DEMONSTRATION OF A HUMAN PYROGEN-INDUCING FACTOR DURING MIXED LEUKOCYTE REACTIONS*

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Fever is mediated by an endogenously produced low molecular weight protein called leukocytic pyrogen (LP),[1] which acts in or near the thermoregulatory center of the pre-optic anterior hypothalamus (1). Human LP is synthesized by phagocytic leukocytes after stimulation by a variety of substances; however, with the exception of certain pyrogenic steroids, these substances have been microbial agents or their products (2). In studies on immunologically mediated fever, the use of soluble antigens or antigen-antibody complexes to stimulate LP in sensitized animals is well established (3–5). On the other hand, antigens have not been demonstrated to stimulate human LP production even when antigens such as tetanus toxoid, penicillin, or tuberculin are incubated with leukocytes from known sensitized individuals in the presence of autologous serum. Thus, the mechanism of LP production in immunologically mediated febrile disease in humans remains unexplained. The present investigations concern the role of alloantigens in the stimulation of LP production and the results demonstrate the release of a soluble factor in the mixed leukocyte reaction (MLR) that induces the synthesis of LP from human monocytes. This factor was produced in the absence of materials of microbial or plant origin, and its ability to induce LP synthesis may be important in the pathogenesis of fever in humans with certain immunologically mediated diseases.

Materials and Methods

Cell Cultures. Methods concerning pyrogen-free materials and pyrogen testing have been described elsewhere (6, 7). Heparinized human blood was obtained from donors, mixed with pyrogen-free saline and separated on Ficoll-Hypaque gradients in 50-ml tubes (8). The mononuclear cell layer was aspirated, washed twice in saline, and resuspended in Eagle's minimum essential medium (MEM; Microbiological Associates, Walkersville, Md.). The cell concentration was adjusted to either 5×10^6 or 5×10^5 cells/ml in MEM containing 10% heat-activated AB serum and then aliquoted into 1-ml volumes in 17- × 100-mm polypropylene tubes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.). Cells from each donor were then combined to produce a MLR; the final volume for the MLR or autologous individual control cultures was 2 ml/tube. Additional controls were 2 ml MEM or MEM with

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[1] *Abbreviations used in this paper*: Con A, concanavalin A; HBSS, Hanks' balanced salt solution; LP, leukocytic pyrogen; LPS, lipopolysaccharide; MEM, minimum essential medium; MLR, mixed leukocyte reaction.

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10% AB serum. All tubes were incubated for 2–7 d in a stationary incubator at 37°C in 5% CO_2. In other experiments, mononuclear cells were incubated in 16-mm diameter flat-bottomed wells (Costar, Data Packaging, Cambridge, Mass.) in 2-ml volumes. Under these conditions, cells were aliquoted and mixed in tubes and then transferred to flat-bottomed wells for incubation. For the in vitro production of LP, blood and processing methods have been described (9, 10). To stimulate LP production with MLR supernates, these supernates were first diluted with Hanks' balanced salt solution (HBSS) and then mononuclear cells from a third donor were added to make a final concentration of 5×10^6/ml at the appropriate dilution of the MLR supernate. As controls for the ability of these cells to be activated for LP production, phagocytosis of heat-killed *Staphylococcus albus* was used (9). After 18 h, all supernates were removed, spun at 2,500 *g* for 30 min, and tested in rabbits. 3–4 rabbits were each given an intravenous injection of the supernate from 2.0×10^6 monocytes. Plateletphoresis by-products were occasionally used for LP production. These cell mixtures were comprised of 40–50% monocytes, 30–40% lymphocytes, and 5–10% neutrophils. Because of centrifugation methods used in plateletphoresis, cells consistently contained a high percentage of monocytes. Because previous studies had demonstrated that the monocytes were essentially the only source of LP in these preparations (9) and that lymphocytes do not produce LP (10), the monocytes were not separated from lymphocytes for the production of LP.

Lymphocyte Studies. Incorporation of [^{3}H]thymidine was used to determine lymphocyte proliferation in MLR and autologous leukocyte cultures. After the mononuclear cells from different or the same donors were combined in 17- × 100-mm tubes, 200-μl aliquots were removed and placed in round-bottomed microtiter plates (Falcon Labware) in quadruplicate. These were incubated at 37°C in 5% CO_2 for the same number of days as the MLR tube cultures. The amount of [^{3}H]thymidine incorporated into DNA-protein was determined by liquid scintillation and expressed as the difference of the mean number of counts per minute in four wells subtracted from wells with media alone.

For adherence studies, mononuclear cells from donors were suspended in MEM with 10% heat-inactivated AB serum at 5×10^6 cells/ml. These were warmed at 37°C, plated out in 5-ml volumes onto 85-mm diameter plastic petri dishes (Falcon Labware) and incubated for 2 h at 37°C. Nonadherent cells were poured off and the plates were washed with 5 ml of warm medium. Cells from the wash were pooled with the first harvest nonadherent population and the concentration of cells was adjusted to 5×10^6/ml before combining individual donors in the MLR. Nylon wool adherence was carried out in columns filled with MEM containing 10% AB serum and incubated at 37°C for 60 min. 1×10^8 mononuclear cells in 2 ml of MEM/serum were applied to columns and allowed to enter the nylon wool. They were incubated for 1 h at 37°C and nonadherent cells were eluted with 30 ml of warmed medium/serum.

Results

Presence of a Pyrogen-inducing Factor in Two-Way MLR Supernates. MLR supernates obtained after 2–7 d of incubation were diluted with HBSS and then added to human monocytes from a third donor not used in the original MLR. These suspensions were incubated in 25-ml flasks at 37°C for 18 h and tested for the presence of LP. Fig. 1 illustrates such an experiment and Table I depicts 14 separate MLR experiments that were studied in this manner. As shown in Table I, when MLR supernates were injected into rabbits they occasionally produced fevers (4 out of 14). The fevers were characterized by rapid onset and monophasia, indicating that various quantities of LP were present in these supernates. However, when MLR supernates were diluted (1:10) to subpyrogenic levels, added to the monocytes of a third donor, and incubated for 18 h, LP was induced in 12 of the 14 MLR supernates studied (Table I). Fever observed in these 18-h incubations was not due to small amounts of LP present in the MLR supernate itself. These MLR supernates could be diluted 1:20 and still induce significant amounts of LP (Fig. 1). Additional experiments were carried out in which the cell concentration of the MLR was reduced from 5×10^6/ml to 5×10^5/ml.

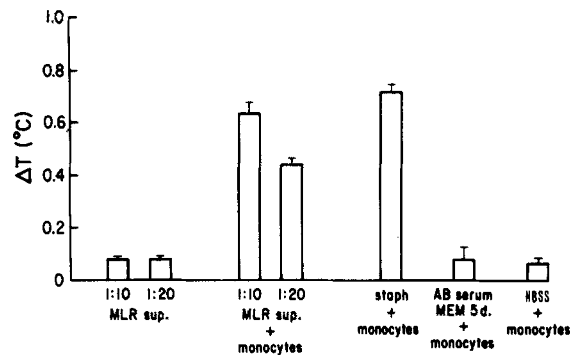

FIG. 1. Mean ± SEM peak rectal temperature, ΔT, (°C) over baseline (3–4 rabbits/experiment) during a 2-h period after injection. This experiment represents a single MLR supernate obtained after 3 d at 37°C. Dilutions (1:10 and 1:20) of supernate from MLR with and without mononuclear cells are present in the first four bars. Each rabbit received the supernate from 2.0×10^6 monocytes stimulated with materials indicated on the abscissa.

TABLE I

Febrile Responses of Rabbits to Human LP Induced by MLR Supernates

Donors*	Time‡	MLR supernate§	Monocytes§ + MLR supernate‖	Monocytes§ + staphylococci
L.R. × D.B.	2	0.00¶	0.51 ± 0.11	0.72 ± 0.13
R.M. × A.D.	3	0.50	0.67 ± 0.03	0.65 ± 0.05
K.M. × S.S.	3	0.00	0.85 ± 0.09	0.65 ± 0.05
A.D. × T.B.	3	0.00	0.52 ± 0.13	1.00 ± 0.00
E.K. × P.W.	4	0.83 ± 0.15	0.93 ± 0.08	0.75 ± 0.06
P.P. × R.C.	5	0.61 ± 0.07	0.63 ± 0.10	0.76 ± 0.08
P.A. × S.B.	5	0.00	0.64 ± 0.13	0.93 ± 0.09
C.D. × B.D.	5	0.00	0.53 ± 0.12	0.85 ± 0.05
K.G. × R.S.	5	0.00	0.00	0.71 ± 0.06
R.M. × L.B.	5	0.00	0.00	0.65 ± 0.02
R.C. × J.H.	7	0.00	0.96 ± 0.05	0.82 ± 0.10
H.B. × S.B.	7	0.28 ± 0.12	0.95 ± 0.11	1.03 ± 0.13
C.D. × C.F.	7	0.64 ± 0.09	0.92 ± 0.08	1.12 ± 0.15
R.M. × A.D.	7	0.57 ± 0.07	0.52 ± 0.08	0.65 ± 0.05

* Initials of the normal volunteer donors whose cells were used in MLR.
‡ Number of days of MLR incubation.
§ 5×10^6 cells/ml.
‖ Diluted 1:10 before addition to monocytes.
¶ Data are expressed as the mean ± SEM peak temperature (°C) elevation over baseline in 3–4 recipient rabbits.

Under these conditions, MLR supernates did not contain detectable levels of LP but nevertheless stimulated LP production.

Because of the possibility that subpyrogenic quantities of LP produced in the MLR supernate could induce subsequent LP production, experiments were designed to remove or destroy any LP present. Supernates from two separate MLR cultures were tested for their ability to induce LP before and after passage over a rabbit antibody to human LP coupled to Sepharose 4B (11). In addition, the MLR supernate was heated at 56°C for 30 min, which destroys LP. As shown in Fig. 2, neither procedure

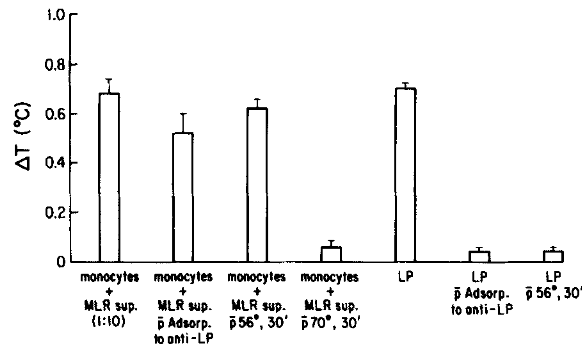

FIG. 2. Mean ± SEM peak febrile responses, ΔT, (°C) over baseline of 6–8 rabbits. Each rabbit received the supernate from 2.0×10^6 monocytes stimulated with materials indicated under the abscissa. A pool of MLR supernates was used in these studies. This pool was passed over an anti-LP immunoadsorbent (see Materials and Methods) on two separate occasions. This immunoadsorbent had the ability to bind 200 rabbit pyrogen doses. (A rabbit pyrogen dose is defined as the amount of LP that produces a peak temperature rise of 0.6–0.9°C over baseline [7]). The LP used as control in these experiments (three bars on the right) was obtained from crude supernates of human monocytes stimulated by phagocytosis of heat-killed staphylococci.

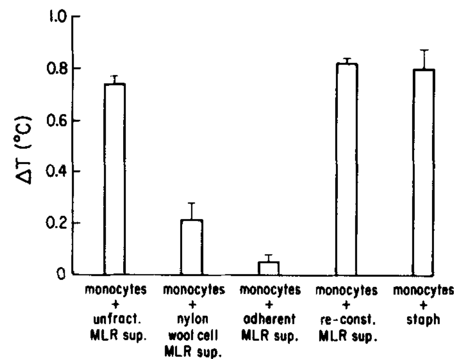

FIG. 3. Mean ± SEM peak febrile responses, ΔT, (°C) over baseline of nine recipient rabbits injected with supernates from human mononuclear cells stimulated for 18 h with materials indicated on the abscissa. These results represent the mean of three separate MLR experiments using six individual donors. MLR supernates prepared from unfractioned mononuclear cells were compared to supernates of the same paired donors prepared from cells purified over adherence to plastic and passage over nylon wool. In addition, the plastic adherent cells from each donor were also used in three paired MLR experiments. In each of the three experiments, cells were reconstituted in an MLR. Each rabbit was injected with the supernate of 2.0×10^6 monocytes obtained from another donor. Three rabbits were used to assay the LP in each supernate for a total of nine rabbits for each bar shown (see text).

altered the ability of MLR supernate to induce LP production. Therefore, the pyrogen-inducing factor was not due to LP itself.

Source of the Pyrogen-inducing Factor. Several experiments were carried out to determine the cellular requirements necessary for the release of this pyrogen-inducing factor. Removal of adherent cells by incubation of plastic surfaces either had no effect or only slightly diminished the amount of the pyrogen-inducer released into the supernate. In further experiments, removal of cells using adherence to plastic, followed by exposure to nylon wool, was employed. Nylon wool-purified cells did not produce this factor when cultured in the MLR (Fig. 3). To determine whether the adherent

cell population was the source of the pyrogen-inducing factor, adherent cells were removed from the plastic at 4°C using a rubber spatula. Adherent cells from two separate donors did not produce this factor when mixed together and incubated for 3 d, although these adherent cells were still viable because they phagocytized staphylococci. However, when adherent cells were added back to nylon-purified cells at a ratio of 1:10 in a reconstituted MLR, the factor reappeared (Fig. 3). These results indicate that both T cells and monocytes are necessary for the release of this factor and are consistent with antigen presentation by macrophages and the elaboration of a lymphokine from T cells.

Characterization of the Pyrogen-inducing Factor. The pyrogen-inducing factor present in the MLR supernates was retained by autoclaved dialysis tubing against 4,000 vol of medium at 4°C for 24 h. Heating supernates at 70°C for 30 min destroyed their ability to induce LP production, whereas heating for 56°C for 30 min had no effect (Fig. 2). MLR supernates were consistently negative in the Limulus amebocyte assay test. In addition, MLR incubated at 4°C for 3 d did not release the factor.

Although LP from human cells produces fever in many species (rabbits, monkeys, mice, and cats), species specificity for the pyrogen-inducing factor is not known. Experiments were designed to incubate rabbit monocytes with human MLR supernates and test for rabbit LP production. Rabbit blood mononuclear cells were obtained from Ficoll-Hypaque gradients of rabbit blood as described for human cells in Materials and Methods. A pool of human MLR supernate was diluted 1:10 with rabbit mononuclear cells at 5×10^6 cells/ml. In addition to stimulation by phagocytosis, human AB serum (10% vol/vol) was used as a control. Supernates from these incubations were injected into rabbits at volumes comparable to the supernate from 2.5×10^7 rabbit monocytes. As shown in Fig. 4, human MLR supernates were capable of stimulating rabbit LP production from monocytes.

Comparison of Lymphocyte DNA Synthesis with Elaboration of Pyrogen-inducing Factor. The two-way MLR is associated with increased lymphocyte proliferation presumably induced by the presence of surface membrane glycoprotein alloantigens. In an attempt

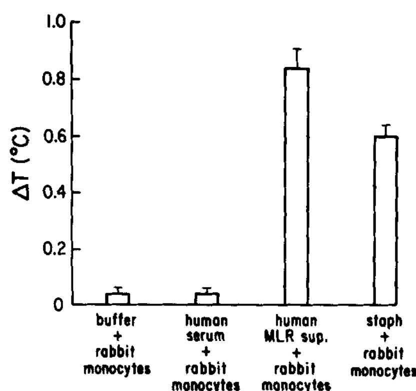

FIG. 4. Mean ± SEM peak febrile response, ΔT, (°C) of six recipient rabbits injected with supernates from 2.5×10^7 rabbit monocytes incubated with various materials as indicated on the abscissa. These results are the mean of two experiments using a pool of MLR supernate diluted 1:10 with monocytes obtained on two separate occasions from four different rabbits. Because human AB serum may stimulate rabbit LP production, a control of AB serum to a final concentration of 10% was added.

to correlate lymphocyte proliferation with the release of the pyrogen-inducing factor, increased thymidine incorporation into new DNA-synthesis was measured in the two-way MLR. The data in Table II indicate that lymphocyte proliferation occurs, and in three of the five experiments was associated with, the elaboration of the pyrogen-inducing factor. However, this factor was present in an MLR supernate before a significant proliferative response took place, and was absent in two experiments in which significant proliferation had occurred. Therefore, the release of this factor does not always correlate with lymphocyte proliferation in the MLR.

In another set of experiments, the production of this factor from individual leukocyte cultures was examined. In early studies, small quantities of this factor were detected occasionally in 5-7-d cultures of the individual donors, but this finding was attributed to nonspecific stimulatory effects. Using heat-inactivated autologous serum in these cultures, it was observed that certain individuals occasionally produced the pyrogen-inducing factor in the absence of allogeneic stimulation. When these individuals were restudied at a later time, this finding could not always be repeated. However, autologous leukocyte cultures of two other donors were tested at concentrations of 5×10^6 and 5×10^5/ml in either round-bottomed tubes or flat-bottomed wells. Under the latter conditions, the pyrogen-inducing factor was not present after culture of autologous leukocytes. However, regardless of cellular concentration, the factor was present in the supernates of the MLR of these donors.

TABLE II

[^{3}H]Thymidine Incorporation and Presence of Pyrogen-inducing Factor in the MLR

Donor*	Time‡	Δ CPM	Monocytes§ + MLR supernate‖
R.M.	2	605 ± 124	0.00¶
D.F.	2	361 ± 126	0.00
R.M. × D.F.	2	2,935 ± 424	0.65 ± 0.12
P.A.	3	642 ± 192	0.00
S.B.	3	1,229 ± 32	0.00
P.A. × S.B.	3	15,415 ± 2,015	0.64 ± 0.13
C.D.	5	19,456 ± 891	0.00
G.T.	5	11,260 ± 793	0.00
C.D. × G.T.	5	141,913 ± 18,460	0.00
K.G.	5	2,310 ± 328	ND**
D.E.	5	247 ± 55	ND
K.G. × D.E.	5	20,516 ± 4,157	0.00
B.S.	5	345 ± 75	0.00
D.D.	5	1,428 ± 142	0.00
B.S. × D.D.	5	25,642 ± 1,281	0.53 ± 0.14
A.M.	5	1,612 ± 51	0.00
T.B.	5	511 ± 73	0.00
A.M. × T.B.	5	6,468 ± 137	0.80 ± 0.11

* Initials of the normal volunteer donors whose cells were used in the MLR.
‡ Number of days of MLR incubation.
§ 5×10^6 cells/ml.
‖ Diluted 1:5 before added to cells.
¶ Data are expressed as the mean ± SEM peak temperature (°C) elevation over baseline in 3-4 recipient rabbits.
** Not done.

Discussion

The pathogenesis of fever in humans has been studied using microbial and nonmicrobial substances that induce the release of LP from human phagocytic cells in vitro. In general, agents such as bacterial lipopolysaccharide (LPS) from Gram-negative bacteria, bleomycin, and etiocholanolone have been shown to produce fever in vivo in humans and induce LP production in vitro (2). Studies on nonmicrobial, immunologically mediated fever, such as drug allergy, have not been reported, although there is hardly a dearth of patients with fever associated with hypersensitivity to specific antigens. Therefore, there is presently no model for studying the mechanism of immunologically mediated fever in humans. On the other hand, several experimental models exist in animals. These include sensitizing animals with specific antigens and after subsequent challenge, evoking a febrile response. At least two mechanisms seem to be involved in the pathogenesis of immune fever in animals. One involves the requirement for circulating antibody in which antigen-antibody complexes bring about the production of LP (5). The other is cell-mediated and can be transferred with sensitized cells (12). Further studies on cell-mediated immune fever have established that a factor is liberated from sensitized lymphocytes when exposed to an antigen that stimulates LP production (13). However, whether these mechanisms play a role in immunologically mediated fever in humans remains unclear, and the inability of several antigens (tetanus toxoid, penicillin, and old tuberculin) to induce LP production from cells to sensitized individuals has hampered these efforts.

The current experiments demonstrate that LP synthesis from human monocytes can be induced by soluble factor (or factors) produced in the human MLR. The stimulation for the release of this substance appears to be the presence of allogeneic cells in the two-way MLR. The importance of the present study, in addition to the fact that materials from humans were employed, is that alloantigens, not microbial or plant products, were used to stimulate the release of the pyrogen-inducing factor. Thus, the present experiments may be similar to allogeneic stimulation seen in graft-vs.-host disease or transplant rejection. In these immunologically mediated diseases, fever is a prominent sign and the production of this factor from human lymphocytes in the MLR may aid in understanding the pathogenesis of fever in these and other immunologically related diseases.

The physical nature of the pyrogen-inducing substance is unknown except that it is retained by autoclaved dialysis membrane and destroyed at 70°C for 30 min. LP was occasionally detected in the MLR supernate after 3–5 d, and most likely represents the stimulation of monocytes present in the MLR. However, removal of LP using an immobilized antibody to LP demonstrated that the pyrogen-inducing substance was not due to LP itself. This latter finding is important because there is evidence that large amounts of LP infused into rabbits induce the production of additional LP in vivo (14). Furthermore, the fact that LP is inactivated at 56°C for 30 min is further evidence that this factor is not LP itself. The fact that the human pyrogen-inducing lymphokine released during an MLR also induced the production of LP in rabbit monocytes suggests that this factor may not be species specific.

The present experiments also demonstrate that the pyrogen-inducing factor is a product of lymphocytes, most likely T cells. Other lymphokines, namely macrophage migration inhibitory factor, has been shown to be a product of B and T cells (15). Adherence of cells to plastic had no significant effect upon the generation of this

factor. With the double technique of adherence to plastic, followed by purification over nylon wool, the pyrogen-inducing factor was consistently absent from the MLR supernates in several experiments. Reconstitution of nylon wool-purified lymphocytes with adherent cells from each donor resulted in the reappearance of this factor in the MLR supernates. These findings are consistent with other reports demonstrating the requirement of adherent cells in the MLR (16, 17), and other T cell recognition studies (18). Although the stimulation for the release of this material appears to be the presence of allogeneic cells in the two-way MLR, release of this pyrogen-inducing factor was occasionally observed in the absence of allogeneic stimulation. This latter observation may be due to conditions of in vitro culture. Such conditions have been described by others and include cell density and geometry of the culture vessel, both of which may contribute to cellular interaction and stimulation in vitro (19). Reduction of cell concentration or the use of flat culture surfaces prevented the spontaneous production of the pyrogen-inducing factor.

Although these studies are the first to show the presence of a pyrogen-inducing factor in the supernates of MLR from human cells, they are consistent with the findings of Bernheim et al. (20) and Atkins et al. (13), in which a pyrogen-inducing product of concanavalin A (Con A) or antigen-stimulated lymphocytes was demonstrated. However, the use of plant products such as Con A in fever studies, and, in particular, in inducing LP production in vitro, depends on the availability of preparations free of LPS (21). Recent reports from the present laboratory have provided evidence that sequentially purified human LP activates murine T cells and thymocytes, properties similar to those of lymphocyte activating factor (22, 23). Other investigations have also shown similar results using rabbit LP (24). Although the characterization and identification of the specific pyrogen-inducing lymphokine in the MLR is presently unknown, activators of other macrophage functions by lymphokines have been described (25, 26) and should be carefully investigated for their ability to contain LP or induce LP production.

Summary

The role of lymphocytes in the pathogenesis of fever was investigated by stimulating human blood mononuclear cells in a two-way mixed leukocyte reaction (MLR). After 2–7 d of incubation, MLR supernates contained a factor that was not pyrogenic itself when injected into rabbits; however, these supernates, when incubated with human blood monocytes from a third donor, induced the synthesis of LP. The pyrogen-inducing activity was stable at 56°C, destroyed at 70°C, and was neither dialyzable nor removable by adsorption by anti-human leukocytic pyrogen (LP) attached to Sepharose 4B. Production of this factor was not always correlated with increased thymidine incorporation in the MLR. Its production was absent when peripheral lymphocytes were purified over nylon wool. The concentration of mononuclear cells in the MLR varied from 5×10^5 to 5×10^6/ml in round-bottomed tubes. Under the latter conditions, some donor cells produced this factor without stimulation in the MLR culture, but when these cells were cultured on flat-bottomed containers at low cell concentration, autologous production was not observed. These experiments demonstrate the production of a human lymphocyte factor (lymphokine) that induces LP synthesis. This pyrogen-inducing lymphokine may be important in the pathogenesis of fever in certain immunologically mediated diseases.

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