

RELEASE OF ENDOGENOUS PYROGEN-ACTIVATING FACTOR  
FROM CONCANAVALIN A-STIMULATED HUMAN  
LYMPHOCYTES\*

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The mechanisms involved in the production of fever during states of delayed hypersensitivity have only recently begun to be understood. Atkins et al. (1) originally showed that certain tissues from sensitized rabbits that contained both phagocytes and lymphocytes released the fever-inducing protein endogenous pyrogen (EP) when incubated with the sensitizing antigen. Later, supernates from sensitized rabbit lymphocytes incubated with specific antigen were shown to contain a soluble agent or lymphokine that activates macrophages, but not polymorphonuclear (PMN) leukocytes to release EP (2). Similar results have been obtained with lymphocytes from sensitized guinea pigs incubated with homologous antigen and normal peritoneal exudate cells (PEC) but evidence for a soluble agent or lymphokine has been equivocal in this model (3).

Our study shows that when human lymphocytes are incubated with the mitogen concanavalin-A (Con-A) a nonpyrogenic lymphokine is released into the supernate. This factor activates normal human macrophages, but not PMN leukocytes, to release EP and, hence, we have named it EP-activating factor (EPAF). Because Con-A activates a larger proportion of lymphocytes than does specific antigen, it should now be possible to generate significant amounts of human EPAF, thus making possible purification and characterization of this lymphokine.

**Materials and Methods**

All materials, glassware, reagents, and solutions were rendered or determined to be pyrogen-free by previously described techniques (1). Pyrogen assays were conducted as before in rabbits (4).

*Activators.* To provide a positive control for EP release, one aliquot of cells in each experiment was incubated with heat-killed staphylococci (in a ratio of 20:1 bacteria:cells) (4).

Con-A (lot 58C-7330; Sigma Chemical Co., St. Louis, Mo.) was used exclusively. It was determined to be free of detectable endotoxin contamination (in a concentration of 100 µg/ml) by the Limulus amebocyte lysate assay (kindly performed by Dr. John Ryan, West Haven Veterans Administration Hospital, West Haven, Conn.).

*Incubation of Human Blood Leukocytes with Con-A.* Refrigerated human blood previously passed through a cell separator (Haemonetics No. 30; Haemonetics Corp., Natick, Mass.) and depleted of granulocytes was obtained from the Red Cross, (Hartford, Conn.). 50 ml of this blood (20 U heparin/ml) was added to three volumes of saline and the erythrocytes and remaining PMN

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leukocytes removed by centrifugation through Ficoll-Hypaque density gradients (4). The monocyte-lymphocyte fractions were washed twice in Krebs-Ringer phosphate buffer (KRP) and the pellets resuspended in 10–20 ml Eagle's minimum essential medium (Auto Pow; Flow Laboratories, Inc., Rockville, Md.) with 10% fetal bovine serum (Flow Laboratories, Inc.) (MEM/FBS). Cell counts were determined with a Coulter Particle Counter (model Z<sub>r</sub>; Coulter Electronics Inc. Hialeah, Fla.) and differential smears were made (75–95% lymphocytes and 5–25% monocytes). Only preparations with viabilities  $\geq 90\%$  by eosin red exclusion were used. Aliquots of  $1 \times 10^8$  lymphocytes contained either  $4.3 \times 10^6 \pm 0.9 \times 10^6$  or  $3.3 \times 10^7 \pm 0.68 \times 10^7$  monocytes suspended in MEM/FBS ( $1 \times 10^7$  cells/ml). Like other preparations described below, the cells were incubated for 18 h at 37°C in 5% CO<sub>2</sub> in 30-ml tissue culture flasks (Falcon Labware, Div. Becton, Dickinson, & Co., Oxnard, Calif.) with or without (control) Con-A ( $10 \mu\text{g/ml}$ ). The cells were then centrifuged and the supernates assayed for EP by intravenous injection of 10- to 14-ml doses into rabbits.

In some experiments, 200 ml heparinized blood (10 U/ml) was drawn by venipuncture from normal volunteers. One-half was subjected to Ficoll-Hypaque sedimentation; the other one-half to dextran (100,000–200,000 mol wt; ICN Nutritional Biochemicals, Cleveland, Ohio) sedimentation and hypotonic lysis of erythrocytes. Monocyte-lymphocyte fractions contained 80–90% lymphocytes and 10–20% monocytes. Leukocyte fractions showed 30–40% lymphocytes, 55–65% PMN leukocytes, and 5–12% monocytes. Both preparations (in a final concentration of  $1 \times 10^7$  cells/ml) were incubated for 18 h in MEM/FBS with and without Con-A ( $10 \mu\text{g/ml}$ ). The supernates were removed and assayed for EP in dosages equivalent to  $\sim 5 \times 10^7$  PMN,  $4 \times 10^6$  monocytes, and  $2 \times 10^7$  lymphocytes (derived from dextran-sedimented cells) or  $4 \times 10^6$  monocytes and  $2 \times 10^7$  lymphocytes alone (derived from Ficoll-Hypaque centrifugation). The cells were then resuspended in MEM/FBS and reincubated for an additional 18 h. Supernates from these second harvests were tested for EP in the same dosages used initially.

*Production of EPAF.* 10 ml heparinized blood (50 U/ml) that had been passed through a cell separator (see above) was diluted with 10 ml Plasmagel (Associated Biomedic Systems, Inc., Buffalo, N. Y.) and then allowed to settle at 37°C for 20 min. The plasma portion was decanted, diluted to 40 ml with MEM/FBS, and 1 g of carbonyl iron powder (GAF Corp., New York) was added. The suspension was rotated at 37°C for 25 min and the iron and iron-containing macrophages drawn down with a magnet until the plasma portion was clear (5). The lymphocytes and the few residual monocytes remaining after centrifugation of the supernates through Ficoll-Hypaque gradients were washed three times in KRP and resuspended in 10 ml MEM/FBS. The cells (96–99% lymphocytes and 1–4% monocytes) were suspended to a final concentration of  $1 \times 10^7$  cells/ml in MEM/FBS and incubated for 18 h in the presence or absence (control) of Con-A ( $10 \mu\text{g/ml}$ ). The cells were then centrifuged and the supernates dialyzed against distilled water for 48 h and lyophilized to a powder. Material derived from  $1 \times 10^8$  lymphocytes was dissolved in individual doses of 2 ml MEM/FBS and assayed for EP directly (by intravenous injection) or for EP-inducing activity (see below).

*Assay for EPAF.* 200–300 ml heparinized blood (10 U/ml) were drawn from each of two normal adult volunteers by venipuncture. Mononuclear cells (8–15% monocytes) were separated by Ficoll-Hypaque density centrifugation and suspended in MEM/FBS ( $1 \times 10^7$ – $2 \times 10^7$  cells/ml). Aliquots containing  $2 \times 10^7$  monocytes were layered on 30-ml tissue culture flasks, incubated for 2 h, and the adherent cells then washed three times with MEM/FBS. The number of monocytes that adhered to the flask (one-third to one-fifth the initially layered cells) was determined by subtracting the number of monocytes (2–15% of the nonadherent population) from the total number of monocytes added to each flask.

After removal of the nonadherent cells, lyophilized preparations of EPAF or control material were reconstituted in 4 ml MEM/FBS (as two doses derived from  $2 \times 10^8$  lymphocytes) and added to the adherent cells in the flasks. Con-A was added to the control material in the same concentration ( $10 \mu\text{g/ml}$ ) as in the experimental sample of lymphocytes incubated with Con-A. The adhered macrophages were then incubated for 18 h. After this time, the supernates (in two doses, each derived from  $\sim 1 \times 10^7$  macrophages) were assayed for EP. The adhered cells were then washed once and reincubated in 4 ml fresh MEM/FBS for additional 18 h and the supernates assayed as before for EP.

In similar experiments, leukocytes from dextran-sedimented fresh human blood were sus-

pended in MEM/FBS ( $1 \times 10^7$  cells/ml; containing 25–35% lymphocytes, 55–65% granulocytes, and 5–10% monocytes). Aliquots containing  $4 \times 10^7$  PMN leukocytes were layered on 30-ml tissue culture flasks and incubated for 2 h. After this period, the adherent cells were washed three times with MEM/FBS. The number of adhered granulocytes was determined, as with monocytes, by subtracting the number of granulocytes (10–25% of the nonadherent population) from the number of granulocytes initially layered on the tissue culture flasks. After adherence, reconstituted preparations containing EPAF or control material (with added Con-A) were added to each flask, as in the preceding experiments, and the flasks incubated for 18 h. Supernates (in two doses, each derived from  $2 \times 10^7$  PMN leukocytes) were then assayed for EP. The adhered cells were reincubated for an additional 18 h in fresh MEM/FBS as before, and the supernates assayed (as a second harvest) for EP.

## Results

*Release of EP by Human Monocytes Incubated with Lymphocytes and Con-A.* To determine whether human monocytes could release EP when cocultured with lymphocytes in the presence of Con-A, mononuclear cells derived from granulocyte-depleted banked blood were suspended in MEM/FBS and incubated 18 h with and without Con-A. (Materials and Methods). The supernates were then assayed for EP. As can be seen from Fig. 1, doses of  $\sim 3 \times 10^7$  monocytes, when cocultivated with Con-A and  $1 \times 10^8$  lymphocytes, released substantial amounts of EP. Little if any EP was released with lower numbers of monocytes ( $\sim 4.5 \times 10^6$ ), although the same number of monocytes released significant amounts of EP in response to phagocytosis of heat-killed *Staphylococcus albus*.

*Release of a Soluble EPAF by Lymphocytes Incubated with Con-A.* To determine if human lymphocytes secrete a lymphokine (EPAF) that activates phagocytes to release EP, lymphocytes isolated from banked blood were incubated for 18 h with and without Con-A. Supernates (dialyzed, lyophilized, and reconstituted in a small volume of MEM/FBS) were added to tissue culture flasks containing freshly adhered macrophages (Materials and Methods). After 18 h incubation, the supernates were removed for EP assay and the macrophages reincubated 18 h in fresh MEM/FBS for a second harvest. As shown in Fig. 2A, material derived from  $1 \times 10^8$  lymphocytes (though containing no detectable EP itself) activated  $1 \times 10^7$  macrophages to release significant amounts of EP during the first and second incubations. Supernates from lymphocytes incubated without mitogen, to which Con-A was added after separation of cells, did not activate adherent macrophages to release EP, indicating that Con-A does not directly activate these cells to produce EP.

The target cell from human EPAF appears to be the macrophage rather than the PMN because EPAF derived from the same number ( $1 \times 10^8$ ) lymphocytes failed to activate  $2 \times 10^7$  adherent PMN leukocytes (containing a small contaminating number of monocytes) to release EP (Fig. 2B). These cell populations were, however, activated to produce EP by *S. albus*. In four additional experiments (data not shown), similar amounts of pyrogen were released after phagocytosis of *S. albus*, both in the presence and absence of mitogen ( $0.78 \pm 0.26^\circ\text{C}$  and  $0.63 \pm 0.18^\circ\text{C}$  with and without Con-A [ $10 \mu\text{g}/\text{ml}$ ], respectively). Con-A, therefore, in concentrations present in preparations containing EPAF, does not appear to alter the ability of this population of cells to generate EP when incubated with another pyrogenic stimulus.

*Inhibition of EP Release by Monocytes Cocultured with Lymphocytes in the Presence of Con-A: Role of the PMN Leukocyte.* To determine if PMN leukocytes can release EP when cocultured directly with lymphocytes in the presence of Con-A, leukocytes were

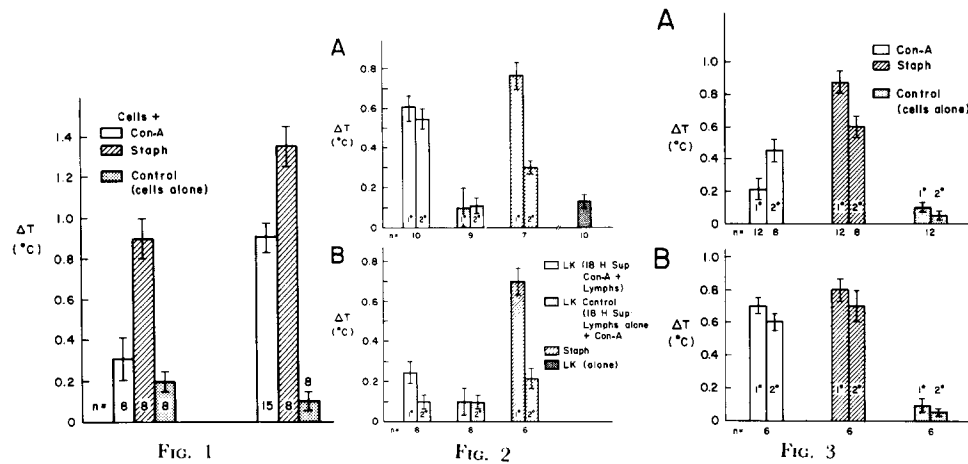


FIG. 1. Mean febrile responses ( $\pm$  SEM) to EP released by mixtures of human lymphocytes and monocytes (from banked blood) incubated 18 h either alone or with Con-A or staphylococci (Staph) (see text). Detectable amounts of EP were released only with the higher dose of monocytes when Con-A was used as the stimulus. Each dose was derived from  $1 \times 10^8$  lymphocytes and either  $4.5 \pm 1.3 \times 10^6$  monocytes or  $30 \pm 10.2 \times 10^6$  monocytes. n, number of recipient rabbits.

FIG. 2. (A) Mean febrile responses ( $\pm$  SEM) to EP released by adherent human monocytes incubated for two successive 18-h periods (1<sup>o</sup> and 2<sup>o</sup> harvest) with a lymphokine (LK) produced by prior 18-h incubation of human lymphocytes with and without (LK control) con-A (see text). Febrile responses to the LK alone and to adherent human granulocytes (B) under the same conditions are shown for comparison. EP production by both populations of adherent cells (monocytes and granulocytes) when incubated with heat-killed staphylococci (Staph) is also shown. In (A), each dose was derived from  $9.7 \times 10^6 \pm 2.1 \times 10^6$  adherent monocytes; in B, from  $1.8 \times 10^6 \pm 0.38 \times 10^6$  granulocytes with  $2.2 \times 10^6 \pm 0.54 \times 10^6$  monocytes. The LK was derived from  $\sim 1 \times 10^8$  lymphocytes incubated with Con-A. Con-A was added to the LK control before incubation with the adherent cells in both (A) and (B).

FIG. 3. Mean febrile responses ( $\pm$  SEM) to EP produced by leukocytes from fresh human blood incubated for two successive 18-h periods (1<sup>o</sup> and 2<sup>o</sup> harvest) with and without Con-A or with staphylococci (Staph). In (A), each dose was derived from  $5.2 \times 10^7 \pm 0.15 \times 10^7$  granulocytes,  $0.43 \times 10^7 \pm 0.14 \times 10^7$  monocytes, and  $1.9 \times 10^7 \pm 0.3 \times 10^7$  lymphocytes. In B, each dose was derived from  $0.36 \times 10^7 \pm 0.08 \times 10^7$  monocytes and  $2.3 \times 10^7 \pm 0.2 \times 10^7$  lymphocytes only.

isolated from fresh venous blood by dextran sedimentation (Materials and Methods). Aliquots of cells ( $\sim 5 \times 10^7$  PMN leukocytes,  $2 \times 10^7$  lymphocytes, and  $4 \times 10^6$  monocytes) were then suspended in MEM/FBS ( $1 \times 10^7$  cells/ml) with or without  $10 \mu\text{g/ml}$  of Con-A, and incubated for 18 h. The supernates were then assayed for EP. Under these conditions, PMN leukocytes released little or no EP (Fig. 3A). In fact, the granulocytes appeared to inhibit the release of EP by the same number of monocytes, derived from the same source and cocultivated alone with lymphocytes and Con-A (Fig. 3B).

### Discussion

Although Con-A is known to activate unsensitized lymphocytes to secrete several lymphokines (6), its role in the pathogenesis of fever has not been previously demonstrated with material known to be free of contaminating endotoxins (7). We believe that our experiments are the first to show that human lymphocytes incubated with Con-A release a soluble factor that activates human phagocytes to release EP.<sup>1</sup>

<sup>1</sup> Dinarello et al. (8) have recently reported that supernates from human mixed lymphocyte reactions (MLR) contain a soluble mediator that activates human macrophages to release EP.

We have called this factor EPAF. The target cell appears to be the macrophage rather than the granulocyte. This limitation of action to a particular cell type is not unique among lymphokines, as macrophage inhibitory factor (MIF) and leukocyte inhibitory factor have as their target cells the macrophage and PMN leukocyte, respectively (9, 10). Recent work suggests that pure populations of PMN leukocytes produce little if any EP when incubated with a standard pyrogenic stimulus such as staphylococci (11). In the case of this EP-producing lymphokine, PMN leukocytes actually appeared to inhibit the release of EP from cocultured macrophages incubated with lymphocytes and Con-A. The nature of this inhibitory action is unknown. Possibilities to be investigated include competition for EPAF by granulocytes and macrophages (which were present in a ratio of 10:1, respectively) or release of a substance by granulocytes that inhibits either the production of EPAF by lymphocytes or its action on its target cell, the macrophage.

The type(s) of lymphocyte(s) (T and/or B) which secretes human EPAF is unknown, but a considerable body of evidence indicates that Con-A differentially activates T cells (6). Recent work in our laboratory has shown that populations of specifically sensitized guinea pig lymphocytes depleted of B cells, when incubated with antigen, will activate peritoneal macrophages to produce EP in vitro (3). However, because of the necessity of obtaining relatively large numbers of cells under pyrogen-free conditions for this assay, we have not yet obtained a sufficiently purified population of B cells to determine if these lymphocytes can also activate phagocytes to release EP. Because Rocklin et al. (12) have shown that both human T and B cells are capable of producing MIF, there is a clear possibility that both types of lymphocytes may similarly release human EPAF.

EPAF appears to be the first human lymphokine to be clearly associated with a known pathologic phenomenon, fever. Because fever is known to decrease the mortality rate of infected lizards (13) and, possibly, of infected mammals as well (14), this factor may be important in the host's defense against microbial pathogens. Its characterization, therefore, may have great clinical significance. Because as little as  $2 \times 10^7$  fresh lymphocytes produce a detectable dose of EPAF, in contrast to a minimum dose of  $\sim 1 \times 10^8$  lymphocytes from banked blood, it should be possible to generate large amounts of this lymphokine. Partial purification and characterization of this important activator of monocytes now appear to be feasible.

### Summary

Evidence is presented that the mitogen concanavalin A stimulates human lymphocytes to produce a nonpyrogenic lymphokine (LK) that is capable of activating human monocytes but not granulocytes to produce endogenous pyrogen (EP) in vitro. The potency of this preparation should facilitate further studies to purify and characterize this agent and determine its relation to other known LK.

It seems likely that this factor, which we have called EP-activating factor (EPAF), plays a significant role in the development of fever in states of delayed hypersensitivity in man.

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