Observations on Trace Proteins in Plasma of Febrile Patients by Cationic Disc Electrophoresis in Acrylamide Gel at pH 3.8

Charles W. Young, Sadie Hodas, Wilner Dessources, and Leonhard Korngold

SUMMARY

Cationic disc electrophoresis at pH 3.8 in 6 M urea-containing acrylamide gels permits analysis of plasma and other body fluids for the presence of trace proteins with pI ≥ 5 and M.W. < 60,000. These charge and size characteristics would include rabbit and human granulocytic pyrogen, human monocyctic pyrogen and, by inference, other similar candidate pyrogenic proteins. Semiquantitation of the trace protein content can be achieved by densitometric scanning of gels stained with Amido schwarz. Duplicate analyses were performed on plasma samples from 133 individuals: normal, 15; afebrile advanced cancer, 18; febrile Hodgkin’s disease, 30; febrile Hodgkin’s disease, 33; other febrile lymphoma, 13; febrile advanced cancer without infection, 12; and pyogenic fever, 12. Plasma from most of the febrile patients, particularly from febrile Hodgkin’s disease patients, contained trace proteins not detectable in the afebrile individuals studied. In patients with Hodgkin’s disease the quantity of trace proteins present in plasma correlated well with overall severity of Hodgkin’s pyrexia, but not with spontaneous hr to hr fluctuations in the fever. Marked reduction in plasma levels of the trace proteins occurred with response to antitumor therapy. Elevated plasma levels of these proteins can be induced by intratumoral inoculation of Bacillus Calmette-Guérin. They appear concomitant with the febrile response.

INTRODUCTION

The pathophysiological mechanisms that produce fever in advanced Hodgkin’s disease and, to a lesser extent, in other neoplasms of the reticuloendothelial system are obscure (13, 18). Clinical evidence, i.e., defervescence after effective anticancer therapy, suggests that the fever results from a product of tumor metabolism. Granulocytes (5, 8) and macrophages (2, 5, 6) can release pyrogen(s), specifically a low-molecular-weight protein or proteins, which produce fever by an undefined action on the hypothalamus (1, 4). The abundant histiocytes, i.e., tissue macrophages, both normal and neoplastic (15), present in the lesions of advanced Hodgkin’s disease might provide a source for a similar pyrogen. The release of unusual amounts of a physiological substance by this neoplastic tissue would be analogous to the production of immunoglobulin by myeloma cells.

We have previously noted (25) that cycloheximide, an inhibitor of protein synthesis, has an acute antipyretic effect in febrile patients with Hodgkin’s disease or other reticuloendothelial neoplasms. A perturbation of protein metabolism occurred with doses of cycloheximide which produced defervescence, leading us to propose that continued synthesis of protein was required to sustain fever in Hodgkin’s disease (24). A simple model that could account for these observations would be to propose the existence of a circulating pyrogenic protein characterized by a short plasma lifetime. If release of the pyrogen from tumor tissue were dependent upon protein synthesis, drug-induced slowing of protein formation would cause pyrogen levels to fall, resulting in defervescence.

In 1967, Sokal and Shimaoka (19) reported that concentrated urine from febrile Hodgkin’s disease patients was pyrogenic in rabbits; while comparably prepared normal urine was inactive. In light of the established existence of pyrogenic proteins and of the proposed relationship between protein synthesis and Hodgkin’s fever, we conjectured that Sokal and Shimaoka’s urinary pyrogen might well be a protein. Accordingly, by means of cationic electrophoresis in urea-containing acrylamide gels, we have compared protein excretion patterns of febrile Hodgkin’s patients with those of afebrile patients with advanced cancer and apparently normal individuals. The methods used placed particular emphasis on detection of proteins with size and charge characteristics similar to those reported for human and rabbit granulocyte pyrogens, i.e., M.W. 10,000 to 40,000, and an isoelectric point between 5 and 9 (5, 9, 14). Urines from febrile patients contain a number of low-molecular-weight proteins not found in normal urines or in afebrile controls with advanced cancer (22). Prominent among these was a relatively cationic protein that was also well visualized in electrophoretograms of cerebrospinal fluid. By applying the same techniques to plasma from febrile patients we have demonstrated therein a characteristic protein electrophoretically identical to that previously seen in urine. A preliminary report on the plasma observations has appeared (23). In this and the following communication (21), we provide details on: (a) the methods used; and (b) the results of a partial survey on the trace protein content of plasma obtained from patients with a variety of conditions, and changes therein in response to therapy. We have analyzed...
samples electrophoretically over 2 pH ranges; 3.8 and 6.0. Detailed comparison of the buffers, reagents, and the results with known standard proteins is given in the following report (21). The order of appearance of the articles herein is due in part to the chronology of the study and to the fact that internal inconsistencies demonstrated in parallel observations on blood, urine, and cerebrospinal fluid at pH 3.8 provide necessary background for the studies at pH 6.0.

MATERIALS AND METHODS

Reagents and methods for electrophoretic analyses on plasma and sera, modified from the method of Reisfeld et al. (16), are detailed elsewhere (21).

Chemicals. Antiserum (and the immunoglobulin fraction therefrom) raised in rabbits against human CRP,2 Research Division, Miles Laboratories, Inc., Elkhart, Ind.; and Affi-Gel 10 was from Bio-Rad Laboratories, Richmond, Calif. Purified CRP was generously provided by Dr. Emil Gotschlich, Rockefeller University, New York, N. Y.

Affinity Chromatography. The γ-globulin fraction of rabbit anti-human CRP antiserum was adsorbed onto Affi-Gel 10 by incubation for 4 hr at 4° in 0.1 m phosphate buffer, pH 7.0; and then it was poured into a 5- x 1.5-cm column and washed until the eluant was free of U.V. (260 nm)-absorbing material. Two ml of CRP- and doublet-containing serum were dialyzed overnight against 0.05 m phosphate buffer in 0.14 m NaCl, pH 7.6, and was percoluted through the Affi-Gel column at a rate of 1 ml/hr at room temperature.

Patients. The patients studied were carefully selected to fall into clear pathophysiological or physiological categories. Febrile patients, with simultaneous neoplastic and bacterial-induced suppurative processes were not studied. The afebrile patients with advanced cancer had had no elevations greater than 37.4° for 10 to 14 days prior to testing. Agonal patients or patients under active radiation therapy were excluded.

RESULTS

When plasma is subjected to electrophoresis toward the cathode through a urea-containing 7% acrylamide gel, the vast bulk of protein is retained within the sample gel or the upper portions of the separation gel permitting visualization of trace components of M.W. < 50,000 and pI ≥ 5. Urea disassociates protein aggregates that are caused by hydrogen bonding and provides a firmer and tighter gel than that obtained with 7% acrylamide alone. Thus, since native hemoglobin (M.W. 60,000) is broken into 4 globin monomers, each with M.W. 15,000 and pI 7, that can enter the separating gel, it is necessary to exclude hemolysis in preparing samples.

Extensive studies of the urine of febrile patients with Hodgkin’s disease have disclosed the presence of a tubular proteinuria characterized by a variety of low-molecular-weight proteins (C. W. Young, S. Hodas, and W. Dessources, manuscript in preparation). Two bands present in urine (Fig. 1, γ and γ) were also demonstrable in spinal fluid. The most distal of these, “γ,” has size and charge characteristics similar to β2-microglobulin (3, 20), a protein commonly observed in tubular proteinurias. Somewhat proximal to the β band lies “γ,” a protein of slightly larger molecular weight but higher isoelectric point as judged from its behavior during both ion-exchange chromatography on QAE-Sephadex and isoelectric focusing in acrylamide gels (unpublished observations). The γ protein was more prominent in spinal fluid than β, but less prominent in the urines of febrile patients with Hodgkin’s disease (Fig. 1).

When plasma samples were examined according to the same technique, febrile Hodgkin’s plasma was characterized by a doublet band in the area of the more cationic protein (γ) described above and by a relative decrease in the presumed β2-microglobulin band (Fig. 2). Normal plasma, on the other hand, displayed a strong β2-microglobulin band and nondetectable staining in the area of the more cationic band (Fig. 2). Plasma from a series of patients with advanced cancer, selected for the absence of fever or any evident inflammatory state for the 2 weeks prior to examination, disclosed patterns quite like those of normal plasma; the doublet band was absent or below the level of detectability (Fig. 2). When a sample of the cationic protein that was purified from urine was subjected to coelectrophoresis with the febrile plasma, it yielded a migration of identity with the distal component of the plasma doublet (Fig. 3).

Densitometric Quantitation. A densitometric scanning technique has been used to measure the protein content in the doublet area (Chart 1). Studies have been carried out on a variety of patient categories: febrile Hodgkin’s disease;

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2 The abbreviations used are: CRP, C-reactive protein; DP, doublet proteins.
active but afebrile Hodgkin’s disease; afebrile Hodgkin’s disease in remission; acute sepsis; chronic infection; fevers of unknown origin in the presence of neoplastic disease; normals; and late-stage advanced cancer in the absence of fever. The results of these observations are shown in scattergram fashion in Charts 2 and 3.

There was a statistically significant correlation between the severity of pyrexia and the plasma content of DP when patients with active Hodgkin’s disease were compared. DP content in plasma of patients with severe pyrexia [99.2 ± 11.6 (S.E.)] significantly exceeded that present in moderate pyrexia (52.1 ± 5.8); p < 0.005. In both, the plasma DP content exceeded that of afebrile patients with active Hodgkin’s disease (21.4 ± 2.3); p < 0.001. Approximately one-half of a small group of patients that were studied in clinical remission on therapy showed persistent traces of DP, while only 1 of 10 patients with apparently inactive Hodgkin’s disease in complete remission without therapy for greater than 2 years, had detectable DP in his plasma.

The excellent correlation between DP and the severity of the febrile phenomenon did not extend to acute fluctuations in body temperature. Although the majority of the daily fluctuations occurred in response to antipyretic medication, some patients with moderate pyrexia experienced spontaneous daily changes that were not reflected in the content of DP within their plasma.

When afebrile normals, afebrile cancer patients, febrile Hodgkin’s patients, febrile leukemia or non-Hodgkin’s lymphoma patients, febrile solid tumor patients, and patients with chronic pyrogenic infections were compared, it
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was readily apparent that the content of DP correlated better with the febrile state than it did with a specific diagnostic entity (Chart 3).

Effects of Therapy. A decline in plasma content of DP was seen when defervescence and marked improvement of other manifestations of Hodgkin’s disease activity occurred in response to anticancer therapy. This change in plasma was observed in as little as 2 days (see Fig. 4, legend). In contrast to anticancer agents, the antipyretics, aspirin, acetaminophen, and indomethacin produced no apparent acute effect on plasma levels of DP. Prednisone appeared to augment levels slightly. This may be a factor in the strikingly elevated DP levels in the patients with severe pyrexia, since that group had been characterized by pyrexia in the face of steroid therapy. In contrast, the antipyretic effect of cycloheximide has been accompanied by a significant decrease in plasma DP levels within 9 hr in a close parallel to the effect of the drug on body temperature and other aspects of protein metabolism (Fig. 4; Table 1).

The effect produced by pyrogenic stimuli on plasma DP levels has been explored only in a preliminary fashion. However, both bleomycin and Bacillus Calmette-Guérin have produced elevations in plasma DP concentrations concurrent with induction of fever. Detailed studies have not yet been done on the relationship between fever of bacterial infection and plasma DP content. A few preliminary observations in postoperative fevers have given erratic results; DP levels have not been consistently elevated. On the other hand, when they have been present in the context of bacterial infection, they have declined promptly when defervescence was produced by antibiotics and wound drainage (Table 2).

Demonstration that the DP are not CRP. Although CRP is relatively large with a molecular weight of ~120,000, its monomeric subunits might enter the separating gel if the native hexameric structure were disassociated by the 6 M urea used in this assay. Because of this, a direct experimental comparison of CRP and DP seemed appropriate. In electrophoretic analyses, the DP and purified CRP differed markedly (Fig. 5). The DP are not removed from serum by Affi-Gel-bound anti-CRP antibody, and their presence in serum does not inhibit the removal of CRP itself (Fig. 5 and 6). Therefore, there is no antigenic overlap between the 2 species.

DISCUSSION

Cationic electrophoresis in urea-containing acrylamide gels has demonstrated trace amounts of a protein (DP) in the plasma (and serum) of febrile patients that was not detected in afebrile individuals. We have not established an identity between DP and any well-characterized plasma protein. Muramidase is not retained in the gel in this assay, having run off the cathodal end. Because of their size and charge characteristics, the acute-phase serum proteins @ antitrypsin, a 1-glycoprotein, haptoglobin, ceruloplasm, fibrinogen, and CRP fail to enter the separating gel or else they are retained in its proximal portion.

LeRoy et al. have described increased concentrations of a hydroxyproline-containing plasma protein, “hyproprotein,” in patients with inflammatory disorders (11) and Hodgkin’s disease (10). The bulk of hyproprotein in plasma was excluded from Sephadex G-200 suggesting a molecular weight of >400,000 (12). However, these observations were carried out in media that would permit formation of aggregates; thus, they offer no prediction for the behavior of the hyproprotein in the presence of 6 M urea. Because of the strong similarity between the clinical conditions that induce

### Table 1

<table>
<thead>
<tr>
<th>Time</th>
<th>Temperature °C</th>
<th>Muramidase (µg/ml)</th>
<th>RNase (µg/ml)</th>
<th>DP density units</th>
</tr>
</thead>
<tbody>
<tr>
<td>−30 min</td>
<td>38.7</td>
<td>0.85</td>
<td>0.75</td>
<td>62</td>
</tr>
<tr>
<td>5 hr</td>
<td>38.1</td>
<td>0.95</td>
<td>0.74</td>
<td>50</td>
</tr>
<tr>
<td>9 hr</td>
<td>36.7</td>
<td>0.55</td>
<td>0.74</td>
<td>21</td>
</tr>
</tbody>
</table>

**Acute changes in pyrexia and in trace protein concentrations in plasma in response to cycloheximide**

A 6-hr infusion of cycloheximide, 0.2 mg/kg/hr, was initiated at 0 hr. The patient was a 22-year-old woman with Stage IV-B Hodgkin’s disease and “severe” pyrexia. She was icteric and pancytopenic at the time of the study. Muramidase (7), RNase (17), and DP levels are the mean ± 0.05 of triplicate determinators.

### Table 2

**Sequential changes in fever and plasma DP concentration in 2 patients after treatment of postoperative infections**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Date</th>
<th>Peak temperature °C</th>
<th>Plasma DP*</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>7/17/72</td>
<td>39.9°</td>
<td>87</td>
<td>This 48-yr-old female had been febrile for 7 days from an infected seroma which was drained on 7/18/72.</td>
</tr>
<tr>
<td></td>
<td>7/20/72</td>
<td>37.2</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7/24/72</td>
<td>37.2</td>
<td>13 (3 doublet)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>7/14/72</td>
<td>38.4</td>
<td>200</td>
<td>This 41-yr-old woman had been febrile for 3 wk due to a pelvic abscess that was drained on 7/15/72. Defervescence ensued over the following 72 hr.</td>
</tr>
<tr>
<td></td>
<td>7/20/72</td>
<td>37.2</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7/25/72</td>
<td>37.2</td>
<td>18 (3 doublet)</td>
<td></td>
</tr>
</tbody>
</table>

* DP values are the mean ± 0.05 of triplicate determinators.
Trace Proteins in Plasma of Febrile Patients

DP and hyproprotein, further comparison of the 2 in the course of plasma fractionation seems indicated.

While it seems likely that the DP are linked in some way to the complex processes that generate fever, a simple identity between all of the plasma DP and leukocyte pyrogen seems out of the question. The purest granulocytic pyrogen preparations from rabbit peritoneal exudates produce fever when injected into rabbits in submicrogram amounts (14); such quantities could not be visualized by postinjection by this electrophoretic method. The force of this argument may be weakened somewhat by noting that the electrophoretic assay in urea visualizes not only "free" molecules of proper size and charge but also similar molecules (in size and charge) that may be linked in vivo to larger proteins by hydrogen bonding.

The precise relationship between the DP in plasma of febrile patients and the cationic protein excreted in the urine of these same patients requires further clarification. The discordant observation that a single protein is present in the doublet area in urine and in cerebrospinal fluid, but 2 closely linked proteins are seen in plasma argues that multiple species of proteins may be involved that are not distinguishable from one another in this assay. In some electrophoretic runs the doublet bands can apparently be preshadowed by another protein (or proteins) advancing slightly distal to the main component. This can be noted as a small shoulder on the distal component of the doublet in the densitometric trace in Chart 1.

Pursuing this possibility, we have developed a cationic disc electrophoresis system operating closer to neutrality (21). In this technique the resolution of proteins with pI's ranging between 7.5 and 9 was improved; furthermore, they were separated almost entirely from proteins with pI's below that level. By combining the new electrophoretic technique with a more sensitive stain for protein, Coomassie blue, we have at least partly resolved the plasma-urine discrepancy and detected further trace components not visualized by this technique (21).

ACKNOWLEDGMENTS

The authors note with gratitude the continuing support and encouragement of Dr. Marguerite P. Sykes, Dr. Irwin H. Krakoff, and Dr. Bayard Clarkson throughout these studies. Material assistance was provided by Maureen O’Hehir and coworkers in obtaining blood specimens and by Ruth Russell in correlating the clinical and laboratory observations.

REFERENCES

Fig. 1. Polyacrylamide gel electrophoretograms of urine concentrates from normal and febrile individuals and of concentrated cerebrospinal fluid (CSF) stained to detect trace proteins in the distal aspects of the gels. The significance of $\beta$ and $\gamma$ is discussed in the text. H. D., Hodgkin’s disease.

Fig. 2. Polyacrylamide gel electrophoretogram examples of the pattern displayed by plasma samples from febrile patients with Hodgkin’s disease (a, b); normal individuals (c, d), and afebrile patients with advanced cancer (e, f). Sample onlay, 10 $\mu l$.

Fig. 3. Polyacrylamide gel electrophoretograms of extensively purified cationic protein urine (CPu) from a patient with Hodgkin’s disease (H. D.), Hodgkin’s disease plasma without and with cationic protein urine added, and cerebrospinal fluid (CSF) without and with cationic protein urine added.

Fig. 4. Polyacrylamide gel electrophoretograms illustrating the effect of therapy upon the DP content of patients with Hodgkin’s disease. Plasma onlay, 20 $\mu l$ in A and A’, and 10 $\mu l$ in B, B’, C, and C’. Time intervals: A to A’, 9 h; B to B’, 6 days; C to C’, 18 days. DP content of 10 $\mu l$ plasma in density units: A (62); A’ (21); B (73), B’ (not detected) (DP content at 48 hr postonset of therapy was 28; at 96 hr postonset it was 7); C (76), C’ (not detected). Therapy: A, cycloheximide infusion (0.2 mg/kg/hr for 6 hr) produced defervescence from 38.7° to 35°. B procarbazine (300 mg/day) produced rapid lysis of fever and regression of hepatosplenomegaly. C, vinblastine (0.1 mg/kg/wk) produced regression of nodes, liver, and lung lesions and defervescence.

Fig. 5. Acrylamide gel electrophoretograms of serum before (a) and after (c) removal of CRP (by anti-CRP antibody and affinity chromatography) and of purified CRP (b).

Fig. 6. Double diffusion in agar. The center well contained anti-CRP antiserum; Wells a, b, and c contained untreated serum, purified CRP, and anti-CRP-treated serum, respectively.
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