Interleukin-1β in Human Plasma: Optimization of Blood Collection, Plasma Extraction, and Radioimmunoassay Methods

JOSEPH G. CANNON,1,2 JOS W.M. VAN DER MEER,1 DOMINIC KWIATKOWSKI,3 STEFAN ENDRES,1 GEROHARD LONNEMANN,1 JOHN F. BURKE,2 and CHARLES A. DINARELLO1

1New England Medical Center—Tufts University, Boston, MA 02111
2Massachusetts General Hospital, Boston, MA 02107
3Medical Research Council Laboratories, Fajara, The Gambia

ABSTRACT

Current immunoassays for interleukin-1β (IL-1β) are effective for analyzing fluids derived from cultured cells. However, IL-1β determinations in human plasma or serum samples are technically complicated by higher protein and lipid concentrations, physicochemical differences which exist between samples from healthy subjects and those experiencing acute phase responses, and by the fact that IL-1β can be produced and degraded in the blood collection tube after the sample is drawn. A simple chloroform extraction process has been developed which eliminates several of the interfering factors from plasma samples and increases the amount of IL-1β detected by radioimmunoassay and lymphocyte activation assay. In the radioimmunoassay, rabbit sera was found to influence the accuracy and variability of plasma measurements. Improvements in radioimmunoassay reagents and methods are reported which reduce this influence. Finally, different concentrations of IL-1β were measured depending on whether serum or plasma was tested. We propose that plasma samples collected with EDTA and aprotinin provide a better determination of free circulating IL-1β in vivo than serum samples, which may contain IL-1β secreted from blood leukocytes during the clotting process.

INTRODUCTION

Endogenous circulating factors which mediate host responses to infection were first demonstrated by passive transfer experiments of Atkins and Wood (1). These experiments showed that an endogenous pyrogen in plasma taken from a febrile animal caused fever when injected into another animal. The work of Kampschmidt and colleagues (2) demonstrated that an endogenous mediator from leukocytes was responsible for acute phase responses occurring in diverse tissues, which reinforced the idea that circulating systemic factors mediated a whole-body response to infection.

It is now known that several cytokines (interleukin-1α and β, tumor necrosis factor α, interferon α, and interleukin-6) are pyrogenic and can induce other manifestations of the acute phase response (3, 4). Most of the information regarding the roles of these cytokines in inflammation and disease has been inferred from experiments in which exogenous cytokines were injected into laboratory animals. Relatively little is known about the production of cytokines in vivo, their tissue distribution, metabolism or clearance in response to infection or trauma.
Recent investigations have reported tumor necrosis factor (TNF) levels in serum from patients with parasitic infections (5), meningococcemia (6), and allograft rejection (7). In addition, the time course of circulating TNF in human volunteers after administration of endotoxin has been studied (8). Measurements of circulating TNF have been possible because bioassays and immunoassays for TNF are not significantly inhibited by human plasma or serum. In contrast, plasma factors have been serious impediments to bioassays for interleukin-1 (IL-1) (9, 10). Modified IL-1 bioassays of plasma samples are only semiquantitative and laborious for large clinical studies (11-13).

The present report describes the development of an extraction method and modifications of radioimmunoassay (RIA) procedures suitable for measuring IL-1β in human plasma. Several considerations were taken into account. For example, like most peptide hormones (14), IL-1β may be bound to large carrier proteins in the circulation. In addition, unlike the sources of other peptide hormones, IL-1β-producing leukocytes are collected in the blood sample, and may secrete IL-1β in the collection tube. Finally, the primary sequences of rabbit and human IL-1β are very similar (15) and have common antigenic determinants (16). Therefore, rabbit sera used in the RIA may contribute rabbit IL-1β or carrier proteins which interfere with the measurement of human IL-1β.

METHODS

**Blood Processing.**

Blood samples (10 cc) from healthy laboratory personnel were collected in sterile, vacuum blood collection tubes containing EDTA (1.5 mg/ml of blood) (Terumo Medical, Elkton, MD) to which aprotinin was added (0.67 TIU/ml of blood) (Sigma, St. Louis, MO). The tubes were centrifuged within 10 minutes of venipuncture (400g for 10 minutes), or placed on ice until centrifugation (2 hours maximum). The plasma was removed without disturbing the buffy coat, aliquoted in 1.5 ml microfuge tubes and spun at 10,000g at 4°C for 1 minute to pellet the platelets. The plasma was then transferred to new microfuge tubes and frozen at -20°C until assay. Seven acute phase blood samples were obtained from Gambian patients who presented at the Medical Research Council clinic at Fajara with acute symptoms of malaria. All were infected with Plasmodium falciparum and were febrile. These samples were processed in the same manner as those from healthy donors.

**Plasma Extraction.**

Because human plasma samples caused precipitation of radioactivity in the RIA in excess of the 100% standard in buffer (B0), a number of procedures were evaluated for removal of interfering plasma factors (17). Chloroform extraction was found to provide the best recovery of exogenous IL-1β. Plasma (0.5 ml) and 1.0 ml of chloroform (Fisher Scientific, Fair Lawn, NJ) were added to 1.5 ml microfuge tubes. The tubes were mounted horizontally on a multitube vortexer (American Hospital Supply, Miami, FL), agitated for 5 minutes, transferred to a microfuge and spun for 5 minutes at 10,000g, 4°C. The aqueous phase was recovered, extracted a second time, then assayed.

**IL-1β Radioimmunoassay.**

Initial studies employed the competitive inhibition assay method developed by Lisi et al. (18). The present assay used polyclonal rabbit antisera raised against a mixture of recombinant human IL-1β from two sources (Cistron, Pine Brook, NJ, and Glaxo, Geneva Switzerland; the latter material was kindly provided by Dr. Alan Shaw). The assay buffer consisted of 0.01 M sodium phosphate in 0.9% saline (pH 7.4) with 0.25% bovine serum albumin (BSA, Sigma A-7030) and 0.25% sodium azide (Fisher). Reference standards ranging in concentration from 5,000 to 10 μg/ml were prepared by serial two-fold dilution of human recombinant IL-1β (Glaxo). Reference standards and extracted plasma samples (100 μl/tube) were tested in 12 x 75 mm polystyrene tubes (Stockwell Scientific, Walnut, CA) with 100 μl of diluted antiserum and 300 μl of buffer.

Five major modifications were necessary in order to optimize this RIA for human plasma samples. First, the rabbit anti-human IL-1β was partially purified by precipitation with 40% saturated ammonium sulfate, resuspended in 0.05 M phosphate buffered saline (PBS), and dialyzed against PBS. This preparation was diluted to 5.5 μg/ml, and 100 μl was added to each tube. Second, the RIA was performed as a sequential assay in which the initial incubation of samples and standards with rabbit anti-human IL-1β was lengthened to 48 hours. This was followed by an 18 hour incubation with 125I-IL-1β (100 μl/tube). Third, in addition to iodination by the chloramine T method (19), some 125I-IL-1β lots were prepared with Bolton-Hunter reagent (20) (New England
Nuclear, Boston, MA), and by a peroxidation method using lactoperoxidase on a solid phase support (Enzymobead reagent, Bio-Rad, Richmond, CA) as specified by the manufacturer's directions. All results presented were obtained with tracer prepared by the chloramine T method unless stated otherwise. Fourth, precipitation of the anti-IL-1β and bound 125I-IL-1β was accomplished by adding 0.75 ml of a solution containing 1.3% sheep anti-rabbit IgG (Cambridge Medical Technology, Billerica, MA) and 4% polyethylene glycol (16-20 kD, Sigma), incubating at room temperature for 1 hour, and centrifuging at 1000 g for 30 minutes. Fifth, pooled normal rabbit serum (2 μl/tube), which was used in the original method to provide carrier proteins for the formation of a large precipitate, was eliminated from the method optimized for plasma.

The radioactivity pelleted in the assay tubes was counted, nonspecific binding in the absence of anti-IL-1β (NSB) was subtracted, and the result expressed as a percentage of B₀ minus NSB. The detection limit was set at 95% binding, or at the deviation from linearity of the standard curve (plotted on a logistic probability graph) if this occurred at a lower percent binding.

**Plasma Chromatography.**

Selected plasma samples and extracts (0.3 ml) were applied to 1 x 30 cm gel filtration columns packed with either Sephadex G-50 (Pharmacia, Piscataway, NJ) or Biogel A1.5m (Bio-Rad). The gels were equilibrated and the samples eluted with 0.9% saline. Fractions of 0.6 or 1.0 ml were collected.

**Lymphocyte Activation Assays.**

Thymus cells from C3H/HeJ mice (Jackson Laboratories, Bar Harbor, ME) were suspended at a final concentration of 2.5 x 10⁶ cells per ml of RPMI 1640 medium supplemented with L-glutamine (0.3 mg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml)(all from Gibco, Grand Island, NY), 10 mM HEPES (Microbiological Associates), 2-mercaptoethanol (Eastman Kodak, Rochester, NY) and 2.5% heat-inactivated fetal bovine serum (HyClone, Logan UT). The cells were incubated with 1 μg/ml phytohemagglutinin (PHA)(Difco, Detroit, MI) and either plasma fractions or various concentrations of human IL-1β in 96 well microtiter plates at 37 °C, 5% CO₂. The rates of proliferation were assessed by adding 1 μCi of 3H-thymidine (6.7 Ci/mmol, New England Nuclear) to each well after 48 hours, then harvesting the cells and counting incorporated radioactivity at 72 hours. A subclone (D10S) of the murine T-helper cells D10.G4.1 (21) was also employed in similar assays except these cells were plated at a lower density (4 x 10⁵ cells per ml) and cultured without PHA.

**Immunoadsorption.**

Individual plasma samples (7 ml) from four subjects were mixed with 0.5 ml of anti-IL-1 bound to Sepharose 4B (Pharmacia) in 15 ml tubes which were rotated end over end at 37 °C for 4 hours. The Sepharose-bound IL-1 preparation was a mixture of 3 different Sepharose/antibody conjugates, directed against recombinant human IL-1α, IL-1β and monocyte-derived IL-1. Controls were prepared by mixing plasma with Sepharose-bound human serum albumin. The optical density (280 nm) of the plasma was measured before and after incubation with the Sepharose conjugates to determine the dilution due to mixing.

**Data Analysis.**

Data are presented as mean ± standard error. Samples below detection were assigned IL-1β concentrations equal to the detection limit of the assay. Statistical significance was determined by appropriate nonparametric tests for paired or multiple groups using Stat-View software (Abacus Concepts, Inc., Calabasas, CA) on a Macintosh computer (Apple Computer, Inc., Cupertino, CA).
RESULTS

Effect of Extraction on Normal Plasma.

After agitating and centrifuging the mixture of plasma and chloroform, an upper aqueous layer which contained IL-1, and a lower chloroform layer which often contained some pigment from the plasma sample was observed. A white, 1 mm thick precipitate separated the two phases. Extracting the aqueous phase a second time increased the IL-1 immunoreactivity by approximately 35%, but a third extraction yielded no further increase (data not shown).

To determine if the aqueous phase retained IL-1 biological activity, unextracted and extracted plasma samples from normal subjects were chromatographed with Sephadex G-50 and each fraction tested for lymphocyte activation (Figure 1A). Unextracted plasma exhibited a characteristic pattern of suppressive activity eluting at the void volume, as previously described (12). Extracted plasma applied to the G-50 column exhibited less inhibitory activity and more IL-1-like stimulatory activity. This stimulatory activity was neutralized by adding a mixture of rabbit anti-human IL-1α and anti-human IL-1β (each at 0.5% by volume) to each fraction and incubating at 22°C for 2 hours before assay (Figure 1B).

The high molecular weight plasma constituents were resolved by chromatography with Biogel A1.5m (1.5 x 10^6 dalton exclusion limit). Whole plasma was applied to the column and each fraction divided into two portions. One portion was further divided in three for measuring optical density, bioactivity and immunoreactivity without extraction. The other portion was extracted and then divided for measurements of optical density and immunoreactivity. The optical density measurements indicated that the extraction process removed proteins in the 300 to 800 kD range (Figure 2A). Endogenous bioactivity eluted at >700, 600-200, 17 and <2 kD (Figure 2B). Immunoreactivity was observed in unextracted fractions eluting at 17 kD (Figure 2C). After extraction, immunoreactivity was also observed in >700 and 600-200 kD fractions.

Antisera Purity and Assay Variability.

Extracted plasma samples retested in consecutive assays exhibited a coefficient of variation of 109% when whole rabbit anti-human IL-1β was used (Figure 3). The coefficient of variation using ammonium sulfate precipitated IL-1β was 16%.

Immunoreactivity of Absorbed Plasma.

To verify that the immunoreactivity detected in plasma by RIA was due to competition between the tracer and native IL-1β in the plasma, four plasma samples were absorbed with anti-IL-1 bound to Sepharose, then extracted and tested in the RIA. The absorbed plasma did not exhibit any competition with 125I-IL-1β in the radioimmunoassay (99 ± 2 % binding, Figure 4). Portions of the plasma which had not been absorbed exhibited 87 ± 1 % binding. Incubation of extracted plasma with albumin/Sepharose showed only a slight change in binding which was attributable to the dilutional effect of adding the Sepharose suspension to the plasma.
Figure 3. Influence of antiserum on assay variability. Retests are expressed as percent of initial measurement.

Effect of Plasma and Extraction on the Standard Curve.

Serial two-fold dilutions of recombinant human IL-1β were prepared in the assay buffer and in EDTA/aprotinin-treated plasma which had been absorbed with rabbit anti-human IL-1β bound to Sepharose 4B. The plasma was then extracted twice and each set of dilutions assayed. The standard curves were virtually indistinguishable over a concentration range of 80 pg/ml to 5 ng/ml (Figure 5), indicating that the chloroform extraction did not affect the immunoreactivity of the IL-1β, and that the extracted plasma, EDTA and aprotinin did not alter the standard curve.
Alternative Iodination Methods.

The Enzymobead tracer preparation bound poorly (<10%) at the antibody dilutions used in these assays. These results, along with previous data that peroxidation destroyed the biological activity of IL-1 (22) led us to abandon this method.

Assays performed with tracer freshly prepared with Bolton-Hunter reagent exhibited standard curves with greater sensitivity (32 ± 7 pg/ml), but reduced binding (15 ± 1.6%), compared to the chloramine T tracer (Table 1). The sensitivity declined over several weeks, however, due to breakdown of the tracer. Removing free I25I by chromatography restored the sensitivity.

Influence of Blood Processing.

Six blood samples were drawn into empty, sterile polypropylene syringes and each sample was immediately distributed into three vacuum blood collection tubes, one containing EDTA and aprotinin, the second containing heparin, and the third containing no anticoagulant. Platelets were removed from the EDTA/aprotinin-treated plasma, but not the heparinized plasma. The IL-1p concentrations measured in the heparinized samples were within 11 ± 10% of the levels measured in the EDTA/aprotinin samples (Figure 6). The IL-1p concentrations measured in serum samples were significantly higher (75 ± 36%) than corresponding EDTA/aprotinin samples.

Altered Binding in Normal Rabbit Serum.

At this stage of assay development, IL-1p immunoreactivity measured in plasma from healthy subjects averaged >200 pg/ml. From cloning and expressing rabbit IL-1 (15), we had found that recombinant rabbit IL-1p cross-reacted with rabbit anti-human IL-1p. We became concerned about undesired interactions between human plasma and the pooled normal rabbit serum which was added to the assay to enhance precipitation of the primary-secondary antibody complex. The precipitation procedure was re-optimized as described in the methods in order to achieve effective precipitation without normal rabbit serum in the assay buffer. The effect of these changes on assay parameters is presented in Table 1. Plasma IL-1p immunoreactivity in normal human plasma was significantly reduced from 212 ± 13 pg/ml to 62 ± 4 pg/ml in the absence of normal rabbit serum (Figure 7).

**Figure 6.** The influence of blood processing on IL-1p levels. The IL-1p concentrations measured for each of 6 blood samples were normalized to the value measured in the EDTA/aprotinin-treated sample (=100%).

**Figure 7.** Influence of normal rabbit serum on immunoreactivity. Aliquots of plasma samples were tested in parallel assays using normal rabbit serum (NRS) to enhance antibody precipitation and assays optimized to recover antibody/tracer without NRS.
Figure 8. Dilution of immunoreactivity. Extracted plasma samples were diluted with buffer, then assayed. The results are expressed as a percentage of the IL-1β concentration measured in the undiluted plasma. At a 1:3 dilution, all samples were below the detection limit of the assay. The dashed, diagonal line represents ideal dilution behavior.

Dilution of Endogenous Immunoreactivity.

Seven plasma samples (mean IL-1β concentration: 186 ± 26 pg/ml) were diluted 1:1.5 and 1:3. The mean IL-1β concentration in the samples diluted 1:1.5 was 127 ± 18 (68%, Figure 8), and at a 1:3 dilution, all were below 78 pg/ml.

Recovery of Exogenous Natural IL-1β.

In 3 separate assays, 10 μl of natural IL-1β (Genzyme, Boston, MA) was added to 0.3 ml plasma samples (final intended concentration: 250-330 pg/ml), incubated for 1 hour at 22°C, then extracted. Endogenous IL-1β measured in unspiked samples was subtracted from the levels found in the spiked samples and the difference divided by the measured value for the spike in buffer (% recovery). For 9 plasma samples tested, recovery was 71 ± 3%.

Fasting and Postprandial Plasma IL-1β Levels.

Blood was sampled from seven subjects in the morning after an overnight fast. Plasma IL-1β levels in these samples were 63 ± 6 pg/ml. The subjects then ate breakfast (orange juice, coffee and pastries) and additional blood samples were collected 90 minutes later. In these samples, the IL-1β levels were essentially unchanged at 58 ± 3 pg/ml. Therefore, simple interventions such as a light meal or a previous venipuncture do not appear to significantly affect plasma IL-1β levels.

Effect of Extraction on Acute Phase Plasma.

In contrast to unextracted plasma from healthy subjects, which usually exhibits >100% binding in the RIA, several plasma samples from malaria patients exhibited very low percent binding (Figure 9). The 60% and 1% binding of the two extreme samples correspond to immunoreactive IL-1β levels of 700 pg/ml and >10,000 pg/ml, respectively. Extracting the plasma eliminated the apparent high levels of IL-1β. This anomalous effect of extraction and the unexpected magnitude of the IL-1β levels in unextracted plasma were ultimately explained upon examination of the centrifuged and decanted test tubes which had contained the unextracted plasma samples. These tubes retained little primary/secondary antibody precipitate whereas the tubes used to measure the extracted plasma samples contained well-formed, adherent pellets. Assays were repeated on the unextracted acute phase plasma, and the tubes examined after the secondary antiserum and polyethylene glycol solution was added and the tubes were centrifuged, but before the supernatant was decanted. Precipitate was suspended in the aqueous phase. Centrifugation for an additional 45 minutes at 1500g failed to pellet the precipitate in the tube. Therefore, the high levels of apparent immunoreactivity (i.e., reduced radioactivity in the test tubes) observed in some unextracted plasma samples of malaria patients did not result from competition between plasma IL-1β and 125I-IL-1β for binding with rabbit anti-human IL-1β, but rather, from incomplete precipitation of antibody/antigen complexes.
DISCUSSION

This investigation sought to develop a practical method for measuring IL-1β in human plasma. The presence of interfering and inhibiting substances in human plasma required the adoption of an extraction process. In a 1973 review (23), Chard listed useful criteria for validating extraction of peptide hormones for radioimmunoassay. First, the method should be fast and simple. Chloroform extraction of a large number of samples can be accomplished in less than 30 minutes. Second, the hormone should be concentrated into a smaller volume to improve sensitivity. The chloroform extraction precipitates high molecular weight proteins which reduces the sample volume about 20%, but leaves the IL-1β in solution. Third, the procedure should not concentrate non-specific inhibitors or proteolytic enzymes. The chloroform extraction apparently removes a considerable amount of the material which inhibits the biological assay (Figure 1A). Fourth, recovery should be greater than 50%. Recovery of natural, monocyte-derived IL-1β was 71 ± 3 %. We tested recovery with natural IL-1, rather than recombinant, in case the natural molecule was altered by post-translational processing (24) which might render it more susceptible to degradation by the chloroform extraction. Fifth, the immunoreactivity of the extract should dilute in parallel to the standard curves. The IL-1β immunoreactivity in extracted plasma was within 4 ± 10 % of ideal dilution. Sixth, extraction materials should be readily available and not vary from batch to batch. Chloroform meets these criteria. Seventh, the extract should be physicochemically similar to the pure hormone. The majority of the immunoreactive IL-1β eluted from gel filtration columns at 17 kD, the molecular weight of the mature form of IL-1β. Eighth, the amount of extracted protein measured should change in response to appropriate physiological circumstances. This criterion is the subject of a separate report (25).

The use of serum was rejected a priori because leukocytes which produce cytokines are in the blood sample and may continue to synthesize and secrete cytokines during clotting. In fact, the clotting process itself may be a stimulus for cytokine production and release (26). This is particularly worrisome when serum is obtained from patients with circulating activated leukocytes. This possibility was supported by measurements of plasma and serum derived from the same blood samples which showed 75% higher levels of IL-1β in the serum than the plasma (Figure 6). Heparin may interfere with antigen-antibody binding (27), therefore EDTA, which has the additional benefit of inhibiting calcium-dependent proteases, was the preferred anticoagulant. The influence of proteases was further reduced by adding the protease inhibitor aprotinin, and removing platelets which release proteolytic enzymes when ruptured by a freeze-thaw cycle.

Plasma from several donors (in addition to the individual data in Figure 2) has been chromatographed with Biogel A1.5m. Immunoreactivity in unextracted fractions was usually found at 17 kD and sometimes at lower molecular weights. After extraction, the fractions at the void volume often exhibited immunoreactivity, and fractions eluting between 600 and 200 kD sometimes exhibited immunoreactivity. The 17 kD peak was not significantly affected by extraction, but the <2 kD material was often destroyed. The >700 kD immunoreactivity revealed by extraction may represent bound IL-1β which is released from large proteins denatured by chloroform. One candidate is α2-macroglobulin (725,000 kD), which has been proposed to be an IL-1 binding protein (28). The immunoreactivity found in the 600 to 200 kD fractions after extraction may also be released from larger protein carriers: in one experiment (data not shown) the 600 to 200 kD extracts were pooled and reapplied to a Biogel column, the immunoreactivity then eluted in a peak around 17 kD.

Assay reproducibility was markedly improved by using rabbit anti-human IL-1β precipitated by ammonium sulfate, rather than whole antiserum. The dilution of the whole antiserum in the RIA (1:6,000) may have resulted in insufficient dilution of other factors in the rabbit serum which caused interference in the presence of human plasma.

The levels of IL-1 estimated in fractionated normal plasma tested in bioassay were in the 10 to 100 pg/ml range (Figures 1A and 2B). When normal rabbit serum was included in the RIA, immunoreactive IL-1β levels averaged 212 ± 13 pg/ml, however, eliminating the normal rabbit serum brought the immunoreactive IL-1β measurements more in line with the bioassay results. Series et al. (29) detected 0.3 to 6 pg/ml of IL-1 activity in the post-operative plasma of surgical patients which had been extracted with silica and measured in a two stage cell line bioassay. The recovery of exogenous IL-1 from the plasma by this method was rather low, 30 to 40% and levels in healthy subjects were not reported. In contrast, Keuter et al. (30) found in excess of 400 pg/ml of IL-1β immunoreactivity in unextracted samples. These high levels may be due to testing serum rather than plasma.
Whether normal rabbit serum was used in the assay was not specified.

Although the number of plasma samples tested from patients with malaria were insufficient to characterize the role of IL-1 in this disease, these samples have demonstrated that unextracted plasma from patients with severe infections cannot be expected to behave in the RIA in a manner similar to plasma from healthy subjects. The fact that some of the malarial samples contained factors which interfered with the precipitation of the antibody/tracer complex is consistent with the changes known to occur in plasma during the acute phase response. Several proteins, including lipoproteins such as serum amyloid A, can increase over one thousand-fold. Free fatty acid concentrations are markedly elevated as well. Such factors could influence precipitation by changing the viscosity of the solution or interfering with hydrophobic interactions between proteins, including interactions during antigen-antibody association. The ability of chloroform to separate lipids from solution may account for the removal of the interference by extraction. These findings strongly suggest that immunoreactive IL-1β found in plasma samples from different pathological conditions should be verified through immunoabsorption, dilution and bioactivity experiments.

ACKNOWLEDGEMENTS

The authors thank Drs. Jeffrey Gelfand, Ronald Tomkins, Laurie Miller, Burton Clark and Jeffrey Tatro for their helpful advice and discussions. The authors also thank Kathleen Kimball, Reza Ghorbani and Scott Orencole for valuable technical assistance.

These studies were supported by grants P50-GM21700, T32-GM07035, AI 15614 from the National Institutes of Health and travel funds from the World Health Organization.

REFERENCES


Please address all correspondence to:

Joseph G. Cannon
Dept. of Medicine - Box 68
New England Medical Center
750 Washington Street
Boston, MA 02111