Presence of High Levels of Leukocyte-Associated Interleukin-8 upon Cell Activation and in Patients with Sepsis Syndrome

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In inflammatory and infectious diseases, the presence of circulating cytokines in plasma strongly suggests, following their exacerbated production, that saturation of specific binding sites has occurred or that an equilibrium between receptor-bound and free cytokines has been reached. In this report, we demonstrate that in addition to circulating interleukin-8 (IL-8), high levels of cell-associated IL-8 were detected in blood samples from patients with sepsis syndrome. The following analysis will reveal that in addition to erythrocytes, which have been dubbed a "sink" for IL-8, peripheral blood mononuclear cells (PBMC) and polymorphonuclear cells (PMN) contributed to the detection of cell-associated IL-8. On a per cell basis, 2,000 to 7,000 times the amount of IL-8 was found associated with PMN than with erythrocytes. In addition, circulating cells may well be the source of the leukocyte-associated form of IL-8. Similarly, in vitro experiments, such as whole-blood stimulation assays or the addition of exogenous IL-8 in blood samples, demonstrated that a large proportion of the IL-8 was associated with leukocytes. This suggests that the trapping of free cytokines onto the cell surface and the internalization of the IL-8 bound to its receptor, occurring both in vitro and in vivo, allows the detection of this cell-associated form. This analysis of cell-associated cytokines was extended to IL-1ra, another component of the inflammatory response, which, in contrast to IL-8, has been demonstrated to exist as an intracellular form. Indeed, cell-associated IL-1ra was also detected in septic patients. The measurement of cell-associated proinflammatory and anti-inflammatory cytokines in patients is clearly a more reliable reflection of their production than is the simple measurement in plasma and may provide useful indication to further understand the inflammatory process.

Interleukin-8 (IL-8) is an α-chemokine that is produced by many cell types, including monocytes/macrophages, fibroblasts, epithelial cells, endothelial cells, and neutrophils. IL-8 has a chemotactic activity for T lymphocytes, eosinophils, and basophils and is a potent chemoattractant factor for polymorphonuclear neutrophils (PMN). In addition, IL-8 is able to stimulate many PMN activities, including oxidative burst, exocytosis of specific granules, and release of proteases, as well as to enhance the expression of integrin on the surface of PMN (1). As a result, IL-8 is an important proinflammatory cytokine, as demonstrated by the significant reduction of immune complex-induced alveolitis and glomerulonephritis and of lipopolysaccharide (LPS)-induced dermatitis and arthritis with anti-IL-8 antibodies (23, 35). Its level in inflammatory fluids often correlates with the severity of the disease and the outcome of the patients, as found in bronchoalveolar lavage samples from patients with acute respiratory distress syndrome (6, 14, 32) and in plasma of patients with sepsis syndrome (15, 20, 31). Circulating cytokines represent the "tip of the iceberg" (5), and investigations at the cellular level can provide further information, such as that which we obtained when following up on the levels of cell-associated IL-1 and tumor necrosis factor alpha (TNFα) in hemodialyzed (22) and septic (36) patients. In the case of IL-8, the investigation was particularly relevant, as had

previously been described that erythrocytes can efficiently trap exogenous IL-8 and thereby function as a "sink" for IL-8 (12). Erythrocytes can specifically bind to IL-8 and other chemokines (38) via the Duffy antigen (25). The number of sites detected on erythrocytes has been estimated at between 800 and 9,000, with a K_d of 5 to 9.5 nM (12, 25, 38). That erythrocyte-bound IL-8 is detectable in humans undergoing IL-1 or IL-2 immunotherapy long after IL-8 had disappeared from plasma suggests that such assessments more precisely reflect the IL-8 production over time in vivo (49, 50). However, it has been observed that a large amount of IL-8 could be found as a cell-associated form following lipopolysaccharide (LPS) activation of leukocytes in a whole-blood assay (4). In in vitro experiments, cell-associated IL-8 could be found following activation of isolated alveolar macrophages (28), monocytes (16), and PMN (37). In this report, we further analyze the nature of the peripheral blood cells which contribute to this detection of cell-associated IL-8 upon activation by endotoxins. We postulate that in addition to the Duffy antigen, the receptors on the surface of leukocytes contribute in part to the detection of the cell-associated IL-8. Indeed, all neutrophils, all monocytes, a majority of NK cells and 5 to 25% of lymphocytes express IL-8 receptors (IL-8 RA and IL-8 RB) (9, 33). These receptors are expressed in great numbers on PMN (n = 14,000 to 75,000) and have a great affinity for this cytokine (K_d = 0.2 to 4 nM) (19, 34, 40–42). Furthermore, upon analysis of septic patients, both circulating IL-8 in plasma and large amounts of cell-associated IL-8 were observed. We further characterized the relative contribution of leukocytes and erythrocytes to the detection of this cell-associated IL-8.

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MATERIALS AND METHODS

Patients. Twelve patients with sepsis syndrome, as defined by Bone et al. (2), were studied upon admission to intensive care units and, in some cases, for several days following their admission. The study included eight men and four women, the average age being 60 ± 5 years (range, 21 to 74 years) and the mean simplified acute physiology score being 18 ± 2. There were four cases of septicemia, two of septic shock, one of cellulitis, two of pneumonia, one of peritracheostomial abscess, and two of peritonitis. The outcome was survival for five patients.

Isolation of human mononuclear and polymorphonuclear cells and erythrocytes. Blood was drawn on heparin (20 IU/ml) from volunteers or from patients. Then 1 volume of MSL (Milieu de Séparation des Lymphocytes; Eurobio, Les Ulis, France) was layered on 1 volume of Polymorphoprep (Nycomed; Pharma AS, Oslo, Norway), and then 1 volume of blood diluted 1:2 in RPMI 1640 medium (Bio-Whittaker) was layered on MSL. After centrifugation for 30 min at 15°C and $500 \times g$, the two cell layers containing either the mononuclear cells or the polymorphonuclear cells and the erythrocyte pellet were washed and centrifuged once for 5 min at $300 \times g$. The cells were then counted in 0.1% eosin. In some experiments, as mentioned above, leukocytes and erythrocytes were recovered following a 30-min incubation of whole blood with increasing amounts of recombinant human (rh) IL-8.

In vitro culture. Whole-blood assays were performed with undiluted blood cultured in flasks (Filter Vent Flasks, 25 cm^2; Nunclon, Rosklide, Denmark) at 37°C in a 5% CO_2 incubator for 30 min or 2 h depending on the experiment. Polymorphonuclear cells (2×10^6 cells/ml) were prepared as followed. First, 10 volumes of blood was mixed with 2 volumes of glucose dextran (3% glucose, 3% Dextran T250 [Pharmacia, Uppsala, Sweden]), and the leukocytes were recovered following a 40-min sedimentation at room temperature. Then the leukocytes were diluted 1:2 in RPMI 1640 medium and layered on Ficoll-Hypaque (MSL; Eurobio, Les Ulis, France). The ratio was 2 volumes of leukocytes to 1 volume of MSL. After centrifugation for 25 min at 15°C and $500 \times g$, the cell pellet was washed and centrifuged once for 5 min at $300 \times g$. Contaminating erythrocytes were lysed following a 5-min incubation of the cell pellet at 4°C, resuspended in 5 ml of lysis buffer (8.32 g of NH_4Cl per liter, 0.84 g of $NaHCO_3$ per liter, 43.2 mg of tetrasodium EDTA per liter). Lysis was stopped by adding a large excess of RPMI 1640 medium, and the cells were washed and centrifuged for 10 min at $200 \times g$. The viability of PMN was assessed by counting the cells in 0.1% eosin. A nonspecific esterase staining was performed to evaluate monocyte contamination, which never exceeded 0.5%. PMN were cultured in RPMI 1640 medium supplemented with L-glutamine (300 mg/liter), antibiotics (penicillin, 100 IU/ml; streptomycin, 100 μg/ml), and 5% heat-inactivated normal human serum (a pool of sera from healthy volunteers). A 0.5-ml portion of PMN suspension per well was incubated in a 5% CO_2 incubator in 24-well multidish plates (Nunclon) for 24 h at 37°C. The cell viability after 24 h ranged from 64 to 84%. Stimuli were added at the beginning of the culture in a volume not exceeding 10 μl. At the end of the culture, the supernatants were harvested, centrifuged for 10 min at $300 \times g$ and 15°C, and kept at -20°C before cytokine assessments.

Assessment of cell-associated forms of cytokines. In whole-blood samples, 1 ml of blood was centrifuged for 10 min at $500 \times g$. Plasma was harvested and kept at -20°C before cytokine assessments. The cell pellet was lysed in 100 μl of lysis buffer (TRAx buffer; T-Cell Sciences, Inc., Needham, Mass.) before addition of RPMI 1640 medium to achieve a 1-ml total volume. One volume of this lysate was then diluted 1:2 in TRAx buffer diluent reagent (T-Cell Sciences, Inc.).

Pellets of isolated PMN, peripheral blood mononuclear cells (PBMC) or erythrocytes were lysed in 100 μl of TRAx lysis buffer before addition of RPMI 1640 medium to achieve 500 μl and addition of 500 μl of diluent reagent. At the end of the culture period, the PMN pellets were lysed by adding 100 μl of TRAx lysis buffer before adding 100 μl of diluent buffer and 300 μl of RPMI 1640 medium.

Reagents. *Escherichia coli* (O111:B4) LPS was purchased from Sigma (St. Louis, Mo.). TNFα was obtained from Rhône Poulenc (Vitry/Seine, France), *Neisseria meningitidis* LPS was the generous gift of Martine Caroff (Institut de Biochimie, Orsay, France), and recombinant human IL-8 was purchased from Immugenex (Los Angeles, Calif.).

Cytokine ELISA. The IL-8 enzyme-linked immunosorbent assay (ELISA) was performed as previously described (31) with a monoclonal anti-human IL-8 antibody and a rabbit polyclonal anti-IL-8 antibody graciously provided by N. Vita (Sanofi Recherche, Labège, France). The sensitivity of the ELISA was 3 pg/ml. We ensured that the TRAx buffer did not interfere with the accuracy of the ELISA. In experiments where defined amounts of IL-8 were added to blood, the total recovery after the treatment with the TRAx buffer was close to 100%. The IL-1 receptor antagonist (IL-1ra) ELISA system was purchased from R&D Systems and used as specified by the manufacturer.

RESULTS

Detection of cell-associated IL-8 upon cell activation. Little or no IL-8 could be detected in plasma from healthy donors, whereas small amounts of cell-associated IL-8 were always
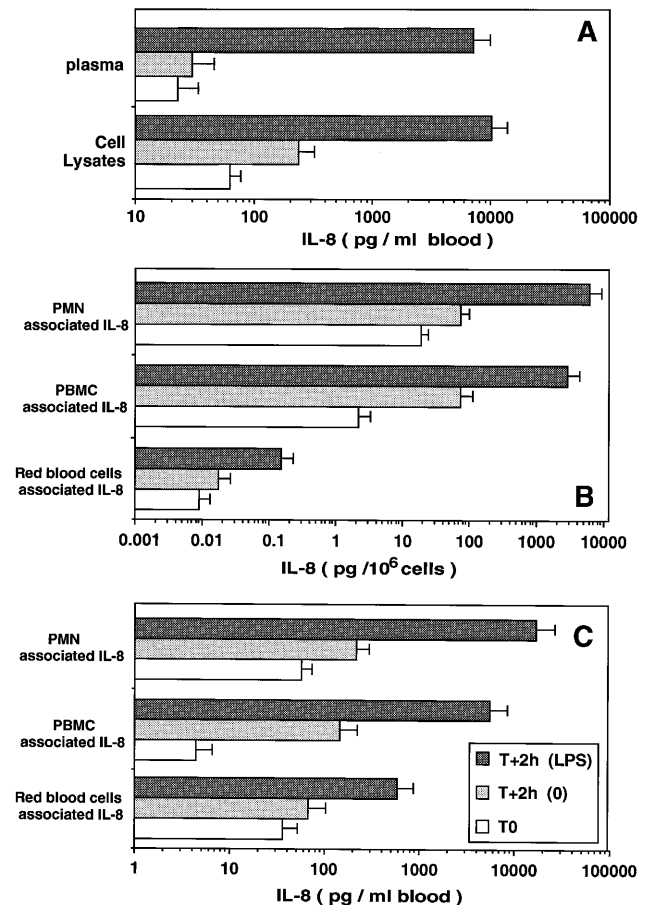

FIG. 1. (A) IL-8 levels measured in plasma or in culture supernatants and in cell lysates after blood sampling (T0) or following whole-blood culture in the absence [T+2h (0)] or in the presence [T+2h (LPS)] of 10 μg of *N. meningitidis* LPS per ml. (B and C) IL-8 levels in cell lysates of PMN, PBMC, or erythrocytes prepared immediately after blood sampling or after whole-blood culture for 2 h at 37°C in the absence or in the presence of LPS. Results are expressed either per 10^6 cells (B) or as the amount of cell-associated IL-8 per milliliter of blood (C). The results are the mean $\pm$ SEM of three different experiments.

found following blood sampling of the volunteers (Fig. 1A). Stimulation of leukocytes by LPS in a whole-blood assay leads to the production of IL-8. Significant and similar amounts of IL-8 could be detected in the cell supernatants as well as associated with the whole-cell pellet. To allow efficient leukocyte recovery and particularly to obtain a good yield of PMN, short stimulation (i.e., 2 h) of whole blood was performed before cellular purification. Further analysis on isolated cells revealed that the highest levels of cell-associated IL-8 were detected with the PMN and the PBMC whereas very low levels were detected in association with the erythrocytes on a per-cell basis (Fig. 1B and C). The levels of PMN-associated IL-8 was 3×10^4-fold higher than that recovered with the erythrocytes. Even when taking into account the relative number of erythrocytes, PMN, and PBMC per milliliter of blood, the contribution of erythrocytes to the detectable cell-associated IL-8 remained low.

To determine whether released and cell-associated forms could be detected in cultures of isolated cells, we further analyzed isolated PMN. As shown in Fig. 2, a significant amount of cell-associated IL-8 was observed in unstimulated cells. A 24-h activation of isolated PMN by LPS or TNF-α allowed the
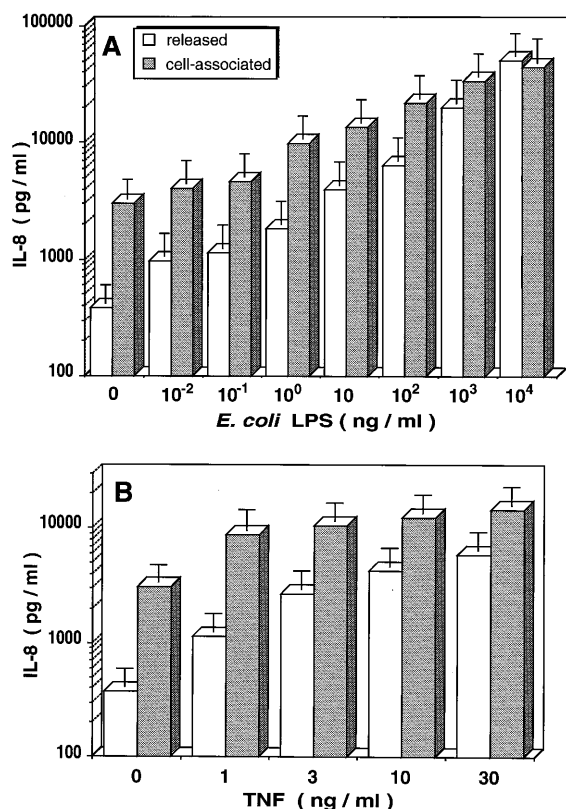
FIG. 2. (A) Levels of released and cell-associated IL-8 in 24-h cultures of isolated PMN triggered by either increasing amounts of *E. coli* LPS or TNF-α. The results are the mean ± SEM of three and five experiments, respectively.
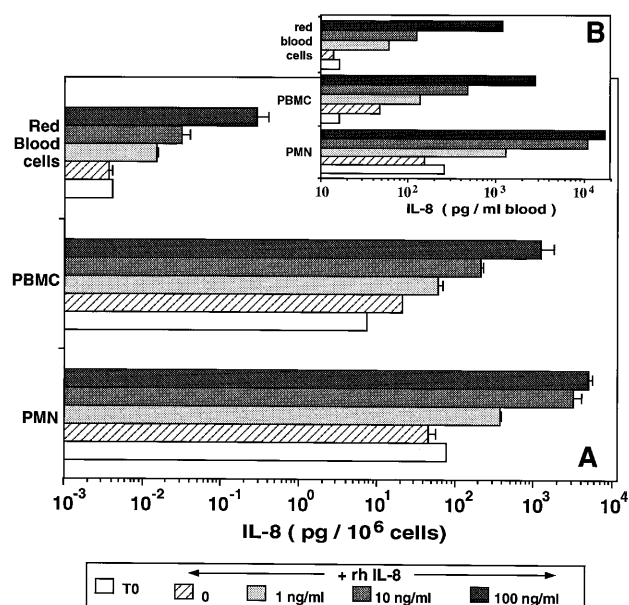

FIG. 3. Levels of IL-8 found associated with erythrocytes, PBMC, and PMN following incubation of increasing amounts of rh IL-8 in whole blood for 30 min at 37°C. Results are expressed as amounts of cell-associated IL-8 per 10^6 cells (A) or per milliliter of blood (B). The results are the mean ± SEM of two experiments.

detection of large amounts of IL-8 in both supernatant and cell-associated compartments. It is worth noting that a plateau of saturation for the cell-associated form was reached at a lower concentration of activators than had been observed for the free form found in the cell supernatants.

Interaction of exogenous IL-8 with leukocytes and erythrocytes. To estimate the contribution of the different IL-8 receptors on the circulating cells, we analyzed the interaction of IL-8 with blood cells by adding exogenous recombinant human IL-8 to blood samples. As shown in Fig. 3, IL-8 associated rapidly with erythrocytes as well as PBMC and PMN in a dose-dependent fashion. When small amounts of rh IL-8 were added (1 to 10 ng/ml), most of the cytokine was linked to the cells and only 4 to 6% was recovered in the supernatant 30 min later. The largest amount was associated with PMN, and a saturation plateau was reached with 10 ng/ml, whereas levels of PBMC- and erythrocyte-linked IL-8 continued to augment with increasing amounts of added rh IL-8. On a per-cell basis, within the range of 1 to 10 ng of rh IL-8 per ml, the amount of the chemokine associated with PMN (300 to 3,000 pg/10^6 cells) was 2×10^4- to 1×10^5-fold larger than that observed with erythrocytes (0.015 to 0.04 pg/10^6 cells). It should be noted that 1 to 10 ng of IL-8 per ml corresponds to concentrations compatible with the physiological quantities found in plasma of septic patients.

Assessment of cell-associated IL-8 and IL-1ra in septic patients. While circulating IL-8 was found in the plasma of all 12 patients with sepsis syndrome upon admission to the intensive care unit (mean ± standard error of the mean [SEM] = 145 ± 40 pg/ml), high levels of IL-8 could also be found associated

with whole blood cells (mean ± SEM = 3,488 ± 1,188 pg/ml). These levels of free and cell-associated IL-8 correlated significantly ($r = 0.81$; $P = 0.0013$).

In following up on three of the patients, both forms of IL-8 were detected over a long period after admission (Fig. 4). The ratio between cell-associated IL-8 and plasma IL-8 was within the range of 3.4 to 41.2. The increase and decrease of cell-associated IL-8 found during the follow-up of the patients paralleled that of plasma IL-8 on a more pronounced scale. PBMC, PMN, and erythrocytes were fractionated from blood samples obtained from septic patients to further analyze the nature of the IL-8-associated cells. As shown in Fig. 5, the presence of IL-8 was essentially associated with leukocytes on a per-cell basis. Depending upon the patient, the amounts of erythrocyte-associated and PMN-associated IL-8 were 0.014 to 0.118 pg/10^6 cells and 13.5 to 183 pg/10^6 cells, respectively.

As shown in Table 1, a similar technical approach could be used to measure and compare levels of circulating and cell-associated IL-1ra. In contrast to IL-8, levels of cell-associated IL-1ra were not always higher than those of circulating IL-1ra. The ratio between the levels of the two forms ranged from 0.3 to 18.7.

DISCUSSION

In inflammatory and infectious diseases, the presence of circulating cytokines in plasma strongly suggests, following their exacerbated production, that either saturation of specific binding sites has occurred or an equilibrium between receptor-bound cytokines and free cytokines has been reached (5). In the case of IL-8, many cells within the vessel can bind this chemokine. This is the case with endothelial cells, which specifically bind to IL-8 via the IL-8 RA (44) and the Duffy antigen (21). In addition, most circulating blood cells have the capacity to bind to IL-8. This is the case for erythrocytes, via the Duffy antigen (12, 25, 26, 38), and leukocytes, via IL-8RA
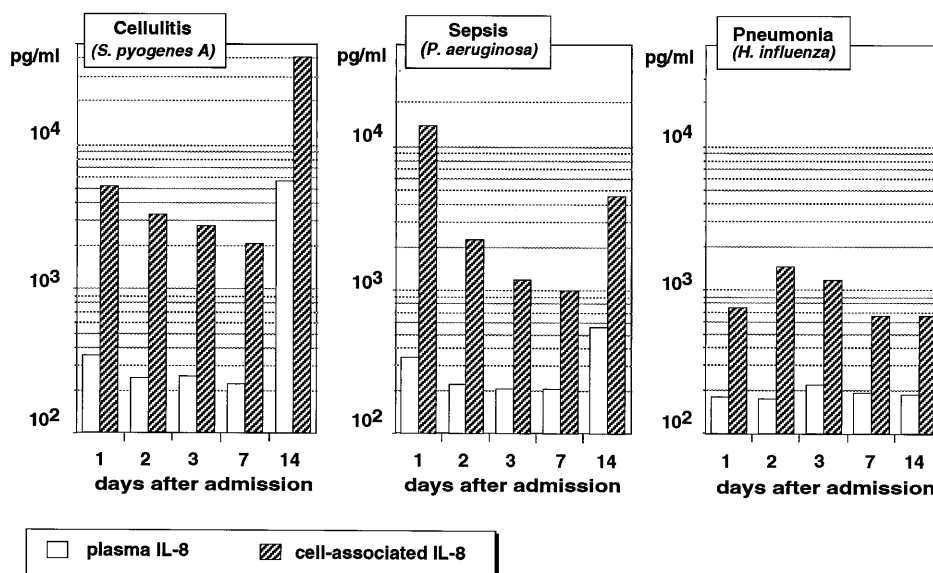
FIG. 4. Levels of circulating and whole-blood-cell-associated IL-8 in three patients with different types of infection, assessed on days 1, 2, 3, 7, and 14 after admission to the intensive care unit.

and IL-8RB receptors (9, 33). Consequently, the presence of detectable free IL-8 in plasma could be associated with that of IL-8 on the surface of these cells. Because the endothelium compartement is difficult to analyze in patients, we focused our attention on circulating cells in an attempt to analyze the relative contribution of these cells to the detection of cell-associated IL-8. Darbonne et al. (12), who proposed that erythrocytes were a "sink" for IL-8, focused most of their attention on the erythrocyte compartment. In agreement, Tilg et al. (49, 50) described the presence of such erythrocyte-bound IL-8 in IL-1- and IL-2-treated patients. It was only recently that the cell-associated IL-8 was investigated with other cell types such as neutrophils, monocytes, and alveolar macrophages by using in vitro activation to allow the detection of cell-associated IL-8 (16, 28, 37). We demonstrated that following PMN activation by LPS and TNF-α, IL-8 could be measured in the cell supernatants as well as associated with the PMN. These results are in agreement with those reported by Kuhns and Gallin (28), who also showed that cell-associated IL-8 was demonstrable from neutrophils harvested from skin lesions of normal human volunteers. These authors specified that the accumulation of IL-8 within PMN was localized to a subcellular fraction of heterogeneous light membranous organelles.

There is very little data available that describes the presence of cell-associated IL-8 from human diseases. However, the influence of cardiopulmonary bypass on cell-associated IL-8 has been reported (27). Interestingly, 24 h after cardiopulmonary bypass, significant levels of leukocyte-associated IL-8 were documented while no plasma IL-8 was detected. We have undertaken the analysis of cell-associated IL-8 in septic patients, who are well known to have circulating IL-8 (15, 20, 31). Thanks to the TRAx buffer, generously provided by T-Cell Sciences, Inc., we have demonstrated the presence of large amounts of IL-8 within the whole-cell lysates of blood samples from septic patients. Depending on the patients and the sampling time during the evolution of the sepsis syndrome, the levels of cell-associated IL-8 were 3.4 to 41.2 times higher, per milliliter of blood, than were the levels in plasma. Furthermore, we analyzed the nature of the circulating cells contrib-

uting to the detection of cell-associated IL-8 measured in whole blood. This is the first report which clearly demonstrates that in addition to the expected erythrocyte-bound IL-8, leukocytes do contribute to an important level of cell-associated IL-8. Moreover, on a per-cell basis, the amount of IL-8 associated with PMN was twice that found with mononuclear cells and 4,680 $\pm$ 2,470 times that found with erythrocytes. Taking into account the relative number of circulating cells, 78.5 to 92% of the detectable cell-associated IL-8 in blood are linked to PMN. While this study does not investigate the possible capture of IL-8 by platelets, the recent report on IL-8 in stored platelet concentrates (47), the description of IL-8 receptors on platelets from patients with ulcerative colitis and Crohn's disease (43), and our preliminary observations in whole-blood assays all suggest that trapping of circulating IL-8 could also occur at the platelet level.

The contribution of circulating IL-8 and that associated with peripheral leukocytes to the clinical symptoms observed during sepsis syndrome is unclear. IL-8 is involved in PMN activation, and evidence of neutrophil activation in septic patients has been reported (39, 51). Furthermore, the contribution of PMN to the tissue damage and organ dysfunction associated with sepsis syndrome has been well established in animal models (24, 30). However, IL-8 found in the circulating compartment desensitized PMN and possibly lymphocytes as well to further responsiveness to chemoattractant signals, delivered consecutively within the tissues (10, 11, 18, 45, 46). Such deactivation may limit the margination of inflammatory cells toward organs and reduce the possible development of multiple organ failure often associated with sepsis syndrome.

The cellular origins of this circulating IL-8 are probably multiple. For example, the endothelial cells, difficult to analyze in patients, are a putative potent source of IL-8 (48). It is worth noting that anti-inflammatory cytokines such as IL-4 and IL-10 enhance the release of IL-8 by LPS-activated endothelial cells (13). In addition, monocytes, lymphocytes, and neutrophils can produce IL-8 upon activation by exogenous microbial products and inflammatory cytokines such as IL-1 and TNF (see reference 1 for a review) or, in the case of T lymphocytes, by IL-8
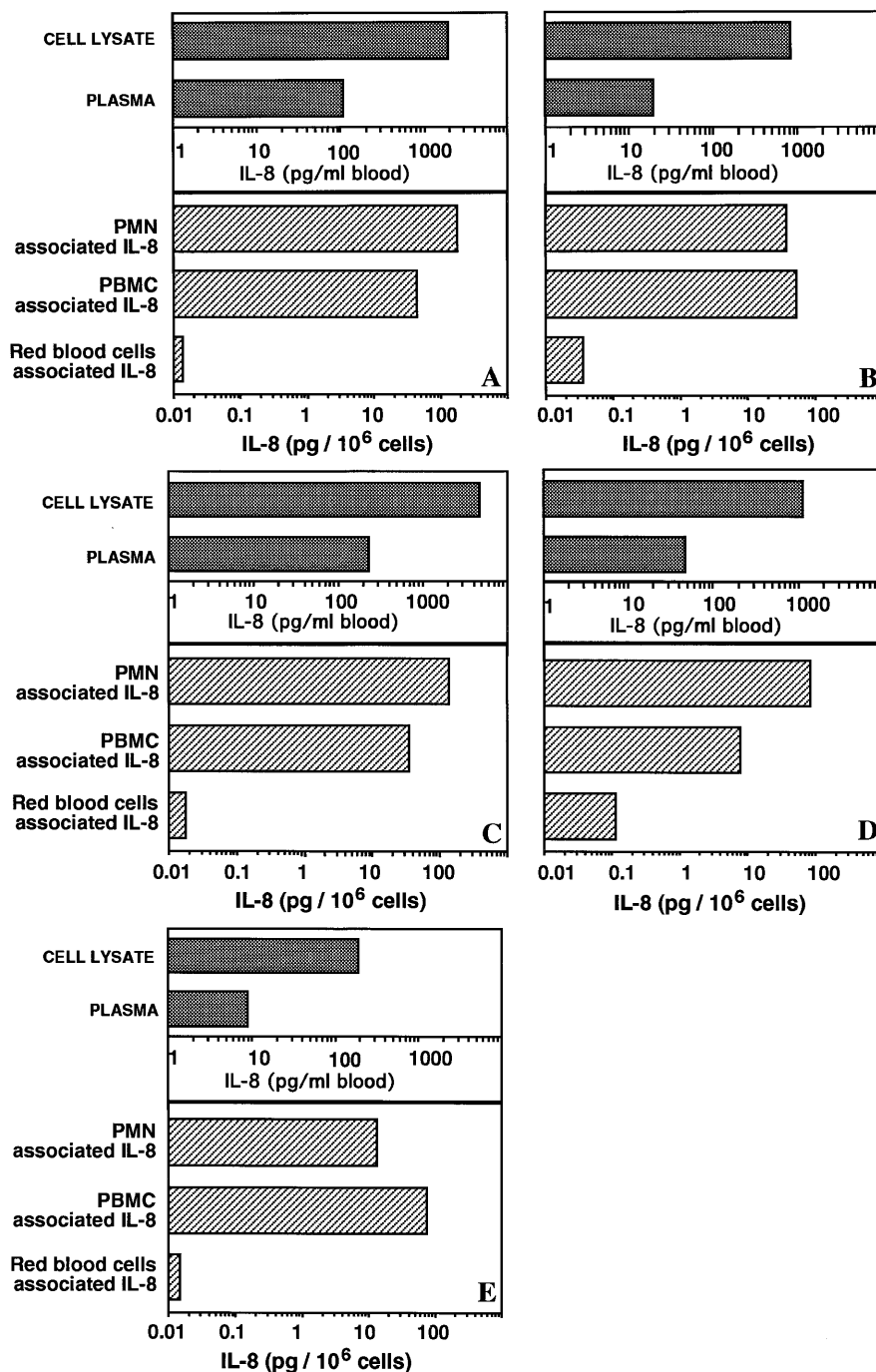
FIG. 5. Levels of circulating, whole-blood-cell-, PMN-, PBMC-, and erythrocyte-associated IL-8 in patients with sepsis syndrome (A), peritonitis (B and C), septic shock (D), and pneumonia (E).

itself (17). The presence of IL-8 mRNA in the leukocytes of septicemic patients has already been reported (15). We also detected IL-8 as well as IL-1ra mRNA (7) in PBMC and PMN from some septic patients and from some healthy controls (data not shown), thus illustrating that circulating mononuclear leukocytes and PMN can contribute to the production of IL-8 and IL-1ra.

In vitro experiments involving whole-blood assays show similar results to those observed in patients. Activation of whole blood with LPS led to the detection of large amounts of cell-associated IL-8. As in the in vivo case with septic patients, the amounts of PMN-associated IL-8, on a per-cell basis, were close to those on PBMC; in contrast, they were 3×10^4-fold the amounts found associated with the erythrocytes. Similarly, when exogenous rh IL-8 was added to blood samples, a majority of the chemokine was found associated with PMN and 2×10^4- to 1×10^5-fold-smaller amounts were found associated with the erythrocytes. In view of the relative number of the receptors (less than a 10-fold difference) and the relative affinity constants of IL-8RA, IL-8RB, and the Duffy antigen

TABLE 1. Plasma and cell-associated IL-1ra levels in
septic patients

Patient and IL-1ra type[a]	Level of IL-1ra (ng/ml) on day:				
	1	2	3	7	14
Patient 1					
Circulating IL-1ra	136	54.0	35.2	12.2	152.6
Cell-associated IL-1ra[b]	74.0	48.1	45.6	18.9	49.5
Patient 2					
Circulating IL-1ra	26.5	16.4	13.0	16.7	17.4
Cell-associated IL-1ra[b]	79.6	52.3	37.3	23.9	21.7
Patient 3					
Circulating IL-1ra	3.9	3.6	4.3	4.8	4.9
Cell-associated IL-1ra[b]	42.7	67.4	42.0	23.6	28.9

[a] Patients 1, 2, and 3 are the same as those in Fig. 4.
[b] The data are expressed as amount per milliliter of blood.

(less than a 10-fold difference) (12, 19, 26, 34, 38, 40–42), such a discrepancy remains difficult to explain. The relatively reduced contribution of PMN to the detection of cell-associated IL-8 in septic patients compared to that in the in vitro experiments may reflect the involvement of different mechanisms. To allow a sufficient recovery of PMN following blood activation with LPS or incubation with recombinant IL-8, short incubation periods were required. Indeed, the yield of recovered PMN diminished with increasing lengths of incubation of the in vitro blood samples at 37°C (data not shown). One can infer, as well, that in vivo circulating cells are in contact with plasma IL-8 for a longer period and that trapping of the circulating cytokines, internalization, and modulation of receptor expression had occurred. Indeed, the expression of the IL-8 receptors on the surface of PMN is itself down-regulated by IL-8 (8, 42), and in septic patients, other factors such as LPS can contribute to the lower expression of the IL-8 receptors (29). The range of PMN-associated IL-8 found in different patients (from 14 to 183 pg/10^6 cells) suggests that such regulation had occurred in patients who have different infectious processes and are at different stages of their sepsis syndrome.

The fact that a similar ratio between PMN-associated and erythrocyte-associated IL-8 was found in the experiments where IL-8 was produced upon in vitro cell activation or in experiments where exogenous IL-8 was added strongly suggests that most of the observed cell-associated IL-8 is a result of the interaction of the chemokine with its various receptors. As it has been found that bound IL-8 is rapidly internalized by PMN (42), one can speculate that following cell lysis by the TRAx buffer, intact IL-8 linked to its receptor and most internalized IL-8 are both detectable in our ELISA. Indeed, only one-fifth of the cell-associated IL-8 measured following lysis of the cells by the TRAx technique could be recovered following treatment of the cells with a glycine-HCl (pH 2) buffer, which is known to release ligands from their receptors (data not shown). However, one cannot exclude the fact that part of the cell-associated IL-8 corresponds to newly synthesized cytokine remaining within the cells. However, among cytokines and growth factors, only IL-1, IL-1ra, and ciliary neurotrophic factor, lacking signal peptides, can accumulate within the cells. To our knowledge, no such accumulation during the synthesis process has been demonstrated for IL-8, although it is possible that IL-8 exists in some PMN granules. A proportion of the newly synthesized IL-8 remaining within the cells could be estimated by blocking the internalization of the released IL-8 by either anti-IL-8 RA and RB, by IL-8 antagonists, or by

anti-IL-8 antibodies. In contrast, it is most likely that levels of cell-associated IL-1ra reflected other mechanisms than those proposed for IL-8, since it is known that a great part of the IL-1ra production remains cytoplasmic and is not released (3).

The measurement of cell-associated cytokines in patients is clearly a more reliable reflection of its production than is their measurement in plasma. ELISAs were used, but similar information can be provided by flow cytometry, as recently demonstrated for cytoplasmic IL-1β in mononuclear cells from patients in the intensive care unit (52). Both analysis can be extended to many components of the inflammatory response. For example, the use of the TRAx buffer allowed the detection of cell-associated IL-1ra. Similarly, approaches have been undertaken to evaluate the relative ratio between a given receptor in its soluble form and on the surface of the cells (data not shown). This determination of the ratio between an inhibitor and an effective signal transmitter may provide useful information to further understand the inflammatory process.

REFERENCES

1. **Baggiolini, M., B. Dewald, and B. Moser.** 1993. Interleukin-8 and related chemotactic cytokines. Adv. Immunol. **55:**97–179.
2. **Bone, R. C., C. L. Sprung, and W. J. Sibbald.** 1992. Definitions for sepsis and organ failure. Crit. Care Med. **20:**724–726.
3. **Butcher, C., A. Steikasserer, S. Tejura, and A. C. Lennard.** 1994. Comparison of two promoters controlling expression of secreted or intracellular IL-1 receptor antagonist. J. Immunol. **153:**701–711.
4. **Cavaillon, J.-M., C. Marie, C. Pitton, and C. Fitting.** 1995. The production of TNFα and IL-8 in whole blood assays are differently regulated upon LPS activation by LPS. Prog. Clin. Biol. Res. **392:**433–439.
5. **Cavaillon, J.-M., C. Muñoz, C. Fitting, B. Misset, and J. Carlet.** 1992. Circulating cytokines: the tip of the iceberg? Circ. Shock **38:**145–152.
6. **Chollet-Martin, S., P. Montravers, C. Gibert, C. Elbim, J. M. Desmonts, J. Y. Fagon, and M. A. Gougerot-Pocidalo.** 1993. High levels of interleukin-8 in the blood and alveolar spaces of patients with pneumonia and adult respiratory distress syndrome. Infect. Immun. **61:**4553–4559.
7. **Chomczynski, P., and N. Sacchi.** 1987. Single step method of RNA isolation by acid guanidinium thiocyanate phenol chloroform extraction. Anal. Biochem. **162:**156–159.
8. **Chuntharapai, A., and K. J. Kim.** 1995. Regulation of the expression of IL-8 receptor A/B by IL-8: possible functions of each receptor. J. Immunol. **155:**2587–2594.
9. **Chuntharapai, A., J. Lee, C. A. Hébert, and K. J. Kim.** 1994. Monoclonal antibodies detect different distribution patterns of IL-8 receptor A and IL-8 receptor B on human peripheral blood leukocytes. J. Immunol. **153:**5682–5688.
10. **Cunha, F. Q., and W. M. S. Cunha Tamashiro.** 1992. Tumor necrosis factor and interleukin-8 inhibit neutrophil migration in vitro and in vivo. Mediat. Inflamm. **1:**397–401.
11. **Cunha, F. Q., G. E. P. Souza, C. A. M. Souza, B. C. S. Cerqueira, and S. H. Ferreira.** 1989. In vivo blockage of neutrophil migration by LPS is mimicked by a factor released from LPS-stimulated macrophages. Br. J. Exp. Pathol. **70:**1–8.
12. **Darbonne, W. C., G. C. Rice, M. A. Mohler, T. Apple, C. A. Hébert, A. J. Valente, and J. B. Baker.** 1991. Red blood cells are a sink for interleukin-8, a leukocyte chemotaxin. J. Clin. Invest. **88:**1362–1369.
13. **De Beaux, A. C., J. P. Maingay, J. A. Ross, K. C. H. Fearon, and D. C. Carter.** 1995. Interleukin-4 and interleukin-10 increase endotoxin-stimulated human umbilical endothelial cell interleukin-8 release. J. Interferon Cytokine Res. **15:**441–445.
14. **Donnelly, S. C., R. M. Strieter, S. L. Kunkel, A. Walz, C. R. Robertson, D. C. Carter, I. S. Grant, A. J. Pollok, and C. Haslett.** 1993. Interleukin-8 and development of adult respiratory distress syndrome in at-risk patient groups. Lancet **341:**643–647.
15. **Friedland, J. S., Y. Suputtamongkol, D. G. Remick, W. Chaowagul, R. M. Strieter, S. L. Kunkel, N. J. White, and G. E. Grifin.** 1992. Prolonged elevation of interleukin-8 and interleukin-6 concentrations in plasma and of

leukocyte interleukin-8 mRNA levels during septicemic and localized *Pseudomonas pseudomallei* infection. Infect. Immun. **60:**2402–2408.

16. **Fujishima, S., H. Nakamura, Y. Waki, K. Soejima, M. Ogawa, M. Shiozawa, Y. Hiraoka, T. Kawashiro, S. Aiso, M. Kanazawa.** 1996. Cell-associated IL-8 in human blood monocytes: analysis by flow cytometry. Cytometry **24:**382–389.

17. **Gezzer, B., B. Deleuran, M. Lund, C. Vestergard, N. Lohse, M. Deleuran, S. L. Jensen, S. S. Pedersen, K. Thestrup-Pedersen, and K. Larsen.** 1995. Interleukin-8 induces its own production in CD4+ T lymphocytes. A process regulated by interleukin-10. Biochem. Biophys. Res. Commun. **210:**660–669.

18. **Gimbrone, M. A., Jr., M. S. Obin, A. F. Brock, E. A. Luis, P. E. Hass, C. A. Hébert, Y. K. Yip, D. W. Leung, D. G. Lowe, W. J. Kohr, W. C. Darbonne, K. B. Bechtol, and J. B. Baker.** 1989. Endothelial interleukin-8: a novel inhibitor of leukocyte-endothelial interactions. Science **264:**1601–1603.

19. **Grob, P. M., E. David, T. C. Warren, R. P. DeLeon, P. R. Farina, and C. A. Homon.** 1990. Characterization of a receptor for human IL-8. J. Biol. Chem. **265:**8311–8316.

20. **Hack, C. E., M. Mart, R. J. M. Strack van Schijndel, A. J. M. Eerenberg, J. H. Nuijens, L. G. Thijs, and L. A. Aarden.** 1992. Interleukin-8 in sepsis: relation to shock and inflammatory mediators. Infect. Immun. **60:**2835–2842.

21. **Hadley, T. J., Z.-H. Lu, K. Wasniowska, A. W. Martin, S. C. Peiper, J. Hesselgesser, and R. Horuk.** 1994. Postcapillary venule endothelial cells in kidney express a multispecific chemokine receptor that is structurally and functionally identical to the erythroid isoform which is the Duffy blood group antigen. J. Clin. Invest. **94:**985–991.

22. **Haeffner-Cavaillon, N., J.-M. Cavaillon, C Ciancioni, F. Bacle, S. Delons, and M. D. Kazatchkine.** 1989. Induction of interleukin-1 production during hemodialysis. Kidney Int. **35:**1212–1218.

23. **Harada, A., N. Sekido, T. Akahoshi, T., Wada, N. Mukaida, and K. Matsushima.** 1994. Essential involvement of interleukin-8 in acute inflammation. J. Leukocyte Biol. **56:**559–564.

24. **Hewett, J. A., E. Schultze, S. VanCise, and R. A. Roth.** 1992. Neutrophil depletion protects against liver injury from bacterial endotoxin. Lab. Invest. **66:**347–361.

25. **Horuk, R., CE. Chitnis, W. C. Darbonne, T. J. Colby, A. Rybicki, T. J. Hadley, and L. H. Miller.** 1993. A receptor for the malarial parasite Plasmodium vivax: the erythrocyte chemokine receptor. Science **261:**1182–1184.

26. **Horuk, R., T. J. Colby, W. C. Darbonne, T. J. Schall, and K. Neote.** 1993. The human erythrocyte inflammatory peptide (chemokine) receptor. Biochemical characterization, solubilization and development of a binding assay for the soluble receptor. Biochemistry **32:**5733–5738.

27. **Kalfin, R. E., R. M. Engelman, J. A. Rousou, J. E. Flack III, D. W. Deaton, D. L. Kreutzer, and D. K. Das.** 1993. Induction of intereulkin-8 expression during cardiopulmonary bypass. Circulation **88:**401–406.

28. **Kuhns, D. B., and J. I. Gallin.** 1995. Increased cell-associated IL-8 in human exudative and A23187-treated peripheral blood neutrophils. J. Immunol. **154:**6556–6562.

29. **Lloyd, A. R., A. Biragyn, J. A. Johnston, D. D. Taub, L. Xu, D. Michiel, H. Sprenger, J. J. Oppenheim, and D. J. Kelvin.** 1995. G-CSF and lipopolysaccharide regulate the expression of interleukin-8 receptors on polymorphonuclear leukocytes. J. Biol. Chem. **270:**28188–28192.

30. **Mallick, A. A., A. Ishizaka, K. E. Stephens, J. R. Hatherill, H. D. Tazeloor, and T. A. Raffin.** 1989. Multiple organ damage caused by tumor necrosis factor and prevented by prior neutrophil depletion. Chest **95:**1114–1120.

31. **Marty, C., B. Misset, F. Tamion, C. Fitting, J. Carlet, and J.-M. Cavaillon.** 1994. Circulating interleukin-8 concentrations in patients with multiple organ failure of septic and non-septic origin. Crit. Care Med. **22:**673–679.

32. **Miller, E. J., A. B. Cohen, S. Nagao, D. Griffith, R. J. Maunder, T. R. Martin, J. P. Weiner-Kronish, M. Sticherling, E. Christophers, and M. A. Matthay.** 1992. Elevated levels of interleukin-8 are present in the airspaces of patients with the adult respiratory distress syndrome and are associated with increased mortality. Am. Rev. Respir. Dis. **146:**427–432.

33. **Morohashi, H., T. Miyawaki, H. Nomura, K. Kuno, S. Murakami, K. Matsushima, and N. Mukaida.** 1995. Expression of both types of human interleukin-8 receptors on mature neutrophils, monocytes and natural killer cells. J. Leukocyte Biol. **57:**180–187.

34. **Moser, B., C. Schumacher, V. von Tscharner, I. Clark-Lewis, and M. Bag-**

giolini. 1991. Neutrophil-activating peptide-2 and gro/melanoma growth stimulatory activity interact with interleukin-8 receptors on human neutrophils. J. Biol. Chem. **266:**10666–10671.

35. **Mulligan, M. S., M. L. Jones, M. A. Bolanowski, M. P. Baganoff, C. L. Deleppe, D. M. Meyers, U. S. Ryan, and P. A. Ward.** 1993. Inhibition of lung inflammatory reactions in rats by an anti-human IL-8 antibody. J. Immunol. **150:**5585–5595.

36. **Muñoz, C., B. Misset, C. Fitting, J.-P. Blériot, J. Carlet, and J.-M. Cavaillon.** 1991. Dissociation between plasma and monocyte-associated cytokines during sepsis. Eur. J. Immunol. **21:**2177–2184.

37. **Nakamura, H., S. Fujishima, Y. Waki, T. Urano, K. Sayama, F. Sakamari, T. Terashima, K. Soejima, S. Tasak, A. Ishizaka, T. Kawashiro, and M. Kanazawa.** 1995. Priming of alveolar macrophages for interleukin-8 production in patients with idiopathic pulmonary fibrosis. Am. J. Respir. Crit. Care Med. **152:**1579–1586.

38. **Neote, K., W. Darbonne, J. Ogez, R. Horuk, and T. J. Schall.** 1993. Identification of a promiscuous inflammatory peptide receptor on the surface of red blood cells. J. Biol. Chem. **268:**12247–12249.

39. **Nuijens, J. H., J. J. Abbink, Y. T. Wachtfogel, R. W. Colman, A. J. M. Eerenberg, A. Dors, A. J. M. Kamp, R. J. M. Strack van Schijndel, L. G. Thijs, and C. E. Hack.** 1992. Plasma elastase α1-antitrypsin and lactoferrin in sepsis: evidence for neutrophils as mediators in fatal sepsis. J. Lab. Clin. Med. **119:**159–168.

40. **Petersen, F., H. D. Flad, and E. Brandt.** 1994. Neutrophil-activating peptides NAP-2 and IL-8 bind to the same sites on neutrophils but interact in different ways. J. Immunol. **152:**2467–2474.

41. **Samanta, A. K., J. J. Oppenheim, and K. Matsushima.** 1989. Identification and characterization of specific receptor for monocyte-derived neutrophil chemotactic factor on human neutrophils. J. Exp. Med. **169:**1185–1189.

42. **Samanta, A. K., J. J. Oppenheim, and K. Matsushima.** 1990. IL-8 dynamically regulates its own receptor expression on human neutrophils. J. Biol. Chem. **265:**183–189.

43. **Schaufelberger, H. D., M. R. Uhr, C. McGuckin, R. P. H. Logan, J. J. Misiewicz, E. C. Gordon-Smith, and C. Beglinger.** 1994. Platelets in ulcerative colitis and Crohn's disease express functional interleukin-1 and interleukin-8 receptors. Eur. J. Clin. Invest. **24:**656–663.

44. **Schönbeck, U., E. Brandt, F. Petersen, H. D. Flad, and H. Loppnow.** 1995. IL-8 specifically binds to endothelial but not to smooth muscle cells. J. Immunol. **154:**2375–2383.

45. **Simonet, W. S., T. M. Hughes, H. Q. Nguyen, L. D. Trebasky, D. M., Dalinenko, and E. S. Medlock.** 1994. Long-term impaired neutrophil migration in mice overexpressing human interleukin-8. J. Clin. Invest. **94:**1310–1319.

46. **Somomkin, J. S., R. C. Bass, H. S. Bjornson, C. J. Tindal, and G. F. Babcock.** 1994. Alterations of neutrophil responses to tumor necrosis factor and interleukin-8 following human endotoxemia. Infect. Immun. **62:**943–947.

47. **Stack, G., and E. L. Snyder.** 1994. Cytokine generation in stored platelet concentrates. Transfusion **34:**20–25.

48. **Strieter, R. M., S. L. Kunkel, H. J. Showell, D. G. Remick, S. H. Phan, P. A. Ward, and R. M. Marks.** 1989. Endothelial cell gene expression of a neutrophil chemotactic factor by TNF, LPS, and IL-1β. Science **243:**1467–1469.

49. **Tilg, H., D. Pape, E. Trehu, L. Shapiro, M. A. Atkins, C. A. Dinarello, and J. W. Mier.** 1993. A method for the detection of erythrocyte-bound interleukin-8 in humans during interleukin-1 immunotherapy. J. Immunol. Methods **163:**253–258.

50. **Tilg, H., L. Shapiro, M. A. Atkins, C. A. Dinarello, and J. W. Mier.** 1993. Induction of circulating and erythrocyte-bound IL-8 by IL-2 immunotherapy and suppression of its in vitro production by IL-1 receptor antagonist and soluble tumor necrosis factor receptor (p75) chimera. J. Immunol. **151:**3299–3307.

51. **Waydhas, C., D. Nast-Kolb, A. Jochum, A. Trupka, S. Lenk, H. Fritz, K. H. Duswald, and L. Schweiberer.** 1992. Inflammatory mediators, infection, sepsis, and multiple organ failure after severe trauma. Arch. Surg. **127:**460–467.

52. **Yentis, S. M., A. W. Rowbottom, and P. G. Riches.** 1995. Detection of cytoplasmic IL-1β in peripheral blood mononuclear cells from intensive care unit patients. Clin. Exp. Immunol. **100:**330–335.