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Alpha-1 Antitrypsin Response of Stimulated Alveolar Macrophages

Zhi-An Yuan, Kenneth J. Soprano, and Friedrich Kueppers

Departments of Medicine and Microbiology, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

Abstract Alpha-1 antitrypsin messenger RNA (A1AT mRNA) was determined in alveolar macrophages and in peripheral blood monocytes of healthy individuals using a sensitive RNase protection assay. Determinations were made of bacterial lipopolysaccharide (LPS) stimulated and unstimulated cells. We found that the amount of A1AT mRNA increased 7.3 and 14 times after 4 h of incubation with LPS for monocytes and macrophages, respectively (relative to total RNA). The increase was 12.3 and 14.8 times, respectively, when expressed as increase per cell. In both cell types there was wide interindividual variation in LPS response: 2–36 and 5–12 times for monocytes and macrophages, respectively.

The possible significance of A1AT production of monocytes and macrophages may be the local control of granulocytic proteases such as elastase and cathepsin G.

Key words: alpha-1 antitrypsin messenger RNA, alveolar macrophages, monocytes, LPS stimulation

The major site of synthesis of alpha-1 antitrypsin (A1AT) is the liver [Kaighn and Prince, 1971]. However, as more sensitive methods for the determination of A1AT and specific A1AT messenger RNA (A1AT mRNA) have become available the production of A1AT by other cells has been demonstrated. Monocytes, macrophages [VanFurth et al., 1983; Perlmutter et al., 1985], and, most recently, even intestinal epithelial cells [Perlmutter et al., 1989] have been shown to produce A1AT or at least to contain its specific mRNA.

Northern blotting has been the most common method used to demonstrate the presence of specific mRNA. Depending on the relative abundance of the message, the amount of RNA necessary to obtain a quantitative signal may be quite large. If the cells to be investigated are only available in small numbers, this could be a limiting factor.

We have therefore developed an RNase protection assay which permits us to reliably measure A1AT mRNA levels in peripheral blood monocytes and alveolar macrophages of single donors.

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Address reprint requests to F. Kueppers, Temple University Health Sciences Center, 3401 N. Broad Street, Philadelphia, PA 19140.

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Using this assay we show that both alveolar macrophages and peripheral blood monocytes isolated from individual donors contain low but detectable concentrations of A1AT mRNA and that 4–6 hours following stimulation with LPS steady state levels of A1AT mRNA are significantly elevated. We also demonstrate a considerable amount of apparent interindividual heterogeneity.

METHODS

Isolation of Peripheral Blood Monocytes

One standard unit of blood (approximately 450 ml) or 100 ml heparinized blood from healthy male and female individual donors (2,000 units of heparin in 100 ml of blood) was diluted 1:1 with phosphate buffered saline (PBS), pH 7.6.

Mononuclear cells (PBM) were isolated by Ficoll (type 400: Sigma) Hypaque (Winthrop-Breon) gradient centrifugation [Colotta et al., 1984]. The PBM layer was collected and the cells were washed twice with PBS and then suspended in RPMI-1640 medium containing 10% fetal calf serum in plastic tissue culture dishes (100 mm: Falcon, Oxnard, CA) and incubated at 37°C in 5% CO_2. After 4 h, non-adherent cells were removed with the medium. The adherent cells were gently washed with PBS. RPMI-1640 without serum was added and the cells were

cultured for the time periods indicated with or without bacterial lipopolysaccharide (LPS, 100 ng/ml E. coli 0111 B4 TCA extract: Sigma). The cells were harvested with a rubber policeman and washed with PBS 3 times. More than 95% of these cells were monocytes. Routinely 2.2 ± .69 × 10^7 monocytes were isolated from 100 ml of blood. When units of blood were used as starting material (450 ml) 5.7–9.55 × 10^7 monocytes were isolated.

Isolation of Alveolar Macrophages

Alveolar macrophages were obtained by standard bronchoalveolar lavage usually from the right middle lobe [Reynolds, 1988] of healthy nonsmoking individuals (males and females, age range 22–24 years). A total of 240 ml saline solution was introduced into the middle lobe through the wedged bronchoscope in 60 ml aliquots with a syringe and immediately suctioned back into the same syringe. Routinely 1–3 × 10^7 cells were obtained; 95–98% of the recovered cells were alveolar macrophages.

One hundred milliliters of the lavage fluid was centrifuged for 15 min at 1,500 rpm. The pellet was resuspended in PBS and recentrifuged. Finally the cells were suspended in 20 ml of RPMI-1640 medium containing 10% fetal calf serum. The cells were counted and were plated in 2 tissue culture dishes approximately 5 × 10^6 cells to each dish in 10 ml of medium without fetal calf serum. To one dish LPS was added to a final concentration of 100 ng/ml; the other dish served as control with no addition. The cells were incubated at 37°C in a standard culture incubator in a 5% CO_2 atmosphere. After 4 h the cells were harvested, recounted, and washed twice with PBS. The final pellet was immediately frozen in liquid nitrogen and kept until used for RNA isolation.

Isolation of RNA [Chirgwin et al., 1979; Shen, personal communication]

The cells were resuspended in 4 M guanidine thiocyanate (Fluka, Switzerland) and were lysed by adding a 10% sarcosyl solution as to achieve a final concentration of .5% sarcosyl. The cells were agitated with a Vortex and kept on ice for 20 min during which time the suspension was agitated 2–3 times. Total RNA was isolated by cesium chloride gradient centrifiguration in .8 ml Beckman open top centrifuge tubes. About 400 μl of lysate was layered on top of 310 μl of a CsCl solution (5.7 M CsCl optical grade, BRL, 50 mM sodium acetate, 1 mM disodium EDTA, pH 5.0). Centrifugation was performed at 35K rpm at 20°C for 16–18 h in a Beckman L8-60 ultracentrifuge using a TI 50.1 swinging bucket rotor. Following centrifugation the top portion of the gradient was removed so that 100 μl remained at the bottom of the tube. The 100 μl of solution containing the total RNA was transferred to a 1.5 ml Eppendorf tube. The empty ultracentrifuge tube was rinsed 3 times with 100 μl RNase free water. The washes were combined with the original 100 μl and were precipitated with 1/10 volume of potassium acetate (3 M, pH 5.0) and 2 volumes of ethanol. Routinely 10–20 μg of RNA was obtained from 2–3 × 10^7 monocytes by this method. The total RNA extracted from unstimulated alveolar macrophages was 9.7 ± 1.45 μg/10^7 cells; from blood monocytes the amount was 4.99 ± 1.86 μg/10^7 cells. RNA determinations were made spectrophotometrically at 260 nm. All RNA preparations were submitted to agarose gel electrophoresis under denaturing conditions to verify the occurrence of the characteristic 28S and 18S bands.

RNase Protection Assay

We followed the procedure of Owen et al. [1987] which is a modification of Melton's original method [Melton et al., 1984]. The principal reagent for this assay is a specific antisense RNA that hybridizes with the A1AT message to be detected. The production of this antisense RNA is described below.

Construction of the PGEM-blue PI plasmid. A 1.65 kb H-A1AT DNA fragment (Pst-Pst) spanning the nucleotides 4587 to 6233 was used. The numbers refer to those given by Long et al. [1984]. This fragment consists of 1646 nucleotides containing exon II (649 bp) and parts of the neighboring introns according to the map of Long et al. [1984]. One microgram of PGEM-blue vector DNA was linearized with Pst 1 and treated with bacterial alkaline phosphatase.

Seventy-five nanograms of the digested vector was ligated to 38 ng of A1AT DNA fragment. E. coli strain TB1 was transformed with the ligated plasmid DNA using a standard protocol [Sambrook et al., 1989]. The resulting Ampicillin resistant colonies were screened as described below.

Selection of clone. Several individual clones were selected and grown overnight in 5 ml LB broth at 37°C with shaking. The cells were lysed and the plasmid DNA was isolated. The plasmid

DNA was digested with Pst 1. The presence of the 1.65 kb insert was verified by agarose (1%) electrophoresis. A positive clone was selected and grown overnight in 500 ml LB broth and the plasmid DNA was extracted and digested with Bam H1 to determine the orientation of the insert. Figure 1 shows that the orientation can be deduced from the presence of characteristic DNA fragments as visualized by polyacrylamide gel electrophoresis (5% polyacrylamide, buffer .05 M Tris, boric acid .05 M, Na_2 EDTA .001 M, pH 8.2, 2 h at 80 V) (Fig. 2). A clone that contained the insert placed next to the SP6 promoter in the antisense direction was selected for the assay. The plasmid was designated PGEM-PI-9 (PI for proteinase inhibitor). The plasmid DNA was linearized with Bam H1, extracted with phenol, and precipitated with ethanol. The linear DNA was suspended in water and dialyzed on a millipore filter (type VM, size 0.05 Um) against water for 30 min, then adjusted to a concentration of 0.5 μg/μl. Synthesis of labeled RNA was initiated by adding Sp6 RNA polymerase (20 units/μg DNA) and ^{32}P UTP (50 μC in 5 μl) to 1 μg of linear DNA (PGEM-PI-9/BAM HI) for 1 h at 40°C. The DNA template was removed by digesting with RQI DNase (6 units) for 40 min at 37°C.

The ^{32}P labeled RNA was extracted with RNA phenol and chloroform and precipitated with 1/10 volume of Potassium acetate (3 M, pH 5) and 2 volumes of ethanol. Finally, the quality of the labeled ^{32}P-RNA was assayed by electrophoresis in a 5% denaturing acrylamide/8 M urea mini gel followed by autoradiography.

Hybridization. The total cellular RNA previously obtained from monocytes was vacuum dried and then resuspended in 28 μl of hybridization buffer (40 mM Pipes, pH 6.7, 400 mM NaCl, 1 mM EDTA). The labeled RNA probe was added in 2 μl. Following denaturation at 80°C for 5 min the mixture was incubated overnight at 55°C. In order to degrade non-hybridized single stranded RNA, RNase A 20 μg/ml and RNase T1 (22,600 units/ml) were added in 300 μl of digestion buffer (800 mM NaCl, 10 mM Tris, 5 mM EDTA, pH 7.5); incubation was for 1 h at 45°C. To remove the enzyme proteinase K (50 μg/ml) and 20 μl of 10% SDS were added and incubated for another 15 min at 37°C. RNA was extracted with RNA phenol and precipitated with 20 μg of carrier tRNA and ½ vol 7.5 M ammo-

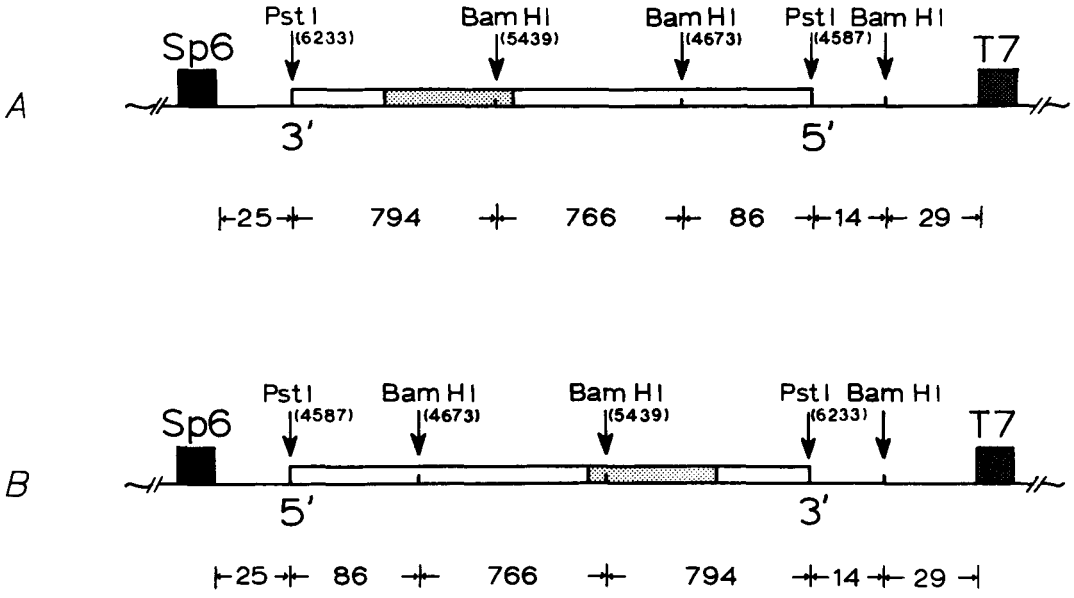

Fig. 1. The 1.6 Kb human A1At fragment can be inserted into the PGEM-blue vector DNA in either of two orientations as shown in **A** and **B**. The two orientations can easily be distinguished from each other by the different fragment patterns on electrophoresis after digestion with Bam H1. In the antisense orientation with the 3' end near the Sp6 promoter there will be 3 fragments of sizes 100, 766, and 3520 bp (A) With the orientation with the 5' end of the insert (sense) near the Sp6 promoter the following fragments result: 766, 808, and 2822 bp (B). The numbers in parentheses refer to the complete nucleotide sequence of the human A1AT gene given by Long et al. [1984].

nium acetate and ethanol. The precipitate was dissolved in 10 μl of H_2O and 10 μl of loading buffer (25% formamide, 75% xylenecyanol, 250 mM EDTA, pH 7.5, and 75 mg/100 ml Bromophenol blue) and analyzed by electrophoresis in a denaturing 10% polyacrylamide/8 M urea gel at 400 V and 30–40 mA, using a heat shield. After the run, the gel was treated with 10% acetic acid for 10 min to remove urea and was washed with water and was then transferred to a dryer. Finally, the dried gel was exposed to XRP-5 Kodak x-ray film for autoradiography in a cassette containing QUATA-3B intensifying screens. Human liver RNA (0.5 μg) was used as positive control. Yeast total RNA was used as negative control. There was never any protection using this RNA. Thus the observed protection was indeed specific for A1AT mRNA. The bands were quantitated with a Zeineh soft laser scanning densitometer.

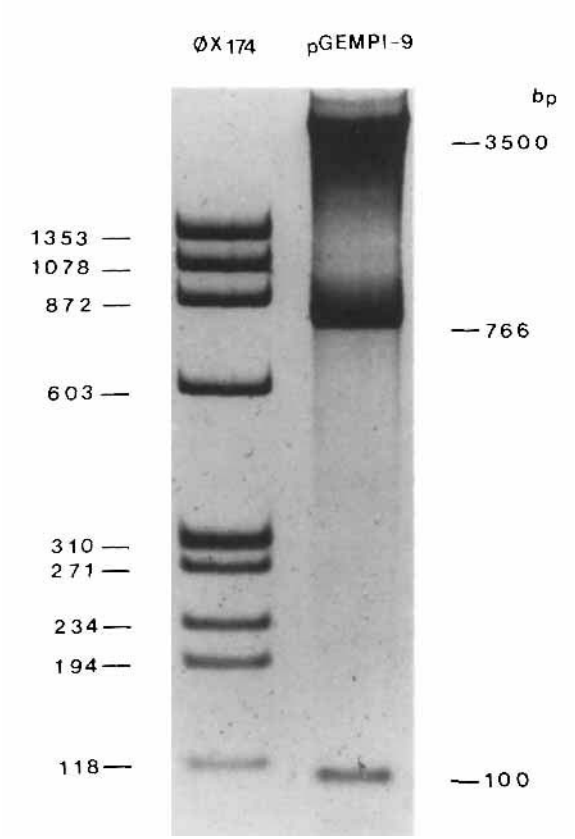

Fig. 2. Polyacrylamide gel electrophoresis of DNA fragments after treatment of PGEM-PI-9 with Bam H1. The appearance of a 766 bp and a 100 bp fragment indicates that the A1AT fragment is incorporated into the plasmid in the antisense direction. See also legend to Fig. 1. Molecular size markers are shown on the left.

RESULTS

Total cytoplasmic RNA from LPS-stimulated and unstimulated monocytes and alveolar macrophages was analyzed by the RNase protection assay using the PGEM-PI-9 probe. (The probe is available to interested investigators upon request.) Figure 3 shows that the lowest levels of A1AT mRNA were found in unstimulated monocytes and macrophages. Although the signals were weak there were sufficient amounts of specific message to yield quantifiable bands by autoradiography (Fig. 3). There was no difference in A1AT mRNA concentration between preparation from monocytes without incubation (0 time) and those that were incubated without LPS.

In response to LPS stimulation for 4 h the amount of specific message increased 7.33 ± 5.3 times in monocytes (cell preparations from 5

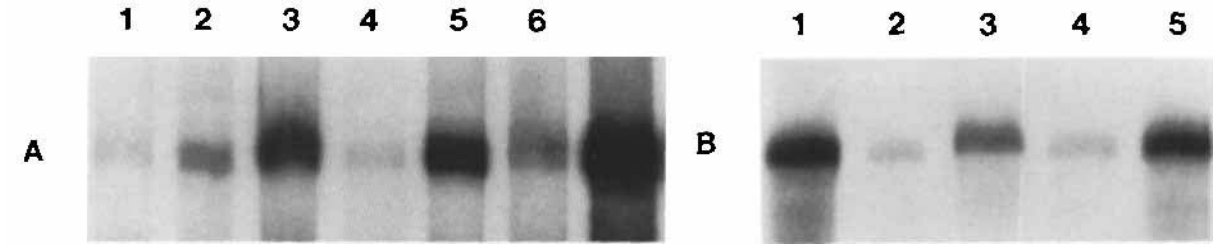

Fig. 3. Autoradiography of the electropherogram of the free labeled probe, the last step of the RNase protection assay. **A:** RNA extracted from monocytes incubated with LPS (100 ng/ml) for 4 h as indicated. Amounts of total RNA applied to gel are given in parentheses in the following. **Lane 1:** No incubation, 0 time (5 μg). **Lane 2:** Same cells as in 1 after 4 h incubation with LPS (5 μg). **Lane 3:** Cells from different donor after 4 h incubation with LPS (5 μg). **Lane 4:** Cells from another donor, 4 h incubation, no LPS (2 μg). **Lane 5:** Cells from individual in 4 after 4 h incubation with LPS (2 μg). **Lane 6:** Same as in lane 4 but 4 μg RNA. **Lane 7:** Human liver RNA (1.5 μg). **B:** RNA extracted from alveolar macrophages incubated for 4 hours with or without LPS (100 ng/ml). **Lane 1:** Liver RNA (.5 μg). **Lane 2:** Control, no LPS (4 μg). **Lane 3:** Same cells as in 2 after 4 h with LPS (3 μg). **Lane 4:** Different cells, no LPS (12 μg). **Lane 5:** Same cells as in 4 after 4 h with LPS (11 μg).

individuals) and 13.9 ± 11.7 times in alveolar macrophages (cell preparations from 4 individuals); this difference between the two cell types was, however, not statistically significant (Fig. 4A,B). These values represent means and standard deviations of all individual samples. Each value of cells from an individual donor was compared to the value of cells from the same donor after stimulation. Although the interindividual variation was considerable, as can be judged by the standard deviation, we always observed an increase in A1AT mRNA following LPS treatment.

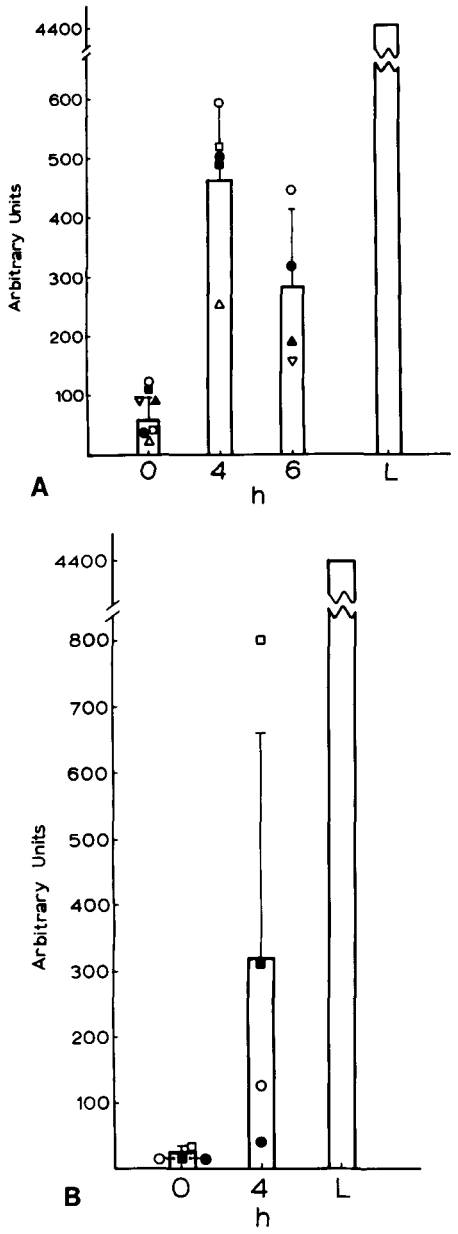

Fig. 4. A: Amount of A1AT mRNA in monocytes. Values are expressed in arbitrary units adjusted for the amount of RNA applied to the gel and related to a constant amount of liver RNA included in each gel. The hours of incubation with LPS are indicated. Identical symbols indicate results of cells from the same individual at different time points. L, A1AT mRNA in human liver RNA. B: Amounts of A1AT mRNA in alveolar macrophages (expressed as in A) after incubation for 4 h with LPS (100 ng/ml) and without LPS. L, A1AT mRNA in human liver RNA.

In the timed experiments with monocytes (Fig. 4A) the increase in message was greatest (7.33 ± 5.27 times greater than the control value) after 4 h of LPS stimulation and was somewhat lower, but still above the controls, after 6 h of LPS stimulation. A few sporadic values that were available (not shown) in some instances in which we had sufficient cells show that after 12 and 18 h the specific message levels were always above baseline values (3.2 times and 2.4 times, respectively) but not as high as the 4 and 6 h levels.

The observed increase in A1AT mRNA levels was not due to a nonspecific increase in total cytoplasmic RNA. RNA was routinely determined in all samples before and after they were subjected to the RNase protection assay. The total RNA extracted from the control monocytes was 4.99 ± 1.86 $\mu g/10^7$ cells. After 4 h incubation with LPS the RNA concentration increased to 8.37 ± 4.31 $\mu g/10^7$ cells (not significantly different from control cells; $P = .09$). After 6 h LPS stimulation the RNA level was 10.77 ± 5.5 $mg/10^7$ cells (significantly different from control cells; $P = .026$). The amount of RNA approximately doubled while the specific A1AT mRNA increased 7 times. The total RNA extracted from the alveolar macrophages was 10.3 ± 5.6 $\mu g/10^7$ cells in the LPS-stimulated cells and 9.7 ± .75 in the unstimulated cells. Thus, in macrophages there was no significant change in the total RNA after LPS stimulation while the specific message increased to almost 14 times the baseline value.

DISCUSSION

Our results show that both human peripheral blood monocytes and alveolar macrophages increase their levels of A1AT mRNA in response to LPS. The amount of specific A1AT mRNA increased 7 and 14 times in monocytes and macrophages, respectively. Although the increase of specific message in alveolar macrophages appeared to be greater than in monocytes the difference was not statistically

significant, most likely due to the large standard deviation.

It should be noted that the extent of induction did vary from individual to individual. The lowest responding macrophages showed a twofold increase in A1AT mRNA after 4 h LPS stimulation while the highest responder showed an increase of 36 times the baseline value. For the monocytes the increase was between five- and twelvefold after 4 h LPS stimulation.

One of the advantages of the RNase protection assay is its great sensitivity which makes it possible to use the relatively small amount of RNA available from cells of individual donors.

A major difference between the two cell types was the amount of total extractable RNA: 4.99 ± 1.86 $\mu g/10^7$ monocytes and 9.7 ± .75 $\mu g/10^7$ alveolar macrophages. After incubation with LPS the total RNA in monocytes nearly doubled while that of alveolar macrophages remained the same. However, even if the increase of specific A1AT mRNA is expressed per cell and not relative to total RNA the increase in both cell types is similar and highly significant ($P < .001$): 12.3 times for monocytes and 14.8 times for macrophages.

This observation is in keeping with current thinking that the monocyte as a less differentiated cell can be induced to produce a wider range of different mRNAs, a potential that the alveolar macrophage in its final stage of differentiation no longer possesses.

The majority of activated alveolar macrophages is probably derived from monocytes that have recently left the vascular compartment and have migrated into the alveolar space where they reach their final stage of differentiation [Hirata et al., 1986]. We may assume that in vivo monocytes rapidly undergo a similar process when they are stimulated by LPS.

Barbey-Morel et al. [1987] reported experiments similar to ours. However, they incubated peripheral blood monocytes with LPS (100 ng/ml) for longer periods of time, from 8 to 24 h, and found only a modest 1.5- to 2.5-fold increase of steady state A1AT mRNA while the A1AT secreted into the medium increased 4.5–8.7 times. In another report by Perlmutter and Punsal [1988] using a similar experimental design with somewhat lower LPS concentration (10–50 ng/ml) no increase in A1AT mRNA was found after 12 and 16 h while the synthesis of A1AT protein increased four- to fivefold.

These discrepancies are possibly due to differences in timing since we made our measurements after 4 or 6 h while the other investigators used longer incubation times of 16 and 24 h. In a few instances when we had sufficient monocytes to measure the LPS effect after 12 and 18 h, we observed that the specific message levels were lower than those at 4 and 6 h but that they were always above the values of untreated control cells. A possible explanation that could accommodate our results as well as those quoted above [Perlmutter and Punsal, 1988] is that early in the LPS response there is indeed a substantial increase of A1AT mRNA but later, after 16 and 24 h, mRNA accumulation is reduced. It remains to be determined whether this change is due to altered transcription, processing stability, transport, or a combination of these factors.

Human liver RNA was always included as a positive control in our assays; yeast RNA was a negative control. The amount of A1AT mRNA in total liver RNA was 90–220 times greater than that found in monocytes or macrophages. These ratios are in good agreement with those of Mornex et al. [1986].

While most A1AT found in peripheral blood is synthesized by the liver [Hood et al., 1980] the small quantities of A1AT of macrophage origin may, however, be important in extravascular compartments like the alveoli. During an inflammatory response activated macrophages present at the site and newly recruited monocytes could contribute directly to protection against proteinases liberated from macrophages and from granulocytes that are abundant at inflammatory sites.

Free leukocytic elastase is generally recognized as a most destructive enzyme that can attack multiple protein substrates and has a major role in causing damage to tissue and structural proteins in inflammatory diseases and in emphysema. Local production of A1AT would counteract its proteolytic activity. Other more specific actions of elastase include exposure of binding sites for bacteria on mucosal surfaces [Dal Nogare et al., 1987] and possibly the conversion of the biologically inactive interleukin 1 beta (IL 1β) precursor to an active form. Black et al. [1988] have shown that cathepsin G and neutrophil elastase can produce active forms of IL 1β. If these forms are important in vivo a major inhibitor of the two neutrophil proteases such as A1AT may contribute to the local control of inflammation.

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