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Human Leukocytic Pyrogen Test for Detection of Pyrogenic Material in Growth Hormone Produced by Recombinant *Escherichia coli*

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Human growth hormone is biosynthetically produced in recombinant strains of *Escherichia coli* as methionyl human growth hormone (met-hGH). When purified from the bacterial culture, met-hGH is biologically active in established assays for growth hormone. Therefore, a phase I trial of met-hGH was carried out in healthy human adults; during the first trial, however, signs, symptoms, and clinical laboratory tests characteristic of an acute-phase response to pyrogenic agents was observed. Prior testing of the met-hGH preparation used in the phase I trial did not reveal evidence of toxicity, and the U.S. Pharmacopeial Convention rabbit pyrogen test, as well as the *Limulus* amoebocyte lysate (LAL) test, had not detected significant levels of exogenous pyrogens or endotoxin. In addition, standard inhibition studies with added endotoxin showed no inhibition by the LAL test. When this preparation of met-hGH was incubated with human blood mononuclear cells, leukocytic pyrogen (LP) was released into the supernatant medium, suggesting that the preparation contained pyrogenic material. Various lots of met-hGH based on different purification and formulating methods were tested by the human LP assay for contaminating pyrogens. The results of these tests aided in the identification of procedures for met-hGH preparations which did not induce LP in vitro. Thus, subsequent lots of met-hGH which had passed the LP test were used in repeat clinical studies, and no inflammatory or pyrogenic reactions were observed. When the LP test was used, experiments revealed that the original lot of met-hGH was contaminated with endotoxin which had not been detected in the LAL or rabbit pyrogen tests. Lyophilization in glycine-phosphate buffer had resulted in a 10- to 20-fold reduction of endotoxin reactivity in the LAL test and the U.S. Pharmacopeial Convention rabbit pyrogen test. These data provide a probable explanation for the negative result from the LAL and rabbit pyrogen tests in the initial lot of met-hGH which induced acute-phase reactions. In addition, these studies demonstrate that the release of LP from human cells is a reliable indicator of the presence of materials that are pyrogenic for humans.

Pyrogenic and inflammatory reactions are well-recognized indicators of microbial contamination in pharmaceutical products manufactured for parenteral use. Humans are particularly sensitive to bacterial products, and several studies have shown that nanogram quantities of bacterial lipopolysaccharide, i.e., endotoxin, produce leukocytosis, hypoferremia, and fever in humans (4, 13, 19). The development of methods which result in the expression of human gene products in recombinant strains of *Escherichia coli* has been a major accomplishment of biological research; because of the large amounts of endotoxin which are produced by *E. coli* and other gram-negative bacteria, however, separation and adequate removal of endotoxin is a formidable task. Nevertheless, products such as human insulin have been produced in *E. coli* and do not elicit inflammatory or pyrogenic responses, nor is there evidence of antibody responses to *E. coli* products in human patients (1).

Human growth hormone (hGH) has been produced in recombinant *E. coli* strains as methionyl hGH (met-hGH), which has biological activity indistinguishable from that of pituitary-derived hGH (14). Although the initial lot of met-hGH which was used in a phase I trial had been thoroughly investigated for purity, when injected into human subjects, it induced several signs and symptoms characteristic of a pyrogenic and inflammatory response to a bacterial product. These included pain at the site of intramuscular injection, chills, and fever and were associated with laboratory findings such as leukocytosis, neutrophilia, hypoferremia, decreased hemoglobin, and increased serum globulins. On the basis of the standard U.S. Pharmacopeial Convention (USP) rabbit pyrogen test and the *Limulus* amoebocyte lysate (LAL) test, both of which are routinely used to detect pyrogen contamination, it was predicted that the lot of met-hGH prepared for the initial phase I trial would not induce pyrogenic reactions. Both tests included classical inhibition studies which revealed no inhibition by the reconstituted product. Thus, despite the results of these accepted tests, the response in human subjects demonstrated that this lot of met-hGH was either contaminated with a pyrogen or the methionyl analog was itself intrinsically pyrogenic.

The pathogenesis of fever and many aspects of the inflammatory response to microbial products involve the synthesis and release of a pyrogenic meditator molecule from the phagocytic cells of the host. The endogenous polypeptide, or leukocytic pyrogen (LP), has been shown to mediate fever and several other aspects of the acute-phase response that results from most, if not all, microbial products and from other pyrogenic molecules of nonmicrobial origin (2, 6, 9). LP is found in the circulation during experimental fevers and can be induced in vitro when phagocytic cells are incubated with exogenous pyrogenic materials. Therefore, we hypothesized that the lot of met-hGH which induced inflammatory responses in human subjects would induce LP from human cells in vitro. In the present study, we demonstrated that

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human mononuclear phagocytes incubated with the offending lot of met-hGH produce LP and that the LP assay test can be used prospectively to detect contaminating pyrogenic materials. In addition to causing the undesirable side effects noted, endotoxins injected into humans under experimental conditions have been shown to induce pituitary hormone release (19); thus, the presence of endotoxin in preparations of any pharmaceutical product synthesized in *E. coli* and intended for parenteral use could also compromise efficacy studies.

Further studies were designed to identify the nature of the pyrogenic contaminant. We determined in this study that the pyrogen contamination in the initial lot of met-hGH was most likely endotoxin. This finding was ascertained under two experimental conditions: (i) the use of polymyxin B, which specifically blocks the biological activity of endotoxins in the human LP assay, and (ii) comparison of the activity of endotoxin before and after lyophilization in the glycine formulation buffer by both the LAL and the rabbit pyrogen test. These results suggest that small quantities of endotoxin in the initial lot of met-hGH were not detected by these tests and account for the clinical response during the initial phase I trial.

MATERIALS AND METHODS

Human mononuclear cells. Human subjects taking no medication were bled after a 12-h fast via an antecubital vein into 60-ml heparinized syringes (final concentration, 10 U of heparin per ml). Blood was diluted with two parts of 0.15 M NaCl and underlaid with Ficoll-Hypaque. After centrifugation, the interphase containing the mononuclear cells (MNCs) was aspirated, washed twice with 0.15 M NaCL, and resuspended at a concentration of 5×10^6 cells per ml in Eagle minimal essential medium (Microbiological Associates, Walkersville, Md.) containing 0.01 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, 100 U of penicillin, and 100 μg of streptomycin per ml. The endotoxin concentration of this medium was <0.02 ng/ml, as measured by the LAL test (see below). Differential counts were carried out by use of a cytocentrifuge and stained with Wright stain. The percentage of monocytes was calculated from a 200 cell count differential. Five milliliters of this cell suspension was dispensed into 25-cm^2 culture flasks (Corning Glass Works, Corning, N.Y.). For each experiment, a cell control (nothing added) and a positive control were included. The positive control consisted of stimulating the mononuclear cells (MNCs) with a suspension of heat-killed *Staphylococcus albus* cells at a particle/leukocyte ratio of 20:1, as described elsewhere (8). Positive controls also contained 5% (vol/vol) human AB serum that had been frozen fresh to maintain its opsonizing capacity.

All preparations of growth hormone were lyophilized and dissolved in Eagle minimal essential medium. The concentration of met-hGH was adjusted so that the addition to the MNC suspension was at a minimal volume (less than 0.5 ml); appropriate controls were also adjusted with the same volume. Initial experiments had indicated that the presence of 5% heat-inactivated (56°C, 30 min) human AB serum during the incubation of met-hGH with MNCs gave consistent LP production when compared with serum-free incubations. Therefore, AB serum was included in subsequent incubations. Cell controls also contained 5% heated AB serum. All MNC incubations were carried out at 37°C in 5% CO_2 for 48 h. After incubation, cell cultures were centrifuged at 2,200 × *g* for 30 min, and the supernatant medium was removed and stored at 4°C until being assayed.

Assay of human LP. To test for the presence of LP in the MNC supernatant medium, we used the rabbit pyrogen test as modified for the detection of endogenous pyrogens. Details concerning the housing and training of rabbits have been reported elsewhere (20). The assay of human LP (5) involves frequent measurement of rabbit rectal temperature by use of a thermistor (Yellow Springs Instrument Co., Yellow Springs, Ohio) inserted 8 to 10 cm into the rectum and secured to the tail of the rabbit with an elastic band. Recordings were made every 12 min with a thermometer and recorder (Yellow Springs Instrument Co.) or every minute with a Digistrip recorder (Kaye Instruments, Bedford, Mass.). Peak rectal temperature elevation was obtained after calculating the mean baseline level during the 60-min period before and 12 min after the injection. Peak temperature elevations of 0.3°C above baseline are considered significant fevers. Peak fevers occurring after 60 min were not used in the data analyses, since LP fever reaches peak elevation 40 to 55 min after intravenous injection. All injections were made into the lateral ear vein. The volume was calculated as the supernatant medium derived from 10^6 monocytes per kg of rabbit body weight. All rabbits were New Zealand-derived albino females weighing 2.5 to 3.0 kg each. The dose-response relationship for human LP assayed in rabbits has been determined in several studies (3, 8, 16); peak fevers of 0.4 to 0.9°C above baseline are located on a straight line and hence have been used to quantify the amount of LP in various supernatant from human cells. For these assays, the supernatant medium from each incubation, i.e., MNCs plus stimulant, was injected into three rabbits, and the mean fever peak was used for data analyses. Mean peak fever (± standard error of the mean) was used for these studies.

Additional controls were carried out during this study to enhance the accuracy of the LP rabbit pyrogen test. Rabbits were prescreened for their response to a standard dose of human LP. A single preparation of human LP was used which produced peak fevers of 0.6 to 0.9°C, also called a rabbit pyrogen dose (7). Thus, rabbits which on three successive days had mean peak fevers below 0.6°C or above 0.9°C were not used in the study. Because rabbits develop antibodies to human blood products, rabbits were not used to assay human LP after seven consecutive daily injections.

LAL test. LAL testing was performed at Tufts University, Boston, Mass., and at Genentech, Inc., San Francisco, Calif. For LAL testing at Tufts University, lysate with a sensitivity of 0.025 ng/ml was obtained from Associates of Cape Cod, Woods Hole, Mass. The procedure for the LAL test was as previously described (12). Endotoxin used for controls was RE-2, National Reference *E. coli* endotoxin, kindly supplied by the Bureau of Biologics, Bethesda, Md. In the LAL testing at Genentech, we employed lysate obtained from Mallinckrodt, Inc., St. Louis, Mo., with a routine sensitivity of 0.025 ng/ml. All preparations of met-hGH were diluted in pyrogen-free water at a concentration of 1 mg/ml.

Endotoxin. Endotoxin used for the USP rabbit pyrogen test and to induce human LP production in vitro was RE-4 obtained from the USP, Rockville, Md. The USP rabbit pyrogen test was carried out by North American Science Associates of California, Irvine, Calif. Polymyxin B was obtained from Pfizer Inc., New York, N.Y.

RESULTS

Production of LP from human MNCs incubated with met-hGH. Lot GH002A was prepared in December 1980 and in several animal studies had demonstrated no toxic effects.

The lot (lyophilized finished product) was assayed by the USP rabbit pyrogen test and the USP LAL test. Inhibition studies were carried out by the recommendations of the USP, and no inhibitory effects were observed; the lot passed both tests. Thus, this lot was selected for the first phase I trial of met-hGH, in which it produced a constellation of signs, symptoms, and clinical laboratory data indicative of an acute-phase response to a pyrogenic agent. Since repeat pyrogen and LAL testing remained unchanged, lot GH002A was evaluated for its ability to induce human cells to produce LP. The initial experiments revealed that concentrations of lot GH002A greater than 0.1 mg/ml were necessary to induce LP production in vitro (data not shown). Furthermore, incubating the MNCs in the presence of lot GH002A required 48, rather than the usual 24, h of incubation to release significant amounts of LP into the supernatant medium. Nevertheless, it was shown that lot GH002A consistently induced human LP.

Figure 1 illustrates the results of subsequent studies on LP production induced by met-hGH. Unstimulated control cells released no detectable LP, whereas cells incubated with *S. albus* or lot GH002A produced significant amounts of LP. The glycine buffer control and pituitary-derived hGH at similar concentrations did not induce LP in these experiments (Fig. 1). This latter finding is of considerable significance, since growth hormone can function as an immunostimulatory substance under certain in vitro conditions. Finally, a preparation of met-hGH which was subjected to isoelectric focusing before formulation and lyophilization did not induce LP. This latter experiment provided evidence that the *N*-terminal methionine did not provide an explanation for the pyrogenicity of lot GH002A.

Effect of various lots of met-hGH in the LAL and the human LP test. Extensive testing of several lots of met-hGH was carried out subsequent to the demonstration that lot GH002A induced human LP production (Table 1). In these studies, LAL testing was also performed at both Tufts University and Genentech. There was substantial agreement in the LAL tests from both laboratories; the results from Genentech are shown in Table 1. No correlation between the concentration of LAL-positive material and the induction of LP from the various lots assayed was found (Table 1). Thus, we concluded that the LP test was measuring a pyrogenic contaminant which was not being detected by the standard LAL test.

Four lots of met-hGH, however, did not induce LP production (Table 1). These lots were prepared under different purification and formulating conditions. When retested, these lots consistently did not induce LP production (data not shown). Thus, based on the conditions of preparation of these lots, another lot of met-hGH was prepared for use in a repeat phase I trial in human subjects. The repeat phase I trial was designed to detect evidence of inflammatory responses by use of several parameters of the acute-phase response, such as falls in iron and zinc levels in serum (4, 13). Before the repeat phase I trial, the selected lot of met-hGH was repeatedly tested in the LAL, USP rabbit pyrogen, and human LP tests; these all yielded negative responses. After intramuscular injection of the second clinical lot of met-hGH into human subjects, no signs, symptoms, or laboratory test results characteristic of the acute-phase response or pyrogenic reaction were observed (manuscript in preparation). A similar absence of these responses has been observed in all subsequent clinical trials of met-hGH for growth hormone replacement therapy (17).

Comparison of human LP induction by initial and subsequent clinical lots of met-hGH. We sought to identify the nature of the pyrogenic contaminant in the initial lot GH002A of met-hGH. Approximately 15 months after its preparation, lot GH002A was reevaluated for its ability to induce LP production from the same donor MNCs as those used to retest subsequent lots of met-hGH used in clinical trials. Each donor whose cells had not been previously used to test lots of met-hGH was selected. Eleven donors (six males and five females) were selected (Table 2). The responses to the apyrogenic lots of met-hGH used in clinical trials were uniformly negative in inducing LP, whereas the MNCs from 9 of 11 donors produced LP when incubated with lot GH002A. The results also indicate that the presence of human AB serum during these incubations results in greater amounts of LP released when compared with serum-free incubations. Thus, 15 months after its preparation, lot GH002A retained its ability to induce LP in vitro, whereas lots of met-hGH which produced no inflammatory side

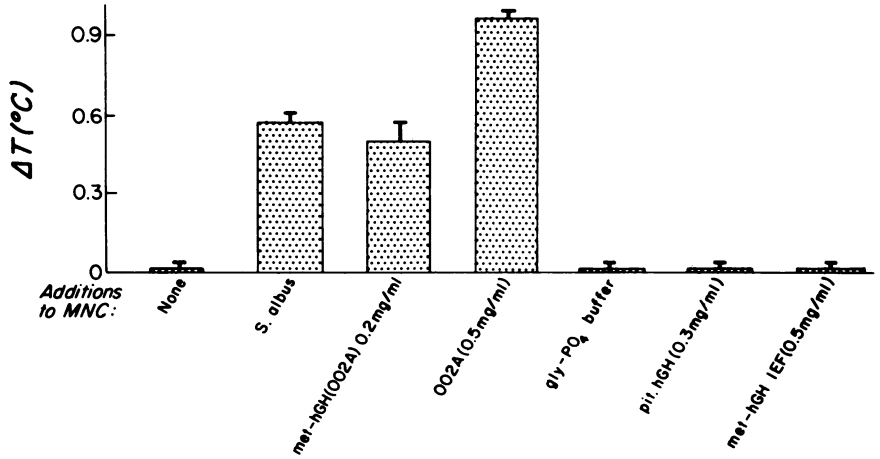

FIG. 1. Mean fever peak above baseline (± standard error of the mean) produced in rabbits after intravenous injection of supernatants derived from human MNCs incubated with materials indicated under the abscissa. Control injections of met-hGH without prior incubation with MNCs given at a comparable dose did not produce fever. met-hGH IEF, met-hGH purified by isoelectric focusing; pit. hGH, pituitary-derived hGH; 002A, lot GH002A; gly-PO_4, glycine-phosphate. MNCs from several human donors were used in these experiments, and data have been combined.

TABLE 1. Comparison of LAL reactivity with LP production by using various lots of met-hGH[a]

met-hGH lot	LAL (ng/ml)	LP
GH002A	0.10	0.63 ± 0.03
GH003A	0.10	0.56 ± 0.03
0111-26	0.40	0.60 ± 0.15
0111-28	0.40	0.56 ± 0.03
GH004A	0.20	0.43 ± 0.03
CS-363	0.2–0.40	0.63 ± 0.03
RP-390	0.15	0.36 ± 0.06
KO-386	0.2–0.40	0.00
316-2-1	0.10	0.00
316-2-2	<0.10	0.00
316-2-3	0.30	0.00
316-2-3a	0.40	0.53 ± 0.03
316-2-4	0.60	0.60 ± 0.03
316-2-4a	0.20	0.40 ± 0.13
316-2-5	0.20	0.28 ± 0.11

[a] Lots of GH002A through to and including KO-386 were formulated and lyophilized in glycine-phosphate buffer; lots 316-2-1 and the remaining lots were formulated and lyophilized in mannitol buffer (see text). Each lot was assayed with the MNCs from two separate donors, except lots 0111-26 and 0111-28. All incubations were in 5% heat-inactivated AB serum; the concentration of met-hGH was 0.5 mg/ml. The supernatant from each incubation was assayed in rabbits in triplicate, and the mean peak fever (± standard error of the mean) is shown.

effects in ongoing clinical trials (17) were inactive in the LP test.

Polymyxin B prevention of LP production by initial lot of met-hGH. The ability of endotoxin to cause fever in rabbits, the gelation of LAL, the activation of complement, and the induction of LP production in vitro are due to the strongly anionic lipid A moiety (15). Polymyxin B and other cationic antibiotics have been shown to be highly effective inhibitors of the many biological activities of endotoxin by blocking the reactive lipid A group (10). Recently, polymyxin B has been shown to prevent endotoxin-induced rabbit LP production in vitro (11, 18). Therefore, experiments were undertaken to test the ability of polymyxin B to block the induction of human LP by lot GH002A. As part of these studies, we first carried out a dose-response experiment using the reference *E. coli* endotoxin, RE-4, incubated with human MNCs with or without polymyxin B. LP was induced by concentrations of RE-4 endotoxin of 0.5 to 50 ng/ml in the presence of 5% heat-inactivated AB serum (Fig. 2). Significantly less LP was released in serum-free medium. Polymyxin B prevented LP production even at the highest endotoxin concentration used (50 ng/ml), whereas it had no effect on *S. albus*-induced LP. In these studies, all MNC supernatants were incubated with polymyxin B (12.5 μg/ml) for 1 h before injection into rabbits to prevent residual endotoxin from interfering with the rabbit pyrogen test.

When lot GH002A of met-hGH was incubated with MNCs in the presence of 12.5 μg of polymyxin B per ml, no LP was detected in the supernatant medium (Fig. 3). Thus, from the results of these experiments, it seems likely that the pyrogenic contaminant present in lot GH002A is similar to the activity of endotoxin, since LP production was enhanced in the presence of 5% AB serum and polymyxin B inhibited LP production in vitro.

Reduction in endotoxin activity after lyophilization in glycine. The above data suggest that lot GH002A was probably contaminated with an endotoxin-like material; however, LAL and USP rabbit pyrogen testing did not detect quantities of endotoxin sufficient to account for the spectrum of clinical and laboratory findings observed during the phase I trial in which this lot of met-hGH was used. In the course of investigating the pyrogenicity of lot GH002A, reference endotoxin RE-4 was purposely assayed before and after lyophilization in the glycine-phosphate buffer used to formulate lot GH002A. The results of these studies (Table 3) include data obtained by using a mannitol buffer, which was used to formulate subsequent clinical lots of met-hGH. Neither formulating buffer contains significant concentrations of endotoxin. Liquid glycine spiked with either 5 or 50 ng of RE-4 endotoxin per ml produced gelation at 3.2 and 25 ng/ml, respectively, in the LAL test. At 50 ng/ml, RE-4 in liquid glycine retained its pyrogenicity in the USP rabbit pyrogen test. When these same preparations were then subjected to lyophilization in the glycine buffer, however, LAL reactivity was reduced from 3.2 to 0.2 and from 25 to 3.2 ng/ml. These data indicate that a 10-fold reduction in detectable activity was taking place during lyophilization in glycine. Furthermore, the preparation became nonpyrogenic in rabbits. On the other hand, a similar experimental design in which the mannitol formulating buffer was used showed no reduction in the LAL test as a result of lyophilization. Thus, these data provide evidence that the ability of the LAL test, as well as the USP rabbit pyrogen test, to detect endotoxin is altered by lyophilization in glycine.

To provide further evidence that the biological activity of endotoxin is reduced during lyophilization in glycine, studies were carried out with the human LP test. The design of these studies included the assay of endotoxin in liquid glycine or liquid mannitol before and after lyophilization. Three human donors were selected, and the MNCs containing 5% AB serum were incubated as described above. There was a marked reduction in LP production after lyophilization in glycine but a minimal reduction when the mannitol buffer was used (Fig. 4). In subsequent experiments, we were unable to induce human LP in vitro from MNCs incubated with RE-4 lyophilized in glycine at 10 ng/ml (data not shown). Thus, these experiments confirm that lyophilization in the presence of glycine reduces the biological activity of endotoxin in the three assays used.

DISCUSSION

The results of these studies suggest that the initial clinical lot of met-hGH (lot GH002A) which had induced in human subjects several components of the acute-phase response was probably contaminated with bacterial endotoxin. This finding was ascertained by the demonstration that this lot consistently induced human mononuclear phagocytes to

TABLE 2. Comparison of LP production induced by lots of met-hGH used in initial and repeat phase I trials

Addition to MNCs	LP production at following temp (no. of donors)[a]	
	<0.3°C	>0.3°C
None	0.04 ± 0.01 (11)	
ABS	0.09 ± 0.01 (11)	
met-hGH[b]	0.05 ± 0.01 (11)	
met-hGH + ABS	0.12 ± 0.03 (11)	
GH002A	0.11 ± 0.03 (7)	0.37 ± 0.04 (4)
GH002A + ABS	0.16 ± 0.02 (2)	0.55 ± 0.05 (9)

[a] Results are shown as mean peak fever (± standard error of the mean) in rabbits. Numbers in parentheses represent numbers of donors (of a total of 11) showing reaction. Each supernatant derived from the cells of each of 11 donors was assayed in three rabbits.

[b] Lots of met-hGH which had produced no side effects in repeat phase I and clinical trials for replacement therapy. In these human trials, the dose of met-hGH was 0.125 mg/kg. All concentrations of met-hGH used in vitro were at 0.625 mg/ml. ABS, 5% heat-inactivated AB serum added to MNC cultures.

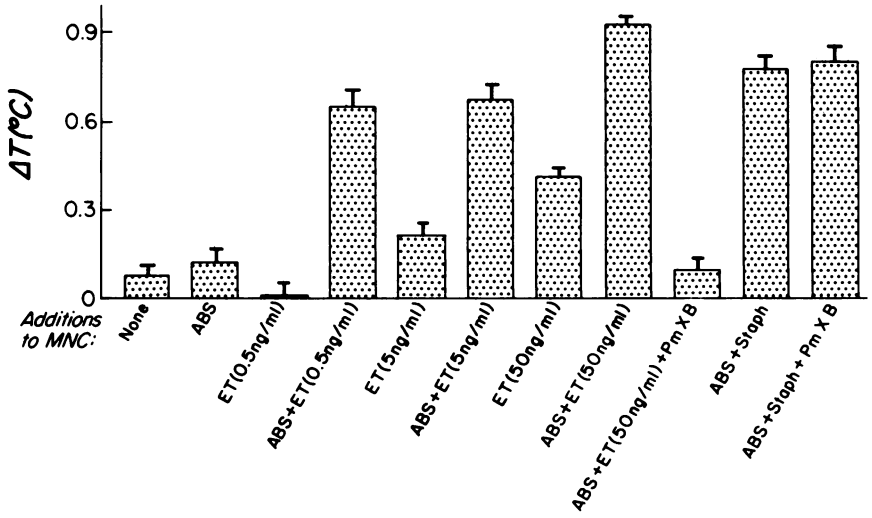

FIG. 2. Mean fever peak above baseline (± standard error of the mean) produced by supernatants from human MNCs incubated with materials indicated under the abscissa. The concentration of polymyxin B used during these incubations was 12.5 μg/ml. This concentration was also added to all supernatants 1 h before injection into rabbits. For these studies, MNCs were obtained from three donors and each supernatant was assayed in three rabbits. ABS, Human AB serum; ET, endotoxin; Staph, *S. albus*; PmXB, polymyxin B.

produce LP in vitro. The ability of the human LP test to detect the presence of contaminating pyrogenic materials confirms the hypothesis that exogenous agents which are pyrogenic for humans stimulate the production of LP from appropriate human responder cells (in this case, blood monocytes). met-hGH itself, when purified by isoelectric focusing, did not induce LP production and provided laboratory evidence that the *N*-terminal methionine imparts no intrinsic biotoxicity to growth hormone. This was confirmed in subsequent clinical trials in which met-hGH was administered without side effects.

Until further experimentation had established the probable source of the pyrogenic nature of the initial lot GH002A, the LP test proved to be useful in screening several lots of met-hGH prepared by various procedures. From the onset of these screening tests, it became clear that there was no correlation between the ability of a lot to induce human LP production and the concentration of LAL-reactive material (see Table 1). This finding suggested that either endotoxin was not the source of the contamination or that the LAL and USP rabbit pyrogen tests were not detecting the pyrogen. The well-established sensitivity of the LAL and rabbit pyrogen tests for detecting nanogram quantities of endotoxin made the latter hypothesis unlikely. Humans and rabbits are highly sensitive to the fever-, neutrophilia-, and hypoferremia-inducing properties of many endotoxins (4, 13, 19). Thus, attention was focused on the possibility that the offending lot of met-hGH might be contaminated with another bacterial product, for example, peptidoglycan. Peptidoglycans from both gram-negative and gram-positive bacteria

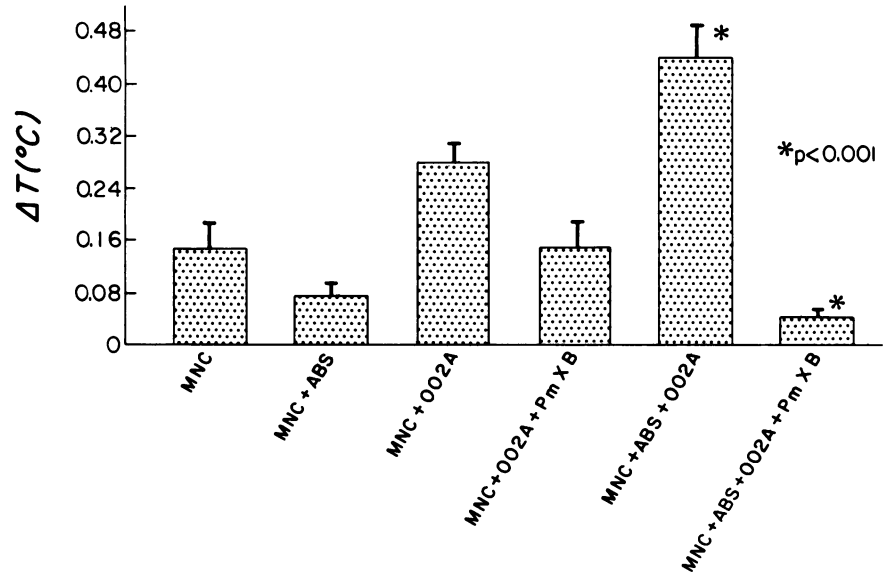

FIG. 3. Mean fever peak above baseline (± standard error of the mean) produced by supernatants from human MNCs incubated with materials indicated under the abscissa. For these studies, MNCs were obtained from five donors and the supernatant from each incubation was assayed in three rabbits. ABS, Human AB serum; 002A, lot GH002A; PmXB, polymyxin B.

TABLE 3. Effect of lyophilization on endotoxin in the LAL and USP rabbit pyrogen tests

Material[a]	LAL (ng/ml)	Result of rabbit pyrogen test[b]
Liquid glycine	<0.025	NT
5 ng/ml in liquid glycine	3.2	NP
50 ng/ml in liquid glycine	25.6	P
5 ng/ml lyophilized in glycine	0.2	NP
50 ng/ml lyophilized in glycine	3.2	NP
Liquid mannitol	<0.025	NT
5 ng/ml in liquid mannitol	1.6	NP
5 ng/ml lyophilized in mannitol	1.6	NP

[a] Endotoxin used in these studies was RE-4. Glycine and mannitol buffers were the same as those used to formulate met-hGH.

[b] NT, Not tested; NP, nonpyrogenic (refers to the USP rabbit pyrogen test in which the temperature increases of three rabbits is added and is <0.6°C); P, pyrogenic.

are also capable of inducing pyrogenic and acute-phase responses indistinguishable from those produced by endotoxin (10). However, several parameters to determine the purity of the preparation did not reveal the presence of such contaminants. Furthermore, peptidoglycans, unlike endotoxins, require microgram-per-kilogram quantities to induce acute-phase responses. Nevertheless, lots of met-hGH which consistently did not induce human LP production in vitro were used in subsequent phase I and clinical trials without evidence of pyrogenic or inflammatory responses (17).

While investigating the effects of certain procedures on the reproducibility of the LAL test, we clearly demonstrated that lyophilization of purified *E. coli* endotoxin (RE-4) in the presence of glycine-phosphate buffer resulted in a 10-fold reduction in activity. This reduction was demonstrated in both the LAL and the USP rabbit pyrogen test. Further testing with the human LP test confirmed that lyophilization in glycine-phosphate buffer reduces the biological activity of endotoxin. The fact that the initial lot GH002A was lyophilized in glycine suggested that the LP test may detect endotoxin altered by the glycine lyophilization process. We were unable, however, to demonstrate this using concentrations of RE-4 of 10 ng/ml before lyophilization. Thus, it seems likely that the concentration of endotoxin in lot GH002A may have been greater than 10 ng/mg of met-hGH or that the native endotoxin (as compared with purified RE-4) is highly reactive with human MNCs.

The most convincing data that lot GH002A was contaminated with endotoxin was derived from experiments in which polymyxin B was used. There is considerable evidence that the lipid A moiety of gram-negative endotoxins contains the biological and toxic properties of the molecule (15). This has been demonstrated in several experiments in which the lipid moiety is cleaved from the parent molecule and then mixed with albumin (for solubility); under these conditions, all biological and toxic activity can be demonstrated in these preparations. Furthermore, chemical modification which esterifies the lipid A-reactive groups also results in loss of activity. For tissue culture conditions, however, these methods are not practical. The ability of polymyxin B to inhibit the lipid A moiety enables this antibiotic to block most, if not all, of the bioactivity of endotoxin in vivo as well as in vitro. Of particular importance is that polymyxin B also prevents endotoxin from stimulating LP production in vitro (11, 18). In the present studies, polymyxin B was added to the human MNC culture when stimulated by lot GH002A and prevented the production of LP. In control experiments, polymyxin B completely prevented 50 ng of RE-4 endotoxin per ml from stimulating LP production. The specificity of polymyxin B in blocking endotoxin was demonstrated by the fact that *S. albus*-induced LP production was unaffected by the presence of this antibiotic. Others have shown that polymyxin does not block the pyrogenicity or LP-inducing property of synthetic polynucleotides (18). Additional evidence that lot GH0002A was contaminated with endotoxin was provided by the consistent finding that both lot GH002A and RE-4 endotoxin

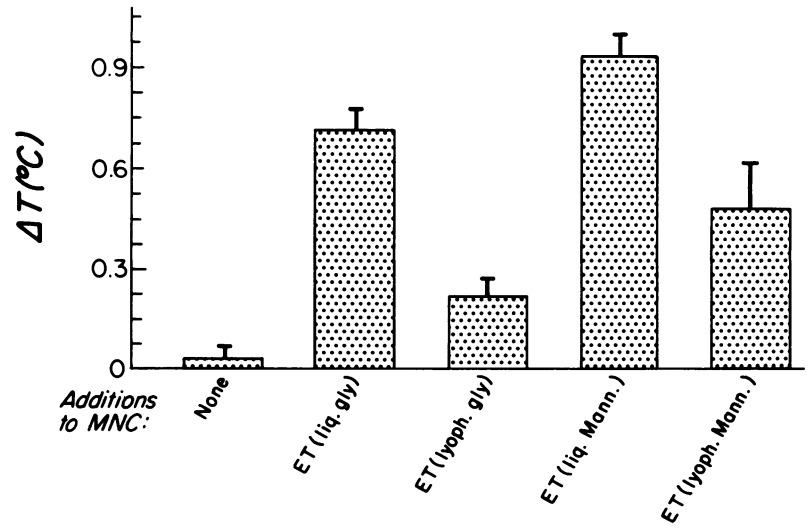

FIG. 4. Mean fever peak above baseline (± standard error of the mean) produced by supernatants from human MNCs incubated with materials indicated under the abscissa. For these studies, all incubations were in 5% heat-inactivated human AB serum. These data represent the results of MNCs obtained from three donors. Each incubation supernatant was assayed in three rabbits. ET (liq. gly), Endotoxin in liquid glycine; ET (lyoph. gly), endotoxin in lyophilized glycine; ET (liq. Mann.), endotoxin in liquid mannitol; ET (lyoph. Mann.), endotoxin in lyophilized mannitol.

induced more LP in the presence of heat-inactivated human AB serum than under serum-free conditions.

The above results obtained by employing polymyxin B and together with the data from the lyophilization experiments, suggest that lot GH002A probably contained sufficient levels of endotoxin to induce the inflammatory response in human subjects during the initial phase I trial but that these levels of endotoxin were not detected in standard pyrogen assays because of the inhibitory effect of glycine. Both the LAL and USP rabbit pyrogen tests are rapid (the LAL test takes 1 h and the rabbit pyrogen test takes 3 h). On the other hand, the LP test required 48 h of incubation to obtain significant LP production. This latter finding suggests the possibility that a certain amount of dissociation of endotoxin from the glycine may have taken place during this time.

It is presently unclear as to whether the pyrogen contamination of lot GH002A was from the *E. coli* strain used to produce the growth hormone or from a water or procedural source. Because numerous lots of met-hGH have been produced since the production of lot GH002A without evidence of pyrogen contamination or clinical side effects, it seems less likely that the recombinant *E. coli* strain was the source. Nevertheless, because of the large amounts of endotoxin produced by these bacteria and because humans are the most sensitive species to its toxic effects, effective detection systems should be carefully monitored. It is unlikely that the human LP assay should be used as part of a monitoring system, but from the results of the present study, it appears that the use of this assay may prove useful at some point in the development of purification procedures for future products.

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