes. The B cells were an enriched cell population (> 75 % sIg positive by immunofluorescence) prepared from peripheral blood mononuclear cells by sequential depletion of monocytes by adherence to plastic petri dishes and of ER+ T cells by rosetting to SRBC. Null cells (< 13 % sIg positive, < 10 % ER+ and < 6 % OKT 3 positive by immunofluorescence) were prepared from nylon wool nonadherent cells by depleting the T cells by SRBC rosetting. Immunofluorescence studies were performed by indirect immunofluorescence on a Zeiss microscope using the monoclonal antibodies given above. The monocyte cell line U-937 [25] was obtained from Dr. John Atkinson, Washington University School of Medicine, and was maintained in RPMI 1640 with 10 % FBS in spinner culture.

Cell cultures, biosynthetic labelling and harvesting of cell supernatants

The medium was either EBSS containing 2 % (v/v) AB serum, 2 mM-sodium pyruvate, 2 mM-glutamine, 1 mM nonessential amino acids, 1 mM essential amino acids, (without methionine or leucine depending on the radio-label), penicillin (100 μ g/ml) and streptomycin (100 μ g/ml) or an identical medium without 2 % AB sera but supplemented with bovine albumin (400 μ g/ml), phosphatidylcholine (75 μ g/ml), cholesterol (25 μ g/ml), and transferrin (1 μ g/ml) as described by Iscove *et al.* [26]. Purified monocytes were suspended at a density of 6.25×10^6 /ml in medium containing [35 S]methionine or [3 H]leucine and incubated at 37 °C in a 5 % CO $_2$ atmosphere in plastic 50 ml centrifuge tubes (Fisher Scientific). Incubations were performed for 0-8 h as indicated in the Results section. [35 S]Methionine or [3 H]leucine were added to final concentration of 100-200 μ Ci/ml. Cells were incubated with or without *E. coli* LPS, Con A or PMA at the concentrations indicated in the Results section. All cultures were performed under sterile conditions. Cell viabilities (Trypan Blue exclusion) were > 95 % and did not change during the course of the experiments.

To determine if any of the cell types that might contaminate the enriched monocyte preparations were

possible sources of the biosynthetically labelled supernatant products or were perhaps making indirect contributions through interactions with monocytes, suspensions of T cells, null cells, B cells, PBM or monocytes to which small numbers of PBM had been added were also biosynthetically labelled in the presence and absence of LPS. The labelling conditions were the same as those used for monocytes except that the cell concentration was 0.4×10^6 /ml for null cells and 3.0×10^6 /ml for B cells. T cells were cultured in the presence and absence of $20 \mu\text{g}$ of Con A/ml as well as with LPS.

At the completion of the labelling period, the cell suspensions were centrifuged and the cell supernatants were made 0.5 M in phenylmethanesulphonyl fluoride, 1 M in EDTA, $1 \mu\text{g}/\text{ml}$ in pepstatin and $10 \mu\text{g}/\text{ml}$ in leupeptin. They were then dialysed versus 1000 vol. of EBSS for 24 h at 4°C to facilitate the removal of non-protein bound radioactivity. Protein-bound radioactivity was determined in triplicate for each sample following dialysis by precipitation with trichloroacetic acid (10%, w/v, final concentration) and incubation at 4°C for 30 min followed by centrifugation at 10000 rev./min. Radioactivity of the pelleted fraction was measured by scintillation counting. Based on trichloroacetic acid precipitation, 0.44–1.1% of the original radioactivity used for cell labelling was incorporated into supernatant proteins in various experiments.

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed by a modification of the method of O'Farrell [27,28]. Isoelectric focusing was carried out in rods (105 mm $\times$ 2.5–5 mm) containing 9.2 M-urea, 2% (v/v) NP-40; 2% (w/v) ampholines (1.6% pH range 5–8, 0.4% pH range 3.5–10) prepared from 3.9% (w/v) acrylamide and 0.1% (w/v) bisacrylamide, at 400 V for 10–14 h followed by 1 h at 800 V. Within a single experiment each rod was loaded with a constant amount of protein-bound radioactivity from cells of a single donor, usually $(1-3) \times 10^6$ c.p.m.; 75–90% of the trichloroacetic acid-precipitable radioactivity entered the isoelectric focusing gels. After focusing, rod gels were equilibrated for 45 min in 15 ml of equilibration buffer containing 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2.3% (w/v) SDS, and 0.0625 M-Tris/HCl, pH 6.8. The second dimension was the discontinuous SDS gel system of Laemmli [29] in which the separating gel (a 160 mm $\times$ 140 mm $\times$ 1 mm slab) was prepared from a linear gradient of 6%–20% (w/v) acrylamide. No detectable radioactivity remained in the isoelectric focusing rod following this step. After fixing and staining, the gels were impregnated with 2,5-diphenyloxazole (EN³HANCE; New England Nuclear) prior to radioautography to determine areas containing radioactivity. The isoelectric points given are uncorrected for effects of urea on isoelectric focusing behaviour [30] which is apparently due to a suppressing effect of urea on hydrogen ion activity. Parallel isoelectric focusing rods were run in selected experiments with calibrated proteins of predetermined pI to determine pH values. M_r standards were included on parallel gels.

Immunoprecipitation

Dialysed monocyte supernates from LPS-stimulated or unstimulated cells containing 1×10^6 c.p.m. of [³⁵S]-methionine (for IL-1) or 1.5×10^6 c.p.m. of [³H]leucine

(for TNF) were incubated for 2 h at 4°C with rabbit antibodies. For IL-1, duplicate reactions containing rabbit anti-(human IL-1) at a 1:400 dilution with or without 300 half-maximal units of human IL-1/ml as unlabelled inhibitor were incubated and antigen-antibody complexes were separated by adding 0.5 vol. of formalin-fixed *Staphylococcus* A cells and harvested by centrifugation. The precipitates were washed three times with 5 vol. of 0.01 M-sodium phosphate/0.15 M-NaCl, pH 7.4, and radioactivity was eluted by incubation in a buffer of 9.3 M-urea, 0.64 M-2-mercaptoethanol, 2% (v/v) Nonidet P-40, and 2% ampholytes. For TNF, the assay was performed identically except rabbit anti-TNF was used at a 1:100 dilution, and reactions were incubated with or without 9×10^5 units of rTNF. After immunoprecipitation, radioactivity in the supernates and precipitates was analysed by two-dimensional gel electrophoresis and radioautography as described above.

Time course studies

Mononuclear cells were cultured at 6.25×10^6 cells/ml in the presence of [³⁵S]methionine (200 $\mu\text{Ci}/\text{ml}$) for 0, 1.0, 1.5, 4 and 6 h periods at 37°C in a 5% CO_2 atmosphere. Equal volumes of supernatant were obtained following centrifugation, dialysed as described and equivalent volumes prepared for two-dimensional electrophoresis. Trichloroacetic-acid-precipitable radioactivity was determined in triplicate for each sample. Following a 30 min period, trichloroacetic-acid-precipitable radioactivity increased linearly for all cultures during a 6 h incubation period. Following electrophoresis, radioautography exposure time was normalized dependent on the radioactivity applied to each two-dimensional electrophoretogram.

RESULTS

Supernatants of unstimulated monocytes

In two-dimensional gels of supernatants of monocytes incubated for 4 h or longer with [³⁵S]methionine in the absence of LPS, a number of prominent bands were consistently (30 of 30 experiments involving 30 different leukocyte donors) observed (Table 1, bands 1, 2, 3, 5, 6, 7, 8, 9, 11, 12, 13, 14, 15, 17, 18, 19, 20 and 24) (Fig. 1a). The most prominent of these was a group of bands with M_r of 20000–24000. In Fig. 1(a) there are three bands of M_r 24000 with approximate pI values of 5.6, 5.8 and 6.0 (bands 1–3), and a protein at M_r 22000, pI 6.0 (band 9). As described below, there was sometimes a fourth band with an apparent pI of 6.4 also present in the M_r 24000 region. Two other prominent bands (5 and 6) were present as diffuse spots at an average pI of 5.6 and average M_r values of 64000 and 80000. Bands 11–15, 17–20 and 24 were consistently and prominently radiolabelled in all donors (Fig. 1a). Other bands less prominently radiolabelled (bands 10, 16, 21, 22 and 23) were detectable dependent on radioautograph exposure time (Fig. 1a).

Supernatants of stimulated monocytes

Monocytes are known to be activated by a variety of exogenous stimuli, including LPS, PMA, lectins, and lymphokines [1,9,19,32]. Stimulation of adherent cells with PMA (15 ng/ml) or Con A (20 $\mu\text{g}/\text{ml}$) during a 6 h labelling period did not produce significant changes in the labelling pattern (compare Figs. 1b and 1c with Fig.

Fig. 1. Two-dimensional gels of adherent cell supernatants

(a) Control cells; (b) PMA (50 ng/ml); (c) Con A (20 μ g/ml); (d) LPS (20 μ g/ml); (e) LPS (20 μ g/ml) with 90% adherent cells + 10% mononuclear cells. Labelling time, 6 h. Gel electrophoresis was performed as described in the Methods section and radioautographs were obtained following exposure of Kodak XAR-5 film. In these and the other figures, the basic side is on the left, the acidic on the right and the high- M_r region on top.

1a). PMA did induce the synthesis and accumulation of five proteins, labelled 25, 30, 31, 33 and 34 (Fig. 1b), which were also induced by LPS (see below). Con A had little effect on the secreted proteins except for an increase in the band at M_r 42000 (band 13, Table 1) which is probably actin (Fig. 1c).

In contrast with PMA and Con A, LPS, at 20 $\mu\text{g/ml}$, produced a substantial change in the labelling pattern with the appearance or marked accentuation of major bands at M_r 17000, 32000, 45000, 37000 and 45000 (Table 1, bands 25, 33, 34, 26 and 28) (Fig. 1d). In addition, minor bands at M_r 23000 and 24000 and pI of 7.2 or greater (bands 30 and 31, respectively, in Table 1) were induced by LPS. Band 27, at M_r 46000 and pI 4.8, and band 29, at M_r 34000 and pI 5.8, also were accentuated in the presence of LPS. Variable stimulation was seen with bands 32 (M_r 25000, pI 6.8), and faint bands 35 (M_r 100000, pI 5.6) and 36 (M_r 120000, pI 5.7).

Mixtures of monocytes with other cell types

It has been reported that some monocyte products may be dependent on the presence of small numbers of T lymphocytes [1-3,10,23]. Therefore, experiments were performed in which 10 % of the monocytes were replaced with unfractionated PBM from the Ficoll-Hypaque gradients (Fig. 1e). Aside from a few obvious lymphocyte products, the pattern observed was strikingly similar to that obtained with highly purified monocytes. Band 4 (M_r 24000, pI 6.4) was prominent but its accumulation was unchanged by LPS. However, at least three new products (M_r 33000, pI 6.0; M_r 24000, pI 6.0; M_r 22000, pI 5.7) (Table 1, bands 37, 38 and 39, respectively) were seen in LPS-stimulated monocyte-mononuclear cell mixtures which were not present when monocytes, T cells, B cells or null cells were incubated separately, with and without LPS. Although these bands were faint, their presence was observed in four separate donors and did not appear to be due simply to resolution. In addition, bands 33 and 34 (Table 1) were less prominent in cultures radiolabelled in the presence of 10 % unfractionated PBM. Whether this reflects absorption of these molecules or more rapid degradation has not been determined.

Taking the results of these studies together, with the exception of bands 4 and 37-39, the proteins observed in the supernatants of adherent cells in the presence and absence of LPS did not appear to be due to contamination or require indirect contributions by other leukocytes.

Supernatants of nonadherent cells

While the monocyte preparations used in these studies were greater than 90 % pure, the possibility had to be considered that some of the bands were derived from contaminating cells. However, direct comparisons of the patterns obtained with purified monocytes and those for B cells, null cells, and T cells (not shown) led to the conclusion that all of the bands, with the exception of band 8 (which is more prominent in supernatants from T cells and is presumably β_2 -microglobulin) were of monocyte origin.

Supernatants of monocytes prepared by centrifugal elutriation

Monocytes purified by centrifugal elutriation from PBM, thus avoiding adherence to plastic surfaces, gave similar patterns of secreted proteins on radioautographs of two-dimensional polyacrylamide gels. This indicates

Fig. 2. Two-dimensional gels of U-937 cell supernatants

(a) Control cells; (b) LPS (20 $\mu\text{g/ml}$). Labelling time, 6 h.

that the products studied are not unique to the method of preparing monocytes. Monocytes prepared by centrifugal elutriation and adherence to plastic surfaces resulted in cell preparations demonstrating 99 % non-specific esterase positivity and resulted in identical patterns of secreted proteins (not shown). The results are consistent with the interpretation that monocytes are the source of these products.

Effect of LPS on the monocyte-like cell line U-937

Since the question of cell contamination can be avoided completely by using suitable cell lines, U-937, a monocyte-like cell line derived from a human histiocytic lymphoma [25], was studied. While some variation in

Fig. 3. Effect of actinomycin D on the labelling patterns of monocyte supernatants in two-dimensional gels

(a) LPS at 20 µg/ml; (b) LPS (20 µg/ml) with actinomycin D (14 µg/ml) added at 90 min. Labelling time, 6 h.

results was obtained depending on the culture conditions and source of the U-937 cells, almost all of the bands seen in supernatants of unstimulated purified peripheral blood monocytes were also seen in supernatants of labelled U-937 cells (Fig. 2a). Moreover, as with the peripheral blood monocytes, the prominent bands 25, 26, 28, and 29 in U-937 cells were all stimulated by LPS (Fig. 2b). Additionally, the less prominent LPS-induced bands 30–34 (Table 1) were evident in the cell supernatants.

The U-937 cell line has been previously shown to be activated by human lymphokines to express surface macromolecules, antibody-mediated cytotoxicity and antibody-dependent phagocytosis, but, under the same

Fig. 4. Summary (see Table 1) of secreted proteins of human mononuclear phagocytes by two-dimensional analysis

Shaded areas represent those proteins whose secretion is unaffected by LPS; outlined areas represent those proteins whose synthesis is increased by LPS or LPS plus actinomycin. Bands 25, 30, 31, 33 and 34 were also increased by PMA.

culture conditions, produces little or no IL-1 [31,33]. However, we find that band 25, identified as IL-1 β by immunoprecipitation (see below), is prominent in supernatants radiolabelled for 6 h from these cells both in the presence and absence of LPS stimulation. Additionally, assays of IL-1 biological activity demonstrate readily detectable levels in U-937 supernatants (results not shown).

Accentuation of the LPS response by actinomycin D

When the production of lymphokines or monokines is induced by an exogenous agent, it is sometimes possible to enhance markedly the response by the delayed addition of inhibitors of RNA or protein synthesis, presumably because proteins that normally suppress the response are no longer synthesized (see for example [12,13,32,34]). The possibility that this might also be true for the biosynthetic labelling response to LPS in adherent cells was examined by using a cell donor whose responses to LPS in terms of the M_r 17000, 37000, 45000, 46000 and 34000 labelled products was unusually modest (bands 25, 26, 28 and 29) (Fig. 3a). As seen in Fig. 3(b), the delayed addition of actinomycin D at 90 min considerably enhanced the response to LPS in all of these proteins. Indeed, the enhancement of labelling in band 25 (M_r 17000, pI 6.6) was so marked that it became the most prominent of all of the labelled supernatant bands. As shown below, band 25 crossreacts with antibodies to human IL-1 β . Actinomycin D alone did not produce this effect. (Note the four heavy bands in the M_r 24000 region instead of the three bands seen in Fig. 1.) The selective augmentation of labelling of these bands by actinomycin D was confirmed in two additional experiments with different cell donors.

Protease inhibitors

Since monocytes are a potential source of proteolytic enzymes, some of which might be released into the

Fig. 5. Immunoprecipitates of LPS-stimulated monocyte supernatants labelled with [35 S]methionine for 6 h (1×10^6 c.p.m. in protein) following reaction with rabbit anti-human IL-1 at a 1:400 dilution

(a) No added human IL-1; (b) identical reaction containing 300 half-maximal units of IL-1/ml. Antigen-antibody complexes were separated by addition of 0.5 vol. of formalin-fixed *Staphylococcus* A cells and washed three times with 5 vol. of 0.1 M-sodium phosphate/0.15 M-NaCl, pH 7.4. Radioactivity was eluted with a buffer containing 9.3 M-urea, 0.64 M-2-mercaptoethanol, 2% (v/v) NP-40 and 2% ampholytes.

supernatants under the conditions of our experiments, monocytes were also cultured in the presence of phenylmethanesulphonyl fluoride (0.5 mM), an inhibitor of serine proteases, and leupeptin (10 μ g/ml), another protease inhibitor. No significant effect on the labelling pattern was observed. Also, no major change in the labelling pattern was produced when 0.2 vol. of dialysed supernatant from monocytes labelled for 4 h in the presence or absence of LPS was incubated for 10, 30 or 60 min at 37 °C with fresh unlabelled monocytes at the usual cell concentration (not shown).

Reproducibility of the LPS response in monocytes from different donors

Similar results were obtained with monocytes from a total of 30 leukocyte donors; however, the degree of stimulation of labelling of bands 25–27, 30 and 31, and a band of M_r 125 000, pI 5.5 by LPS did vary to some extent with the cell donor. The LPS-induced proteins were seen in all donors except for band 37 and band 38 which were absent from five of 30 donors. A summary of the constitutive and LPS-stimulated secreted proteins from human mononuclear phagocytes demonstrates these effects (Fig. 4, Table 1).

Identification of IL-1 β in monocyte supernatants

To test the hypothesis that radiolabelled monocyte supernatants prepared by this method contain proteins with potential monokine activity, it should be possible to demonstrate well-characterized immunoreactive monokines. Immunoprecipitates of monocyte supernatants stimulated by LPS and radiolabelled with [35 S]-methionine following reaction with anti-IL-1 in the absence of excess human IL-1 revealed four prominent bands with M_r and pI values as follows: 25 000, pI 5.9; 25 000, pI 6.0; 17 000, pI 6.6; 24 000, pI 7.1 (Fig. 5a). In the presence of excess unlabelled human IL-1, only the M_r 17 000 pI 6.6 (band 25) and the M_r 24 000 pI 7.1 (band 31) protein bands were reduced in intensity (Fig. 5b). Monocyte supernatants not stimulated with LPS but treated with anti-IL-1 demonstrated only the M_r 25 000 pI 6.0 molecule, which was not reduced by excess unlabelled ligand (not shown).

Identification of TNF in monocyte supernatants

To evaluate which supernatant proteins are immunologically related to TNF, [3 H]leucine-labelled supernatants from LPS-stimulated monocytes were reacted with anti-TNF in the presence or absence of unlabelled rTNF. The immunoprecipitates were analysed by two-dimensional gel electrophoresis. Several proteins were precipitated by anti-TNF and inhibited in immunoprecipitates performed with excess unlabelled rTNF. Protein bands of M_r 25 000, pI 6.3, M_r 23 000, pI 6.5 and M_r 20 000, pI 6.5 were specifically precipitated by anti-TNF (Figs. 6a and 6b). These results were seen in three of three donors with all samples performed in duplicate. Analysis of the supernatants following immunoprecipitation demonstrated quantitative absorption of the M_r 20 000, 23 000 and 25 000 bands in the absence of unlabelled rTNF (not shown).

Kinetics of appearance of radiolabelled monocyte proteins

Since this assay measures the net effects of synthesis and degradation on supernatant proteins, relative accumu-

Fig. 6. Immunoprecipitates of LPS-stimulated monocyte supernatants labelled with [^3H]leucine for 6 h (1.5×10^6 c.p.m. in protein) following reaction with anti-(human TNF) at a 1:100 dilution

(a) No added rTNF; (b) identical reaction containing 9×10^5 units of rTNF/ml. Separation of antigen-antibody complexes, gel electrophoresis and autoradiography were performed as described in Fig. 5.

lation rates can be compared by performing the experiments at selected time points. Monocytes from a single donor were equally divided and radiolabelled with [^{35}S]methionine in the presence of LPS and 10% unfractionated PBM for 0, 1.0, 1.5, 4.0, and 6.0 h time periods. The kinetics of appearance for the prominent

Table 2. Kinetics of appearance in adherent cell supernatants of radiolabelled bands following lipopolysaccharide stimulation

Band number	Time of appearance (h)
1-5, 7-9, 15, 19, 20, 25, 26, 28	1.0
6, 26, 24, 29, 32	1.5
11, 13, 14, 18, 23, 30, 31, 33-39	4.0
29	6.0

radiolabelled proteins demonstrated that bands 1-5, 7-9, 15, 19, 20, 25, 26, and 28 were clearly detectable at 1.0 h (Table 2). Bands 6, 16, 24, 29, and 32 were detectable after 1.5 h of culture. After 4 h bands 11, 13, 14, 18, 23, 30, 31 and 35-39 were detected whereas band 29 became detectable only after a 6 h incubation.

By comparing relative intensity of the bands during

Fig. 7. Two-dimensional gels of adherent cell supernatants

(a) Control supernatant without neuraminidase; (b) supernatant incubated with 0.2 units of neuraminidase/ml in sodium acetate (60 mM), pH 5.5, for 15 min at 37 °C. Control and neuraminidase supernatants contained 1×10^6 c.p.m. of protein-bound [^{35}S]methionine.

each labelling period it was evident that maximal steady-state levels of bands 4, 25, and 26 occurred during the 1.0 h incubation, while bands 7, 20 and 28–30 were maximal at 1.5 h. The remainder of the bands listed in Table 2 were maximally accumulated during the 4.0 h labelling period, except bands 16, 32 and 33 which were maximal at 6 h. Cells labelled for 0 h demonstrated no detectable bands on two-dimensional electrophoretograms.

Treatment of monocyte supernatants with neuraminidase

Monocytes stimulated with LPS are known to secrete certain glycosylated proteins, such as the complement components, α_1 -proteinase inhibitor and colony stimulating factors [35–37]. LPS-stimulated monocyte supernatants labelled with [35 S]methionine were analysed before or after incubation with excess neuraminidase (Fig. 7). The neuraminidase-treated supernatants demonstrated an alteration in pI of only bands 3, 32 and 36 (Fig. 7b; cf. the untreated sample, Fig. 7a). Bands 29, 33 and 34 were insufficiently labelled in this experiment, precluding their evaluation. To ensure that an excess of active neuraminidase was present, the pI values of serum components present in the monocyte supernatants and stained with Coomassie Blue demonstrated quantitative shifts to more basic values. These are the only proteins visualized on Coomassie Blue stained gels (results not shown).

DISCUSSION

The resolving power of two-dimensional gels is a considerable advantage when a complex mixture of proteins is present and genetic heterogeneity is a possible source of variation. Two-dimensional gel electrophoresis has been particularly useful in studies of the major histocompatibility antigen complex, where proteins that are very closely related structurally to one another have been resolved using this technique and the differences in electrophoretic behaviour related to specific immune response patterns [38–40]. This method was successfully used to determine the specific effects of LPS on biosynthetically labelled proteins produced by murine peritoneal macrophages [19]. Those authors studied total cellular proteins and defined proteins whose levels both increased or decreased in response to LPS stimulation. The results of the two-dimensional gel electrophoresis studies in monocytes described in the present paper indicate that human peripheral blood monocytes biosynthetically labelled with radioactive amino acids release a variety of labelled products into the medium, some of which are selectively stimulated by LPS. Parallel studies with other cells present in the peripheral blood as well as with the monocyte-like cell line U-937 [25] indicate that these products are produced by monocytes rather than contaminating cells. The possibility is not fully excluded that some of the lower- M_r products are derived from larger ones by proteolysis. However, very similar patterns are obtained when protease inhibitors were present in the medium during labelling. Moreover, no major changes are observed when labelled supernatants were separated from cells and incubated further with unlabelled cells for various time periods at 37 °C.

It should be emphasized that the present observations are restricted to proteins labelled during the first 1–8 h of culture of freshly isolated human monocytes. The

intensity of the bands on radioautographs represent the net result of synthesis and degradation; pulse-labelling and cold chase studies are needed to measure net synthesis rates of specific proteins. It also seems likely that additional products would be obtained if the culture period had been extended. However, problems in interpretation sometimes arise when longer labelling periods are used. Cell death and release of cytoplasmic proteins into the media may complicate analysis of secreted products. We find that the major radiolabelled cytoplasmic proteins are absent from the supernatant and, from cell viability studies, it appears that non-specific cell lysis does not occur to a measurable extent under the conditions of our experiments.

Whether or not the products seen in the medium of 'unstimulated' monocytes are indeed produced and released constitutively in the absence of an exogenous stimulus remains to be established. Mononuclear phagocytes that have been freshly isolated from plastic surfaces, as was done in the studies described in this paper, are probably not truly resting cells either in a metabolic or functional sense. On the other hand, mononuclear phagocytes prepared by elutriation from PBM without adherence to plastic surfaces demonstrate very similar patterns of secreted proteins (not shown).

Regardless of their precise functional activities, several of the labelled monocyte-secreted proteins seen in our two-dimensional gels (for example, those at M_r 24000, 64000 and 70000, Table 1) are sufficiently prominent that they may be useful as markers for mature monocytes or tumours of monocytic origin. While much effort has been directed to the development of monocyte surface markers using monoclonal technology, an ability to measure secreted monocyte products in the blood or inflammatory fluids may also prove to be of considerable interest. Several of the most prominent radiolabelled monocyte proteins (bands 1–3, 4 and 9) are stable when incubated for prolonged periods (up to 96 h) in human plasma. Antibodies directed against these products have been produced in our laboratory as tools to measure these products from human samples.

The relationship of the various products demonstrated in monocyte supernatants in this study to previously described activities will require further studies. Human peripheral blood monocytes have been labelled biosynthetically previously, but electrophoretic studies of their unfractionated supernatants have been primarily limited to one-dimensional gels [4]. Monocytes are known to make a wide variety of secreted products [1–3, 21, 41–44]. Two of the most interesting LPS-stimulated products are IL-1 and TNF. Human IL-1 has been described as having an M_r of 17000 with pI values ranging from 5 to 7 [21, 44]. We have identified immunoreactive IL-1 in monocyte supernatants with an M_r of 17000 and a pI of 6.6, and a second molecule of M_r 24000, pI 7.0. These molecules are easily detected in LPS-stimulated monocyte supernatants following stimulation for 6 h, but are absent from unstimulated supernatants. Auron *et al.* [45] have also described a precursor of IL-1 present in monocyte supernatants with M_r of 24000 and pI of 6.8–7.2. Radiolabelled proteins which cross-react with antibodies directed against human TNF- α are present in LPS-stimulated adherent cell supernatants. Proteins with M_r values of approx. 20000–25000 with pI values of 6.5 and 6.3 are specifically immunoprecipitated. These results suggest the possible

presence of precursor products containing epitopes common to TNF. TNF precursor molecules have been demonstrated in murine supernatants; however, this is the first report of probable precursor molecules secreted by human monocytes [46]. It is known that TNF contains a long leader sequence (76 amino acids), but its role in TNF secretion has not been defined [41].

The presence of IL-1 and TNF in human adherent cell supernatants following LPS stimulation suggests that the bands described herein are specific to LPS stimulation and may represent secreted proteins capable of functioning as monokines. Fifteen proteins have been described which are either specifically secreted, or at least released, from human adherent cells following this stimulus (Fig. 4, Table 1). Certain of these LPS-specific proteins are overproduced by sequential treatment of human monocytes with LPS and actinomycin D (bands 25, 26, 28 and 29) (Figs. 3 and 4). This induction of certain mononuclear-phagocyte-secreted proteins by protein synthesis inhibitors has been observed with other monokines, and suggests that some secreted products are controlled by protein repressor molecules [13].

The ability to study products secreted from human monocytes following LPS stimulation, and to analyse these products by immunoreactivity, provides a powerful tool to study potential human monokines. The advantage of two-dimensional electrophoresis is that it allows the rapid assay of monocyte products with minimal manipulation of the conditioned medium, and, theoretically, minimum loss of proteins. The use of biosynthetic labelling methods makes this an extremely sensitive assay for LPS effects on human monocytes. Additionally, the ability to transfer proteins electrophoretically from two-dimensional gels to media suitable for protein sequencing to obtain direct structural data makes this methodology extremely valuable [47].

We thank Aaron D. Gilden and Theresa Schectel for expert technical assistance, and Carolyn Davinroy for typing this manuscript. J.R.P. was supported by National Institutes of Health Research Service Awards. K.F. was supported by the Ishizaka Foundation and by United States Public Health Service Research Grant (CIRID) in portions of this work.

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Received 30 March 1987/13 July 1987; accepted 21 September 1987