IDENTIFICATION OF A SPECIFIC INTERLEUKIN 1 INHIBITOR IN THE URINE OF FEBRILE PATIENTS

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Interleukin 1 (IL-1)[1] is a 15,000-dalton macrophage-derived protein that is defined by its ability to induce the differentiation of immature T lymphocytes (1). In addition, recent work has demonstrated that IL-1 or closely related molecules act on the anterior hypothalamus to produce fever (2); act on the liver, to induce the production of acute phase proteins such as the serum amyloid-associated protein (SAA) (3); and act on synovial cells to induce secretion of prostaglandins and collagenase (4). These diverse properties suggest that IL-1 is one of the major mediators of inflammation in the body (5). Little is known about the feedback regulation of the effects of IL-1. However, circulating antiproteinases such as α_1-anti-trypsin are thought to be involved in modulating inflammatory reactions (6), and recent reports suggest that this molecule may inhibit the in vitro effects of IL-1 as well (7).

During an investigation of the functional activities of febrile urinary proteins, it was noticed that urine from febrile patients contained a potent inhibitor of IL-1-induced thymocyte proliferation. In this report, we describe the identification and characterization of this inhibitor.

Methods

Patients. Urine was collected from 61 febrile patients, 23 afebrile patients, and 19 normal volunteers. Febrile patients were classified by their clinical diagnosis and consisted of patients with pulmonary or extrapulmonary tuberculosis, other bacterial infections, viral infections, or patients with presumed noninfectious etiologies for their fever (i.e., acute myocardial infarction, or cerebrovascular accident). 11 ambulatory, febrile patients with either viral infections, or localized bacterial infections (i.e., tonsillitis) but who had received no medication of any kind were also studied. Afebrile patients were selected from a group roughly matched to the febrile group for age, sex, and race, and had diagnoses of hypertension, headache, diabetes, or ovarian cyst. Patients with known urinary tract infections or septicemia were excluded from the study.

Urine Collection and Processing. Urine was collected and stored at 4°C. It was then

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[1] *Abbreviations used in this paper:* CRP, C-reactive protein; ^{3}H-TdR-methyl[^{3}H]thymidine; IL-1, interleukin 1; IL-2, interleukin 2; PHA, phytohemagglutin; PMA phorbol myristic acetate; SAA; serum amyloid-associated.

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dialyzed twice against 100 volumes of phosphate-buffered saline (PBS), pH 7.4, sterilized by filtration through a 0.29-μm Acrodisc filter (Gelman Sciences, Inc., MI) and frozen at −70°C until use.

Mouse Thymocyte Assay. Before bioassay, all specimens were dialyzed against 100 volumes of RPMI 1640 (Flow Laboratories, McLean, VA) and resterilized by filtration. Samples were assayed for IL-1 activity with thymocytes from 4–6-wk old C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, ME). Single cell suspensions of thymocytes were prepared by gently pressing the tissue between the frosted ends of microscope slides. Aggregates were removed by a 5-min unit gravity sedimentation. The cells were washed three times with Hanks' Balanced Salt solution (HBSS) and resuspended to a density of 1.5×10^7 cells/ml in RPMI 1640 containing 10% heat-inactivated fetal calf serum, antibiotics, additional 2 mM glutamine, and 2.5×10^{-5} M 2-mercaptoethanol. Thymocytes were cultured for 72 h at 1.5×10^6 cells/well in flat-bottom Falcon Microtest III tissue culture plates (Becton Dickinson, Oxnard, CA) in the presence or absence of 1 μg/ml PHA (purified phytohemagglutinin, Wellcome Research Laboratories, Beckenham, England), IL-1, and various dilutions of test urine. Cultures were pulsed with 0.5 μCi ^{3}H-TdR (5.0 Ci/mMol, Amersham Corp., Arlington Heights, IL) for the final 4 h of incubation. Cells were collected on glass fiber filter paper using a semiautomated cell harvester (Mash II, Microbiological Associates, Bethesda, MD).

Lymphokines. Murine IL-1 was obtained from P388D$_1$, cells stimulated with phorbol myristic acetate (PMA) (8). The 7-d culture supernatants were centrifuged, concentrated by ammonium sulfate precipitation (40–65% cut), dialyzed twice against 100 volumes each of PBS, and frozen in aliquots at −70°C until use.

Human IL-1 was obtained from the culture supernatant of endotoxin-stimulated peripheral blood mononuclear cells. This was partially purified by fast protein liquid chromatography (FPLC) and was the generous gift of Dr. Robert Newton (Dupont Corp., Wilmington, DE).

Murine IL-2 was prepared from the supernatant of PMA-stimulated EL-4 cells using the protocol of Farrar et al. (9). The supernatant was centrifuged, concentrated by ammonium sulfate precipitation, dialyzed, filtered, and stored as described above.

Human IL-2 was purified by immuno-affinity chromatography of the culture supernatant of Jurkatt cells (10) and was the generous gift of Dr. Richard Robb (Dupont Corp.).

Gel Filtration of Murine IL-1. All biochemical manipulations were carried out at 4°C. The supernatant of PMA-stimulated P388D$_1$ cells was collected and concentrated as described above. 6 ml of the concentrated supernatant was applied to a 2.6- × 74-cm Sephacryl S-200 column equilibrated in buffer (50 mM Tris, 100 mM NaCl, pH 7.5). A flow rate of 30 ml/h was used and 5-ml fractions were collected. Aliquots of the fractions were filter sterilized and assayed for IL-1 activity in the presence or absence of PHA. The column was calibrated by marker proteins of known molecular weight. These included bovine serum albumin (BSA, mol wt 67,000), ovalbumin (OVA, mol wt 45,000), chymotrypsinogen (CHYMO, mol wt 25,000), and cytochrome C (CYTOC, mol wt 12,400).

Purification of Urine Inhibitor. Urine from a febrile patient was adjusted to 80% saturation with ammonium sulfate by the addition of powdered salt and was kept at 4°C overnight. The precipitate was collected by centrifugation at 10,000 rpm for 30 min, and was dissolved in one-tenth of original volume of buffer (50 mM Tris, 100 mM NaCl, pH 7.5), and dialyzed against the same buffer. The urine concentrate was applied to the Sephacryl S-200 column and chromatographed as described above.

Quantification of Interleukins and Urine Inhibitor. Units of IL-1 and IL-2 activity were determined using the reciprocal dilution method of Farrar et al. (11). Units of urine inhibitor were determined by culturing fourfold dilutions of urine in the presence of 0.25 U of an IL-1 standard. The reciprocal of the highest dilution that produced 30% inhibition of IL-1 activity was arbitrarily designated to be the inhibitory units/milliliter of the urine sample. Using this system, a urine that produced 30% inhibition only at the highest concentration tested (¼ dilution) would be assigned a value of 4 inhibitory units/ml.

% inhibition of thymocyte proliferation was calculated using the formula:

$$\% \text{ inhibition} = \left[1 - \frac{\text{CPM } ^3\text{H-TdR in the presence of IL-1 + inhibitor}}{\text{CPM } ^3\text{H-TdR in the presence of IL-1 + medium}} \right] \times 100.$$

Results

Identification of Urine IL-1 Inhibitor. Because of the functional relationship between IL-1 and endogenous pyrogen, and the small size of this molecule, we reasoned that the urine of febrile patients might be a good source of human IL-1. Despite numerous attempts, we were unable to detect significant IL-1 activity in either whole urine, or in urine fractions that were prepared by a variety of chromatographic separation techniques. However, during the course of these experiments it was noted that the febrile urines were inhibitory in the mouse thymocyte assay. We therefore investigated the character and specificity of this inhibitory effect.

Febrile urine was repeatedly dialyzed to remove any nonspecific toxic activity and filter sterilized. This material was tested for inhibitory activity using a PHA-stimulated mouse thymocyte IL-1 assay. 5 U/ml of IL-1 produced a good thymocyte proliferative response (12,500 cpm/culture) (Fig. 1). A 1/64 dilution of febrile urine inhibited this response by ~50% and there was a dose-dependent increase in inhibitory activity. Maximal inhibition was observed at a urine dilution of 1/4 (95% inhibition). In contrast, as seen in Fig. 1, urine from a normal individual was much less inhibitory.

The ability of IL-1 to overcome the effects of the inhibitor (INH) was tested next. A dilution of urine that contained a known amount of inhibitor (16 INH U/ml) was added to culture wells containing increasing amounts of IL-1 (Fig. 2). There was an inverse correlation between the amount of IL-1 in culture and the degree of inhibition. Inhibition was maximal at suboptimal concentrations of IL-

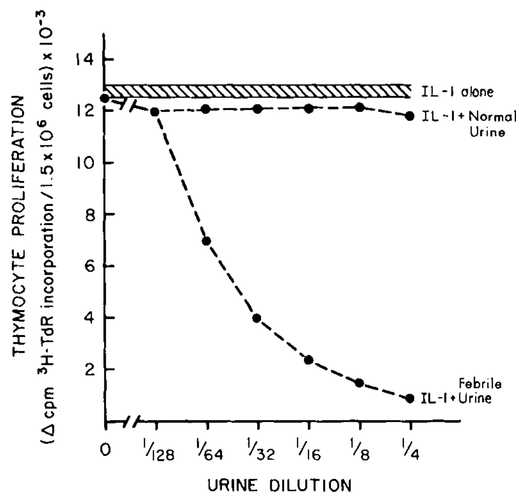

FIGURE 1. Inhibition of IL-1–induced mouse thymocyte proliferation by febrile urine. Mouse thymocytes were cultured in the presence of PHA (1 μg/ml), murine IL-1 (5 μg/ml), and increasing amounts of sterile, dialyzed urine. Results represent the arithmetic means of triplicate cultures.

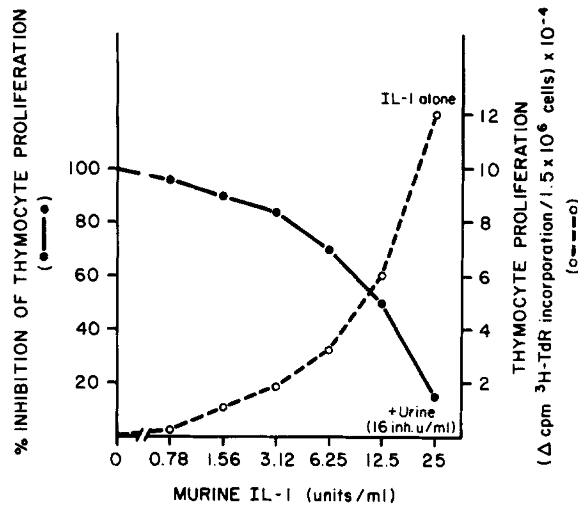

FIGURE 2. Competitive antagonism between IL-1 and the urine inhibitor on mouse thymocyte proliferation. Mouse thymocytes were cultured in the presence of increasing concentrations of murine IL-1 and a fixed amount of sterile, dialyzed febrile urine with known inhibitory activity. Results represent the arithmetic means of triplicate cultures.

1. Nevertheless, 50% inhibition was still observed at a concentration of IL-1 (12.5 U/ml) that produced a proliferative response of 50,000 cpm/well. At the maximum IL-1 concentration tested (25 U/ml), this dilution of urine was still significantly inhibitory (15% inhibition).

Analysis of the Specificity of the Urine Inhibitor. We next sought to determine the specificity of the urine inhibitor. Recent reports suggested that phorbol myristate acetate (PMA) may contaminate IL-1 preparations and be a significant mitogen in some in vitro assays (12). We therefore subjected the crude IL-1 preparation to further purification by Sephacryl S-200 gel filtration. This procedure has been shown to separate IL-1 from contaminating PMA (13). Two peaks were mitogenic for thymocytes in the presence of PHA (Fig. 3). One was ~15,000 daltons, which corresponded to the molecular weight of IL-1, and the second was >50,000 daltons, which corresponded to the presumptive elution profile of PMA bound to protein (13). PMA does not stimulate thymocytes in the absence of PHA, while IL-1 exhibits a diminished but significant thymocyte mitogenic activity (12). We therefore retested the column fractions in the absence of PHA. Under these conditions, only the 15 peak was mitogenic for thymocytes, confirming that it contained the authentic IL-1.

The urine inhibitor was then tested for activity against this partially purified IL-1 preparation. It was found to be as active against the 15-kdalton peak material as it was against the less pure preparation (Fig. 4), indicating that the inhibitor was blocking the effects of IL-1 and not the contaminating PMA.

Several other aspects of this experiment suggested that the inhibitor was acting against IL-1. First, the inhibitor blocked the mitogenic effects of IL-1 in the absence of PHA. Secondly, it was not inhibitory in the absence of IL-1, so that neither the low level spontaneous proliferative activity of thymocytes nor the small amount of proliferation induced by PHA alone was effected by the

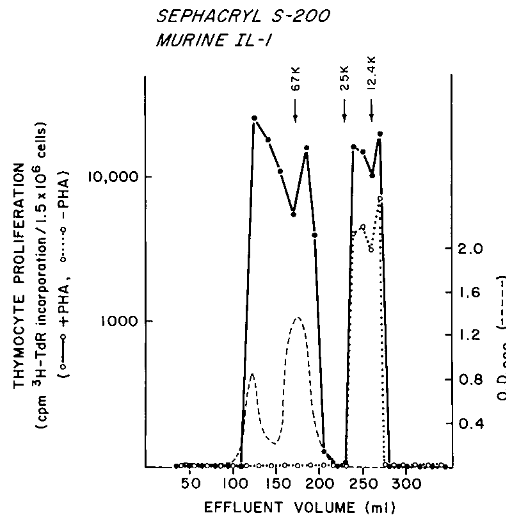

FIGURE 3. Fractionation of crude murine IL-1 by molecular sieve chromatography on Sephacryl S-200. The culture supernatant of PMA-stimulated $P388D_1$, cells was concentrated and applied to the column as outlined in the methods section. Fractions of column effluent were tested for murine thymocyte mitogenic activity in the presence or absence of PHA (1 μg/ml). *K*, kilodaltons.

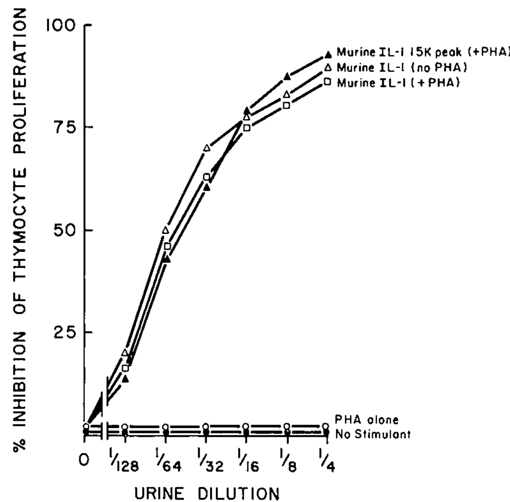

FIGURE 4. Comparative effect of the urine inhibitor on thymocyte proliferation in response to crude or partially purified IL-1. The proliferative response of murine thymocytes under each of the specified conditions was tested in the presence of increasing amounts of sterile, dialyzed febrile urine. Results represent the arithmetic means of triplicate cultures. The thymocyte proliferation (cpm/culture) in response to the various stimulants were: murine IL-1 15K Peak (+ PHA) = 26,132/culture; murine IL-1 (No PHA) = 18,229/culture; murine IL-1 (+ PHA) = 24,473/culture; PHA alone = 1,224/culture; no stimulant = 542/culture.

inhibitor. Taken together these experiments suggest that the inhibitor was specific for IL-1, and was not blocking the effects of PHA, nor was it nonspecifically toxic for thymocytes.

We next investigated the effects of inhibitor on another thymocyte mitogen,

interleukin-2 (IL-2). For these studies we analyzed the effects of the urine inhibitor against four preparations: human and mouse IL-1, and human and mouse IL-2. A fixed amount of urine inhibitor (16 U/ml) had no effect at all on IL-2–induced thymocyte proliferation (Fig. 5). Increasing concentrations of inhibitor were also tested against a fixed amount of IL-2 in separate experiments, but again up to 128 U/ml of inhibitor had no effect on IL-2–induced thymocyte proliferation (data not shown). The inhibitor was also tested against human IL-1, and was found to be more active against this material than against murine IL-1 (Fig. 5). These findings confirm that the urine inhibitor is specific for IL-1.

Characterization of the Urine Inhibitor. A preliminary physicochemical characterization of the urine inhibitor was performed next. Urine was concentrated by ammonium sulfate fractionation, and subjected to Sephacryl S-200 gel filtration. The inhibitor eluted from the gel in a moderately narrow and symmetrical peak with an apparent molecular weight of 20–40 kdaltons (Fig. 6), consistent with the behavior of a low molecular weight urine protein.

Patient Studies. Urine samples were next collected from a number of normal individuals, afebrile patients, and from patients with fevers of diverse etiologies, and tested for IL-1 inhibitor activity.

Normal individuals had low levels of activity, ranging from 0–100 U/ml, with a mean of 36 U/ml (Fig. 7). The activity in afebrile patients was similar to that of controls (mean = 27 U/ml). Febrile patients were subdivided into five groups. The mean urine inhibitory activity in all febrile patients was significantly greater

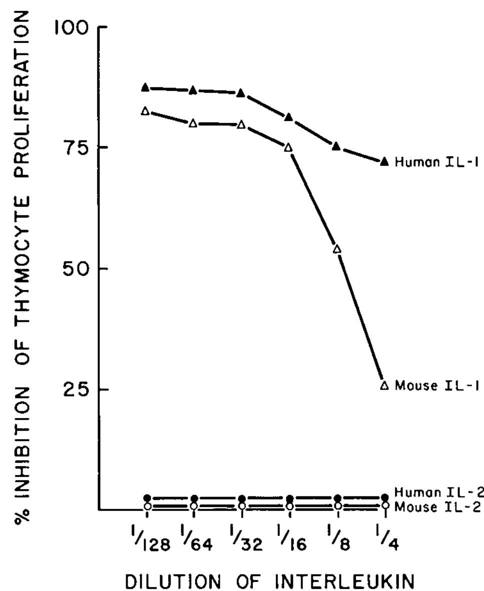

FIGURE 5. Comparative effect of the urine inhibitor on IL-1 and IL-2 induced thymocyte proliferation. Increasing concentrations of interleukin were tested in presence or absence of a fixed amount (16 U/ml) of sterile dialyzed febrile urine. The interleukins were prepared as described in the methods section. Results represent the arithmetic means of triplicate cultures. Thymocyte proliferation (cpm/culture) in response to these interleukins fell into the following ranges (1/128-1/4 dilutions): human IL-1 8,229–36,620; mouse IL-1 12,640–44,820; human IL-2 44.300–152,200; mouse IL-2 26.684–112,400.

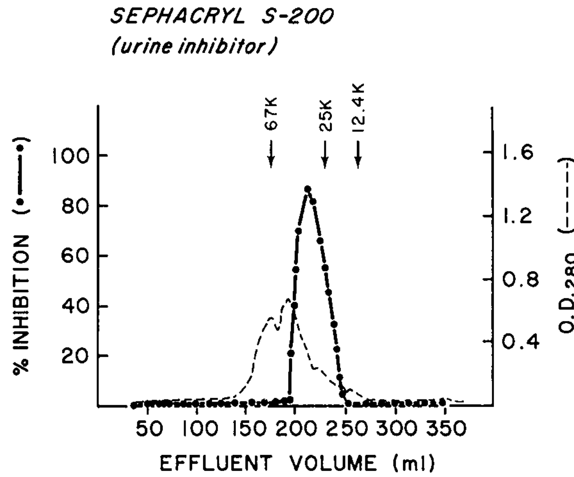

FIGURE 6. Fractionation of the urine inhibitor by molecular sieve chromatography on Sephacryl S-200. Febrile urine was concentrated (10-fold) by ammonium sulfate precipitation, dialyzed and 6 ml was applied to the column. Column effluent fractions were dialyzed, sterilized, and tested for inhibitory activity in the presence of murine IL-1 (2.5 U/ml). Results represent the arithmetic means of triplicate cultures. Thymocyte proliferation in response to IL-1 (+ PHA) = 27,650 cpm/culture.

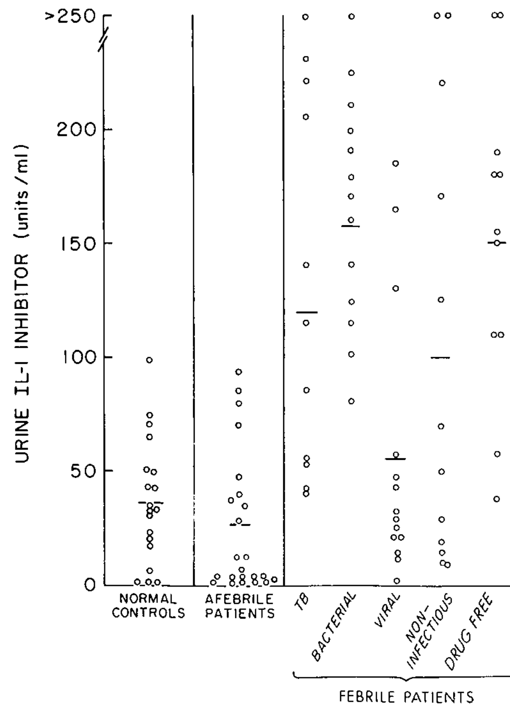

FIGURE 7. Quantification of inhibitor concentrations in the urine of normal and febrile individuals. Urine from each of the patients was dialyzed, sterilized, and tested for inhibitory activity in the presence of murine IL-1 (2.5 U/ml). The inhibitory activity of each sample was calculated using the formula described in the methods section.

than that of the afebrile individuals. The highest activities were found in patients with bacterial infections or tuberculosis, and every patient in this group had an elevated urine inhibitor titer. Inhibitor titers in patients with viral infections or noninfectious fevers were also elevated, but in these groups, titers of some patients fell into the normal range.

Because of the possibility that the urine inhibitory activity was due to protein-bound urinary drug metabolites, a large group of ambulatory, febrile patients were carefully screened for drug usage. 11 patients were found that had not taken any medication including antipyretics or antibiotics for at least 24 h before urine collection. Inhibitor levels in this group ranged from 38–340 μg/ml with a mean of 151 μg/ml.

These findings indicate that the IL-1 inhibitor is a normal constituent of human urine but that the urine levels of this material are significantly increased in febrile states.

Discussion

We have found that the urine of febrile patients contains a potent inhibitor of IL-1–induced thymocyte proliferation. Our data suggest that this material is specific for IL-1 and is not a nonspecific immuno-suppressive molecule, since the inhibitor has relatively little effect of spontaneous or PHA-induced thymocyte proliferation. More importantly, it does not inhibit the proliferative effects of another thymocyte mitogen, interleukin 2. Our findings also indicate that this inhibitor is present in small amounts in the urine of normal individuals, but is increased during febrile states of many etiologies.

The nature of this inhibitor remains to be determined. Gel filtration analysis indicates that it is a molecule of 20–40 kdaltons. Preliminary analysis on a two-dimensional gel suggests that it is a protein or glycoprotein (data not shown). However, definitive identification will require additional purification and analysis. Nevertheless, there is evidence suggesting that the urine inhibitor may be a protease inhibitor and/or a liver-derived acute phase protein. In addition to its relationship to endogenous pyrogen and fever (14), IL-1 has been shown to stimulate hepatic synthesis of several acute phase proteins including the SAA protein (3) and C-reactive protein (CRP) (15). It is also known that SAA will inhibit T cell mitogenesis in vitro (16). Although the urine inhibitor is too small to be either of these molecules, it is possible that it is either a proteolytic fragment or a subunit of SAA, CRP, or another immunosuppressive acute phase protein such as α_2-macroglobulin (17).

Another possibility is that the urine inhibitor is related to α_1-antiprotease, which is also an acute phase reactant (5). This serum protease inhibitor blocks PHA-induced T cell proliferation in vitro (18) and has been recently found to block the effect of IL-1 on thymocytes in vitro (7). Again, the urine inhibitor is too small to be intact α_1-antiprotease, but could be a fragment of this molecule. It is noteworthy that both α_1-antiprotease as well as other protease inhibitors have been found in human urine (19). It is also interesting that fragments of the inter α_1 trypsin inhibitor have been identified in urine, and that these fragments fall within the same molecular weight range as the urine IL-1 inhibitor (20–40 kdaltons) (20).

A number of other inhibitors of T cell proliferation have been identified in humans, but their relationship to the urine IL-1 inhibitor is not clear. Morse et al. (21), described a 20–40-kdalton inhibitor that is present in the urine of pregnant women. This inhibitor acts on peripheral T cells, and has not yet been tested for interleukin-blocking activity. Dinarello et al., (22) identified an inhibitor of IL-1 in the serum of endotoxin-treated human volunteers, that was bound by a polyclonal anti–IL-1 antibody. It is not clear whether this observation indicates that the serum inhibitor is antigenically related to IL-1, if the polyclonal antibody is not monospecific, or if the inhibitor is bound to circulating IL-1. Other T cell–suppressive factors have also been identified in human serum. Kleinhenz et al. (23) have suggested that an immunosuppressive factor in the plasma of patients with miliary tuberculosis is mycobacterial arabinogalactan (23). However, the physicochemical nature of the urine IL-1 inhibitor described in this report as well as its presence in a number of nonmycobacterial and noninfectious febrile states suggest that it is not related to arabinogalactan.

Recently, Amento et al. (24) have identified an inhibitor of IL-1–induced T cell activation that is produced by the human macrophage cell line, U937 (24). This material had an apparent molecular weight of 45–60 kdaltons. The similarity between this molecule and the urine inhibitor has not yet been investigated. However, these findings raise the interesting possibility that the urine IL-1 inhibitor may be a macrophage product.

It is reasonable to assume that the urine IL-1 inhibitor is a part of a physiological feedback mechanism. An inflammatory stimulus would induce macrophage production of IL-1, which in turn would produce fever and stimulate hepatic synthesis of acute phase proteins. One or more of these proteins or a macrophage-derived inhibitor could then act as suppressors of IL-1 and damp-down the inflammatory response. Presumably the urine inhibitor is derived from some low molecular weight (<50 kdaltons) serum IL-1 inhibitory protein that is excreted into the urine or is a fragment of some larger serum protein.

One problem with this interpretation, is that the increased levels of urine inhibitor seen in febrile states may not reflect an increased synthesis of this molecule, but may be the result of the general increased concentration of urine proteins that occurs with fever (25). It is less likely that the inhibitor is a protein-bound urinary drug metabolite or a microbial product, since urines from presumably drug-free febrile individuals, and from febrile patients without known infections also contained significant levels of the IL-1 inhibitor. A more precise identification of the urine IL-1 inhibitor and the ability to measure this molecule in serum should enable us to answer these questions, as well as to clarify the role of the urine inhibitor in the physiological regulation of IL-1–induced inflammation.

Summary

The urine of febrile patients has been found to contain high concentrations of an inhibitor of interleukin 1 (IL-1)–induced thymocyte proliferation. The inhibitor is specific for IL-1 and does not block the effects of interleukin 2 (IL-2) or phytohemagglutin (PHA) on thymocytes, and it is not nonspecifically toxic for these cells. IL-1 inhibitor can be found in the urine of normal individuals and

afebrile patients, but is present in increased concentrations in the urine of patients with fever of diverse etiologies. Preliminary physicochemical characterization indicates that the inhibitor is a 20–40-kdalton protein.

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