THE RELEASE OF AN ENDOGENOUS PYROGEN FROM GUINEA PIG LEUKOCYTES IN VITRO

A New Model for Investigating the Role of Lymphocytes in Fevers Induced by Antigen in Hosts with Delayed Hypersensitivity*

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As early as 1911, von Pirquet noted the association between hypersensitivity reactions and fever (1). However, until recently, very little has been known about the mechanism of fever produced by antigen in specifically sensitized hosts. Recent studies in animal models suggest that there are two separate immune mechanisms for fever. In humoral immunity, antigen-antibody complexes activate certain (presumably phagocytic) cells in the host to produce a circulating endogenous pyrogen (EP)[1] the "messenger molecule" that stimulates the thermoregulatory centers in the brain (2–4). In cell-mediated immunity, on the other hand, antigen appears to stimulate specifically sensitized lymphocytes to release a soluble agent that, in turn, activates phagocytic cells to generate EP (5).

Sensitized lymphocytes have been clearly shown to play an essential role in the events associated with cell-mediated immunity (6). When activated by specific antigens, sensitized guinea pig lymphocytes produce soluble factors, collectively known as "lymphokines," which have varying biological activities as defined by in vitro assays (7). These factors include substances which inhibit the migration of macrophages [migration inhibitory factor (MIF)], and induce chemotaxis, cytotoxicity, and proliferation of lymphocytes (8). Atkins et al. have investigated the role of the lymphocytes in the genesis of fever induced by antigen given intravenously in rabbits with delayed hypersensitivity (DH) (5). Their data suggest that sensitized lymphocytes, when incubated with specific antigen in vitro, release a nonpyrogenic intermediate substance, perhaps a lymphokine, which stimulates rabbit blood leukocytes to produce EP in vitro.

Experiments in guinea pigs have provided much data on DH, as well as extensive evidence for lymphokines. Uhr and his collaborators clearly showed a number of years ago that guinea pigs with DH can develop fever when challenged with various sensitizing antigens (9, 10). However, the mechanism of fever in DH has not been investigated in this experimental host. The current study establishes: (*a*) an in vivo assay for guinea pig EP in rabbits, and (*b*) a new

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[1] *Abbreviations used in this paper:* BGG, bovine gamma globulin; CFA, complete Freund's adjuvant; DH, delayed hypersensitivity; DLN, draining lymph node; EP, endogenous pyrogen; MIF, migration inhibitory factor; PE, peritoneal exudate; PFS, pyrogen-free saline; PMN, polymorphonuclear leukocytes; staph, *Staphylococci;* sups, supernates.

experimental model for studying the presumptive role of lymphokines in the induction of EP production by guinea pig leukocytes in vitro.

Materials and Methods

General. Standard procedures were used for insuring that all materials were pyrogen-free (11). Techniques for assay of EP and for avoiding sensitization of recipient rabbits to antigen in injected supernates (sups) have been described previously (5, 11). Guinea pigs were Hartley-strain females ranging from 250–300 g, obtained from breeding facilities of the Division of Animal Care, Yale University School of Medicine. Tissue culture media (Auto-Pow^R Eagle's minimal essential medium [MEM]; Flow Laboratories, Inc., Rockville, Md.) with 50 U/ml penicillin and 50 μg/ml streptomycin (penicillin/streptomycin solution; Grand Island Biological Co., Grand Island, N. Y.) were used and stored for up to a week at 4°C. Immediately before use, L-glutamine (Microbiological Associates, Bethesda, Md.) (2 mM) was added and the media adjusted to a pH of 7.4 with CO_2.

Bovine gamma globulin (BGG) from a single lot (Becton, Dickinson & Co., Orangeburg, N. J.) was freshly dissolved in normal pyrogen-free saline (PFS) (5 mg/ml) and filtered through a 0.20 μm plain membrane (Disposable Nalgene filter unit; Nalge Co., Nalgene Labware Div., Rochester, N. Y.) was used as the antigen. Commercial complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, Mich.) was fortified with lyophilized tubercle bacilli (*Mycobacterium tuberculosis*, strains C, DT8PN mixed; heat-killed and freeze-dried; Ministry of Agriculture, Fisheries & Food, Central Vet. Lab., Weybridge, Surrey, England, kindly supplied by Dr. Byron Waksman, Department of Pathology) to a concentration of 3 mg/ml. A strain of *Staphylococcus albus*, (heat-killed) was used as a phagocytic stimulus (12).

Sensitization of Guinea Pigs. Guinea pigs were immunized with 100 μg BGG. BGG in PFS was added to an equal volume of CFA to give a final concentration of 250 μg BGG/ml and the mixture emulsified. Each guinea pig received 0.1 ml emulsion in each of four foot pads. Guinea pigs used as control lymph node donors were immunized with the same volume of emulsified CFA in PFS without BGG.

Skin Testing of Sensitized Guinea Pigs. Guinea pigs were skin tested with 50 μg of BGG in 0.1 ml PFS, injected intradermally. All animals had reactions which were considered positive with erythema and induration of greater than 10 mm diameter at 24 h.

Preparation of Lymphocytes. 10 or 11 days after sensitization, donor guinea pigs were sacrificed with ether. With sterile technique, axillary and popliteal draining lymph nodes (DLN) were dissected out, minced, and gently pressed with added media through a wire 40 gauge screen. Cell suspensions were centrifuged at 1,500 rpm for 15 min, washed once, and the cells resuspended to an appropriate concentration of 1–2 × 10^7/ml. Total cell yield ranged from 2 to 5 × 10^8 lymphocytes per guinea pig. Viability assayed by neutral red (1%) dye exclusion was 50–60%. For most experiments, lymphocytes were pooled from 10 animals.

Preparation and Incubation of Peritoneal Exudate (PE) Cells. 7 days after sensitization, donor guinea pigs were injected intraperitoneally with 30 ml light mineral oil (Marcol-52 oil; ESSO, Humble Oil & Refining Co., Houston, Texas) which was sterilized by autoclaving (120°F for 20 min). 3 or 4 days later (10 or 11 days after sensitization) guinea pigs were sacrificed with ether. Food was withheld for 1 day before the procedure to minimize anesthesia vomiting and risk of bowel perforation during peritoneal cannulation.

With sterile technique, the peritoneum was infused with 150 ml of tissue culture media (MEM) and cannulated. PE was drained into siliconized glassware on ice to minimize cell loss. After centrifugation at 1,500 rpm for 20 min, PE cells were separated from the sup and washed twice in MEM. Total cell yield ranged from 0.6 to 1.5 × 10^8 cells per guinea pig. PEs from two or three guinea pigs were pooled for each experiment.

Wright stain of PE cells revealed differentials of about 75% phagocytes (monocytes and polymorphonuclear cells) which were assumed to be the "effector" (EP releasing) cells, and 25% lymphocytes. In all experiments, the number of phagocytes was calculated to be 75% of the total cell count.

After washing, the exudate was resuspended in MEM in concentrations ranging from 2.5 to 5.0 × 10^6 phagocytes/ml. PE cells were incubated ± various activators and DLN cell suspensions (see below) in plastic culture flasks. Single doses were derived from 5 × 10^6 to 5 × 10^7 phagocytes.

Activators were added as follows: (*a*) Nonspecific phagocytic stimulus—heat-killed staph were

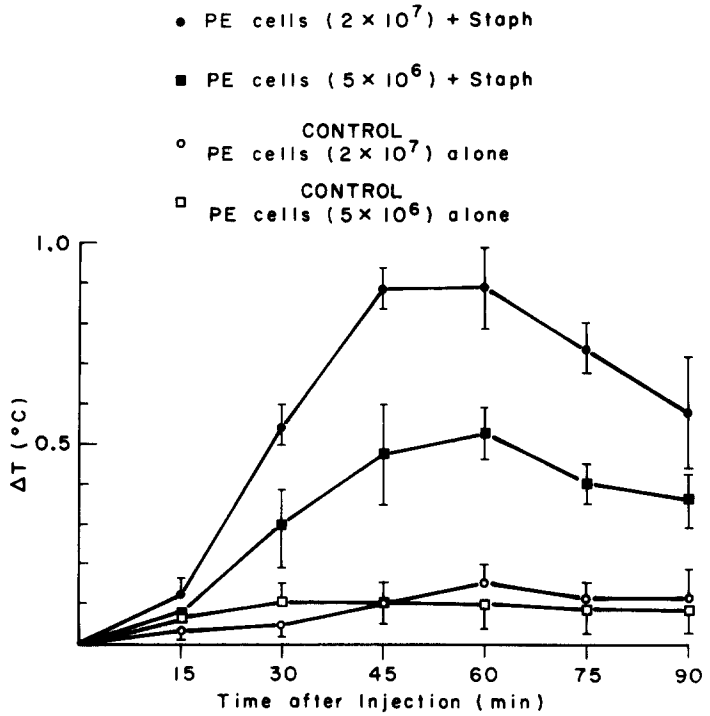


FIG. 1. Mean fever curves (± SEM) induced in eight rabbits by intravenous injection of an EP present in 18 h sups of two doses of PE cells (number of phagocytes per dose) incubated with and without heat-killed staph (20 organisms per phagocyte). (See text for details.)

added in concentration of 20 organisms per phagocyte to provide a "positive" control for production of EP by phagocytes in PEs exposed to a nonspecific stimulus (13, 14). (*b*) Specific antigen – BGG in PFS was added in a concentration of either 0.1 or 1.0 mg/10^7 phagocytes. (*c*) CFA- or BGG-sensitized lymphocytes were added in some experiments in concentrations of 5 or 10 lymphocytes per phagocyte ± BGG (1 mg/10^7 phagocytes). PE cells in these experiments were derived by the same technique from unsensitized guinea pigs.

Cells were incubated at 37°C in a 5% CO_2 atmosphere for 18 h. The sups (1° harvest) were then separated from cells by centrifugation at 2,500 rpm for 20 min and assayed for EP activity (see below). In several experiments, a "second harvest" (2° harvest) of EP was obtained by resuspending cells in the same volume of MEM used initially and incubated at 37°C and in 5% CO_2 atmosphere for another 24 h. Sups were then separated as above and assayed for EP activity. Serum was not used in any experiments. After incubation, all samples were cultured in thioglycollate broth. In the rare instance of bacterial contamination, the results were discarded.

Assay of EP. Procedures used to assay EP were similar to those previously reported (5) except that temperatures were monitored continuously with Rustrak automatic recorders (Timer Service Co., Inc., Norwood, Mass.). For comparison, control and experimental samples from each experiment were usually injected into the same rabbit.

Results

Assay of Guinea Pig EP in Rabbits: Activation of (PE) Cells with Staph. Fig. 1 shows average fever curves induced by Sups of two dosages of guinea pig PE cells incubated 18 h with staph as well as by sups of control cells incubated alone (See Materials and Methods). A dose-related response is clearly evident with a brief latent period and monophasic fever peaking at 45–60 min,

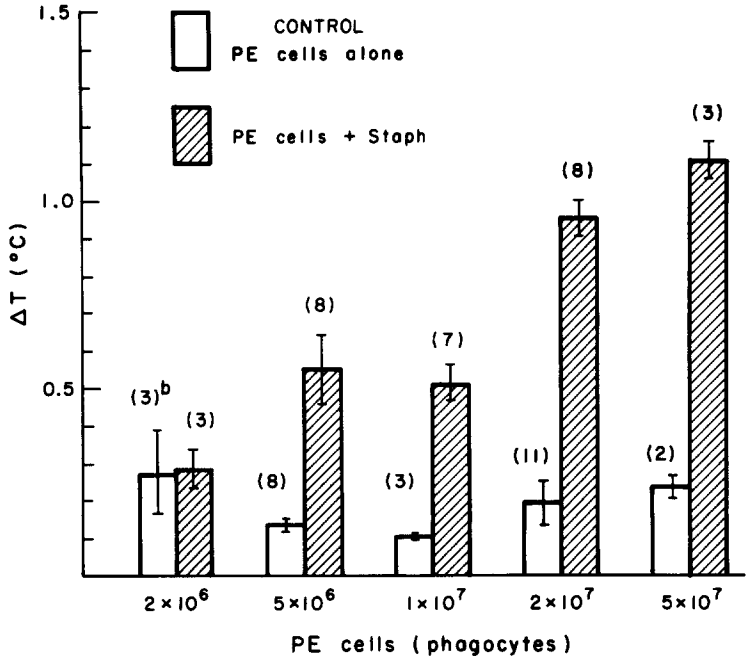


FIG. 2. Mean febrile responses (± SEM) to EP released by various doses of PE cells incubated for 18 h with and without heat-killed staph (20 organisms per phagocyte). In this and following figures, numbers in parentheses indicate number of recipients.

characteristic of the response of the rabbit to EP derived from phagocytic cells of other species (15, 16). Sups of control cells at both doses, on the other hand, did not contain detectable EP.

Fig. 2 shows mean febrile responses induced by sups of PE cells ranging from 2 × 10^6 to 5 × 10^7 phagocytes, incubated 18 h with staph. Over this range the amount of release was directly related to cell dosage. The linear nature of this response was confirmed by assay of increasing amounts of a pool of pyrogenic sups derived from PE cells over an eightfold range (3 × 10^6–2.4 × 10^7 PE phagocytes), see Fig. 3. Further data are needed in order to more precisely determine the dose-response relationship above 2.4 × 10^7 phagocytes.

After resuspension PE cells release EP for at least 42 h. For example, when aliquots of 5 × 10^6 or 2 × 10^7 phagocytes initially incubated with staph were reincubated for an additional 24 h in fresh MEM ("2° harvest") the sups produced significant fevers (mean of 0.83 and 0.89°C, respectively, in five rabbits each), whereas sups from control doses of cells reincubated for 24 h were negative (mean of 0.18 and 0.21°C, respectively).

Release of EP from Specifically Sensitized PE Cells Incubated with Antigen (BGG). PE cells were obtained from guinea pigs sensitized with BGG. These cells were incubated in two different dosages, with and without BGG. Staph were added to cells in some experiments. Results are shown in Fig. 4. No detectable EP was released with the lower dose (5 × 10^6) of cells and BGG; however, with the higher dose (2 × 10^7) a mean fever of 0.51°C was induced. Fever curves were characteristic of those produced by EP derived from staph-

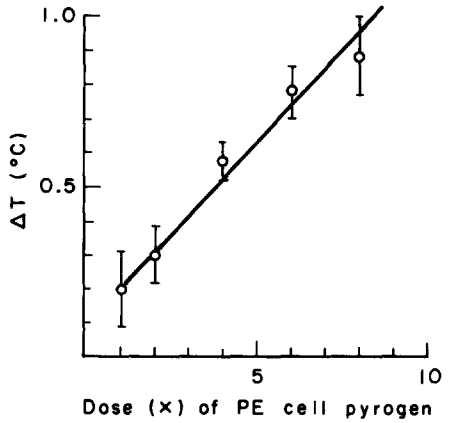


FIG. 3. Varying doses of EP (derived from 18-h sups from staph-stimulated PE cells) plotted against mean febrile responses (± SEM) induced in three or four rabbits. Dose of x pyrogen is equivalent to that produced by 3×10^6 phagocytes.

stimulated cells. Although BGG was inactive as a stimulus with 5×10^6 cells, similar doses of sensitized cells were activated by staph to produce a mean fever of 0.75°C. These data suggest that a specific antigen is not as powerful a stimulus as is phagocytosis for in vitro release of EP by guinea pig PE cells. A similar phenomenon has been noted with rabbit blood leukocytes (5).

In contrast to specifically sensitized PE cells, neither CFA-sensitized nor unsensitized PE cells released EP at 18 or 42 h when incubated with BGG, although these cells were capable of releasing EP when incubated with staph, (Fig. 5).[2] The data demonstrate that BGG is an antigenic stimulus which released pyrogen from specifically-sensitized cells only, in contrast to staph which activated all PE cells equally, irrespective of their source.

Failure of Lymph Node Lymphocytes to Release EP. In view of the mixture of lymphocytes with phagocytes [polymorphonuclear leukocytes (PMNs) and monocytes] in PE cells activated by antigen, we next sought to investigate the possible role of sensitized lymphocytes in this phenomenon.

Axillary and popliteal DLN were obtained from guinea pigs 10 or 11 days after immunization with BGG and CFA or CFA alone. EP was not released in vitro by either pool of lymphocytes in dosages of 2×10^7–4.5×10^8 cells incubated in media for periods of 18 or 42 h alone, or with activators such as staph or BGG, (0.1 or 1.0 mg/10^7 lymphocytes).

EP Production by Unsensitized PE Cells Incubated with Sensitized Lymphocytes plus Specific Antigen. In order to assess the possible role of lymphocytes in activating phagocytic cells in PE when incubated with antigen, unsensitized PE cells were incubated with various dosages of CFA- or BGG-sensitized lymphocytes with and without antigen. When BGG and specifically sensitized lymph node cells were incubated with 2–3 $\times 10^7$ unsensitized PE cells in a ratio

[2] With doses above 3×10^7 phagocytes, PE cells from both unsensitized and BGG-sensitized guinea pigs occasionally released EP in the absence of added activators. However, when such "spontaneous" activation was observed, the amounts of EP released were usually less than the amounts released in response to known activators such as staph or specific antigen.

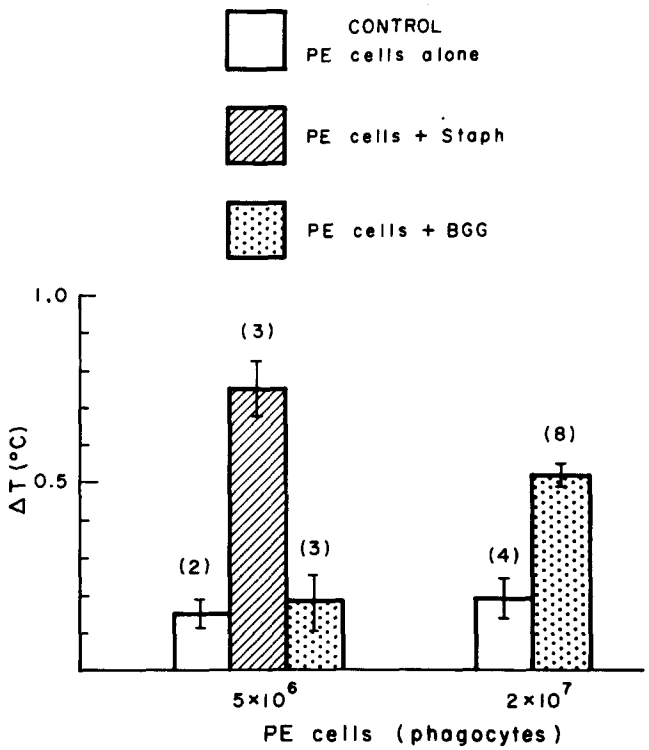


FIG. 4. Mean febrile responses (± SEM) to EP released by two doses of PE cells from BGG-sensitized donors) incubated 18 h with and without BGG (10^2 or 10^3 μg/10^7 phagocytes) and (lower dose of cells) with staph (20 organisms per phagocyte). (See text for details.)

of 10:1 or more, these mixtures produced marginally detectable EP (mean 0.34°C) on 1° harvest (18 h), but released significant amounts of EP on 2° harvest at 42 h (mean febrile response of 0.67°C, see Fig. 6). With lower dosages of either sensitized or control lymphocytes (ratios of 4:1 lymphocytes to PE cells) and BGG, little or no detectable EP was released by normal PE cells at either interval.

Fig. 7 shows the results of two groups of these experiments using a higher ratio of sensitized lymphocytes to unsensitized PE cells (10–14:1). When these cells were incubated with antigen, the 2° harvests clearly contained EP. Control doses (1° and 2° harvests) derived from unsensitized PE cells or lymphocytes incubated alone with BGG, or from unsensitized PE cells incubated with sensitized lymphocytes alone, all failed to release detectable amounts of EP. These data show that co-cultivation of specifically sensitized guinea pig lymphocytes with antigen and normal PE cells cause the release of EP in vitro.

Discussion

In 1958, Uhr and Brandriss described a characteristic febrile response associated with intraperitoneal injection of specific antigen in guinea pigs with DH (9, 10). However, to our knowledge, no further studies have been done on the possible release of EP as a mediator in this, or other forms of experimental fever in the guinea pig.

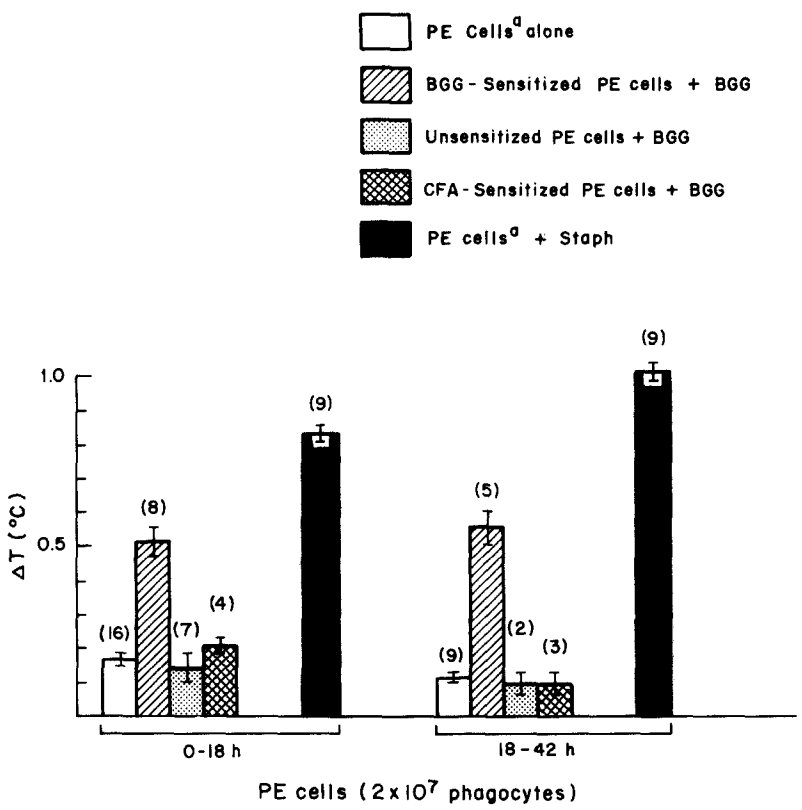
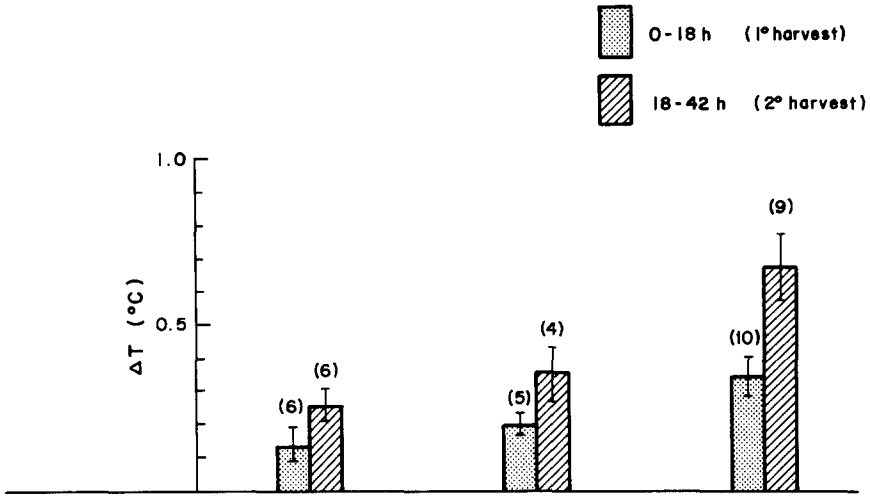


FIG. 5. Mean febrile responses (± SEM) to EP released by three different types of PE cells incubated for two time intervals with and without antigen (10^2 or 10^3 μg BGG/10^7 phagocytes) and with staph (20 organisms per phagocyte). [a]BGG-sensitized, CFA-sensitized, and unsensitized PE cells.

The results reported here demonstrate that guinea pig PE cells produce EP in vitro when presented with a phagocytic stimulus. After intravenous injection in rabbits, guinea pig EP produced prompt monophasic fevers similar to those produced by rabbit and human EP (15). The amount of EP released by PE cells (as reflected in the height of the induced febrile response) was linearly proportional to cell dose at relatively low cell doses.

Specifically sensitized guinea pig PE cells also produced EP in vitro when incubated with antigen. The mechanism of fever associated with DH in the guinea pig, after injection of specific antigen, would thus appear to involve the in vivo mobilization of EP from pyrogen-producing tissues. Since lymphocytes derived from DLN of specifically sensitized guinea pigs like those of rabbits (5) or humans (17) did not produce EP in vitro when incubated either with a nonspecific stimulus (heat-killed staph) or with antigen (BGG), the pyrogen-producing cells in guinea pig EPs are presumed to be those which comprise the nonlymphocyte fraction, macrophages and/or PMNs.

Atkins et al. have described an experimental model of DH in rabbits which suggests that specifically sensitized lymphocytes, when stimulated with antigen, released a soluble lymphokine-like substance in vitro that, in turn, stimu-



PE cells (phagocytes)	2×10^7	3×10^7	$2.2-3 \times 10^7$
BGG sensitized lymphocytes	$0.8-1.0 \times 10^8$	1.2×10^8	3×10^8
BGG	2 mg	3 mg	3 mg
L/P	4-5:1	4:1	10-14:1

FIG. 6. Comparison of mean febrile responses (± SEM) to EP present in 1° and 2° harvest sups derived from 2 to 3 × 10^7 normal PE cells that were incubated with different ratios of BGG-sensitized lymphocytes to phagocytes (L/P) and BGG (1 mg/10^7 phagocytes).

lated unsensitized rabbit blood leukocytes to release EP (5). With the model of DH in the guinea pig described here, the results of these experiments suggest that specifically sensitized DLN cells and antigen similarly activate unsensitized guinea pig PE phagocytes to produce EP in vitro. Although experiments with BGG and higher doses (10:1) of nonspecifically (CFA) sensitized lymphocytes were not done, it seems almost certain that this reaction is a specific one, since reciprocal studies with DLN lymphocytes of rabbits sensitized to either ovalbumin or human serum albumin have clearly shown that specific antigen was required to evoke EP release from normal rabbit blood monocytes (E. Atkins and L. Francis, unpublished data).

An interesting difference was noted in number of sensitized lymphocytes required from DLN, as compared with those in sensitized PEs themselves, to produce detectable EP from PE cells. PEs from sensitized guinea pigs containing 2×10^7 phagocytes were active when incubated with antigen. Since three quarters of the total number of cells in such exudates were phagocytes, these PE cells contained less than 1×10^7 lymphocytes. However, ratios of 10:1 DLN lymphocytes to phagocytes ($2-3 \times 10^8$ lymphocytes) were required to activate 2–3 $\times 10^7$ normal PE phagocytes to produce comparable amounts of EP. Since antigen does not appear to activate sensitized rabbit adherent blood monocytes alone to produce EP (E. Atkins and L. Francis, unpublished data), this function would seem to be a property of activated lymphocytes, presumably "T." On this assumption, then, lymphocytes in these guinea pig PEs are at least 20 times

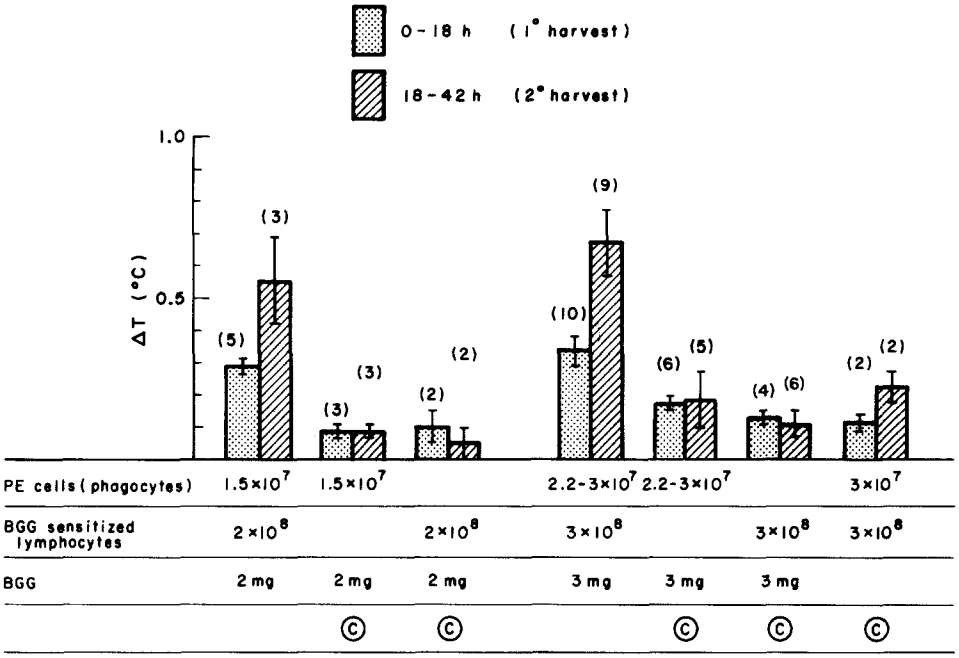


FIG. 7. Mean febrile responses (±SEM) induced by EP present in 1° and 2° harvest sups of normal PE cells incubated with BGG-sensitized lymphocytes and BGG (1 mg/10^7 phagocytes). Responses to various control (C) sups are shown as indicated. (See text for details.)

more effective than are those in DLN. A similar differential in antigen responsiveness has been noted between lymphocytes derived from guinea pig PE and DLN in other in vitro assays of cellular immune function: production of macrophage MIF and antigen-induced lymphocyte proliferation, suggesting that the PE lymphocyte pool is a highly enriched source of antigen-reactive lymphocytes that mediate cellular immune responses (18).

In states of DH, specifically sensitized lymphocytes, when stimulated by antigen, produce soluble substances known collectively as "lymphokines" which have various biological activities (6, 7). These activities are defined by methods of in vitro assay developed by different investigators, e.g. assays for MIF, chemotactic factor, lymphotoxin, etc. (6–8, 19, 20). That these in vitro biological effects are correlated with aspects of the inflammatory responses observed in vivo in DH states can in most cases only be assumed at present. The experimental model described here provides the opportunity, unavailable in most other assays, to study the relationship between a substance produced by sensitized lymphocytes exposed to antigen in vitro and a well-known clinical phenomenon, fever, that develops in hosts immunized for DH and challenged with specific antigen in vivo.

Summary

Guinea pig peritoneal exudate (PE) cells incubated overnight in vitro with heat-killed *Staphylococci* released an endogenous pyrogen (EP) that could be assayed by intravenous injection in rabbits. The febrile responses were linearly related to the dosage of EP over an eightfold range.

PE cells derived from guinea pigs with delayed hypersensitivity (DH) to bovine gamma globulin (BGG), also released EP when incubated with antigen in vitro. This reaction was specific and did not occur with PE cells from normal or complete Freund's adjuvant-sensitized guinea pigs. Studies indicated that monos and/or polymorphonuclear leukocytes rather than lymphocytes were the source of EP.

However, when incubated with BGG and sufficient dosages of BGG-sensitized lymphocytes, normal PE cells released EP over a 42 h period. These results suggest that antigen stimulates specifically sensitized lymphocytes to release an agent (perhaps a lymphokine) that activates phagocytic cells to release EP. This model offers unique advantages for investigating in vitro the role of the lymphocyte in antigen-induced fever in DH as well as the relationship of this lymphocyte-induced activity to other known biologic activities mediated by antigen-stimulated lymphocytes.

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