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Immunology

Interleukin 1 is processed and released during apoptosis

(cytotoxic T lymphocyte/necrosis/cell injury/ATP)

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ABSTRACT **Interleukin (IL-) 1α and 1β are synthesized as 31- to 34-kDa pro molecules. They are released from monocytes and macrophages as proteolytically processed 17-kDa mature molecules that bind with high affinity to specific receptors on target cells. IL-1 is not released via the classic secretory pathway. The pro molecules are synthesized as cytosolic proteins without signal peptides. Although the proteases that convert the pro molecules to the mature forms are cytosolic enzymes, processed IL-1 is not detected associated with the cell but is found only in culture supernatants. We demonstrate here that release of IL-1 is efficiently induced by cell injury. When the injury causes cellular necrosis, IL-1α is released as a mixture of unprocessed and processed molecules but IL-1β is released exclusively as the biologically inactive pro form. In contrast, when cells undergo apoptosis, maturation of both IL-1α and IL-1β is efficient. When apoptosis is rapid, as in macrophages that are targets for allospecific cytotoxic T lymphocytes, processing is observed to occur intracellularly. These findings suggest that cell injury is an important physiologic stimulus for release of IL-1. The nature of the injury profoundly affects the forms of IL-1 that are released.**

Interleukin (IL-) 1α and 1β are proteins that modulate immune and inflammatory responsiveness (1). Because many cells in the body have surface receptors for IL-1 and because soluble IL-1 can be detected in plasma and other fluids during chronic inflammatory diseases, IL-1 has been considered a secretory protein; however, IL-1 has no signal peptide (2) and is not released via the classic secretory pathway (3).

In lipopolysaccharide (LPS)-stimulated cells, the pro forms (≈31 kDa) of IL-1α and -1β accumulate at high levels in the cytosol. Only a small portion of the intracellular IL-1 is released in culture, and it has been suggested that this release is due to cell death (4). Supernatants of LPS-stimulated monocytes and macrophages contain proteolytically processed forms (≈17 kDa) of both IL-1α and IL-1β (5–7). These forms of IL-1 are not detected inside the cell, suggesting that processing occurs concurrent with release. The proteolytic processing of IL-1α and -1β generates forms that have biological activity and are resistant to proteolytic degradation (8). Only the mature form of IL-1β has biological activity; however, both the pro and mature forms of IL-1α are active (9). Monocyte enzymes that can cleave the pro forms of IL-1α (10) and -1β (11, 12) at their natural processing sites (before Leu-119 for IL-1α and Ala-117 for IL-1β) have been characterized. Of considerable interest, although processed IL-1 is observed only extracellularly, both the processing enzymes and the substrate pro-IL-1 molecules are present in the cytosol. This suggests that the cellular processing of IL-1 is itself carefully regulated. Additional enzymes from other sources (13, 14) can cleave IL-1α and -1β at other residues, yielding bioactive mature forms.

IL-1β is present in its mature form in supernatants of freshly isolated monocytes after stimulation with LPS. In contrast, when LPS-stimulated monocytes are killed by freezing and thawing, IL-1β is present in only the unprocessed pro form (3). Some investigators have therefore suggested that the release of IL-1 from LPS-stimulated cells must not be due to cell death but must be the result of the activation of a secretory pathway. Here we demonstrate that both IL-1α and -1β are released as a consequence of cell injury regardless of the insult. All necrotic forms of injury result in release and processing of IL-1α, whereas IL-1β is released only in the unprocessed pro form. In contrast, when the cell injury leads to apoptosis, both IL-1α and IL-1β are efficiently processed and released.

MATERIALS AND METHODS

Isolation and Culture of Peritoneal Exudate Cells (PECs). Female BALB/c or CBA/J mice (The Jackson Laboratory) were injected i.p. with 1 ml of thioglycollate medium (Difco) 4–5 days before harvesting. PECs were harvested by peritoneal lavage with 10 ml of cold R1 [RPMI 1640 containing 10 mM Hepes (pH 7.3) and 1% heat-inactivated fetal bovine serum (FBS) (HyClone)], washed three times, and resuspended at 10^6 cells per ml in R10 (RPMI 1640 containing 10% heat-inactivated FBS, 100 units of penicillin per ml, 100 μg of streptomycin sulfate per ml, and 2 mM L-glutamine). PECs were adhered to plastic culture dishes for 4 hr and then washed with warm R1.

Pulse–Chase Analysis. Monolayers of adherent cells were incubated in R10 with LPS [*Escherichia coli* strain 0127:B8 (Difco)] at 1 μg/ml for 75 min in a humidified 5% CO_2 atmosphere at 37°C. The medium was then replaced with methionine-deficient RPMI 1640 containing 10% dialyzed FBS and LPS. Fifteen minutes later, [^{35}S]methionine (specific activity > 1000 Ci/mmol; Amersham; 1 Ci = 37 GBq) was added at 150 μCi/ml. After an additional 30 min, the cells were washed once with R1 and the chase was initiated by the addition of fresh medium at time 0.

After the chase, culture supernatants were removed, the monolayers were washed with R10, and lysates were prepared by the addition of phosphate-buffered saline (0.15 M NaCl/10 mM phosphate, pH 7.3) containing 1% (vol/vol) Nonidet P-40 and protease inhibitors [iodoacetamide (10 mM), pepstatin (1 μg/ml), EDTA (1 mM), leupeptin (1 μg/ml), and phenylmethylsulfonyl fluoride (0.4 mM), all from Sigma]. Residual cells were removed from the culture supernatants by centrifugation at 500 × *g* for 5 min and were added to the lysate fractions. The culture supernatants were then centrifuged at 10,000 × *g* for 5 min. Finally, protease inhibitors (as above) were added to the supernatants and the samples were frozen at −70°C.

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Abbreviations: IL-1, interleukin 1; PEC, peritoneal exudate cell; CTL, cytotoxic T lymphocyte; FBS, fetal bovine serum; LDH, lactate dehydrogenase.

Lysates were clarified by centrifugation at 10,000 × *g* for 20 min. Lysates and supernatants were precleared with 10 μl of protein A-Sepharose (Sigma). The IL-1 was immunoprecipitated from equivalent fractions of each sample by the addition of purified anti-murine IL-1 monoclonal antibody B122 (5 μg) (15) or 161.1 (3 μg) (16), or K4.7 anti-human IL-1β serum or Endogen (Boston) anti-human IL-1α serum, in a total volume of 0.6 ml. After 1 hr at 4°C, protein A-Sepharose was added and the samples were mixed for 1 hr. The beads were washed three times with phosphate-buffered saline containing 1% (vol/vol) Nonidet P-40 and 0.2% SDS, then boiled in SDS/PAGE sample buffer (17), resolved in a 13% polyacrylamide gel, soaked in EN^{3}HANCE (NEN), and autoradiographed. Densitometric scanning of the autoradiographs was performed with an LKB Ultroscan. The area under the curve was divided by the number of methionine residues predicted to be in the molecule, and the amount immunoprecipitated from the supernatant was expressed as a percentage of the total radioactivity incorporated into the IL-1 precursors at time 0.

Protocols for Injury. Macrophages were stimulated with LPS, pulse-labeled, and then injured by addition of 5 mM ATP (Boehringer Mannheim) for 30 min or by continuous incubation with 20 mM H_2O_2 (Sigma) or 0.2% saponin (Calbiochem). Physical injury was achieved by scraping cells off the dish with a rubber policeman. In some experiments, monolayers were frozen at −70°C and thawed for three cycles or were heated at 42°C for 30 min.

Lactate Dehydrogenase (LDH) Assay. LDH activity was measured in cell lysates and culture supernatants by use of a colorimetric kit (Sigma). Media control levels from each time point were subtracted from the values for supernatant LDH activity. The percentage of LDH released equaled the amount released divided by the amount released plus the amount present in cell lysates.

IL-1 Bioassay. IL-1 activity was detected using the T-cell line D10.G4.1 (18). Lysates and supernatants were clarified by microcentrifugation for 10 min prior to assay. One unit of IL-1 activity per ml is defined as that quantity that produces half-maximal [^{3}H]thymidine uptake by D10.G4.1 cells.

Coculture with Cytotoxic T Lymphocytes (CTLs). BALB/c (H-2^d) or CBA/J (H-2^k) PECs were stimulated and pulse-labeled, and then the H-2^d-specific CTL clone OE4 (19) (generously provided by Osami Kanagawa) was added at an effector/target ratio of 10:1. At the indicated times cells and supernatants were separated and IL-1 was analyzed by immunoprecipitation as above.

DNA Fragmentation. Approximately 2.5 × 10^6 cells were lysed by incubation for 10 min in 500 μl of cold 20 mM Tris·HCl, pH 7.4/10 mM EDTA/0.2% (vol/vol) Triton X-100, and the lysate was centrifuged for 10 min at 10,000 × *g*. The supernatant was incubated at 50°C overnight with proteinase K (Boehringer Mannheim) at 0.1 mg/ml, and extracted with a 1:1 phenol/chloroform mixture, and the DNA was precipitated with 50% 2-propanol and 1.1 M ammonium acetate. After digestion with RNase A (Boehringer Mannheim) at 50 μg/ml for 1 hr at 37°C, the samples were heated at 65°C for 10 min and then electrophoresed in a 1% agarose gel and stained with ethidium bromide at 1 μg/ml.

Amino Acid Sequence Analysis. Approximately 2.5 × 10^7 J774 cells (mouse monocyte/macrophage cell line) were incubated in a 100-mm culture dish for 30 min in 5 ml of leucine-free Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and 2 mM L-glutamine. LPS (1 μg/ml) and 1 mCi of L-[4,5-^{3}H]leucine (Amersham, TRK.683) were added. Two hours later, the cells were washed and cultured for 30 min with 5 mM ATP, then washed again and cultured for 6 hr in DMEM containing 10% FBS and 2 mM L-glutamine. The supernatant was harvested, immunoprecipitated as above, fractionated by SDS/12.5% PAGE, and transferred to a poly(vinylidene difluoride) membrane (Schleicher & Schuell) in a dry blot apparatus (American Biosystems, Hayward, CA). The relevant portion of the membrane was excised and subjected to Edman degradation on an Applied Biosystems model 470 gas-phase sequencer using the O3RATZ program.

RESULTS AND DISCUSSION

Necrosis Leads to the Release but Not Maturation of IL-1β. Analysis of release of IL-1 from LPS-stimulated PECs by pulse–chase labeling and immunoprecipitation (Fig. 1*A*) demonstrates that both IL-1α and IL-1β accumulate in substantial quantities within the cell; however, by immunoprecipitation very little release of IL-1 into the culture supernatant can be detected. Experiments reported elsewhere (20) showed, by both bioassay and immunoprecipitation, that IL-1 was released but that the amount released represented a very small percentage of the total IL-1 expressed by the macrophage. For murine and human macrophages, the fractional release values of IL-1α and -1β were equivalent and always <5%. Importantly, fractional release of IL-1 correlated with the fractional release of cellular LDH. Freshly isolated LPS-stimulated human monocytes showed a broader range of IL-1 release (2–25%); however, again IL-1 release was strongly correlated with release of LDH. These data suggest that IL-1 is released from cultured mononuclear phagocytes as a consequence of cellular injury.

To test directly whether cellular injury can result in the release of functional IL-1, we intentionally injured LPS-stimulated murine macrophages by processes that can result either in necrosis or in apoptosis (21). We first analyzed IL-1 release in response to several methods of necrotic injury. Fig. 1*B* shows the rapid release of IL-1α and -1β following

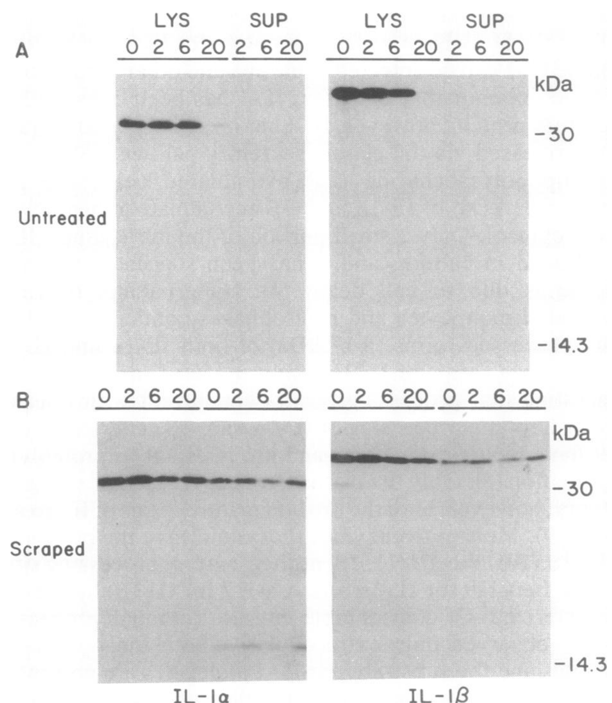

FIG. 1. IL-1α and -1β are released during necrotic cell injury but IL-1β is not processed. Murine PECs were stimulated with LPS and pulse-labeled with [^{35}S]methionine. Monolayers were either left intact (*A*) or gently scraped off the dish at time 0 (*B*). At 0, 2, 6, and 20 hr after injury, IL-1 release was analyzed by immunoprecipitation from the cell lysates (LYS) and culture supernatants (SUP). LDH release at 20 hr was 2% (*A*) and 46% (*B*).

physical injury caused by scraping the cells off the culture dish. Scraping resulted in substantial cell injury with immediate release of 46% of the total cellular LDH. Although both IL-1α and IL-1β were also released immediately upon scraping, only IL-1α was proteolytically processed. The IL-1β released into the culture supernatants was solely the 34-kDa pro form. At 20 hr, this supernatant contained 880 units of IL-1 activity per ml. This activity was completely neutralized by anti-IL-1α antibody (data not shown) consistent with the observation that the pro form of IL-1β is biologically inactive (9). In other experiments, cellular necrosis was induced by oxidative damage with hydrogen peroxide, excessive heat, freezing and thawing, and lysis with the detergent saponin. All of these treatments resulted in the release of IL-1α as a mixture of the processed and pro forms, whereas IL-1β was released only as the unprocessed pro form. These results confirm our previous conclusion that IL-1 is released as a result of cellular injury. They also suggest that cellular necrosis may fail to activate or may destroy the IL-1β-processing enzyme but does not interfere with the proteases that process IL-1α.

Efficient Processing and Release of IL-1α and -1β During Apoptosis. Next we assessed IL-1 release in response to apoptotic stimuli. Some cells can be induced to undergo apoptosis by exposure to extracellular ATP (22). Murine peritoneal macrophages were stimulated with LPS and pulse-labeled, incubated for 30 min with 5 mM ATP, and then given fresh medium. Fig. 2*A* shows the loss of PEC viability after ATP treatment as assessed by the cellular release of LDH. Though the cells were fully viable after the 30-min incubation with ATP, the inductive signal for apoptosis had been delivered and the cells progressively died, so that 80% were dead after an additional 20 hr of culture. Cell death was also assessed by trypan blue exclusion and measurement of total protein remaining in cell monolayers (data not shown). Both methods indicated similar levels of loss of viability in ATP-treated cultures but not in control cultures. DNA fragmentation occurred in the cells undergoing apoptosis (Fig. 3, lanes 7–10), whereas necrotic stimuli, such as hydrogen peroxide (lanes 1 and 2) and scraping, detergent, or freezing and thawing (data not shown) did not lead to DNA fragmentation.

In ATP-treated cells, IL-1α and -1β were progressively released into the culture supernatant (Fig. 2*B*). Notably, both IL-1α and IL-1β were recovered as the proteolytically processed forms after as little as 2 hr of chase. At 20 hr of chase the culture supernatants contained 1200 units of IL-1 biological activity per ml. Antibody neutralization studies showed that this bioactive material was composed of both IL-1α and IL-1β. An excess of monoclonal antibody 161.1 (anti-IL-1α) reduced the supernatant IL-1 activity to 550 units/ml, whereas B122 (anti-IL-1β) reduced the supernatant activity to 600 units/ml. Both antibodies together completely neutralized the supernatant activity. The murine macrophage cell line J774, which is known to be responsive to extracellular ATP (23), exhibited a similar pattern of release (data not shown). N-terminal sequencing of the mature IL-1β immunoprecipitated from these supernatants demonstrated that cleavage was prior to Val-118 of the pro molecule (Fig. 4). This is analogous to cleavage before Ala-117 in human pro-IL-1β, a cleavage known to occur by means of a specific intracellular IL-1β convertase enzyme that has been purified from human monocytes (11, 12). These data provide clear evidence that there is a murine equivalent of the human IL-1β-converting enzyme.

IL-1 Is Released During the Interaction of CTLs with Target Cells. Macrophages also undergo apoptosis when they serve as targets for CTLs (24). The H-2^d-allospecific CTL line OE4 was cocultured with LPS-stimulated, pulse-labeled PECs from BALB/c (H-2^d) or CBA/J (H-2^k) mice. The result of this interaction was apoptotic death of BALB/c but not CBA/J macrophages. The DNA fragmentation that occurred during specific CTL killing of BALB/c macrophages is shown in Fig. 3, lanes 3–6. IL-1α and -1β were not released from

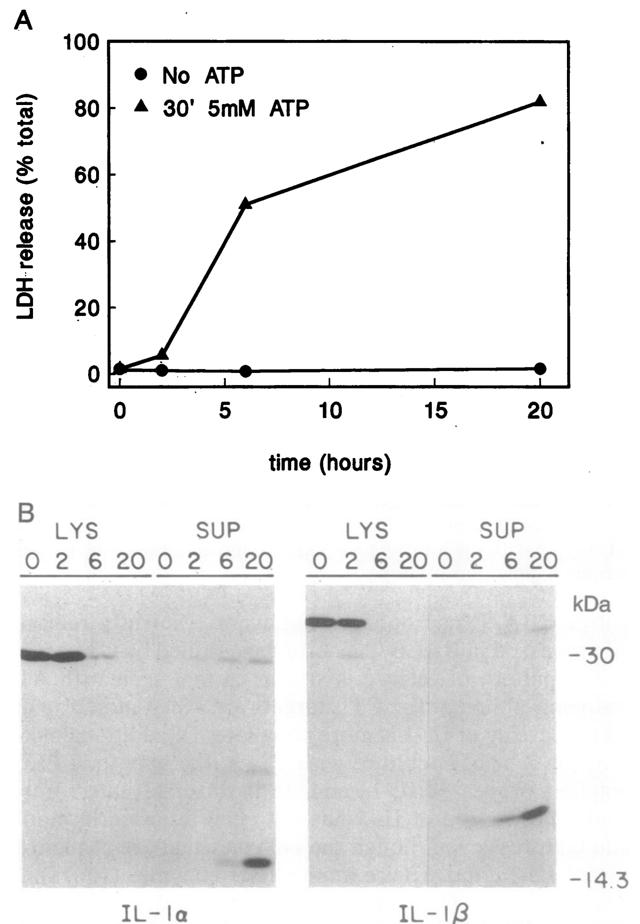

FIG. 2. IL-1α and -1β are released and processed during apoptosis. Murine PECs were stimulated with LPS and pulse-labeled with [^{35}S]methionine. Medium containing 5 mM ATP was added and the cells were incubated for 30 min. Fresh medium was then added (time 0). (*A*) LDH release was measured at 0, 2, 6, and 20 hr in ATP-treated and control cultures. (*B*) IL-1 release was analyzed by immunoprecipitation from the cell lysates (LYS) and culture supernatants (SUP).

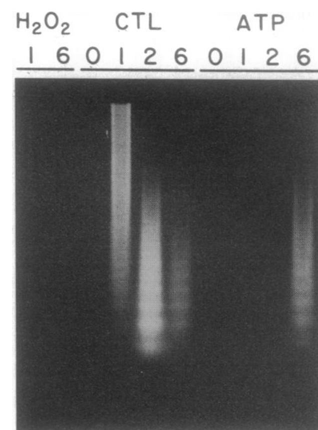

FIG. 3. DNA fragmentation occurs in those cultures in which IL-1β processing is seen. PECs were stimulated and treated with 20 mM H_2O_2, allospecific CTLs, or 5 mM ATP. At 0, 1, 2, and 6 hr after onset of injury, cells were harvested and analyzed for DNA fragmentation.

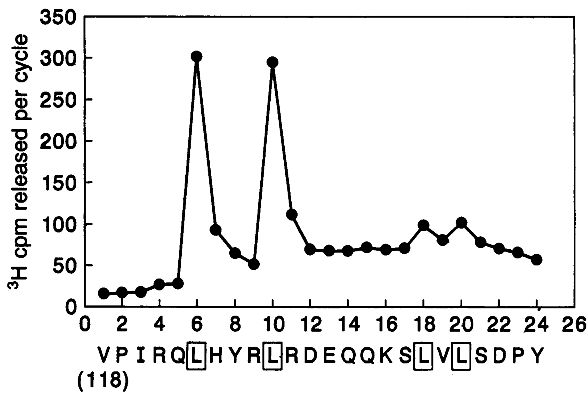

FIG. 4. The N terminus of processed murine IL-1β released from ATP-treated cells is Val-118. [^{3}H]Leucine-labeled processed murine IL-1β was purified from ATP-treated, LPS-stimulated J774 cells and then subjected to automated Edman degradation. Released radioactivity (y axis) was plotted as a function of successive cycles (x axis) and aligned with the murine pro-IL-1β amino acid sequence with the leucines highlighted by boxes.

control CBA/J macrophages but were efficiently released and processed in BALB/c macrophages killed by CTLs (Fig. 5). The pattern of release is similar to that seen with ATP treatment, although the CTL targets are killed more rapidly and processing of IL-1 is more complete. Of additional note, following 2 hr of coculture with CTLs, the apoptotic PECs contained processed IL-1α and -1β in the cell lysates. Intracellular processing of IL-1 has not been reported in monocytic cultures, even though the enzymes that are thought to process IL-1α and -1β are intracellular enzymes (10, 11). In addition, a 28-kDa form of IL-1β was observed in cell lysates. This may have been the product of cleavage of pro-IL-1β between Gly-25 and Ala-26, which is an alternative cleavage site for the IL-1β convertase (12).

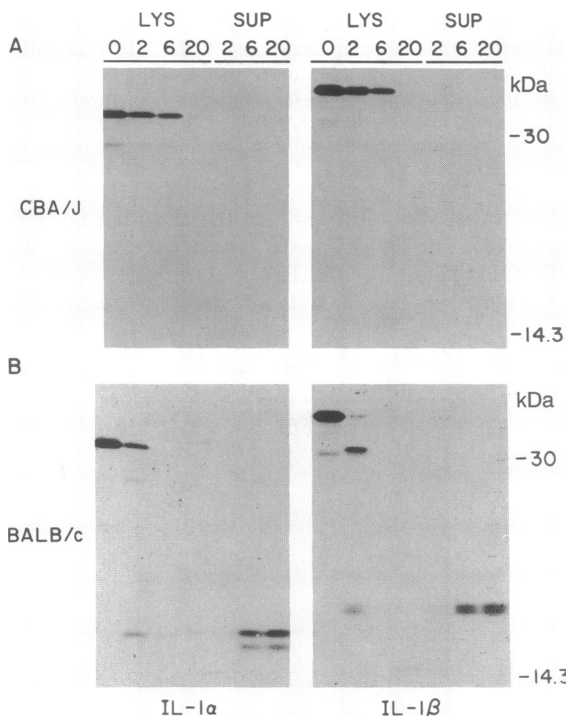

FIG. 5. IL-1α and -1β are processed and released during specific CTL–target interaction. CBA/J (*A*) or BALB/c (*B*) PECs were stimulated with LPS and pulse-labeled with [^{35}S]methionine. CTL allospecific to H-2^d were added at a killer/target ratio of 10:1. At 0, 2, 6, and 20 hr of coculture, IL-1 release was analyzed by immunoprecipitation from the total cell lysates (LYS) and culture supernatants (SUP).

To investigate whether this processing took place in the macrophages or the CTLs, we allowed the coculture to proceed for 30 min and then removed the T cells. Apoptosis of the macrophages, intracellular processing, and release of IL-1α and -1β were seen in this experiment as well (data not shown). This suggests that the processing occurs within the macrophage and that once the apoptotic signal has been delivered, further participation of the CTL is not required. Perhaps the apoptotic stimulus activates the IL-1β convertase from an inactive to an active form. Alternatively, apoptosis may result in an altered compartmentalization of cellular components that permits an interaction of the pro-IL-1α and -1β molecules with their respective proteolytic enzymes.

IL-1 is an important cytokine responsible for many of the biological effects observed during acute inflammation. Its role in directly regulating T-cell activation, however, is less well established. Nevertheless, IL-1 is intimately involved in the interaction of T cells with antigen-presenting cells. In previous studies (25–27), CD4$^+$ T cells were shown to induce IL-1 mRNA and protein production as a result of both direct cellular contact and the secretion of tumor necrosis factor. Another study, by Wasik *et al.* (28), showed the induction of IL-1 after interaction with CD8$^+$ T cells as well. Here we have shown that the interaction of CD8$^+$ T cells and macrophages results in apoptosis and the processing and release of IL-1. Preliminary experiments have been performed to evaluate whether CD4$^+$ T cells capable of inducing IL-1 expression could activate IL-1 release. These studies failed to show release or processing of IL-1α or -1β; however, the cytolytic potential of these cells is not clear. Further analysis of CD4$^+$ clones is needed. The findings thus far suggest there are two central processes for IL-1 production and release: (*i*) contact with microbial components and (*ii*) interaction with T cells.

Release of IL-1 from Freshly Isolated Human Monocytes Correlates with Apoptosis. We (20) and others (29, 30) have observed that freshly isolated monocytes show much higher fractional release of IL-1 in response to LPS than peritoneal or alveolar macrophages. Additionally, monocytes that are maintained in culture for 1 day or more prior to stimulation with LPS show a dramatic reduction in IL-1 release (20). We have suggested that the release of IL-1 seen in fresh monocytes is due to the significant levels of cell death in these cultures, perhaps due to withdrawal from growth factors present *in vivo*. Because other hematopoietic cells undergo apoptosis when withdrawn from growth factors (31, 32), and given that apoptosis can lead to processing, we tested whether the release of processed IL-1β from fresh human monocytes was due to apoptosis in these cultures. Fig. 6 shows that freshly isolated, but not cultured, human monocytes from the same donor demonstrated DNA fragmentation by 20 hr after treatment with LPS. The percentage of cells dying in cultures of fresh monocytes, as assessed by LDH release at 24 hr, averaged 14% among 12 different donors. This correlates with $\approx$10% IL-1 release in these same cells as well as with estimates of the percentage of cells that are releasing IL-1β based on a single-cell assay (29). Fresh monocytes that have not been stimulated with LPS also undergo DNA fragmentation; however, because unstimulated cells produce very little IL-1, release of IL-1 is undetectable (data not shown).

The degree of DNA fragmentation observed in LPS-stimulated monocytes (Fig. 6) is less extensive than observed in the ATP-treated cells or in the macrophages that were CTL targets (Fig. 3). This may reflect the activation of additional proteases or nucleases in monocytes or may simply be a consequence of the lower level or slower time course of cell injury. Additional support for the presence of a population of

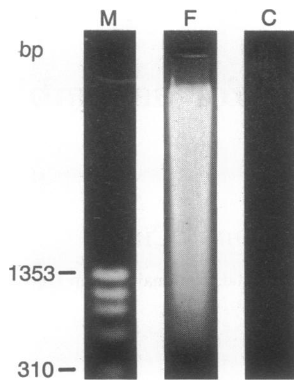

FIG. 6. Freshly isolated human monocytes undergo low levels of DNA fragmentation. Human monocytes purified by elutriation were analyzed immediately (lane F) or after culture for 24 hours (lane C). Fresh or cultured cells were LPS-stimulated for 2 hr. The medium was replaced with medium without LPS and the cells were incubated for an additional 20 hr. Lane M, size markers (bp, base pairs).

apoptotic cells in these monocyte cultures includes the observation that the dying cells were smaller than viable cells as determined by flow cytometric analysis of cells stained with propidium iodide (data not shown). Our data complement studies of IL-1β release from LPS-stimulated human monocytes by Lewis *et al.* (29). Using a plaque assay, they showed that although 90% of LPS-stimulated monocytes produced intracellular IL-1β, the cytokine was released by only 6% of the monocyte population and that the cells that released IL-1β were smaller than the remaining cells. These data should be interpreted with the consideration that apoptotic cells show prominent cytoplasmic condensation (21).

Our data demonstrate that cell injury can result in the release of high levels of biologically active IL-1α and -1β. Although we cannot exclude the existence of other mechanisms for release of IL-1 *in vivo*, we feel that cell injury can account for the IL-1 release observed *in vitro*. Cell injury has also been suggested to be a mechanism for release of basic fibroblast growth factor, a protein with structural features similar to IL-1 (33). The accumulation of substantial quantities of these cytokines in the cytoplasm with no detectable release from viable cells has suggested that they may have important intracellular activities as well. Recent data support these concepts (34, 35); however, these cytokines also have potent activities as extracellular mediators. Our data support a vital role for IL-1 as a signal that cellular injury has occurred and as a mediator that initiates the inflammatory response to facilitate tissue repair.

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