

# INTERLEUKIN 1 $\beta$ IS LOCALIZED IN THE CYTOPLASMIC GROUND SUBSTANCE BUT IS LARGELY ABSENT FROM THE GOLGI APPARATUS AND PLASMA MEMBRANES OF STIMULATED HUMAN MONOCYTES

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The recent cloning of cDNA for murine (1), human (2–4), and rabbit (5) IL-1 molecules has shown that there are two classes of IL-1 known as IL-1 $\alpha$  and IL-1 $\beta$ . The cDNAs encode precursor molecules with molecular masses of 33 kD, whereas the mature, biologically active molecules found in culture supernatants have molecular masses of 14–18 kD (6–11). NH<sub>2</sub>-terminal sequence analysis of human IL-1 $\alpha$  (9), IL-1 $\beta$  (8), and murine IL-1 $\alpha$  (1) has shown that each mature molecule is derived from the COOH-terminal domain of its respective precursor. Processing of IL-1 precursors appears to occur in conjunction with externalization, since only 33-kD precursors are found intracellularly whereas the mature 18-kD form is exclusively extracellular (12–14). The precursor NH<sub>2</sub>-terminal domains removed during processing are large compared to most lymphokines, and they lack hydrophobic domains typical of leader or membrane anchor sequences (1–5). The apparent absence of a leader sequence in addition to the observed accumulation of human IL-1 (12, 13, 15) and murine IL-1 (14, 16) within activated monocytes and macrophages suggest that the mechanism of IL-1 secretion is distinct from that of typical secretory proteins, and may represent a novel pathway of protein secretion. For example, IL-1 clearly differs from the monokine cachectin (TNF), which has a secretory leader and is efficiently secreted without any intracellular accumulation (17).

Early attempts to define the subcellular localization of IL-1 molecules have relied heavily on bioassay of paraformaldehyde-fixed cells. The observation that activated murine macrophages (18, 19), B lymphocytes (20), and dendritic cells (21), when fixed, are capable of stimulating murine thymocyte proliferation, has been interpreted widely as evidence that IL-1 is bound to the plasma membrane of these cells. Unfractionated membranes prepared from murine macrophages (18) and human monocytes (15) have also been found to stimulate murine thymocyte proliferation and the activity found in membranes of murine macrophages was blocked by anti-IL-1 antiserum (18). Subsequent immunofluorescent studies of human monocytes with anti-IL-1 $\beta$  antisera (12) failed to confirm the presence of plasma membrane-bound material and gave staining patterns consistent with the cytosolic localization of the IL-1 $\beta$  precursor.

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In the current study, immunoelectron microscopy with specific antibodies to human IL- $1\beta$  was used to define the subcellular localization of the IL- $1\beta$  precursor within activated human monocytes, at considerably higher resolution than obtainable with immunofluorescence microscopy. In particular, the purpose of this study was to determine whether IL- $1\beta$ , the major species of IL-1 produced by human monocytes, is present on plasma membranes and whether IL- $1\beta$  gains entry to the secretory apparatus of the cell. The results indicate that IL- $1\beta$  is not anchored to the plasma membrane but localized in the cytosolic ground substance. Furthermore, the pathway of IL- $1\beta$  secretion appears to be distinct from lysozyme and most hormones in that passage through the Golgi apparatus and endoplasmic reticulum (ER)<sup>1</sup> does not occur.

### Materials and Methods

*Cells.* Peripheral blood leukocytes were obtained from healthy human subjects by leukapheresis, and the mononuclear cell fraction was isolated in a discontinuous Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient. Mononuclear cells were resuspended in RPMI 1640 medium supplemented with 1% FCS (endotoxin-free; Gibco Laboratories, Grand Island, NY), 20 mM Hepes, 4 mM glutamine, 100 U/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin. For immunoelectron microscopy, the cells were seeded into 10-cm diameter tissue culture dishes (No. 25020; Corning Glass Works, Corning, NY) at  $10^8$  cells per dish, and the monocytes were allowed to adhere for 1.5 h at  $37^\circ\text{C}$  in a humidified atmosphere of 5%  $\text{CO}_2$  and air. Most of the contaminating platelets and lymphocytes were removed using three washes (5 ml each) of  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS. These cultures were stimulated with LPS (1  $\mu\text{g}/\text{ml}$  *Escherichia coli*, strain 055.B5P; Sigma Chemical Co., St. Louis, MO) for either 4 or 20 h, washed three times with HBSS, and fixed as described below. For immunofluorescence microscopy, mononuclear cells were isolated as detailed above, and plated into 16-mm diameter tissue culture wells (No. 3524; Costar, Cambridge, MA) containing poly-L-lysine-coated 12-mm diameter coverglasses at a concentration of  $2 \times 10^6$  cells per well. After 18–20 h of LPS stimulation, these preparations were rinsed once with 1 ml of HBSS and fixed as described below.

*Antibodies and Peptides.* Rabbit polyclonal antisera were raised against an internal IL- $1\beta$  peptide (amino acid residues 197–215) as previously described (13), and shown to be specific for mature IL- $1\beta$  (18 kD) in concentrated monocyte-derived culture supernatants (13). We purified the IgG fraction of this IL- $1\beta$  antiserum, as well as the preimmune serum, on a protein A-Sepharose column (Pharmacia Fine Chemicals) using standard techniques, and used this material in all of our localization experiments. The relevant IL- $1\beta$  peptide (p197–215) and an irrelevant IL- $1\beta$  peptide (p117–128) were synthesized and purified as previously described (13) and used in blocking experiments to assess the levels of specific staining of our immunoelectron microscopic preparations (see below). Rabbit anti-human lysozyme IgG that neutralized purified lysozyme in solution, formed a single precipitation line in double immunodiffusion tests, and specifically stained sections of human tonsil, was obtained from Accurate Chemical & Scientific Corp. (Westbury, NY). Affinity-purified goat anti-rabbit IgG (CooperBiomedical, Inc., Malvern, PA) was conjugated to either 5 or 10 nm colloidal gold particles as previously described (22, 23) for our immunoelectron microscopic experiments. In addition, we obtained a commercial preparation of 10 nm colloidal gold conjugated to affinity-purified goat anti-rabbit IgG, in which >80% of the conjugated gold particles were singlets (Janssen Life Sciences Products, Piscataway, NJ). Just before use, all colloidal gold conjugates were diluted 1/5 with a solution of 1% BSA (crystallized, globulin-free; Sigma Chemical Co.) in 100 mM KCl, 20

<sup>1</sup> Abbreviations used in this paper: ER, endoplasmic reticulum; Ve, vesicles.

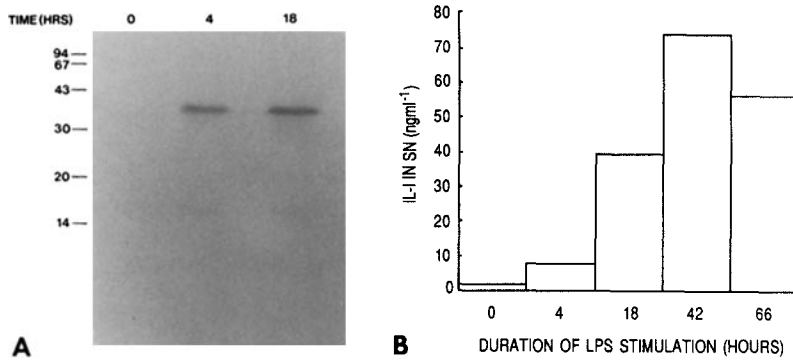
mM Tris-HCl, pH 8.3, with 0.1% NaN<sub>3</sub>, and centrifuged at 11,000 *g* for 30 min at 4°C to further reduce aggregates. For immunofluorescence microscopy, a FITC-conjugated affinity-purified goat anti-rabbit IgG was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN).

**Measurement of Intracellular IL-1 $\beta$ .** The intracellular accumulation of 33-kD IL-1 $\beta$  precursor was detected by immunoblotting. Cultures containing ~10<sup>8</sup> human mononuclear cells (see above) were stimulated with LPS for 0, 4, or 18 h. After two washes with ice-cold PBS, the adherent cells were scraped into PBS containing 5 mM *N*-ethylmaleimide, 2 mM PMSF, and 2 mM EDTA. The mononuclear cells were pelleted, resuspended in Laemmli sample buffer containing 1% dithiothreitol, boiled for 5 min, and analyzed by SDS-PAGE in 15% gels. Immunoblotting analysis was performed as previously described (13), except that nonspecific binding was blocked with a 5% nonfat dry milk solution (24).

**Assay of Secreted IL-1 $\beta$ .** Previous immunoblotting experiments have shown that the IL-1 $\beta$  found in monocyte culture supernatants is the 18-kD mature form (12, 13). This material binds with high affinity to the human fibroblast IL-1-R (25), whereas the IL-1 $\beta$  precursor does not bind to this receptor (26). Mature secreted IL-1 $\beta$  can therefore be quantitated in a radioreceptor binding assay. While IL-1 $\alpha$  would also be detected by this method, the low amounts of IL-1 $\alpha$  produced by human monocytes relative to IL-1 $\beta$ , as well as the lower affinity of IL-1 $\alpha$  for the fibroblast IL-1-R (25), suggest that the IL-1-R binding activity in monocyte supernatants is largely due to IL-1 $\beta$ . Culture supernatants were collected after 0, 4, 18, 42, and 66 h of LPS stimulation, centrifuged to remove any nonadherent cells, and frozen until assay. They were then assayed in a competitive receptor binding assay as previously described (25); a standard curve was constructed by assaying known concentrations of human rIL-1 $\beta$  (1–100 pM) (4).

**Immunofluorescence Microscopy.** To assess the effects of various crosslinking fixatives upon IL-1 $\beta$  antigenicity, unsectioned monocyte coverslip cultures (see above) were washed with HBSS and treated with various mixtures of formaldehyde and glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and 4.5 mM CaCl<sub>2</sub> for 30 min at 23°C. Immunoreactivity was monitored via immunofluorescence microscopy. Specifically, the fixed cells were washed, permeabilized with 0.1% Triton X-100 for 4 min at 4°C to permit intracellular penetration of the antibodies, and incubated in 5 mg/ml NaBH<sub>4</sub> in 0.1 M Tris HCl buffer (pH 7.2) for 20 min. The latter treatment was used to reduce free aldehyde groups introduced by the fixative that would otherwise bind antibodies to the cells nonspecifically. To lower the background further, the monocytes were also incubated with 5% nonfat dry milk (24) clarified at 15,000 *g* for 20 min as previously described (27). After washing in 0.1 M Tris-HCl buffer (pH 7.8), the monocytes were treated for 1 h with rabbit anti IL-1 $\beta$  peptide (p197–215) IgG (50–100  $\mu$ g/ml) in 0.1 M phosphate buffer (pH 7.8), 0.1% BSA, 0.1% sodium azide, and clarified 0.1% nonfat dry milk, followed by 1 h in the fluorescein-conjugated goat anti-rabbit IgG diluted 1/25 in the latter buffer mixture. Finally, the coverslip cultures were mounted with 5% *n*-propyl gallate (28) in 0.1 M NaHCO<sub>3</sub> (pH 8.2), 90% glycerol, and photomicrographs were taken with a Zeiss epifluorescence photomicroscope III as previously described (27).

**Ultrathin Cryosectioning and Immunoelectron Microscopy.** LPS-stimulated monocytes in 10-cm diameter culture dishes were fixed as described above, infiltrated in situ with a 7% acrylamide solution, and separated from their substratum by a rubber policeman. Cell pellets were then infiltrated with acrylamide containing ammonium persulfate, and polymerized. Small clumps of embedded cells were dissected out of the hardened acrylamide, and cryoprotected by complete infiltration with a solution of 2.3 M sucrose in 0.1 M phosphate buffer (pH 7.2). These specimens were frozen rapidly in liquid nitrogen-cooled Freon 22. ~80-nm ultrathin frozen sections were cut at -95°C, and placed on formvar-coated 200-mesh nickel grids as previously described (29). Reduction of free aldehydes with NaBH<sub>4</sub>, adsorption with milk supernatant, and labeling of IL-1 $\beta$  antigens with rabbit anti peptide antibodies, were performed directly on the surfaces of the thin sections as described above. This method ensures unobstructed access of our immunoprobes to all sectioned subcellular compartments. For controls, the IL-1 $\beta$  p197–215 antibodies were preincubated with excess (100  $\mu$ g/ml) relevant (p197–215), or irrelevant



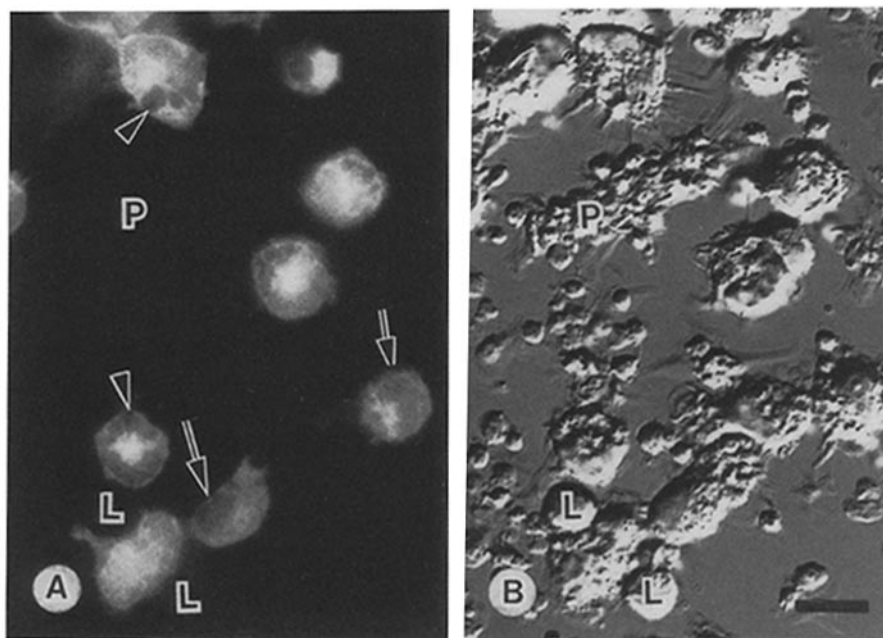
**FIGURE 1.** Time course of intracellular and extracellular accumulation of IL-1 $\beta$  in LPS-stimulated human monocytes. (A) The intracellular accumulation of IL-1 $\beta$  precursor was detected by immunoblot analysis of monocyte lysates using anti-IL-1 $\beta$  (p197–215) IgG at 0, 4, and 18 h of stimulation. (B) Appearance of IL-1 $\beta$  in monocyte culture supernatants (SN) was measured in a competitive radioreceptor-binding assay that was standardized with known concentrations of rIL-1 $\beta$ . Supernatants were collected at 0, 4, 18, 42, and 66 h of LPS stimulation.

(p117–128) peptides, clarified at 15,000 *g*, and used in place of untreated IL-1 $\beta$  antibodies in the staining protocol described above. Additional controls consisted of preimmune rabbit IgG or rabbit anti-human lysozyme IgG. After extensive washing, the bound rabbit antibodies were localized using either 5 or 10 nm colloidal gold-conjugated affinity-purified goat anti-rabbit IgG (see above), and the grids were washed again, fixed with glutaraldehyde, counter stained, and reembedded as previously described (22, 30).

To obtain more customary ultrastructural views of LPS-stimulated monocytes, cultures were prepared as described above, treated with LPS for 20 h, and fixed in situ with a mixture of 3.5% paraformaldehyde and 2% glutaraldehyde in 0.1 M sucrose, 0.1 M cacodylate buffer (pH 7.2), and 4.5 mM calcium chloride for 16 h at 4°C. After refixation in 1% tannic acid, the monocytes were scraped from their substrate, pelleted, and postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer as previously described (31). After embedding in epoxy resin, ultrathin sections were cut and stained with uranyl acetate and lead citrate (31). Electron micrographs were taken at 80 kV using a JEOL 100 CX II electron microscope equipped with a liquid nitrogen-cooled specimen anticontamination device.

## Results

*Time Course of IL-1 $\beta$  Accumulation.* To determine when cultures should be fixed for immunoelectron microscopy, monocytes were stimulated with LPS for 0, 4, 18, 42, or 66 h. Intracellular IL-1 $\beta$  precursor was then assessed by immunoblot analysis, while extracellular IL-1 $\beta$  accumulation was measured in a competitive radioreceptor binding assay (see Materials and Methods). As shown in Fig. 1A, nearly equivalent amounts of the 33-kD IL-1 $\beta$  precursor were specifically recognized by our antipeptide (p197–215) IgG in cell lysates after 4 or 18 h of activation; lower molecular mass IL-1 $\beta$  species were never detected intracellularly by our antibodies. No IL-1 $\beta$  precursor was observed before LPS stimulation. As previously reported for activation with LPS and PHA using immunoblots stained with a different IL-1 $\beta$  antibody recognizing amino acids 117–128 (13), the maximum amount of IL-1 $\beta$  precursor was observed between 18 and 48 h, and its intensity decreased thereafter (data not shown). Moreover, IL-1-R binding activity increased substantially in the culture supernatants be-



**FIGURE 2.** Light micrographs of unsectioned human monocyte cultures that were fixed in 3.5% formaldehyde and permeabilized with 0.1% Triton X-100 at 20 h of LPS stimulation. (A) Immunofluorescence micrograph obtained with rabbit anti-IL-1 $\beta$  (p197–215) IgG. The monocytes are intensely positive for IL-1 $\beta$  while lymphocytes (L), and platelets (P) seen in a corresponding Normarski micrograph (B) are completely unstained. The monocyte cytoplasm is uniformly stained for IL-1 $\beta$ , while their nuclei (arrows) and phagocytic vacuoles (arrowheads) are unlabeled. Bar, 10  $\mu$ m.

tween 0 and 42 h of stimulation (Fig. 1 B). Therefore, monocytes stimulated with LPS for 4 or 20 h were selected for immunoelectron microscopy since active secretion of IL-1 activity into the culture medium appeared to be in progress at those times.

*Immunofluorescent Staining and Preservation of IL-1 $\beta$  Antigenicity.* We assessed the preservation of IL-1 $\beta$  antigenicity as a function of fixation conditions by observing changes in immunofluorescence staining intensity of fixed and permeabilized monocyte cultures at 20 h of LPS activation. Initially, a solution of 3.5% paraformaldehyde was used for periods of 4–30 min. The IL-1 $\beta$  staining intensity was found not to diminish with increasing time of fixation with paraformaldehyde. The IL-1 $\beta$  specific labeling was homogeneously distributed in the monocyte cytoplasm, while their outer cell membranes, phagocytic vacuoles, and nuclei appeared unstained; contaminating platelets and lymphocytes likewise were not labeled (Fig. 2). The anti-IL-1 $\beta$  p197–215 IgG staining intensity seen with paraformaldehyde was similar to that observed with a periodate-lysine-paraformaldehyde fixative using anti-IL-1 $\beta$  p117–128 (12, 32), but the anti-p197–215 IgG produced clearer definition of the nuclei and phagocytic vacuoles (Fig. 2A). When increasing quantities of glutaraldehyde (0.1, 0.3, 0.5, and 1.0% by volume) were combined with 3.5% paraformaldehyde, a sharp decrease in IL-1 $\beta$  immunofluorescence occurred at all glutaraldehyde concentrations ex-

ceeding 0.1%. A mixture of 3.5% paraformaldehyde and 0.1% glutaraldehyde was thus found to preserve optimal levels of IL-1 $\beta$  antigenicity; borohydride reduction and blocking with clarified powdered milk (see Materials and Methods) were observed to minimize nonspecific background labeling. As shown below, this fixative also produced acceptable fine-structural preservation of LPS-activated monocytes for immunoelectron microscopy.

*Immunoelectron Microscopic Localization of IL-1 $\beta$ .* To guide our study and interpretation of the ultrathin frozen sections, we initially characterized the morphology of LPS-stimulated monocytes using conventional electron microscopic techniques described in Materials and Methods. At 20 h of LPS stimulation, monocytes exhibited a well-developed Golgi apparatus associated with numerous secretory vesicles (Ve), and an expanded ER consisting of many anastomosing cisternae (Fig. 3). The secretory Ve diameters ranged from 44 to 96 nm (mean = 59 nm, median = 65 nm,  $n = 75$ , SD = 13.76, SE = 1.58). These cells also contained abundant phagocytic vacuoles and free polysomes in the cytoplasmic ground substance. In addition, their plasma membranes showed microvilli (Fig. 6), and many infoldings that penetrated deeply into the cytoplasm close to the nucleus (Figs. 3 and 5).

Ultrathin frozen sections of monocytes that were glutaraldehyde-fixed at 4 h of activation exhibited 90.1% of their IL-1 $\beta$  antigens localized within the cytoplasmic ground substance (Table I). An immunoelectron micrograph of such a preparation is shown in Fig. 4. Plasma membranes, secretory Ve, ER cisternae, phagocytic vacuoles, and mitochondria were only sparsely labeled, while the Golgi apparatus was completely unstained at the 4-h time point (Fig. 4 and Table I). Most of the remaining grains (7.1%) were associated with the nucleus (Table I). A similar distribution of IL-1 $\beta$  immunostaining was observed at 20 h of LPS activation, and micrographs of these preparations are presented in Figs. 5, 6, and 9. Again, the majority of the anti-IL-1 $\beta$  staining (85.8%; Table I) was confined to the cytoplasmic matrix where it was distributed randomly at loci distant from the Golgi apparatus (Figs. 5 and 9). Despite active IL-1 secretion that took place at this time (Fig. 1B), only occasional grains were associated with the lumen of the ER (Fig. 6), the Golgi apparatus (Fig. 9), and secretory Ve (Table I). Taken together, these grains never accounted for more than 1–2% of the total immunolabeling. In the majority of sections, most of the monocyte surface lacked IL-1 $\beta$  immunostaining, and many transversely sectioned plasma membranes exhibiting a typical bilaminar structure usually were unlabeled (Figs. 4 and 6). However, sparse quantities (1–2.8%) of IL-1 $\beta$  antigens were detected in the vicinity (20 nm) of some plasma membranes at both sampling times (Figs. 4 and 5; Table I). Platelets and lymphocytes present in these sections did not show specific IL-1 $\beta$  labeling.

The anti-IL-1 $\beta$  immunogold labeling occurred as singlets (in 62–54% of the antigenic sites), or as elliptically shaped clusters (in 38–46% of the IL-1 loci) associated in the plane of the section surface at 4 and 20 h, respectively, of LPS activation (Figs. 5–7, 9). The lengths of the major cluster axes ranged from 17–97 nm (mean = 44 nm, median = 50 nm,  $n = 80$ , SD = 19.07, SE = 2.13). Many (49%) of the IL-1 $\beta$  antigen clusters were smaller than secretory Ve and Student's paired  $t$  test showed that the mean major axis length of the clusters was



FIGURE 3. Electron micrograph of an epoxy-embedded human monocyte at 20 h of LPS stimulation, prepared by conventional electron microscopic techniques (see Materials and Methods) for purposes of morphological orientation. An expanded Golgi apparatus (*G*) is seen adjacent to a centriole (*C*) and the nucleus (*N*). Numerous cisternae of ER and *Ve* are found throughout the cytoplasm. The surface membrane (*arrow*) often forms large phagocytic vacuoles (*Va*) which penetrate deeply into the cell. Polysomes (*arrowhead*) and mitochondria (*M*) are also conspicuous. Bar, 250 nm.

significantly smaller ( $p < 0.005$ ) than the mean secretory *Ve* diameter. These morphometric data confirm the observation that secretory *Ve* lacked significant IL-1 $\beta$  labeling (Table I, Figs. 6 and 9). Similar staining patterns consisting of single and clustered immunogold grains were obtained using different sources of anti-rabbit IgG conjugated to various lots of colloidal gold particles (diameters of 5- or 10 nm). To further make certain that the immunogold clusters reflected

TABLE I  
Quantitative Immunoelectron Microscopic Analysis of IL-1 $\beta$  Localization in LPS-stimulated Human Monocytes

| Stimulation time | Fixer*      | Stain <sup>‡</sup> | No. Cells Sampled <sup>§</sup> | Cellular compartments        |          |             |                              |            |              |          |
|------------------|-------------|--------------------|--------------------------------|------------------------------|----------|-------------|------------------------------|------------|--------------|----------|
|                  |             |                    |                                | Cytoplasmic ground substance | ER lumen | Golgi lumen | Plasma membrane <sup>†</sup> | Nucleus    | Mitochondria | Vesicles |
| <i>h</i>         |             |                    |                                |                              |          |             |                              |            |              |          |
| 4                | Form        | Exp.               | 17                             | 2,791 (88.2)                 | 5 (0.15) | 0           | 32 (1.0)                     | 260 (8.2)  | 11 (0.3)     | 66 (2.0) |
| 4                | Form        | Control            | 6                              | 79 (69.3)                    | 0        | 0           | 0                            | 33 (28.9)  | 1 (0.9)      | 1 (0.9)  |
| 20               | Form        | Exp.               | 24                             | 3,273 (88.3)                 | 7 (0.18) | 2 (0.05)    | 39 (1.0)                     | 238 (6.44) | 139 (3.7)    | 8 (0.2)  |
| 20               | Form        | Control            | 15                             | 139 (73.9)                   | 0        | 0           | 0                            | 25 (13.2)  | 24 (12.7)    | 0        |
| 4                | Glut + Form | Exp.               | 14                             | 1,853 (90.1)                 | 0        | 0           | 35 (1.7)                     | 146 (7.1)  | 7 (0.3)      | 15 (0.7) |
| 4                | Glut + Form | Control            | 5                              | 52 (78.7)                    | 0        | 0           | 0                            | 14 (21.2)  | 0            | 0        |
| 20               | Glut + Form | Exp.               | 10                             | 1,412 (85.8)                 | 12 (0.7) | 0           | 47 (2.8)                     | 117 (7.1)  | 52 (3.1)     | 5 (0.3)  |
| 20               | Glut + Form | Control            | 3                              | 29 (70.7)                    | 0        | 0           | 0                            | 10 (24.4)  | 2 (4.8)      | 0        |

Numbers represent the total anti-IL-1 $\beta$  IgG-conjugated colloidal gold particles counted in various subcellular compartments visualized in electron micrographs of immunolabeled ultrathin cryosections of LPS-stimulated human monocytes. The parentheses show the counts per organelle expressed as a percentage of the total number of gold grains determined for each subgroup of cells.

\* After 4 or 20 h of LPS-stimulation, cultures were fixed with either 3.5% paraformaldehyde (Form), or 0.1% glutaraldehyde (Glut) plus 3.5% formaldehyde as described in the Materials and Methods.

<sup>‡</sup> Fixed monocytes were ultrathin cryosectioned, and the monocyte sections placed on grids and stained with rabbit anti-IL-1 $\beta$  IgG (specific for the amino acid sequence 197–215 in mature IL-1 $\beta$ ), followed by affinity-purified goat anti-rabbit IgG conjugated to 10-nm diameter colloidal gold particles (Exp.). For controls, monocyte sections were stained with anti-IL-1 $\beta$  IgG preincubated with 100  $\mu$ g/ml purified peptide (197–215), and the anti-rabbit IgG gold conjugate.

<sup>§</sup> Electron micrographs were taken of the monocyte frozen sections immunolabeled with IL-1 $\beta$  antibodies, and printed at magnifications of  $\times$  47,000–84,000. One section was sampled per cell, and the various groups were blind coded.

<sup>†</sup> Plasma membrane immunolabeling was defined as the presence of gold grains at or within 20 nm of transversely sectioned or obliquely cut cell surface membranes.

a true subcellular concentration of IL-1 $\beta$  antigens, and did not represent a staining artifact, we used the same immunoconjugate and staining protocol in parallel experiments to localize fibronectin with rabbit anti-human plasma fibronectin IgG on ultrathin frozen sections of cultured human fibroblasts. This system provided an appropriate control since fibronectin antigens are evenly distributed throughout the extracellular matrix of fibroblasts (22, 29). The immunogold staining of fibronectin within sectioned matrix cables was very homogeneous and characteristically nonclustered (data not shown), thus confirming the specificity of the clustered labeling pattern for certain IL-1 $\beta$  antigens within monocytes.

We performed several controls to ensure that the immunogold staining was specific for IL-1 $\beta$ . Preincubation of IL-1 $\beta$  (p197–215) IgG with excess irrelevant peptide (p117–128) did not alter the pattern or labeling intensity of IL-1 $\beta$  antigens (Fig. 7A), while pretreatment with the relevant peptide (p197–215) eliminated both singlet and clustered colloidal gold immunostaining (Fig. 7B). The magnitude of this reduction was from 96 to 98% of the immunogold labeling obtained with unblocked anti-IL-1 $\beta$  IgG (Table I). In addition, substitution of preimmune rabbit IgG for anti-IL-1 $\beta$  IgG in our staining protocol resulted in similarly low background levels (not shown).

To control for the capability of our immunoelectron microscopic staining method to detect proteins synthesized in the ER and concentrated in secretory



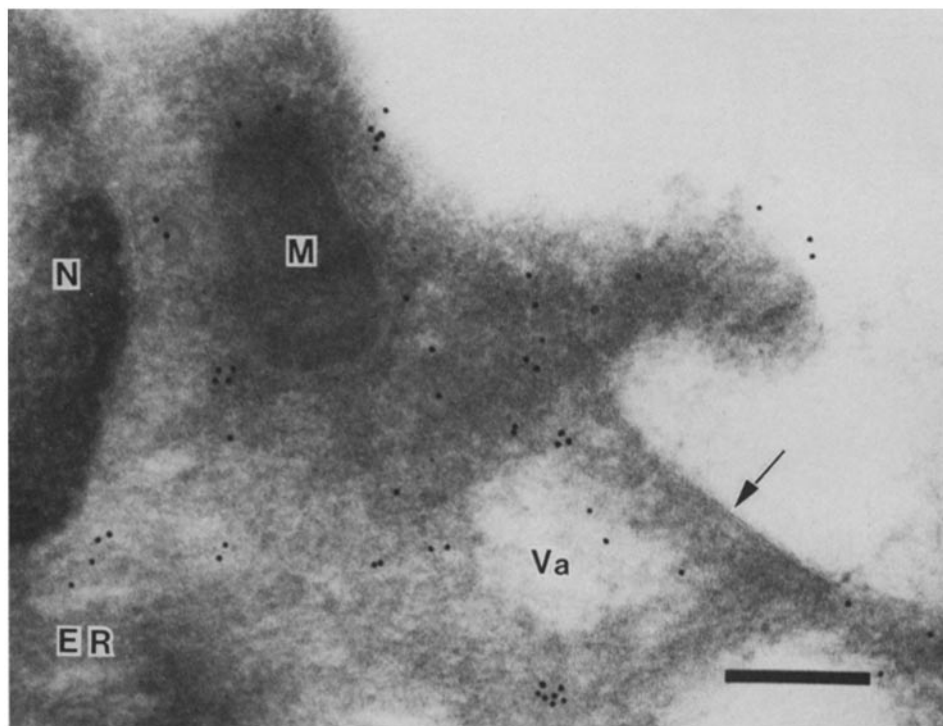


FIGURE 4. This figure and Figs. 5–7 and 9 are immunoelectron micrographs of ultrathin frozen sections of LPS-stimulated human monocytes indirectly labeled for IL-1 $\beta$  with anti-peptide (p197–215) IgG, and a goat anti-rabbit IgG 10 nm colloidal gold conjugate, as described in Materials and Methods. All cells depicted were fixed with 3.5% formaldehyde plus 0.1% glutaraldehyde, except that in Fig. 9 the cells were preserved with 3.5% formaldehyde alone. G, Golgi apparatus; ER, endoplasmic reticulum; M, mitochondrion; Mv, microvillus; N, nucleus; P, cell process; Va, phagocytic vacuole. Bar = 250 nm.

Fig. 4 is a monocyte fixed at 4 h of LPS stimulation, when the intracellular content of IL-1 $\beta$  is high, but only small quantities of hormone have been secreted (Fig. 1). IL-1 $\beta$ -specific labeling is localized in the cytoplasmic ground substance, while ER cisternae, vacuoles (Va), and the transversely sectioned cell membrane (arrow) are relatively unlabeled. Only sparse staining is present at the obliquely sectioned plasma membrane in the upper portion of the micrograph.

granules, ultrathin frozen sections of activated monocytes were stained for lysozyme with monospecific rabbit antibodies in the protocol used above for IL-1 $\beta$ . Striking concentrations of lysozyme antigens were observed within elongated cisternae of the ER (Fig. 8A), and in membrane-bound dense secretory granules ~250 nm in diameter (Fig. 8B).

Since the use of a glutaraldehyde fixative reduced the level of IL-1 $\beta$ -specific immunofluorescence observed in LPS-activated monocytes, we also performed immunoelectron microscopic experiments on stimulated cells preserved with formaldehyde alone. Although the IL-1 $\beta$  labeling density was increased 43–66% relative to comparable monocytes fixed with the 0.1% glutaraldehyde solution, no alterations were observed in the pattern of IL-1 $\beta$  staining reported above (Fig. 9, Table I).

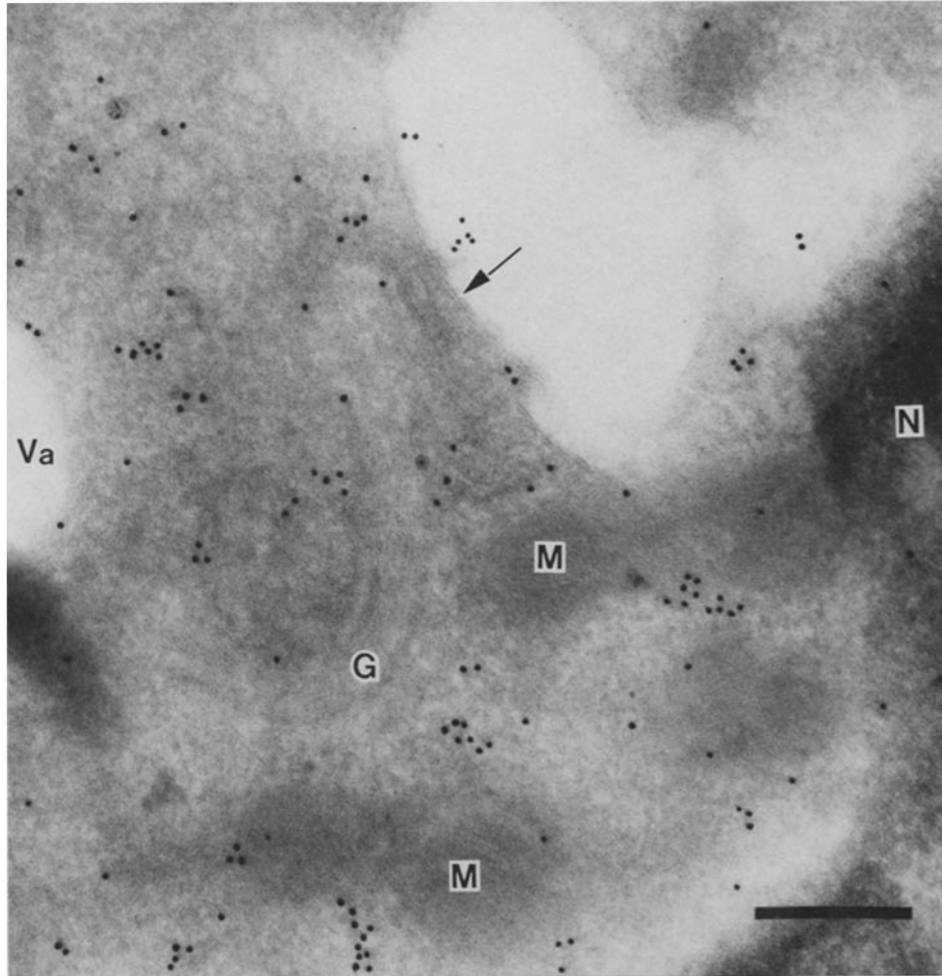


FIGURE 5. At 20 h after LPS activation, when IL-1 $\beta$  secretion is well underway (Fig. 1), the Golgi apparatus (G) completely lacks IL-1 $\beta$  labeling. IL-1 $\beta$ -specific staining is found in the ground substance of the cytoplasm, where it appears as single gold grains or as elliptical clusters confined to the plane of the section. The outer cell membrane (arrow) is only sparsely stained. See first part of legend to Fig. 4.

### Discussion

In the current study we used immunoelectron microscopy to localize IL-1 $\beta$  precursor within activated human monocytes. The cells were studied during a period of active IL-1 secretion as shown by a 40-fold increase in IL-1-R binding activity in the culture supernatants after LPS stimulation (Fig. 1). Also, pulse-chase experiments with [<sup>35</sup>S]methionine have confirmed that IL-1 $\beta$  is actively synthesized and secreted at 4 and 20 h of LPS stimulation (Hall, G., and J. Schmidt, unpublished observations), i.e., at the times when our immunoelectron microscopic observations were made. The highest concentrations of fixatives consistent with optimal antigen retention were used to obtain maximum preservation of subcellular architecture. Furthermore, our interpretation of the frozen

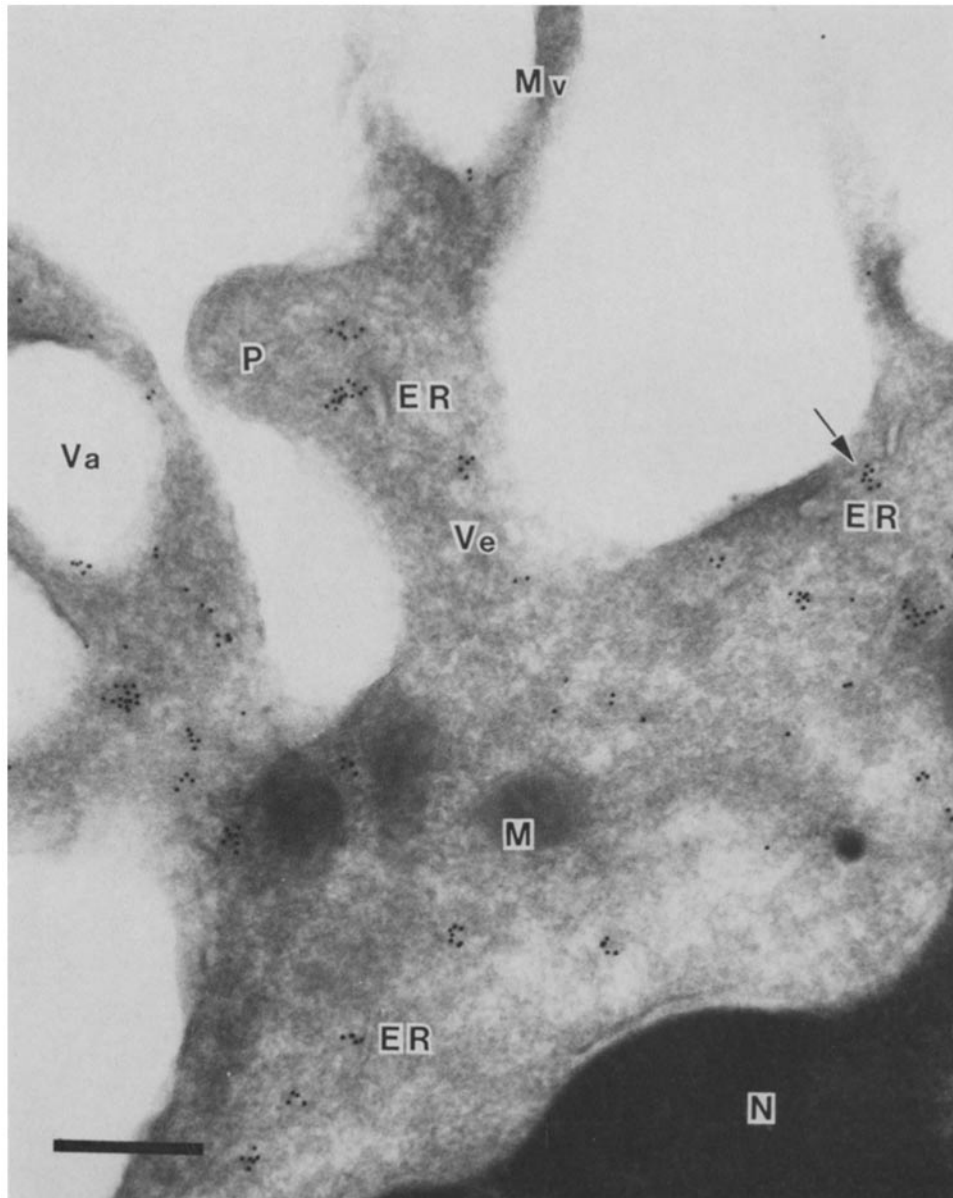


FIGURE 6. A human monocyte prepared as that shown in Fig. 5, illustrating the lack of significant IL-1 $\beta$  staining at the cell surface. The outer cell membrane is unlabeled while IL-1 $\beta$  antigens are found randomly distributed throughout the cytoplasmic ground substance. The lumens of secretory Ve and most cisternae of the ER are not stained, except for the ER shown by the arrow. See first part of legend to Fig. 4.

sections was guided by study of epoxy-embedded sections of identically stimulated cells (Fig. 3). The specificity of the IL-1 $\beta$  staining pattern was demonstrated by: (a) immunoblot analysis of cell lysates (Fig. 1A); (b) the failure of preimmune IgG to give staining; (c) nearly complete inhibition of staining by preincubation

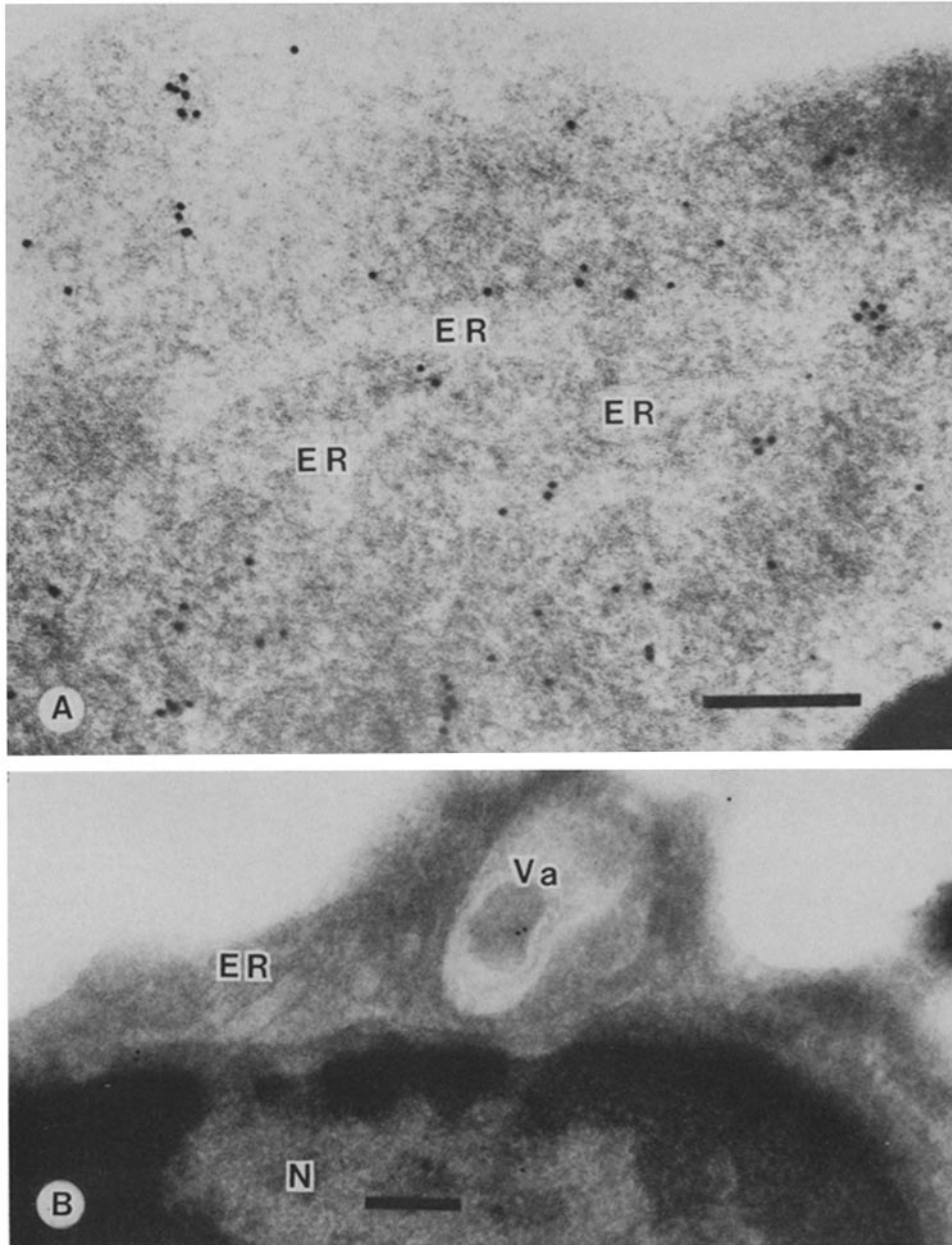
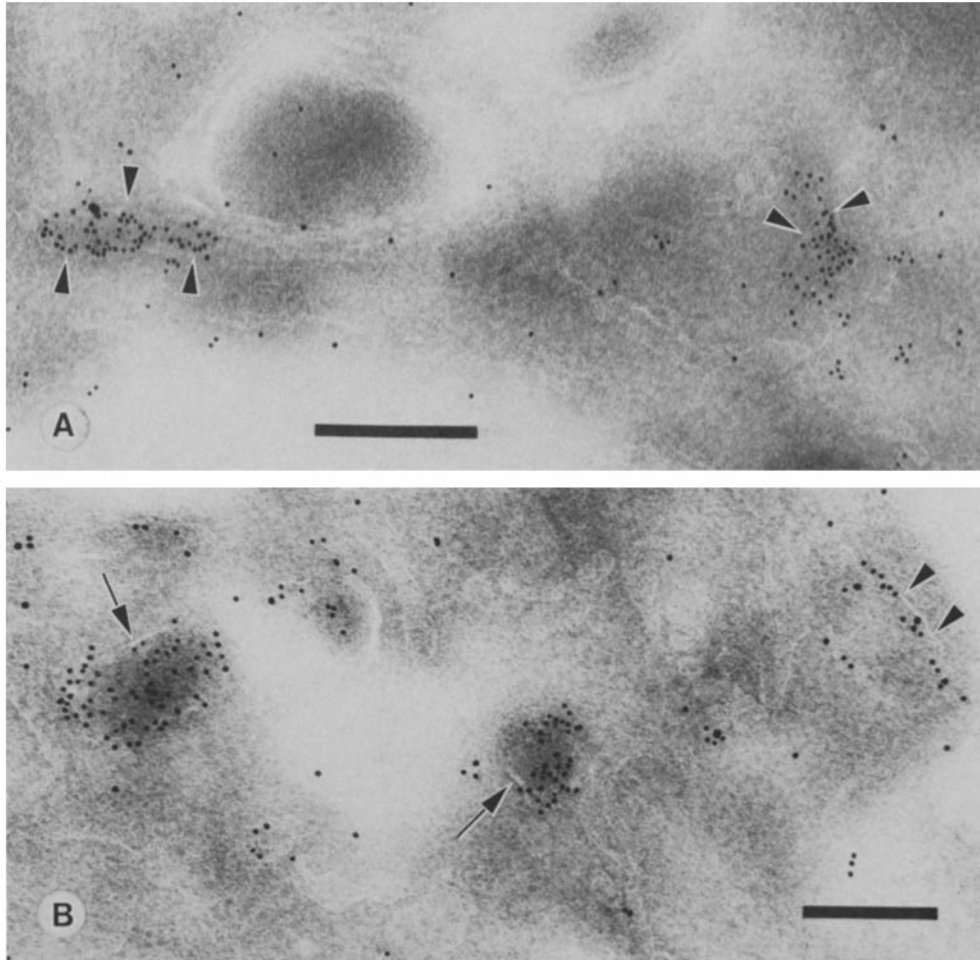


FIGURE 7. Control immunoelectron micrographs demonstrating the specificity of the labeling due to anti-IL-1 $\beta$  (p197-215) IgG by preincubation with excess antigen. (A) The typical cytoplasmic staining pattern outside the ER cisternae is seen despite pretreatment with excess irrelevant antigen (p117-128). (B) Nearly all labeling is abolished when the anti-IL-1 $\beta$  peptide antibodies are preincubated with excess relevant antigen (p197-215).



**FIGURE 8.** Ultrathin frozen sections of human monocytes activated with LPS for 20 h and labeled for lysozyme with a monospecific rabbit antibody using the immunoelectron microscopic staining protocol described for IL-1 $\beta$  (see Materials and Methods). (A) Bulbous and elongate cisternae of the ER (*arrowheads*) exhibit lysozyme-specific staining. (B) Membrane-enclosed dense secretory granules (*arrows*) also exhibit high concentrations of lysozyme; a linear profile of ER is also lysozyme-positive (*arrowheads*).

of immune IgG with relevant, but not irrelevant, IL-1 $\beta$ -derived peptides (Fig. 7); and (d) demonstration of abundant lysozyme antigens within the ER and secretory Ve of monocytes labeled with our immunoelectron microscopic protocol.

The results show that the overwhelming majority of IL-1 $\beta$  precursor is localized to the cytosolic ground substance of the cell at both 4 and 20 h of LPS stimulation. This finding is in agreement with previous light microscopic studies performed by Bayne et al. (12) using antibodies recognizing a different IL-1 $\beta$  antigen. Very few grains were found within the secretory apparatus of the cell, i.e., within saccules of the Golgi apparatus, secretory Ve, or cisternae of the ER. A small but finite amount of specific labeling was associated with the cell nucleus.

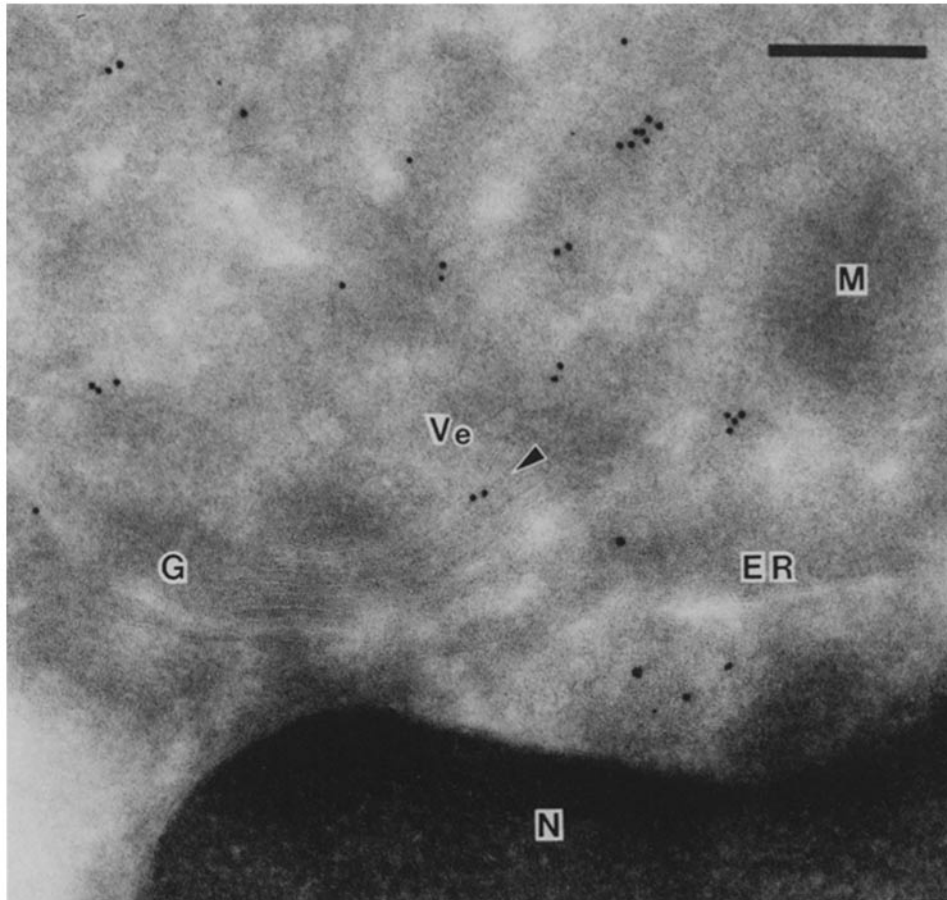


FIGURE 9. Illustration of a human monocyte fixed solely with 3.5% formaldehyde at 20 h of activation with LPS. The random pattern of IL-1 $\beta$  localization within the cytoplasmic ground substance appears very similar to that observed in sections of monocytes fixed with formaldehyde plus glutaraldehyde. Secretory Ve, and cisternae of ER do not contain IL-1 $\beta$  antigens. The arrowhead depicts only slight IL-1 $\beta$  staining in the Golgi apparatus. See first part of legend to Fig. 4.

Most of this labeling was observed at the nuclear periphery and is therefore probably due to superimposition of portions of the juxtannuclear cytoplasm over the nuclear border in tangentially oriented sections. While the possibility of surface-bound IL-1, suggested by previous studies of murine cells (18–20), cannot be entirely eliminated on the basis of our results, <2.8% of all labeled IL-1 $\beta$  antigens were localized to the plasma membrane (Table I). The latter finding is in agreement with our inability to (a) demonstrate binding of radiolabeled IL-1 $\beta$  to LPS-activated human monocytes, and (b) surface label cell-associated IL-1 $\beta$  using standard lactoperoxidase techniques (Chin, J., G. Hall, and J. Schmidt, unpublished observations). Although paraformaldehyde-fixed human monocytes do stimulate murine thymocyte proliferation, all of this activity can be accounted for by IL-1 leaching out of these mildly fixed cells during the course of the thymocyte assay (Hall, G., and J. Schmidt, manuscript submitted for publication).

Therefore, on the basis of morphological and functional studies, IL-1 $\beta$  appears to be localized within the cytoplasm, and not on the plasma membrane of activated human monocytes.

While the IL-1 $\beta$  precursor appeared to be mainly localized in the cytosolic ground substance of activated monocytes, the distribution of label was not always homogeneous (Figs. 4–8). The anti-IL-1 $\beta$  immunogold labeling appeared clustered within the cytosol in 38–46% of the antigenic sites observed in 4 and 20 h specimens. These clusters are probably not an artifact of the labeling technique in that care was taken to eliminate putative immunogold aggregates before staining (see Materials and Methods), and 80% of the immunogold conjugate was composed of singlets. Furthermore, no significant clustering was observed in parallel immunoelectron microscopic experiments on cultured human fibroblasts using the same protocol and goat anti-rabbit IgG gold probe to stain fibronectin. In those cases where clustering of IL-1 $\beta$  labeling was observed, all of the gold grains were in a single plane; i.e., they did not appear as heaped-up, three-dimensional complexes as would be expected for aggregated immunogold conjugates. Finally, the clustered gold particles, like the singlets, were eliminated by preincubation of the primary antibodies with relevant peptide. No clusters were observed with control IgG. We believe that these clusters represent labeling of several IL-1 $\beta$  precursor molecules concentrated at subcellular loci, and that they do not result from the use of polyclonal antibodies in a sandwich technique because (a) the IL-1 $\beta$  antigen sequence is short (18 amino acids); (b) the clusters were planar, and (c) in many cases, the largest dimension was too great to be accounted for by the aggregation of the immunogold particles at a single antigenic site.

There are several possible explanations for the observed clustering of anti-IL-1 $\beta$  label. First, the clusters may represent nascent IL-1 $\beta$  precursor molecules on free polyribosomes. However, the immunoblot analyses performed with this (Fig. 1A) and other (13) anti-IL-1 $\beta$  antibodies suggest that lower molecular weight precursor material is not sufficiently abundant to account for the large number of clusters observed. Although most secretory vesicles did not contain IL-1 $\beta$  antigens, a second possibility is that the clusters are due to enclosure of IL-1 $\beta$  precursor in secretory Ve that have not been adequately preserved by our fixation technique. However, the adequate fixation of many secretory Ve, as well as membranes of the Golgi apparatus and ER (Figs. 4–9), argues against this possibility. Furthermore, the observations that the mean IL-1 $\beta$  antigen cluster size was smaller than the mean diameter of secretory Ve, and that the clusters were elliptical in shape whereas the secretory vesicles were round, suggest that IL-1 $\beta$  precursor does not fill these vesicles. A third possibility is that clusters may form through an interaction of IL-1 $\beta$  precursor with a condensing molecule (33). Sulfated proteoglycans have been suggested as perhaps serving such a role (34). Interestingly, complexes composed of class II MHC molecules and chondroitin sulfate proteoglycan molecules have recently been reported (35, 36). Subcellular fractionation experiments are being performed to test this hypothesis (37).

The mechanism of IL-1 $\beta$  secretion remains unclear. While the absence of a typical leader sequence suggested that IL-1 secretion did not involve the Golgi apparatus, the possibility remained that IL-1 $\beta$  beta possesses an atypical, yet

functional, leader sequence similar to that found in OVAL (38). The current study argues that this is unlikely because no substantial labeling of ER, Golgi apparatus, or secretory vesicles was observed. Similarly, while the absence of a membrane anchor sequence suggested that the IL-1 $\beta$  precursor was not an integral membrane protein, the possibility remained that IL-1 was bound to plasma membranes via covalently linked lipid moieties (reviewed in reference 39). Alternatively, a membrane-bound species of IL-1 $\beta$  may have resulted from a cryptic, alternately spliced mRNA not represented in the cDNAs cloned to date. Membrane and secreted IgM molecules arise from such a mechanism (reviewed in reference 40). The current study is important because it argues that anchorage of human IL-1 $\beta$  to the plasma membranes of monocytes by these or other mechanisms does not occur.

The results of this study suggest that the mechanism of IL-1 $\beta$  secretion is likely to be novel. Since the antibodies used do not recognize human IL-1 $\alpha$  (9), no comment can be made on its localization. It has been previously reported, however, that translation of murine IL-1 $\alpha$  mRNA in rabbit reticulocyte lysates in the presence of dog pancreatic microsomes did not result in processing of the murine IL-1 $\alpha$  precursor (1). This result suggests that the  $\alpha$  class of IL-1 molecules also lacks a functional leader sequence. Interestingly, acidic fibroblast growth factor, which is homologous with human IL-1 $\beta$  (41), and basic fibroblast growth factor, have also recently been found to lack hydrophobic leader sequences (42, 43). It is therefore possible that IL-1 $\beta$  is the prototype of a group of inflammatory and growth mediators using a novel pathway of protein secretion.

### Summary

The subcellular location of IL-1 $\beta$  was determined using a postsectioning immunoelectron microscopic method on ultrathin frozen sections of human monocytes stimulated with LPS. This methodology permits access of antibody probes to all sectioned intracellular compartments, and their visualization at high resolution. Staining was performed with a rabbit antibody that specifically recognized amino acids 197–215 in the 33-kD IL-1 $\beta$  precursor molecule, followed by affinity-purified goat anti-rabbit IgG conjugated to 10 nm colloidal gold particles. ~90% of the IL-1 $\beta$  antigens were localized in the ground substance of the cytoplasm at 4 or 20 h after activation, when both intracellular and extracellular accumulation of IL-1 $\beta$  was well underway. No significant IL-1 $\beta$  staining was observed on the outer cell membrane, nor within the lumens of the endoplasmic reticulum (ER), the Golgi apparatus, or secretory vesicles. In contrast, lysozyme was localized in the ER and dense secretory granules using these methods. Our results suggest that IL-1 $\beta$  is not anchored on the plasma membrane, and that its secretion occurs by a novel mechanism that does not use a secretory leader sequence, nor the classical secretory pathway involving the ER and Golgi apparatus.

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