The Production of Antibody against Human Leukocytic Pyrogen

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ABSTRACT Human peripheral blood leukocytes were stimulated with killed staphylococci in vitro to release leukocytic pyrogen (LP). Supernates from these stimulated leukocytes were concentrated, emulsified in Freund's complete adjuvant, and injected intradermally into rabbits. After seven monthly booster injections, rabbit antiserum destroyed the pyrogenic activity of human LP, and the titer of this neutralizing ability increased in the subsequent 7 mo. The pyrogen-neutralizing capacity of the rabbit antiserum was recovered in the globulin fraction, the IgG and IgM peaks of Sephadex G-200, and the acid-eluted fraction of a goat anti-rabbit IgG immunoadsorbant. The neutralizing antibody was specific for human LP inasmuch as it had no effect on rabbit, guinea pig, or monkey LP. When coupled to Sepharose, this antibody bound human LP; after acid elution from this immunoadsorbant, LP was recovered without loss of biologic or chemical characteristics. The antiserum was also absorbed with stimulated leukocyte supernates which did not contain LP, and this had no effect on the titer of anti-LP. Crude human LP, eluted from immunoadsorbant columns prepared from absorbed antiserum, contained significantly reduced contaminating protein when evaluated by polyacrylamide gel electrophoresis. These studies have established that specific antibody to human leukocytic pyrogen can be produced. This antibody is useful in the further study and purification of leukocytic pyrogen and its role in the pathogenesis of human fever.

INTRODUCTION

Ever since its discovery in rabbits, leukocytic pyrogen (LP)[1] has been considered to be the mediator of fever in all species (1). However, this substance has proven to be highly labile and difficult to purify from the large quantity and variety of proteins present in crude supernates from stimulated leukocytes (2, 3). Understandably, to define its role in the pathogenesis of fever, purification of this molecule is a necessity. This is particularly the case with human LP in that LP has yet to be definitively demonstrated in sera from patients with febrile diseases (4, 5). Even the rabbit, which responds to human LP, has failed to be useful in detecting LP in human febrile plasma (6). Clearly, a biological assay of human LP is not practical. Thus, techniques such as immunoassays should provide a sensitive method for measuring this substance.

In this paper, we report the first successful method for developing antibodies to human LP in the rabbit. Besides being a prerequisite for an immunoassay, this antibody has provided evidence that this molecule has antigenic identity despite its widespread biological cross-reactivity in rabbits, monkeys, and mice (7–9). In addition, this antibody is highly useful in the purification of human LP.

METHODS

Materials. All glassware, needles, syringes, media, and solutions were sterile and pyrogen-free.

Preparation of LP. 450 ml of fresh human blood was obtained from individual normal volunteers in acid citrate dextrose (Transfer Pak, Fenwall Laboratories, Morton Grove, Ill.). Buffy coat concentrates were made by removing the uppermost 50-ml of packed erythrocytes after a 1,500-g centrifugation for 3 min. Buffy coats were either sedimented in 3.6% dextran (mol wt 200,000, Sigma Chemical Co., St. Louis, Mo.) in 0.85% NaCl, or the mononuclear layer was separated on a Ficoll-Hypaque gradient (10, 11) (Ficoll, Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.; Hypaque, Winthrop Laboratories, New York). By using either preparation of leukocytes, the cells were washed, and 20 million cells were resuspended in Hank's balanced salt solution, with 100 U of penicillin G, 8 μg of gentamicin, and 2 U of heparin per ml. AB serum stored at −70°C was added to make a final concentration of 10%. Heat-killed *Staphylococcus albus* was also added at a bacteria:leukocyte ratio of 30:1. Leukocyte suspensions were shaken gently at

This work was presented in part at the Annual Meeting of The American Society for Clinical Investigation, Atlantic City, N. J., May, 1976.

Received for publication 8 September 1976 and in revised form 14 April 1977.

[1] *Abbreviations used in this paper*: LP, leukocytic pyrogen; PBS, phosphate-buffered saline.

37°C for 30 min, centrifuged at 250 *g* for 10 min, and resuspended in fresh Hank's balanced salt solution without serum or bacteria at a concentration of 5 million leukocytes per ml. After 18 h at 37°C in a stationary incubator, the suspensions were centrifuged at 2,200 *g* for 30 min and stored at 4°C in 0.02% sodium azide.

Pyrogen assay. New Zealand albino rabbits weighing 2–3 kg were used in all pyrogen assays. Specific information about training and temperature recording has been reported previously (12). After intravenous injection into a lateral ear vein, a peak rise of 0.6-1.0°C above base line was considered a single rabbit pyrogenic dose of human LP. To determine this, a two-point dose response was carried out on each preparation of LP using six rabbits (injections were given in triplicate).

Immunization. New Zealand albino rabbits of either sex weighing 3–4 kg were used for all immunizations. Crude LP obtained from dextran-sedimented buffy coat leukocytes was concentrated, dialyzed against H_2O, lyophilized, and emulsified in Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.). Primary immunization was made into the footpads and the skin of the dorsal neck. Each rabbit received the equivalent of 25 pyrogenic doses (12.5 mg protein) determined before lyophilization. Monthly booster injections were given intramuscularly in Freund's incomplete adjuvant. Bleedings were obtained 4 wk after a booster immunization, and the antisera were stored at −20°C.

Concentration techniques. Crude supernates containing LP were placed in autoclaved dialysis tubing and concentrated to 1/20th the initial volume at room temperature by rapid evaporation in front of a fan. Using these same dialysis tubings, concentrates were dialyzed at 4°C against phosphate-buffered saline (PBS) or in preparation for lyophilization, against H_2O.

Gel filtration. Preparations of human LP made from the mononuclear layers of Ficoll-Hypaque gradients were concentrated and chromatographed over Sephadex G-50 (fine) in a 165 × 5.6-cm column (Pharmacia Fine Chemicals). This procedure separated the two molecular species of LP (10). In addition, a Sephadex G-75 (fine) 60 × 2.6-cm column was used for molecular weight determinations. A similar column containing Sephadex G-200 was used to separate immunoglobulins.

Protein determinations. The Lowry method (13) or absorbance at 280 nm was employed to estimate protein content. In both cases, crystallized bovine serum albumin was used as a standard.

Immunoadsorbants. Antiserum was precipitated at 50% $(NH_4)_2SO_4$ saturation (14) and dialyzed against PBS. This globulin fraction was then coupled to Sepharose 4B (Pharmacia Fine Chemicals) (15, 16). Sepharose 4B was washed thoroughly and allowed to settle. After decantation, it was resuspended in an equal volume of 0.1 M $NaHCO_3$. At 4°C, 4 N NaOH was added to raise the pH of the mixture to 11. 15 g cyanogen bromide (Eastman Kodak Co., Rochester, N. Y.) was dissolved in 15 ml of dimethylformamide, and this was added slowly per 100 ml settled Sepharose with constant stirring while maintaining the pH at approximately 11. Thereafter, the material was washed free of cyanogen bromide on a coarse sintered funnel with cold PBS. At room temperature, 4 N NaOH was added to adjust the pH to 7.8–8.2, and 4–6 mg of the globulin fraction was added per milliliter of activated Sepharose. This slurry was kept at room temperature for 2 h and then overnight at 4°C with gentle mixing. The mixture was then washed several times with PBS to remove unbound protein. The adsorbant was kept in PBS with 0.02% sodium azide at 4°C for at least 10 days. Before use, the material was poured into sterile columns, washed with 0.1 N HCl, and reequilibrated to neutrality with PBS.

Preparations of human LP were applied to these columns at room temperature and washed with PBS. When protein was no longer detectable in the washes, columns were eluted with 0.1 N citrate buffer, pH 2.5 (15). The immunoadsorbant was washed with 0.1 N HCl before being stored in PBS with sodium azide.

Neutralization test. A simple test was used to determine the neutralizing ability of the rabbit antiserum. One dose of LP was incubated with antiserum for 2 h at room temperature followed by 48 h at 4°C. Formed precipitates were removed by centrifugation at 2,000 *g* for 30 min at 4°C, and the supernatant material was assayed for pyrogen activity. Controls with normal rabbit serum or no added serum utilized the same source of LP. All tests were done in duplicate.

Polyacrylamide gel electrophoresis. Preparations of human LP which were dialyzed against H_2O were incubated in 100 mM Tris-borate buffer, pH 8.6; 1% sodium dodecyl sulphate, and 2 M urea for 1 h at room temperature. These preparations were then applied to 7.5% polyacrylamide gels (125 × 5 mm) and run in the presence of 0.1% sodium dodecyl sulphate and Tris-borate buffer, pH 8.6 (17). Gels were subjected to 1.5–2 mA for 2–3 h, removed, and stained in 0.2% Coomassie Blue. Gels were destained in a diffusion destainer (Bio-Rad Laboratories, Richmond, Calif.) and scanned with an automatic recording device at 525 nm (Helena Laboratories, Beaumont, Tex.).

RESULTS

Early bleedings. Despite the formation of precipitates when antiserum and crude pyrogen were mixed, there was no loss of pyrogenic activity with antiserum obtained during the first 5 mo after primary immunization. Immunodiffusion confirmed the presence of precipitating antibody to a variety of human serum proteins. By using pooled antisera from these early bleedings, a globulin fraction was made with $(NH_4)_2SO_4$ and coupled to Sepharose. Crude human LP, which was concentrated by evaporation and dialyzed against PBS, was applied to this immunoadsorbant. As shown in Fig. 1, the pyrogenic activity was recovered in the early, nonadhering protein peak. Recovery of pyrogenic activity was nearly 100%. No pyrogen was detected in citric acid-eluted fractions. A similar elution pattern of pyrogen activity was observed when either the 15,000- or 38,000-mol wt pyrogen isolated by gel filtration was passed through this column.

Late bleedings. Neutralizing ability of the rabbit antiserum to crude human LP was observed first in serum obtained 7 mo after primary immunization. The amount of antiserum required to neutralize one dose of human pyrogen fell from 1 ml obtained at 7 mo to 0.003 ml obtained 13 mo after the primary immunization (Fig. 2). The final concentration of antiserum in each reaction mixture had no effect on its ability to neutralize the pyrogen. Crude human LP in volumes as large as 6 ml/pyrogenic dose or as small as 0.5 ml/pyrogenic dose was neutralized by 10^{-2} or 10^{-3}

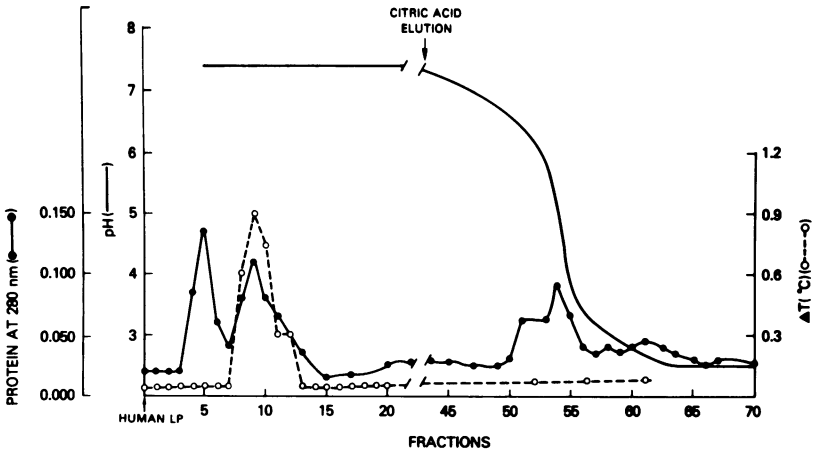

FIGURE 1 50 pyrogenic doses of crude human LP were applied to a rabbit antiserum immunoadsorbant column (3 × 15 cm). Source of globulin was pooled early bleedings (3–6 mo after primary immunization). Rabbits were injected in duplicate, and each point (○) represents mean change in temperature. Each fraction is 10 ml. pH is indicated as a solid line. Protein (●) was measured at 280 nm. Acid elution with citrate buffer, pH 2.5.

ml of antiserum. Similarly, the purity of the pyrogen had no effect in that neutralization occurred when pyrogen preparations of high specific activity (up to 10 μg protein per pyrogenic dose) were used. Also, neutralization was observed with either the 15,000- or 38,000-mol wt pyrogen which had been prepared from human mononuclear cells and subsequently isolated by gel filtration (10). In addition, inasmuch as eosinophils have been shown to be a source of human LP (18), a preparation of crude human LP was also made from a buffy coat containing 72% eosinophils, 10% neutrophils, and 18% lymphocytes. This LP was also neutralized by antiserum obtained after 7 mo of immunization (cf. Table II).

Characteristics of the antiserum. Antiserum of proven neutralizing ability against human LP was precipitated at 50% $(NH_4)_2SO_4$ saturation. All neutralizing activity was recovered in this globulin fraction. The $(NH_4)_2SO_4$-precipitated protein was then chromatographed over Sephadex G-200, and the protein peaks corresponding to IgM and IgG were isolated and pooled separately. Although significant neutralizing activity against human LP was found in the IgM peak, most of the neutralizing capacity was in the IgG peak. In addition, goat anti-rabbit IgG (ICN Pharmaceuticals Inc., Life Sciences Group, Cleveland, Ohio) was coupled to activated Sepharose 4B. When antiserum containing pyrogen-neutralizing ability was passed over this immunoadsorbant material, no neutralizing activity was found in the unbound protein which passed through the column. All the pyrogen neutralizing activity was in the fraction which had bound to this anti-rabbit IgG and was later eluted with citric acid. These results are summarized in Table I. Thus, the observations of rising titers, presence in the $(NH_4)_2SO_4$-precipitated fraction, and elution from Sephadex G-200 in the IgM and IgG peaks, as well as adherence to an anti-rabbit IgG immunoadsorbant,

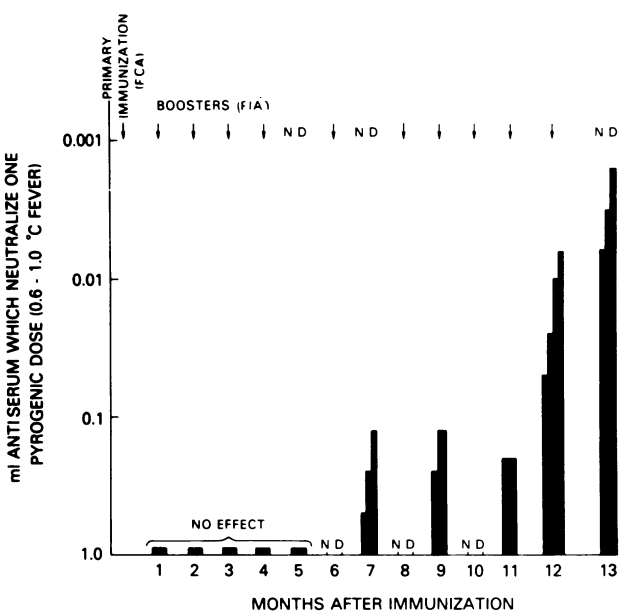

FIGURE 2 Neutralization of human LP with rabbit anti-human LP antiserum. Each bar represents the milliliters of antiserum which neutralized a single rabbit pyrogenic dose. Multiple bars at any given month represent different quantities of antiserum tested which neutralized a single pyrogenic dose. Experiments were done in duplicate. ND denotes not done. FCA denotes Freund's complete adjuvant; FIA, Freund's incomplete adjuvant. Each arrow represents an immunization injection.

TABLE I
Nature of Human LP-Neutralizing Property of Rabbit Antiserum

Experiment	Number of neutralizing doses			
	In antiserum*	Precipitated in 50% $(NH_4)_2SO_4$	Eluted from Sephadex G-200 column	Eluted from goat anti-rabbit IgG affinity column
A	200	200‡	ND¶	ND
B	200	200	150§	ND
C	50	ND	ND	25‖

* Determined by incubation with human LP 24–48 h at 4°C and assayed in rabbits.
‡ Repeated several times with various batches of antiserum having higher neutralizing titers, and recovery was consistently 100%.
§ 75% recovered from IgG peak. 25% recovered from IgM peak.
‖ Nonspecific losses due to concentration procedures and loss of IgM.
¶ ND = Not done.

all support the fact that these antisera contain neutralizing antibody against human LP.

Specificity of antibody against human LP. Rabbits and guinea pigs were given intraperitoneal injections of sterile mineral oil. After 27 h, peritoneal macrophages were harvested with saline washes. Rabbit and guinea pig macrophage LP were made by adding killed staphylococci in the usual manner (see Methods), but fresh rabbit or guinea pig serum was substituted for human serum. Similarly, rabbit and monkey *(Macaca mulatta)* blood LP was made by using both dextran sedimentation and Hypaque-Ficoll gradients. Rabbit and guinea pig LP produced fever in the rabbit, but monkey LP, despite large quantities, was nonpyrogenic for rabbits. Thus, the monkey was used for a homologous pyrogen assay (8). Anti-human LP antiserum was added per pyrogenic dose of crude rabbit blood LP, rabbit macrophage LP, guinea pig macrophage LP, monkey blood, or monkey monocyte LP in quantities that would neutralize 100–200 pyrogenic doses of human LP. After 48 h at 4°C, the supernates were injected into appropriate hosts, and no loss of pyrogenic activity was observed with these LP (Table II).

Attachment of anti-human LP to Sepharose. By using $(NH_4)_2SO_4$ precipitation, the globulin fraction from the late bleedings (9–12 mo after primary immunization) was coupled to activated Sepharose 4B. Crude human LP was concentrated and placed on this immunoadsorbant. Fig. 3 illustrates a typical run. Protein from the crude pyrogen, which did not adhere to the immunoadsorbant, had no detectable pyrogenic activity. However, during citric acid elution, nearly all the pyrogenic activity in the crude LP (50 pyrogenic doses) was recovered in four fractions at pH 3.5–2.5. Thus, using the globulin fraction from late bleedings, LP adhered to this immunoadsorbant and was eluted with the acid wash.

TABLE II
Specificity of Anti-Human LP Antibody

Source of LP	No. pyrogenic doses	Antiserum added per pyrogenic dose*	No. recipients‡	No. pyrogenic doses remaining after incubation with antiserum
		ml		
Human blood LP, dextran sedimentation	12	0.0015	12	0
Human mononuclear LP, Ficoll-Hypaque separation	16	0.003	16	0
Human eosinophil LP, dextran sedimentation	4	0.01	4	0
Human LP, 15,000 mol wt	8	0.003	8	0
Human LP, 38,000 mol wt	8	0.005	8	0
Guinea pig peritoneal macrophage LP	6	0.5–1.0	6	6
Rabbit blood LP, dextran sedimentation	4	0.5	4	4
Rabbit peritoneal macrophage LP	4	0.5	4	4
Monkey blood LP, dextran sedimentation	4	1.0	4	4
Monkey mononuclear LP, Ficoll-Hypaque separation	4	1.0	4	4

* Same source of antiserum used throughout these experiments.
‡ Rabbits were used as recipients for human, rabbit, and guinea pig LP; monkeys (*Macaca mulatta*) were used to assay monkey pyrogen (8).

Either 15,000- or 38,000-mol wt human LP (obtained from Sephadex G-50 gel filtration of crude LP from human mononuclear cells) was applied to this anti-human LP column. In separate experiments, both molecular species of LP bound to the immunoadsorbant and eluted in the acid wash. In addition, after recovery from the immunoadsorbant, both the 15,000- and 38,000-mol wt pyrogens were individually rechromatographed over Sephadex G-75 with no observed change in their respective elution pattern or molecular weight.

Rabbit and guinea pig macrophage LP were applied to this anti-human LP column, and all pyrogenic activity was recovered in the nonadhering protein peak. These results support the specificity of the neutralization experiments in which anti-human LP antiserum was unable to inactivate these animal pyrogens (Table II).

Absorption of undesired antibodies from anti-human LP antiserum. In using immunodiffusion and immunoelectrophoresis, we observed that the rabbit anti-human LP antiserum contained large quantities

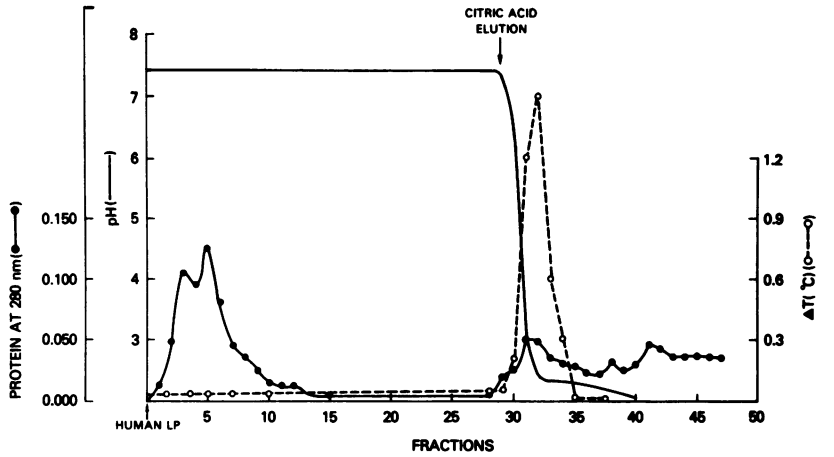

FIGURE 3 50 pyrogenic doses of crude human LP were applied to a rabbit anti-human LP-immunoadsorbant column (3 × 15 cm). Source of globulin was pooled late bleedings (7–12 mo after primary immunization). Rabbits were injected in duplicate, and each point (O) represents the mean change in temperature. Each fraction is 10 ml. pH is indicated by a solid line. Protein (●) was measured at 280 nm. Acid elution with citrate buffer, pH 2.5. When the four fractions containing pyrogenic activity were pooled and assayed in rabbits, nearly all 50 pyrogenic doses were recovered.

of anti-human albumin, anti-human immunoglobulins, and antibody against other human serum and leukocyte products. Because cycloheximide, in concentrations as low as 2.5 μg/ml, has been shown to prevent the synthesis of human LP without interferring with phagocytosis (19), we made preparations of LP in the presence of cycloheximide. All materials were identical to that used for making LP, with the exception that 2.5 μg cycloheximide per ml was present in these incubations. After concentration, there was no detectable pyrogenic activity, but proteins normally found in crude LP (as detected by immunoelectrophoresis) were still present. This concentrated material, made in the presence of cycloheximide, was added to the globulin fraction of anti-human LP, at weekly intervals over a 6-wk period. Absorption attempts were stopped when visible precipitates failed to form. This absorbed globulin, which retained its neutralizing

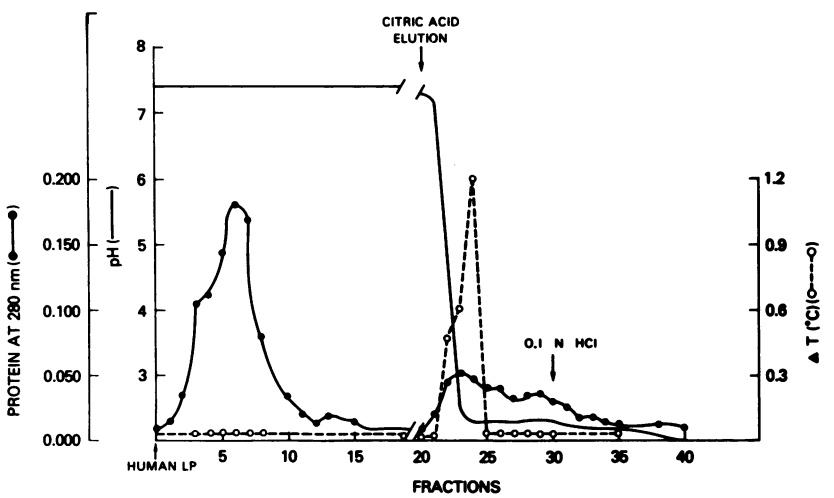

FIGURE 4 50 pyrogenic doses of crude human LP were applied to a rabbit anti-human LP-immunoadsorbant column (3 × 5 cm). Source of globulin was pooled late bleedings (7–12 mo after primary immunization), which had been absorbed for 6 wk with crude human leukocyte supernates which had been made in the presence of cycloheximide. Rabbits were injected in duplicate, and each point (O) represents the mean peak fever. Each fraction is 10 ml. pH is indicated by a solid line. Protein (●) was measured at 280 nm. Acid elution with citrate buffer, pH 2.5. When the three fractions containing pyrogen were pooled and assayed, nearly all 50 doses were recovered.

titer property, was then coupled to Sepharose. The same source and quantity of crude LP depicted in Fig. 3 was applied to this absorbed, anti-human LP immunoadsorbant column. As shown in Fig. 4, the nonadhering protein peak is significantly larger in comparison to that from the nonabsorbed immunoabsorbant (Fig. 3), and once again, the pyrogen eluted in a single peak during acid wash. Because of dilutional effects, the protein readings during the citric acid elution of pyrogen are too low for significant comparisons. In addition, in separate experiments, the 15,000- and 38,000-mol wt pyrogens eluted at pH similar to that for crude LP.

Use of immunoadsorbants in purification of human LP. A source of human LP was divided into three equal aliquots. Aliquot A was assayed in rabbits, and the specific activity (micrograms protein per pyrogenic dose) was determined by using the Lowry technique (13). Aliquot B was passed over an unabsorbed, anti-human LP-immunoadsorbant column; the eluted pyrogen peak was concentrated, and the specific activity was determined. Aliquot C was passed over an absorbed, anti-human LP-immunoadsorbant column; the eluted pyrogen peak was similarly concentrated, and the specific activity was determined. Approximately 100 μg from each aliquot was then subjected to electrophoresis in sodium dodecyl sulphate gels (see Methods), stained with Coomassie Blue, destained, and the optical density and distribution of the bands were recorded on a scanning device.

As depicted in Fig. 5, three different protein patterns were observed. Crude pyrogen (tracing A) contains numerous high molecular weight proteins present in serum (left side of gel), and the large peak occuring in the middle of the gel was identified as albumin by immunodiffusion. Human LP, eluted from an unabsorbed anti-human LP-immunoadsorbant column (tracing B), contains less large molecular weight serum proteins but increased quantities of albumin and smaller molecular weight proteins (right side of gel). The presence of Sepharose-bound antibodies to these proteins results in their being eluted along with the LP during the citric acid wash. However, if the antiserum containing these antibodies is first absorbed with crude leukocyte supernates made in the presence of cycloheximide, the LP eluted from these immunoadsorbants is significantly purer. As demonstrated in tracing C, only seven bands were identified in comparison to 19 bands in tracing A. Thus, we were able to absorb out unwanted antibodies to proteins present in crude LP without removing antibody which binds LP. Furthermore, the specific activity of LP eluted from the absorbed immunoadsorbant increased 50-fold (see legend of Fig. 5). Because of the small quantity of LP (in micrograms of protein) necessary to produce fever (2), none of the staining bands represents the LP protein.

DISCUSSION

Since human LP, like LP from other animals, shows little specificity in its ability to produce fever in several species (7–9), the possibility of producing antibody against LP remained in question. It was considered that LP from different species might share antigenic similarities which would prevent the production of antibody against it. However, in this paper we have shown that antibody to LP can be produced in rabbits and, moreover, that the antiserum is capable of neutralizing the fever-producing property of human LP. Evidence that the pyrogen-neutralizing factor in the antiserum behaved like typical antibody was supported by several findings: (*a*) the concentration of neutralizing activity increased with time and booster injections; (*b*) neu-

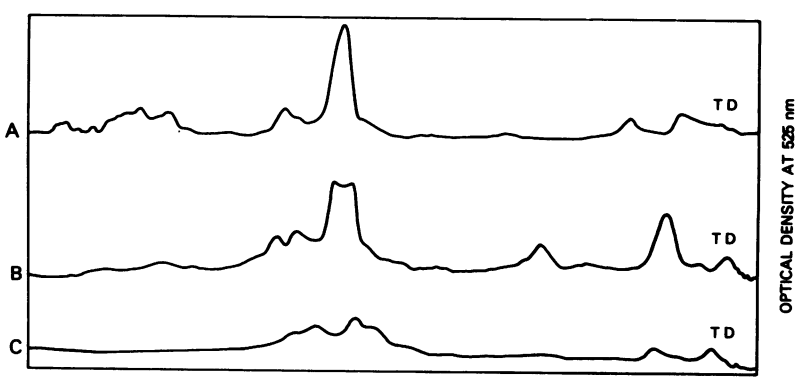

FIGURE 5 (A) Protein tracing from 100 μg of crude human LP equivalent to 0.1 pyrogenic dose. (B) Protein tracing from 100 μg human LP equivalent to one pyrogenic dose eluted from unabsorbed anti-human LP-immunoadsorbant column. (C) Protein tracing from 100 μg human LP equivalent to five pyrogenic doses eluted from absorbed anti-human LP-immunoadsorbant column. TD denotes tracking dye.

tralizing activity was found only in the $(NH_4)_2SO_4$ precipitate of antiserum and the IgG and IgM peaks after Sephadex G-200 gel filtration; (*c*) the factor bound to anti-rabbit IgG and eluted in an acid wash; (*d*) it was recovered after 6 wk of absorption with serum and leukocyte proteins not containing LP; and (*e*) when this factor was coupled to Sepharose, LP was bound and then released without loss of biologic activity or change in molecular weight. These last two findings clearly establish that this factor is not an induced proteolytic enzyme which destroys LP. In addition, this antibody is highly specific, inasmuch as it had no effect on rabbit, guinea pig, or monkey LP. A similar situation can be seen with insulin which is biologically active in several species but retains independent antigenic specificity.

The prolonged period from primary immunization until neutralizing antibody was detected may be related to the low concentration of the molecule in the immunizing material. Each immunization or booster injection with 25 pyrogenic doses of crude LP contained 12.5 mg of protein. However, most of this protein was not LP; in fact, from using estimates of the specific activity of rabbit LP (amount of fever per micrograms of protein) (2), each immunization contained only 1–2 μg of the pure LP. Similarly, by using human interferon, a small molecular weight protein which, like LP, is also biologically active in nanogram quantities, neutralizing antibody has been demonstrated after prolonged booster immunizations (15).

Crude LP in these experiments was the product of neutrophils, monocytes, and eosinophils, since lymphocytes do not produce LP (20). Although monocytes produce a 38,000-mol wt LP in addition to the 15,000-mol wt LP, the larger pyrogen comprises <5% of crude LP after gel filtration (10). Because of its low isoelectric focusing point (pH 5.1), it is likely that the larger LP has an amino acid composition and antigenic specificity different from that of the 15,000-mol wt pyrogen which has an isoelectric focusing point at pH 6.9. Because antiserum was successful in neutralizing or binding both pyrogens, it may indicate that two specific antibodies are present in the antiserum. Nevertheless, the appearance of neutralizing antibody to both molecules at the same time, despite the low concentration of the 38,000-mol wt LP in the immunizations, does not support the development of two specific antibodies. It is more likely that an antibody is present which is directed against a common antigenic site on both pyrogens. This latter explanation is more feasible because both pyrogens may share the same antigenic site for biologic activity.

It was demonstrated in these studies that anti-LP has a high affinity for human LP. As little as 0.003 ml of antiserum neutralized a single dose of both molecular sizes of LP in crude or partially purified states even when volumes were as large as 6 ml. In addition, when coupled to Sepharose, the antibody bound either the 15,000- or 38,000-mol wt LP until it was dissociated and eluted at low pH. The affinity of this antibody for human LP is sufficient for biological neutralization to take place in the fluid phase, but when attached to immunoadsorbants, this antibody does not alter biological activity. Thus, solid-phase immunoadsorbants can now be applied as a purification method of LP and, as demonstrated in this paper, provide a more efficient means of purification than previously shown for gel filtration, ion exchange, and other techniques (3).

The use of inhibitors of mRNA and protein synthesis has provided indirect evidence that human LP is a newly synthesized protein (19). This conclusion is based on the ability of these inhibitors to prevent formation of biologically active LP. The present studies also reveal that cycloheximide prevents the synthesis of antigenic portions of the LP molecule, because we were unable to absorb anti-LP with leukocyte supernates made in the presence of cycloheximide. In addition, these absorption experiments support the fact that anti-LP antibody is directed against LP and not another protein to which LP may bind. For example, noncovalent binding of small molecules, such as LP, to large serum molecules, such as albumin, could have occured in the immunizing material. Under these conditions, antibody to LP may reflect antibody to such a large protein; however, the presence of serum proteins in leukocyte supernates prepared with cycloheximide had no effect on the titer of anti-LP during the absorption experiments. Therefore, it is unlikely that anti-LP is directed against a large protein which binds LP.

In summary, specific antibody to human LP has established antigenic identity for this potent biological molecule and that both 15,000- and 38,000-mol wt components may share a common antigenic site (or sites). This antibody also provides us with an efficient method of purifying LP from crude leukocyte supernates. Production of antibody is the first and most essential step in the development of an immunoassay for this molecule, a technique which seems to be necessary if we are to measure LP in patients. Finally, this antibody may provide a means to study the origin, receptors, and metabolic disposition of LP and its role in the pathogenesis of fever.

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