Physicochemical characterization of a polypeptide present in uremic serum that inhibits the biological activity of polymorphonuclear cells

(uremia/granulocyte dysfunction/protein purification)

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ABSTRACT A granulocyte inhibitory protein was isolated and characterized from uremic serum by using ion-exchange column chromatography, size-exclusion chromatography, and immunochemical procedures. The purification process concentrated the protein 240-fold and to a purity of >95%. An overall recovery of 45% was achieved; the purified protein had a specific activity of 104 units per mg of protein. The polypeptide had a molecular weight of ~28,000 and an isoelectric point of 4.0–4.5. Amino acid sequencing of the NH2-terminus revealed a single sequence (Asp-Ile-Val-Met-Thr-Glu-Ser-Pro-Gly-Thr-Leu-Ser-Val-Ser-Pro-Gly-Glu-Arg-Ala-Thr) that proved to be nonhomologous with other serum proteins that appear during an inflammatory state. The polypeptide inhibited the uptake of deoxyglucose, chemotaxis, oxidative metabolism, and intracellular bacterial killing by polymorphonuclear leukocytes. A specific rabbit polyclonal antibody raised against the protein nullified these inhibitory changes. We contend that the protein is responsible for the leukocyte dysfunction that is commonly seen in patients with uremia.

The increased morbidity and mortality of patients with renal failure has been attributed to the dysfunction of polymorphonuclear (PMN) cells (1, 2). Since PMN cells are metabolically active, uremia could contribute to impaired function by means of disarrangements in their cellular biochemistry and biology. For example, PMN cells from individuals with renal failure demonstrated impaired glycolytic enzyme activity (3), diminished intracellular ATP levels (4), attenuated chemotactic activity (5), and a reduced oxidative metabolism, which leads to impaired bacteriocidal activity (6). Conversely, hemodialysis improved the chemotactic response of PMN cells, which suggests the presence of an inhibitory factor(s) in uremic serum. In this context, factor(s) in the middle molecular weight range may be responsible for the dysfunction of the host’s cellular immune system found in patients with renal failure (7). We report here on the physicochemical and immunochemical characterization of a granulocyte inhibitory protein (GIP) that was isolated from the ultrafiltrate of a patient with chronic renal failure. The protein is capable of inhibiting the uptake of [3H]glucose in PMN cells; it reduces oxidative metabolism, inhibits chemotaxis, and diminishes intracellular killing of bacteria in PMN cells. In addition, a specific heterologous antibody prepared against the protein nullifies these changes.

MATERIALS AND METHODS

Protein Purification. Plasma ultrafiltrates were prepared from a patient with chronic renal failure by using a polysulfone filter (Fresenius, Oberursel, FRG) with a M, cutoff of 30,000–50,000 in the Dialysis Unit of the Department of Medicine of the University of Freiburg, FRG. The filtrates were pooled and stored at −70°C. After thawing, the filtrate was concentrated 25-fold by means of an Amicon ultrafiltration cell with a PM 10 membrane, which has a M, cutoff of ~10,000. The concentrate was dialyzed overnight at 4°C in dialysis buffer [20 mM TrisHCl (pH 8.0)], after which it was chromatographed on an ion-exchange fast protein liquid chromatographic (FPLC) system (Pharmacia). The original concentrate was applied to a Mono Q 5/5 column equilibrated with the dialysis buffer. The material was eluted with a linear gradient of 0–1 M NaCl in 20 mM TrisHCl (pH 8.0) [5% (wt/vol)] at a flow rate of 2 ml/min. Each fraction was tested for biological activity (for details, see below).

The fractions that contained biological activity were pooled and were concentrated by lyophilization. The material was resuspended in water and chromatographed with a high-performance size-exclusion (HPSEC) column. The material was placed on a Zorbax GF 250 (Du Pont de Nemours, Homburg, FRG) column (9.4 mm i.d., 250 mm long) equilibrated with 0.2 M sodium phosphate buffer (pH 7.1) and eluted at a flow rate of 1 ml/min. The biologically active fractions were in the M, range of 25,000–30,000. The active fractions were chromatographed a second time under the same conditions.

Electrophoretic Analysis. The active fractions were analyzed with a gel electrophoresis system according to Laemmli et al. (8) with SDS/12.5% polyacrylamide gels under reducing and nonreducing conditions.

Protein Concentration Determination. The concentration of the protein was measured by using the Lowry et al. method (9).

Western Blot. The electrotransfer of the proteins from SDS/polyacrylamide gels to a nitrocellulose membrane was probed with a rabbit antibody conjugated to horseradish peroxidase (Bio-Rad).

Isoelectric Focusing. The analysis was performed by using a standard agarose gel isoelectric focusing method (10) employing pH 3–10 ampholytes (Pharmacia).

Amino Acid Sequencing. The NH2-terminal sequence determination was done by using the automated Edman degradation procedure. An Applied Biosystems model 470A gas-phase sequencer was employed (11). The respective phenylthiohydantoin derivatives were identified by an on-line reverse-phase HPLC (model 120A) that was fitted with a C18 (2 mm i.d., 25 cm long) column.

Abbreviations: PMN cells, polymorphonuclear cells; GIP, granulocyte inhibitory protein; FPLC, fast protein liquid chromatography; iMet-Leu-Phe, N-formylmethionylleucylphenylalanine; HPSEC, high-performance size-exclusion column.

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Antibody Production. Rabbits were injected subcutaneously at multiple sites with 170 g of purified protein emulsified in Freund’s complete adjuvant. Booster injections of 170 g of purified protein in incomplete adjuvant were administered on days 8, 15, and 22. The animals were sacrificed, and their serum was collected and stored at -70°C. The IgG fraction was obtained by NH4SO4 precipitation and purified by ion-exchange column chromatography on DEAE-Sephacel (Pharmacia) by using 17 mM sodium phosphate (pH 6.8).

ELISA Assay. The assay was used to detect and titer the specific polyclonal antibody. Microtiter plates were coated with the purified GIP antigen to which various concentrations of the antibody were added. The antibody was detected with a goat anti-rabbit peroxidase-conjugated antibody and visualized with 1.2-phenylenediamine as the substrate.

PMN Cell Function Tests. The PMN cells were isolated from venous blood of normal donors according to Metcalf et al. (12). Their viability was determined by the trypan blue exclusion test, and it was found to be 96%. Intracellular killing activity of Staphylococcus aureus was assessed according to Metcalf et al. (12). The chemotaxis assay was performed by using the agarose procedure (13). A chemiluminescence assay was used to measure the oxidative respiratory burst potential of the PMN cells, and it was performed according to Metcalf et al. (12). D-[3H]glucose uptake was assessed according to McCall et al. (14).

GIP Activity. Specific GIP activity was determined in terms of its ability to inhibit the N-formylmethionylleucylphenylalanine (fMet-Leu-Phe)-induced uptake of D-[3H]glucose into PMN cells to basal levels after purification procedures that included dialysis and lyophilization.

Further enrichment of GIP activity was achieved by HPSEC. Pooled fractions from eight gel-filtration experiments (16 mg of protein) were used (Fig. 2). The major activity (500 g of protein), which eluted in fractions 20 and 21 at a retention time of 20.49 min, inhibited the uptake of D-[3H]glucose into PMN cells. The biological activity of the other fractions was insignificant. The fraction containing GIP activity was once again extensively dialyzed and then concentrated by vacuum dialysis; its volume was reduced to 1.9 ml, and it was stored at -70°C.

The purity of the concentrated GIP was determined by gel electrophoresis under reducing and nonreducing conditions. The SDS/PAGE data for the initial material (Fig. 3a, lane 2) and the GIP active fraction after FPLC (Fig. 3a, lane 3) and HPSEC (Fig. 3a, lane 4) are shown in Fig. 3a. Only one major band is present (Mr = 28,000) in the latter lane, which contained reduced protein. Nonreduced GIP migrated a
similar distance, which suggests that it is a polypeptide chain (data not shown).

A Western blot of the gel probes that used an antibody specific for GIP is illustrated in Fig. 3b. A predominant immunoreactive band is present in the same location as GIP. Lanes 3 and 4 in Fig. 3b (reduced protein) correspond to the GIP peak in fractions 15, 16, and 17 shown in Fig. 1 and to fractions 20 and 21 illustrated in Fig. 2. The immunoblot data suggest that the corresponding peaks were predominantly GIP with a $M_r$ of 28,000. The isoelectric point of GIP was between 4.0 and 4.5. A second band, minor and faintly stained, was also noted.

The amino acid sequence analysis of the purified GIP revealed the following NH$_2$-terminal sequence: Asp-Ile-Val-Met-Thr-21,-Ser-Pro-Gly-Thr-Leu-Ser-Val-Ser-Pro-Gly-Glu-Arg-Ala-Thr.

Yields of GIP. The protein content of one ultrafiltrate preparation (9500 ml) was 272 mg of protein; it was estimated by the Lowry procedure (9). The GIP active material obtained from the FPLC Mono Q isolation procedure yielded 16 mg of protein, or $\approx$6% of the initial material. After HPSEC, the active protein fraction yielded 0.51 mg of protein, or 0.19% of the initial material. The methods for the purification of GIP are provided in Table 1. Pure GIP had a specific activity of 104 units/mg in the assay. A 240-fold purification was achieved in these experiments, with a recovery in the 45% range. Comparable data were obtained from six additional individuals with chronic renal failure.

**Biological Activity of GIP and Its Neutralization by a Specific Antibody.** The ability of GIP to block four measured functional changes of PMN cells that were stimulated either by fMet-Leu-Phe or by _S. aureus_ 502 occurred as a function of concentration. Thus the IC$_{50}$ of GIP required for inhibition of half-maximal fMet-Leu-Phe-induced D-[3H]glucose uptake was $5.2 \pm 1.2 \mu$g/ml (mean $\pm$ SEM, $n = 5$); the IC$_{50}$ for inhibiting fMet-Leu-Phe-stimulated chemotaxis was $7.4 \pm 1.7 \mu$g/ml ($n = 6$); the IC$_{50}$ for inhibition of fMet-Leu-Phe-dependent activation of oxidative metabolism was $8.1 \pm 1.4 \mu$g/ml ($n = 5$); and the IC$_{50}$ for inhibiting the intracellular killing of _S. aureus_ 502 by the PMN cells was $7.9 \pm 1.7 \mu$g/ml ($n = 6$). The neutralizing effect of anti-GIP antibody is depicted in Fig. 4.

In addition to these inhibitory changes, GIP blocks the activity of other metabolic enzymes of PMN cells: phosphorylase, cytochrome c, and glycogen synthetase. Moreover, the release of elastase, lactoferrin, and myeloperoxidase was also inhibited by GIP. The results of these inhibitory effects by GIP will be described elsewhere. Boiling the GIP for 20 min destroyed its activity. When PMN cells were exposed to high concentrations (up to 500 $\mu$g/ml) of GIP, their viability was unaffected, although they were biologically dysfunctional; following the removal of the factor by washing, normal biological activity was restored.

**DISCUSSION**

A PMN cell inhibitory protein (GIP) was detected in the serum of a patient with chronic renal failure by using conventional column chromatography followed by HPSEC. When GIP was analyzed for protein purity by SDS/PAGE and Western blotting, the protein was shown to be 95% pure. The observed retention times on HPSEC for GIP purification from various ultrafiltrate preparations were consistent, which allowed a reliable preparative isolation of the protein. The high-resolution analysis excluded the possibility of GIP isoforms and contaminating protein within detection limits of the methods employed.

The degree of GIP purification was $\approx$250-fold if the D-[3H]glucose uptake is considered as a criterion of specific GIP activity. Purified GIP appears to be relatively homogenous, with a molecular weight of 28,000 as was verified by column chromatography, HPSEC, and SDS/PAGE. Isoelectric focusing of the protein indicated that it is acidic. A computer search for probable homology regarding the NH$_2$-terminal sequence excluded the possibility of it being identical to other known serum-derived molecules that occur during an inflammatory response, which include endotoxins, interleukins, interferons, complement factors, colony-stimulating factor, and tumor necrosis factor $\alpha$ and $\beta$ (Protein Identification Resource National Biomedical Research Foundation, release 23). In fact, most of the mediators stimulate PMN cell function rather than inhibit it (data not shown). Moreover, the GIP-specific antibody failed to cross-react with any of the above-mentioned inflammatory mediators. Although of low titer, specific GIP antibodies (IgG fraction) were capable of neutralizing the activity of the protein under four different assay conditions.

Although GIP was isolated from the serum of an uremic patient, its biological implications are far reaching. Evolutionarily, it appears paradoxical that a circulating factor that inhibits the host's cellular defense mechanism occurs under

<table>
<thead>
<tr>
<th>Protein, mg</th>
<th>Volume, ml</th>
<th>Activity, units</th>
<th>Specific activity, units/mg</th>
<th>Fold purification</th>
<th>% recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultrafiltrate</td>
<td>272</td>
<td>9500</td>
<td>119</td>
<td>0.46</td>
<td>1</td>
</tr>
<tr>
<td>PM 10 concentrate</td>
<td>260</td>
<td>380</td>
<td>108</td>
<td>0.42</td>
<td>0.96</td>
</tr>
<tr>
<td>FPLC Mono Q active fractions</td>
<td>16</td>
<td>12</td>
<td>71</td>
<td>4.4</td>
<td>10</td>
</tr>
<tr>
<td>HPSEC active fractions</td>
<td>0.51</td>
<td>1.9</td>
<td>53</td>
<td>104</td>
<td>240</td>
</tr>
</tbody>
</table>

One unit corresponds to 10 $\mu$g of GIP per ml, which inhibits the fMet-Leu-Phe-induced uptake of D-[3H]glucose into $10^8$ PMN after 60 min of incubation.
biological conditions. However, one has to consider that the following events may play an important role in the production of GIP: the direct immunosuppressive effects of uremic toxins, the indirect effects of metabolic anomalies in chronic renal failure, and the contact effect of the dialysis membrane on blood constituents (i.e., cells and complement factors). Furthermore, since most of the patients are suffering from severe infections, there is a possibility that GIP may be derived from one of the infecting microorganisms.

The possibility of a middle-range molecular weight protein present in uremic serum that leads to the impairment of leukocyte function has been described previously (7). This goes along with recent observations in which the pathogenesis of infection in uremic individuals favors a direct immunosuppressive action of the host's cellular defense mechanisms (2). Thus, GIP blocks fundamental PMN cell function, which is relevant for controlling biological homeostasis. As a consequence, the following has to be taken into consideration: (i) GIP is restricted to the uremic syndrome; thus, its early detection may be beneficial for the prognosis of the host, and an appropriate intervention (i.e., immunization) could then be considered as an appropriate therapeutic strategy. (ii) GIP is not restricted to uremic individuals, and one has to consider the role of the factor in the pathogenesis of various clinical disorders associated with an increased susceptibility to infection (seen in immunocompromised individuals). (iii) The IC₅₀ of GIP required to inhibit both the biochemical and functional changes is in the nanomolar range; therefore, the efficacy of the protein is well within the range of physiological effector substances (i.e., lymphokines and cytokines). (iv) The biological activity of PMN cells can be restored after the removal of GIP, and it is therefore possible that the function of the protein is of physiological relevance. For example, it may be capable of counterbalancing the overriding stimulatory effects of inflammatory and immunological stimuli on the PMN cells. In summary, GIP is a protein with multiple biological implications. However, its exact role remains to be elucidated.

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