

STUDIES ON THE MECHANISM OF FEVER ACCOMPANYING
DELAYED HYPERSENSITIVITY*

THE ROLE OF THE SENSITIZED LYMPHOCYTE

By ELISHA ATKINS, JOSEPH D. FELDMAN, LORRAINE FRANCIS, AND
ELLEN HURSH

(From the Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06510, and the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California 92037)

(Received for publication 11 January 1972)

Although fever has long been recognized as a cardinal manifestation of hypersensitivity, surprisingly little is known about the mechanisms by which antigens bring about changes in body temperature in hosts with delayed hypersensitivity (1, 2). There is a growing body of evidence that in this immunologic state antigen reacts with specifically sensitized lymphocytes to produce soluble mediators, collectively known as lymphokines, that induce a variety of biologic effects, both in vivo and in vitro, such as cytotoxicity, inhibition of macrophage migration, transformation of normal lymphocytes, chemotaxis, and others (3, 4). It is not known whether such substances are involved directly or indirectly in fevers induced by injection of antigen into hosts with delayed hypersensitivity. The following studies were designed to determine if lymphocytes play an essential role in the release of endogenous pyrogen (EP)¹ and hence in the pathogenesis of fever in rabbits with delayed hypersensitivity induced by two heterologous proteins, bovine gamma globulin (BGG) or bovine serum albumin (BSA) conjugated to dinitrophenol (DNP) or by unconjugated human serum albumin (HSA).

Materials and Methods

General.—Techniques to insure that glassware, instruments, reagents, and solutions were free of contaminating bacterial pyrogens have been previously reported (5).

* This is publication No. 562 from the Department of Experiment Pathology, Scripps Clinic and Research Foundation, La Jolla, Calif. This work was supported by a US Public Health Service Grant A1-07007 and Atomic Energy Commission Contract AT(04-3)-779. Supported by National Institute of Allergy and Infectious Diseases Special Fellowship Grant 7Fo3 A1 15698-01A1.

¹ *Abbreviations used in this paper:* Ag Ab, antigen-antibody; BGG, bovine gamma globulin; BSA, bovine serum albumin; BSS, balanced salt solution; CFA, complete Freund's adjuvant; DLN, draining lymph nodes; DNP, dinitrophenol; EP, endogenous pyrogen; HSA, human serum albumin; MEM, Eagle's minimal essential medium; MLN, mesenteric lymph nodes; RSA, rabbit serum albumin.

Culture media were determined to be pyrogen-free by injection of at least 10 ml intravenously into each of two or more rabbits. Housing, feeding, and selection of rabbits, both as donors and recipients, as well as techniques of pyrogen assay, were similar to those previously reported (5). Temperatures were taken in La Jolla with Thermistemp Telethermometers (YSI Model 44; Yellow Springs Instrument Co., Yellow Springs, Ohio) and in New Haven with Foxboro rabbit scanning switch and fever recorder (The Foxboro Company, Foxboro, Mass.), both equipped with thermistor probes. Rabbits (New Zealand stock weighing 3.0–4.0 kg each) were obtained from a single breeder for experiments with BGG and BSA carried out at La Jolla, Calif., and from several breeders for experiments with HSA carried out at New Haven, Conn.

Antigens.—

Bovine gamma globulin (BGG): A single lot of BGG (Armour Pharmaceutical Co., Chicago Ill.) was used. Before each experiment it was freshly dissolved in saline and immediately filtered through disposable filter units (0.22 μ without grid [Falcon Plastics, Div. B-D Laboratories, Inc., Los Angeles, Calif.]). Protein content, after filtration, was calculated for each experiment by nitrogen determination and generally varied between 4.4 and 5.8 mg/ml.

Bovine serum albumin (BSA): A single lot was kindly supplied by Dr. W. O. Weigle of Scripps Clinic and Research Foundation. This material was handled in the same manner as BGG.

Human serum albumin (HSA): Normal serum albumin USP salt-poor (250 mg/ml) was obtained from the American National Red Cross. This material was diluted 1:10 in saline or Eagle's minimal essential medium (MEM) before use as an immunogen or when added to tissues in vitro.

Conjugates: Dinitrophenol (DNP) was conjugated to BGG and BSA according to the method of Eisen et al. (6). The number of DNP groups bound to these proteins was calculated to be 41 and 43, respectively.

Antigen-antibody complexes: Two rabbit antisera were used to precipitate antigen-antibody complexes or to form soluble complexes in antigen excess. Both had antibody-binding ratios of 3:3:1. As shown in Table II, precipitated complexes containing varying amounts of DNP-BGG were formed at or near equivalence; soluble complexes were formed with 3 mg of conjugate (30:1 antigen excess) and with 4 mg of conjugate (80:1 excess). The complexes were mixed with normal blood cells in either heparinized whole blood or in media containing 15% fresh autologous plasma. Cell counts ranged from 1.1 to 1.9×10^8 leukocytes in 9–17 ml volumes/dose. The mixtures were shaken 60–70 min for 3 hr at 37°C and incubated overnight at 37°C without shaking. The cells were sedimented 18 hr later by centrifugation and the supernates were tested in rabbits for pyrogens.

Antibody.—Serum was obtained from ear bleedings by arterial puncture of donor rabbits 7–35 days after inoculation of antigens. Circulating antibody to hapten-protein conjugates and to carrier proteins was detected by double diffusion in agar. Where necessary, antibody was determined by quantitative precipitin techniques (7).

Sensitization of Donor Rabbits.—DNP-BGG or DNP-BSA conjugated (10 mg/ml of saline) were incorporated in an equal volume of complete Freund's adjuvant (CFA) containing 10 mg of tubercle bacilli/ml.² Rabbits were anesthetized with 75 mg of intravenous sodium pentobarbital³ and inoculated with a total dose of 10 mg of DNP-BGG/DNP-BSA in CFA (2.0 ml) distributed as follows: 0.2 ml in each front footpad; 0.5 ml in each hind paw; and 0.6 ml in four subcutaneous sites over the haunches.

In experiments with HSA, 50 mg of HSA (2 ml of a 1:10 dilution) were incorporated in 2 ml of CFA containing 6 mg of tubercle bacilli/ml. 0.15 ml of this emulsion (1.9 mg of HSA)

² Tubercle bacilli (H37Ra; Difco Laboratories, Detroit, Mich.).

³ Diabutal (60 mg/ml; Diamond Labs, Des Moines, Iowa).

was then injected into each of the four shaved footpads to give a total immunizing dosage of 7.5 mg of HSA.

Preparation of Tissues and Assay of Supernatant Fluids.—Donor rabbits (3–35 days after sensitization) were lightly anesthetized with 75–90 mg of intravenous sodium pentobarbital followed by 10,000 units of heparin⁴ and exsanguinated by cardiac puncture. Blood was centrifuged (1500 rpm for 30 min), the plasma removed, and the cellular sediment (with some 10–15 ml of overlying plasma) resuspended to its original volume in either Eagle's MEM without Ca⁺⁺⁵ or Medium 199.⁶ Blood from normal donors was usually pooled from two or three animals and treated the same way. Cell counts showed that leukocytes ranged from 0.5 to 1×10^7 /ml.

Draining lymph nodes (DLN) from axillary and popliteal regions, mesenteric lymph nodes (MLN), liver (about 10 g), and spleen were removed aseptically.⁷ Cellular suspensions were prepared by teasing apart nodes or finely dividing liver and spleen in MEM or Hanks' balanced salt solution (BSS) and gently pressing them through a No. 50 tantalum gauze. The cells were then washed once in balanced salt solution (BSS)⁸ and resuspended with 10% fresh rabbit serum in Eagle's MEM for tissue culture (containing Ca⁺⁺ and Mg⁺⁺). Final cell concentrations of lymphocytes were adjusted to contain $1-2 \times 10^7$ /ml with dosages of $1-2 \times 10^8$ in volumes of 10–15 ml. Wet preparations of cells from DLN usually showed 5–10% large mononuclear forms, those from MLN, 1–3%. The other cells appeared to be small lymphocytes. Dosages of 1 g of crude liver suspensions were dispersed in 10 ml of medium and calculated to contain 0.7×10^8 Kupffer cells, an amount sufficient to produce and detect EP. The resuspended cells were incubated overnight in Eagle's MEM, with individual dosages of 4–6 mg of carrier protein (BGG, BSA, or HSA) or 3.5–5 mg of conjugate (DNP-BGG or DNP-BSA) at 37°C, in either Erlenmeyer or tissue culture flasks in an atmosphere of CO₂. All samples (experimental and control) were routinely set up in duplicate in each experiment. The next morning, the cells incubated with antigen, as well as control preparations incubated either without antigen or with other activators, were centrifuged (1200 rpm for 20 min, and the supernatant fluids (10–15 ml/dose) were assayed for fever-inducing activity by injection into normal rabbits. In some instances, $1-2 \times 10^8$ lymphocytes from either DLN or MLN were added directly to $0.5-1.0 \times 10^8$ normal blood cells, all in a volume of 15–20 ml, which, as supernate, was injected as a single dose into test rabbits. Additional heparin (1000 units/flask) was usually added to these preparations to prevent clotting. All samples before and after incubation with antibiotics (penicillin 5 units/ml and streptomycin 5 µg/ml)⁹ were cultured in thioglycollate broth and, in the rare instance of bacterial contamination, the results were discarded.

Other Activators.—Heat-killed, washed staphylococci (see reference 8) (30:1 bacteria to cells) and old tuberculin (see reference 9) (100 mg/dose) were occasionally used to compare ability of various tissues to release EP in vitro in response to both nonspecific and (in case of CFA-immunized donors' tissues incubated with tuberculin) specific microbial stimuli (8).

⁴ Liquaemin Sodium (5000 units/ml; Organon, Inc., West Orange, N.J.).

⁵ Grand Island Biological Co., Grand Island, N.Y., or Flow Labs, Inc., Rockville, Md.

⁶ Prepared in Department of Epidemiology at Yale Medical School and supplied by Dr. Harvey Liebhaver.

⁷ On several occasions alveolar macrophages were flushed from the lungs to be used as a source of EP. These cells could not be activated by standard nonspecific means, such as phagocytosis of bacteria (8), despite variations of media and times of incubation. Because of this, data derived from the use of alveolar macrophages are not recorded in this paper.

⁸ In experiments with HSA, the washing step was omitted.

⁹ Penicillin-streptomycin solution (5000 units of penicillin and 5000 µg of streptomycin/ml; Grand Island Biological Co.).

Flasks with staphylococci were initially shaken (60–70 times/min) for 3 hr to promote phagocytosis before being placed in a stationary incubator overnight.

Recipients.—Normal rabbits were used as recipients of supernates of all tissues and cells incubated with antigens. Because such animals become immunized and eventually respond with fever to repeated intravenous inoculation of antigen, they were not used for more than 7–10 days. Thereafter, they were given only control supernates derived from suspensions without specific antigens.

Skin Tests.—Donor animals were skin-tested at four abdominal sites with the 50 μ g in 0.1 ml of the following agents: BGG (or BSA in animals sensitized to this antigen), DNP-BGG (DNP-BSA, DNP-RSA (rabbit serum albumin), and RSA. Tests were read at 6, 24, and 48 hr and areas of both erythema and induration were recorded. At 7 days and thereafter, characteristic gross lesions of delayed hypersensitivity (erythema without induration) were regularly present at 24 and 48 hr after testing with carrier antigen and conjugate. At 10 days and thereafter, Arthus reactions to conjugate (positive at 6 hr) were evident but did not develop to carrier protein. Skin reactions to either DNP-RSA or RSA did not exceed 3 mm in diameter

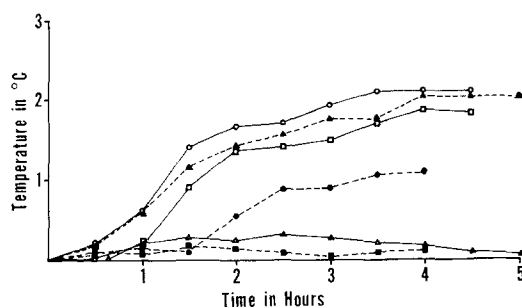


FIG. 1. Febrile responses of normal (N) and DNP-BGG sensitized (HS) rabbits to various dosages of bovine gamma globulin (BGG) and dinitrophenol-bovine gamma globulin (DNP-BGG). Each curve represents an average of two recipients challenged between days 14 and 17 after sensitization (HS rabbits). \triangle — \triangle , N-BGG 550 μ g; \blacktriangle — \blacktriangle , HS-BGG 550 μ g; \bullet — \bullet , HS-BGG 110 μ g; \blacksquare — \blacksquare , N-DNP-BGG 3.16 mg; \circ — \circ , HS-DNP-BGG 474 μ g; \square — \square , HS-DNP-BGG 158 μ g.

RESULTS

Role of Antibody in the Production of Fever.—

Fevers induced by intravenous injection of DNP-BGG and BGG in sensitized rabbits: Fig. 1 shows the responses of sensitized and normal rabbits to intravenous injection of two dosages of DNP-BGG and BGG. The sensitized rabbits were inoculated with DNP-BGG in CFA 14–17 days before testing. They all showed delayed skin reactions of 12–18 mm in diameter to both immunizing and carrier antigens. Note in Fig. 1 that the onset of fever was delayed for 45–60 min, a latency characteristic of febrile responses to microbial antigens given intravenously, such as tuberculin (9) and staphylococcal filtrate (10), as well as to other heterologous proteins such as BSA (11) and HSA (12) in specifically sensitized recipients. The lack of fevers in normal rabbits given the same dosages indicates the specificity of the febrile response, as well as the absence of detectable contaminating bacterial endotoxins in these preparations.

Induction of fever by passive transfer of immune sera to normal rabbits subsequently given antigen: To ascertain the possible role of serum antibody in mediating the previously demonstrated febrile responses to carrier antigen (BGG) and the conjugate (DNP-BGG), sera of donors, immunized with DNP-BGG or DNP-BSA in CFA, were passively transferred to normal rabbits by intravenous infusion. These sera contained circulating antibodies as shown in Table I. 1 or 2 hr later, the recipients were injected with either conjugate or carrier antigen and temperatures were recorded. The results are given in Table I. In only one instance was a significant fever produced. After infusion of 22 ml of plasma into each of two normal recipients, DNP-BGG produced a fever of 1°C with a characteristically delayed onset. BGG was nonpyrogenic in the other

TABLE I
Attempted Passive Transfer of Febrile Reactivity to Specific Antigen with Plasma of Sensitized Donor Rabbits

Donor	No.	Skin test	Day \bar{p} HS	Ab/ml	Vol	Agent	Amt.	No. R	Response
				(μ g)	(ml/R)		(mg/R)		(°C)*
HS	1	Arthus	15	N.D.	22	BGG	5	1	0.0
HS		DH		N.D.		DNP-BGG			
N		—	—	0	25	DNP-BGG	3.4	1	0.0
HS	2	DH	7/8	0/0	40	BGG	5	2	0.15
HS	2	Arthus	15/22	53/86	20-27	BGG	5	3	0.1
		DH							
HS	1	Arthus	25	257	40	BGG	5	1	0.4
HS	2	DH	7/7	0/0	40	BSA	6	2	0.1
HS	2	Arthus	10/13	40/132	40	BSA	6	2	0.1
		DH							

* Change in temperature (or average, where more than one recipient).

DH = delayed hypersensitivity; HS = sensitized (DNP-BGG or DNP-BSA); N = normal; N.D. = not done (Ouchterlony positive); R = recipient.

normal recipient, although there were precipitating antibodies by Ouchterlony analysis to both agents. Further trials with plasma of donors immunized with DNP-BGG 7-25 days before, with and without antibodies to carrier protein, were negative except for a single marginal response (0.4°C). There were also four negative experiments in normal recipients given BSA after transfer of plasma from donors immunized with DNP-BSA.

These data showed that passive transfer of immune sera and injection of carrier antigen into the recipient were ineffective in inducing fever, despite the large volumes used (up to 40 ml) and the presence, in certain instances, of small amounts of precipitating antibody to the carrier antigen. As a possible explanation of the difference between the negative results in these experiments and those reported previously with passive transfer (11, 12), it should be noted that both the route and interval after sensitization were chosen to promote

delayed hypersensitivity rather than antibody formation and that the serum antibody levels were therefore quite low.

Studies in vitro of blood leukocytes from normal and sensitized rabbits with antigen-antibody (Ag Ab) complexes: In vivo, Ag Ab complexes activate cells to produce circulating EP in certain hypersensitivity states and are pyrogenic when injected intravenously into unsensitized rabbits (12). In vitro experiments were carried out to mobilize EP from normal and sensitized blood cells by Ag Ab complexes.

Ag Ab complexes were prepared as particulate and soluble complexes (see

TABLE II
Production In Vitro of EP from Rabbit Blood Cells Incubated with Ag Ab Complexes (DNP-BGG-Anti-DNP-BGG)

Form	Ag Ab Complex (protein R)		No. R	Mean temp. increase† (°C)
	Ag/Ab*	Total protein*		
Ppt.	33/109 µg	142 µg	6	0.15
Ppt.	83/274 µg	357 µg	4	0.18
Ppt.	120/396 µg	516 µg	5	0.06
Ppt.	300/990 µg	1.29 mg	4	0.13
Sol.	3 mg/100 µg (30:1)	3.10 mg	4	0.35
Sol.	4 mg/50 µg (80:1)	4.05 mg	4	0.26
Staph§			2	1.15
Blood cells (control)			11	0.10

* Calculated from quantitative precipitin reaction (7).

† In this and following tables, a mean fever of 0.3°C above control values is regarded as significant.

§ Incubated at a ratio of 30 bacteria to 1 leukocyte.

Ppt. = particulate complex; R = recipient; Sol. = soluble complex.

Table II). They were mixed with normal blood cells and incubated at 37°C overnight. The next morning, after centrifugation, the supernates were removed and infused into normal rabbits to measure fever production. The supernates were 9-17 ml volumes derived from 1.1 to 1.9 × 10⁸ leukocytes suspended in media or whole blood. This number of white cells is sufficient to release EP with known activators (5). Table II shows that normal blood cells were not activated to produce detectable EP.

Three donor rabbits, immunized with DNP-BGG in CFA 6, 15, and 22 days before, were bled for plasma and blood leukocytes. In one, no antibodies were detected by double diffusion in agar (see Table III). In the other two, antibodies to carrier protein were 53 and 86 µg/ml, respectively. To these latter two, 5 mg dosages of BGG were added to 5 ml of plasma to form Ag Ab soluble

complexes in antigen excess, which were incubated at 37°C overnight and then added to aliquots of $1-2 \times 10^8$ normal blood leukocytes for a second overnight incubation. 5 mg dosages of BGG were also incubated with aliquots of $1-2 \times 10^8$ blood leukocytes (resuspended in Eagle's MEM) from the immunized donors. The same procedures were carried out with BGG and both plasma and blood leukocytes from the rabbit without detectable antibody. The next morning, supernates from the blood cells of both the normal and immunized donors were removed and infused into rabbits to measure production of EP.

Table III shows that soluble Ag Ab complexes of BGG anti-BGG did not release EP from normal blood leukocytes, whereas BGG alone did so from blood

TABLE III
Production In Vitro of EP from Blood Cells of Sensitized and Normal Donor Rabbits, Incubated with Antigen (BGG) and Sensitized Plasma

Donor	Time after Sensitization	Ab/ml	Ag/Ab (per R)	Mean temp. increase	
				NB+ HSP + BGG	HSB + BGG
	<i>day</i>			(°C)*	
1	6	0	5 mg/0	0.17	0.52
2	15	53 μg	5 mg/265 μg > 60 times equivalence	0.10	0.85
3	22	86 μg	5 mg/430 μg < 40 times equivalence	0.25	0.65
			Av. (6 R)	0.17	0.68
			HSB-C Av. (6 R)		0.20

* Each number is average of two recipients.

C = control (without Ag); HSB = cells from sensitized donors; HSP = plasma from sensitized donors; NB = normal blood cells; R = recipient.

See text for details.

leukocytes of sensitized donors. Carrier antigen, in the absence of detectable antibody, also released EP from blood leukocytes of a sensitized rabbit, but not from normal blood leukocytes. The results also reveal the lack of pyrogenic factors in the carrier antigen.

Role of Lymphocytes.—

Production in vitro of EP by various tissues incubated with BGG or DNP-BGG: The tissue source of EP was investigated by incubating DNP-BGG or BGG with cell suspensions of blood, spleen, liver, DLN, and MLN. These tissues were removed from rabbits that were inoculated with 10 mg of DNP-BGG in adjuvant 10–20 days before and showed delayed skin test or Arthus reactions, or both, to DNP-BGG and BGG (see skin tests in Materials and Methods). $1-2 \times 10^8$ leukocytes or 1 g of liver suspension were incubated overnight in a volume of 10–12 ml of medium with 3–5 mg of DNP-BGG or BGG. The super-

nates were removed by centrifugation and injected intravenously into unsensitized rabbits and assayed for presence of EP.

The mean febrile response of groups of rabbits to the supernatants of both sensitized and normal tissues incubated with various agents in vitro is shown in Table IV and Fig. 2. Blood leukocytes, spleen, and DLN from sensitized donors were capable of generating EP in vitro when incubated with either BGG or DNP-BGG. Cells from the same sources of normal rabbits did not produce EP when incubated with antigen. The specificity of the response can be seen by

TABLE IV
Production In Vitro of EP by Various Tissues of DNP-BGG-Sensitized (HS) and Normal (N) Rabbits Incubated with Antigens and other Activating Agents

		BGG	DNP-BGG	BSA	OT*	Staph	Control†
		°C‡	°C	°C	°C	°C	°C
Blood cells from:	HS	<i>0.45</i>	<i>0.31</i>	0.05	<i>0.77</i>	<i>1.15</i>	0.07
	N	0.12	0.05	0.06	<i>0.35</i>		0.05
Spleen cells from:	HS	<i>0.52</i>	<i>0.63</i>	0.17	<i>0.63</i>	<i>0.98</i>	0.24
	N	0.15	0.05	0.11			0.14
DLN cells at:	10-14¶	<i>0.57</i>	<i>0.64</i>	0.30		<i>0.77</i>	0.28
	15-35¶	0.12	0.13		0.11	0.12	0.11
MLN cells from:	HS	0.06			0.00	0.25	
	N	0.11		0.11			
Liver	HS	0.11	0.13	0.05	<i>0.43</i>	<i>0.78</i>	0.14

* Test rabbits immunized with complete Freund's adjuvant.

† No antigen or other activating agents used.

‡ Increase in temperatures are averages of four to eight recipient rabbits, except as noted and for "Blood" where averages are of 10 to 15 recipient rabbits.

|| Two recipients only.

¶ Days after sensitization.

In this and following tables, italicized responses are considered significant (see Table II).

lack of EP release from the sensitized tissues when incubated with a heterologous antigen, BSA. The MLN and liver from sensitized donors, on the other hand, were inactive after incubation with specific antigen. Since the liver released EP when incubated with an indifferent activator, heat-killed staphylococci, the difference in this case was not due to a lack of pyrogen-producing cells.

In the initial experiment tissues were used from donor rabbits 10-20 days after sensitization, as there appeared to be no precipitating antibodies to the carrier protein BGG during this interval. However, when larger wells were used for Ouchterlony analysis, precipitating antibodies were found to be present to BGG as well as to DNP-BGG at this stage.

Because of this finding, further experiments with the same tissues were carried out at both earlier and later periods after sensitization of the donor rabbits. The production of EP by these tissues incubated with BGG (and, in analogous experiments, with BSA in rabbits specifically sensitized to this antigen), are shown in Table V, which incorporates the results of the earlier experiments for the intermediate period of 10–20 days after sensitization.

From this table, it is apparent that no tissues were activated to produce EP at 1 wk after sensitization, when there was delayed hypersensitivity only to BGG by skin test and no detectable antibody by Ouchterlony analysis, and that after 3 wk, when circulating antibody was present, there was significantly less EP production by blood, spleen, and DLN which had been previously shown to be active from 10 to 20 days.

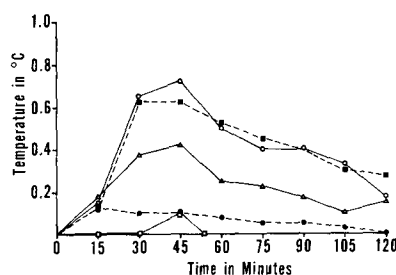


FIG. 2. Febrile responses of normal rabbits to endogenous pyrogen released *in vitro* by sensitized tissues (DNP-BGG-immunized donors) incubated with 5 mg dosages of BGG *in vitro*. See text for details. Each curve represents an average of two recipients. ■-■, blood; ●-●, liver; △-△, spleen; ○-○, DLN; □-□, MLN.

Production in vitro of EP from normal blood cells by interaction of antigen and sensitized lymphoid cells: Tissues that produced EP *in vitro* in the presence of antigen contain both lymphocytes and pyrogen-producing cells (granulocytes or monocytes). MLN and liver, on the other hand, are composed, respectively, almost entirely of lymphocytes or of a mixture of pyrogen-producing Kupffer cells and inactive hepatocytes (13). To test the hypothesis that the production of EP by blood, spleen, and DLN was dependent upon the presence of sensitized lymphocytes capable of activating pyrogen-producing cells, BGG was incubated overnight with a mixture of normal blood cells and MLN lymphocytes from either normal donors or donors sensitized 14–20 days previously. Appropriate controls were prepared consisting of blood cells incubated with lymphocytes alone, as well as both blood and lymphocytes incubated with and without antigen. After incubation, the cells were centrifuged and the supernatant fluids compared for release of EP by intravenous injection into normal rabbits. The results are shown in Table VI. Incubation of the carrier antigen BGG with a mixture of sensitized MLN lymphocytes and normal blood cells resulted in

significant fever, average 0.81°C , as compared with an average fever of only 0.29°C when lymphocytes of normal MLN were substituted for those from a sensitized donor. Since BGG incubated with either normal blood cells or sensitized MLN lymphocytes alone did not release detectable amounts of EP, the results suggested that specific antigen caused MLN cells to release a nonpyrogenic intermediate agent that activated normal blood cells.

Supernates of BGG and DLN cells from donors 1 wk after sensitization did not produce fever in recipient rabbits.¹⁰ This tissue might therefore supply a

TABLE V
*Production In Vitro of EP by Tissues of Sensitized Rabbits, Incubated with Antigen**

Cell source†	Days after sensitization of donor		
	6-8§	10-20	21-35¶
Blood	0/5**	7/12	2/6
Spleen	0/5	3/7	1/5
Liver	0/3	1/11	0/6
DLN	0/5	3/6‡‡	0/10‡‡
MLN	0/3	0/10	0/6

* Rabbits immunized with DNP-BGG or DNP-BSA in CFA.

† Cell suspensions were incubated with antigen overnight at 37°C , and supernatants were removed and injected into test rabbits.

§ Positive delayed skin tests to carrier antigen; no antibody detected.

|| Positive immediate and delayed skin tests to carrier antigen; antibody in circulation of all donors.

¶ Positive immediate skin tests to carrier antigen; antibody in circulation of all donors.

** Number positive/total number. Each number represents a single experiment in which the average of two responses both with and without antigen are compared.

‡‡ Numbers are for 10-14 and 15-35 days, respectively.

Positive = average increase in febrile response of 0.3°C or more to supernates from tissues incubated with antigen as compared with tissues incubated alone. BGG was used for the two right columns; BGG or BSA for 6-8 day column.

source of activated lymphocytes at this time without associated pyrogen-producing cells that would obscure the release of EP from normal blood cells by sensitized lymphocytes and antigens.

Accordingly, suspensions of lymphocytes from both DLN and MLN of donor rabbits sensitized 7 or 8 days previously either with DNP-BGG or DNP-BSA were added with and without specific carrier antigen to normal blood cells and incubated overnight, as previously. The results are shown in Table VII. There was significant release of EP from the samples containing DLN, whereas those

¹⁰ Supernates derived from incubation of lymphocytes of sensitized donors and antigen are incapable of eliciting fever in recipient rabbits within 2 hr of infusion. Such supernates were capable of releasing EP from normal blood leukocytes in vitro after 18 or more hr of incubation.

with MLN from the same sensitized donors were inactive at this early period. EP was released from normal blood cells by DLN incubated with or without antigen. Since antigen was presumed to be present in the DLN at this time (14), it was inferred that the marked EP release in the control preparations was the result of activation of the lymphocytes by antigen (or other sensitizing agents such as Freund's adjuvant) absorbed from the site of inoculation.

These results demonstrating the difference in the capacity of DLN and MLN (1 wk after immunization) to activate normal blood cells were confirmed with another antigen, HSA, in a larger series of experiments that included donor rabbits from the 3rd to 7th day after sensitization to this antigen. As evident in

TABLE VI
*Production In Vitro of EP by Normal Blood Cells Incubated with MLN of Sensitized or Normal Donors, with and without Antigen**

No. of trials	Normal blood cells	Sensitized MLN cells	Normal MLN cells	Ag	Mean temp. increase (°C)
9	+‡	+		BGG	0.81
9	+	+		None	0.45
8	+		+	BGG	0.29
8	+		+	None	0.18
12	+			BGG	0.12
10		+		BGG	0.06
9			+	BGG	0.11

* MLN removed 14–20 days after immunization.

‡ In this and following tables, each + indicates presence of specified cells in the incubation mixture.

Table VIII, nearly all samples of normal blood incubated with sensitized DLN and antigen evoked release of EP, whereas only one-half those incubated with MLN plus antigen were positive. In addition, in this larger series, there was a significant increase in pyrogen release from the samples of DLN incubated with antigen as compared with controls incubated with sensitized DLN without antigen.

Production in vitro of EP from normal blood cells by interaction of viable lymphocytes and antigen: A series of experiments was devised to determine the role of viability of lymphocytes in activating normal blood cells to release EP. It was essential to exclude the possibility that nonspecific activators, e.g. Freund's adjuvant or Gram-negative bacterial endotoxin absorbed from the site of inoculation, might induce EP release.

DLN were removed from rabbits on either the 3rd or 5th day after sensitization with HSA and one-half of the cells from each node were freeze-thawed

TABLE VII

*Production In Vitro of EP by Normal Blood Cells Incubated with Specifically Sensitized Cells of DLN or MLN with and without Antigen**

Normal blood cells	DLN	MLN	Ag	Mean temp. increase (°C)
+‡	+		BGG	0.90§
+	+		None	0.80
+	+		BSA	0.64
+	+		None	0.63
+		+	BGG	0.28
+		+	None	0.23
+		+	BSA	0.36
+		+	None	0.26
+			BGG/BSA	0.19×
	+		BGG	0.21
	+		BSA	0.16
	+		None	0.16×

* DLN and MLN removed 7 or 8 days after immunization.

‡ Each + indicates presence of specified cells in incubation mixture.

§ Each number represents the mean of temperature increase in four rabbits except those marked × where the numbers are means of eight rabbits.

TABLE VIII

Production In Vitro of EP by Normal Blood Leukocytes Incubated with Sensitized Lymphocytes from DLN or MLN with and without Antigen (+HSA/C)

Day (p HS)	Normal blood cells +			
	DLN		MLN	
	+ HSA	+ HSA > Control	+ HSA	+ HSA > Control
3	4/6*	2/6‡	—	—
5	4/5	2/5	1/5	0/5
6	5/5	4/5	3/5	2/5
7	5/5	3/5	3/5	3/5
Total (5-7 days)	14/15	9/15	7/15	5/15
	Normal blood cells +			
DLN + HSA	>	MLN + HSA	9/15	
DLN + HSA	>	DLN Control	9/15 (P = <0.02)	
MLN + HSA	>	MLN Control	5/15	

* Number positive/total number. Each number represents a single experiment in which two responses are averaged. Positive: average febrile response (tissues + HSA) of 0.5 + °C.

‡ Positive: average increase in febrile response (tissues + HSA vs. control) of 0.3 + °C.
HS = sensitization of donor rabbit.

three times before addition to a sample of normal blood. The other half of the cells were mixed with a similar sample of blood from the same donor. Antigen was added in 5 mg doses to one-half of each sample (containing intact or disrupted lymphocytes) at the time when these cells were incubated with normal blood cells. The remaining mixtures received no antigen.

The results in Table IX are expressed, as before, as the number of positive over total number of responses (each number determined from the average fever of two recipients in a single experiment). The data clearly show that at this interval after sensitization, intact lymphocytes are essential to release EP

TABLE IX
Production In Vitro of EP by Normal Blood Leukocytes Incubated with Viable or Disrupted DLN Cells and with or without Antigen (+ HSA/Control)*

	Normal blood cells +			
	Viable DLN		Disrupted DLN	
	+ HSA 7/10‡	Control 4/10	+ HSA 1/10	Control 2/9
Both neg.		3/10		6/9
Both pos.		4/10		0/9
HSA > control		4/10		1/9
Control > HSA		1/10		2/9
Total positive§		11/20		3/19
Controls: viable cells (without blood)			Controls: disrupted cells (without blood)	
	(°C)		(°C)	
DLN + HSA	0.16 (8)		DLN + HSA	0.10 (8)
DLN - control	0.21 (7)		DLN - control	0.13 (7)

* DLN cells were removed 3 or 5 days after sensitization.

‡ Number positive/total number (see Table VIII).

§ Total positive (viable vs. disrupted): $P = <0.01$.

Number in parentheses = number of experiments (two recipients each).

from normal blood cells (total positive 11/20 vs. 3/19 for disrupted cells). Since in none of the three positive instances with disrupted cells (one with antigen, two control) were both the antigen and control sample positive in the same experiment, there seems little likelihood that nonspecific activators associated with or ingested by the cells are responsible for the release of EP from the blood cells.

Production in vitro of EP from normal blood cells by supernates of DLN lymphocytes incubated with antigen: To ascertain whether lymphocytes liberate soluble agents to produce EP from blood leukocytes, or whether the presence of viable cells was necessary, suspensions of lymphocytes derived from DLN, both intact and disrupted by freeze-thawing, and from MLN were incubated overnight with and without antigen. The next day the suspensions were centrifuged

and the supernatant fluids were added to centrifuged cell buttons derived from equivalent volumes of a single pooled sample of normal blood. Each sample was then mixed by pipetting and allowed to incubate for 18 hr. At the end of this period all samples were cleared by centrifugation, and the supernatant fluids were assayed for pyrogenicity as previously. The first of these experiments tested supernates from DLN and MLN, incubated with antigen, and then further incubated with normal blood cells. The results are presented in Table X. Supernates of DLN cells and antigen released significant quantities of EP from blood leukocytes, while supernates of MLN cells and antigen released only barely de-

TABLE X
*Production In Vitro of EP by Normal Blood Cells Incubated with Supernates of DLN or MLN Cells, with and without Antigen**

Normal blood cells	Supernates of		Ag	Mean temp. increase (°C)
	DLN cells	MLN cells		
+‡	+		HSA	1.10
+	+		None	0.40
+		+	HSA	0.18
+		+	None	0.10
+			HSA	0.15
+			None	0.10
	+		HSA	0.10
	+		None	0.05
		+	HSA	0.05
		+	None	0.08

* DLN and MLN cells were removed 7 days after immunization and incubated with or without antigen. Supernates were removed 18 hr later and incubated with normal blood cells or infused directly into recipient rabbits. All numbers are averages of two values of a single experiment.

‡ + indicates presence of specified cells or supernates of cells in incubation mixtures.

tectable EP. In other experiments of similar design, supernates of DLN, viable or disrupted, with and without antigen, were further incubated with normal blood cells to release EP. Table XI shows these and other data derived from cells of donors 5-7 days after sensitization. Supernatant fluids of viable sensitized DLN lymphocytes activated normal blood leukocytes to produce significant amounts of EP, whereas those derived from disrupted DLN lymphocytes were uniformly inactive. In addition, supernates of viable DLN lymphocytes incubated with antigen produced slightly higher fevers than did those from cells incubated without antigen. The results with supernates of viable MLN cells were intermediate, the average fever being only half that of the viable DLN, but higher than those from freeze-thawed cells.

The evolution of this capacity of sensitized lymphocytes plus antigen to acti-

vate normal leukocytes is shown in Table XII. This table comprises representative experiments demonstrating pyrogen release from normal blood cells incubated with lymphocytes from DLN and MLN (both viable cells and supernatant fluids of viable cells previously incubated with and without antigen). From these data, taken at various intervals after immunization, it is apparent that lymphocytes of DLN only released EP from normal blood leukocytes in the 1st wk.

TABLE XI
*Production In Vitro of EP by Normal Blood Cells Incubated with Supernates of Viable or Disrupted DLN Cells or Viable MLN Cells**

No. of trials	Normal blood cells	Supernates of			Ag	Mean temp. increase (°C)
		DLN viable	DLN disrupted	MLN viable		
16	+‡	+			HSA	0.62
16	+	+			None	0.49
8	+		+		HSA	0.15
8	+		+		None	0.13
6	+			+	HSA	0.36
6	+			+	None	0.26
16		+			HSA	0.23
16		+			None	0.24
8			+		HSA	0.09
8			+		None	0.12
6				+	HSA	0.08
6				+	None	0.10

* DLN and MLN cells were removed 5-7 days after immunization and were incubated with or without antigen before supernate was removed.

‡ See Table X.

Thereafter, both cells and supernates of previously incubated MLN became almost equally effective in mobilizing EP from unsensitized blood cells.

DISCUSSION

These studies demonstrate that normal blood cells, when incubated with antigen and viable sensitized lymphocytes, release EP in vitro. Since antigen alone, normal lymphocytes, or disrupted, sensitized cells, with or without antigen, were only weakly or rarely effective in this system, the activator appears to be a product of viable, sensitized lymphocytes and not a nonspecific factor (such as

bacterial endotoxin) associated with such cells.¹¹ On occasion normal blood cells release EP in vitro when incubated with viable sensitized lymphocytes without antigen. In most instances, addition of antigen significantly increased the effectiveness of specifically sensitized lymphocytes in activating normal blood leukocytes, thereby providing evidence that the reaction was, to some degree at least a specific one. The ability of such cells alone to activate leukocytes may be due to the presence of antigen in the node, a finding which has been demonstrated by other techniques (14-17). Since these results were regularly obtained

TABLE XII

Mean Febrile Responses (°C) Induced by Supernates of Normal Blood Cells Incubated with either: (a) Sensitized DLN or MLN Cells, with or without Antigen (+ HSA/C); or (b) Supernates of Lymph Node Cells (from the Same Donors) Previously Incubated with or without Antigen (+ HSA/C)

Donor (day p HS)	Normal blood cells (NBC) +			
	DLN		MLN	
	Cells (+ HSA/C)	Supernate	Cells (+ HSA/C)	Supernate
	°C	°C	°C	°C
3	0.05/0.08	—	—	—
5	1.08/0.35	0.03/0.18	0.43/0.35	—
7	0.75/0.88	1.15/0.40	0.23/0.23	0.18/0.10
12	0.73/0.48	0.80/0.35	0.58/0.33	0.55/0.28
22	—	0.38/—	—	0.70/0.48

All figures are average of two recipients in single experiment.

	°C
NBC + HSA (12)	0.10
DLN + HSA (8)	0.15
MLN + HSA (8)	0.07
NBC + NMLN + HSA (4)	0.19

Number in parentheses = number of recipients.

with lymphocytes from DLN as early as 5 days after sensitization, and occasionally at 3 days (5 days before precipitating antibodies were detectable by Ouchterlony and quantitative precipitin techniques) it seems unlikely that activation of the blood leukocytes was due to union of antigen and small amounts of antibody synthesized by the lymphocytes during incubation.

The effective agent in activating normal blood cells to release EP in vitro appears to be soluble, and was clearly detectable in supernatant fluids from viable

¹¹ Addition of small amounts of endotoxin (filtered typhoid vaccine) to cellular suspensions of normal MLN that were subsequently disrupted by freeze-thawing, regularly evoked release of EP from normal blood cells incubated with these mixtures. It seems unlikely, therefore, that disruption of cells would inactivate this agent if it were initially present in DLN.

lymph node lymphocytes previously incubated with antigen only. Similar results were obtained in a supplementary experiment with nonpyrogenic supernates of sensitized thoracic duct lymphocytes obtained 9 days after sensitization and previously incubated with specific antigen (BGG) (unpublished observations). By contrast, supernates of the same cells incubated alone or with BSA failed to activate normal blood cells.

During the 1st wk after sensitization lymphocytes from DLN were generally more effective in activating normal blood cells than were those from MLN. Since neither blood nor spleen, which contain lymphocytes as well as pyrogen-producing cells, were capable of releasing EP when incubated with antigen at this early period, as shown in Table V, it seems probable that sensitized lymphocytes were present in significant numbers only in DLN at this early time. The lack of pyrogen-producing cells in DLN at 6–8 days appears to explain the failure of antigen to release EP also from DLN. Later, at 10–20 days, spleen and blood, as well as DLN themselves, were activatable by antigen (having at this time presumably both sensitized lymphocytes and pyrogen-producing cells). It therefore seems likely that sensitized lymphocytes, or antigen, migrate from the initial site of sensitization in the DLN to invade other tissues throughout the body. In support of this theory, lymphocytes from MLN after the 2nd wk were regularly effective in activating normal blood cells in the presence of antigen.

By contrast, liver (containing few lymphocytes, but many Kupffer cells)¹² or mesenteric lymph nodes alone (containing mostly small lymphocytes) failed to release EP after incubation with antigen *in vitro*, whether obtained early or late after sensitization. On the basis of these data, it seems reasonable to postulate that during states of delayed hypersensitivity the lymphocyte, though incapable of producing EP itself, plays a critical intermediate role in bringing about release of EP from these effector cells.

The soluble agent released by sensitized lymphocytes was itself nonpyrogenic in the doses used to activate cells and appeared to require incubation of antigen with viable cells for its production. These properties suggest that the activator belongs to the group of substances collectively known as "lymphokines," agents produced by lymphocytes that are stimulated by various immunologic and nonimmunologic means and that possess such diverse physiologic activities as cytotoxicity, inhibition of macrophage migration, transformation of normal lymphocytes, and others (3, 4, 18).

On the other hand, despite clear evidence in other experimental fever models that antigen-antibody complexes are pyrogenic *in vivo* and will produce circulating EP (12), we were unable here and previously to activate normal blood leukocytes to generate EP *in vitro* with various combinations of antigen (DNP-BGG or BGG and, in unpublished studies, HSA) and antibodies, both in the

¹² The hepatocyte does not appear to produce EP (13).

zone of antigen excess and with precipitates of antigen and antibody. Such complexes presumably activate appropriate tissues *in vivo*, perhaps by phagocytosis or by release of mediators from injured tissues.

If the lymphokine nature of this agent is confirmed by more direct techniques, these studies will have provided the first evidence for a truly endogenous activator of leukocytes for pyrogen production,¹³ as distinguished from such exogenous agents as antigen-antibody complexes of heterologous serum proteins and activators of microbial origin. Although an activator of exudate leukocytes has been described from peritoneal fluids of rabbits infused with glycogen, the possibility that the effects were due, at least in part, to small amounts of contaminating Gram-negative bacterial endotoxin could not be eliminated (20).

For the reasons already stated, it seems unlikely that such nonspecific exogenous activators are responsible for the findings presented here. Furthermore, since fever is so regularly associated with inflammation due to causes other than infection, such as infarctions, tumors, acute gouty arthritis, and sickle cell crises, it is reasonable to believe that there must be other endogenous activators of appropriate cells for pyrogen production to account for the fevers appearing clinically in such situations.

SUMMARY

Experiments have been carried out to investigate the possible role of the sensitized lymphocyte in mediating the fevers of delayed hypersensitivity. Rabbits were made delayed hypersensitive to one of several heterologous proteins (bovine gamma globulin, bovine serum albumin, or human serum albumin) by footpad injection of antigen or antigen conjugated with dinitrophenol and incorporated in complete Freund's adjuvant. At intervals after sensitization, various tissues were removed, and single cell suspensions were incubated overnight with either carrier protein or conjugate *in vitro*. Release of an endogenous pyrogen (EP) was assayed by intravenous injection of the supernatant fluid into unsensitized rabbits. Of the tissues tested only those containing both lymphocytes and pyrogen-producing cells, blood, spleen, and draining lymph nodes, released detectable amounts of EP when incubated with antigen *in vitro*. Incubation of normal blood cells with specifically sensitized lymphocytes and antigen also resulted in significant release of pyrogen. Similarly, blood leukocytes released EP *in vitro* after mixture with supernates derived from incubation of sensitized lymphocytes and antigen. Cells and supernatant fluids from draining lymph nodes were usually effective in activating normal blood leukocytes earlier after sensitization than were those from mesenteric lymph nodes, suggesting that such cells, or antigen, had migrated from the original site of sensitization.

The activator was soluble, nonpyrogenic in the dosages tested, and required

¹³ Human (but not animal) leukocytes can be activated by certain endogenous pyrogenic steroids *in vitro* (19).

incubation of viable cells with specific antigen for its production. These properties suggest that it may belong to the class of "lymphokines," biologically active agents released from lymphocytes that have been activated by immunologic or certain nonimmunologic stimuli.

The authors would like to thank Dr. Phyllis Bodel for helpful editorial criticism and assistance with the statistical evaluations.

REFERENCES

1. Uhr, J. W., and M. W. Brandriss. 1958. Delayed hypersensitivity. IV. Systemic reactivity of guinea pigs sensitized to protein antigens. *J. Exp. Med.* **108**:905.
2. Uhr, J. W., and M. Scharff. 1960. Delayed hypersensitivity. V. The effect of X-irradiation on the development of delayed hypersensitivity and antibody formation. *J. Exp. Med.* **112**:65.
3. Dumonde, D. C., R. A. Wolstencroft, G. S. Panayi, M. Matthew, J. Morley, and W. T. Howson. 1969. "Lymphokines": non-antibody mediators of cellular immunity generated by lymphocyte activation. *Nature (London)*. **224**:38.
4. Lawrence, H. S., and M. Landy, editors. 1969. *Mediators of Cellular Immunity*. Academic Press, Inc., New York.
5. Atkins, E., and C. Heijn, Jr. 1965. Studies on tuberculin fever. III. Mechanisms involved in the release of endogenous pyrogen in vitro. *J. Exp. Med.* **122**:207.
6. Eisen, H. N., M. Kern, W. T. Newton, and E. J. Helmreich. 1959. A study of the distribution of 2,4-dinitrobenzene sensitizers between isolated lymph node cells and extracellular medium in relation to induction of contact skin sensitivity. *J. Exp. Med.* **110**:187.
7. Talmage, D. W., and P. H. Maurer. 1953. I¹³¹-labelled antigen precipitation as a measure of quantity and quality of antibody. *J. Infec. Dis.* **92**:288.
8. Atkins, E., P. Bodel, and L. Francis. 1967. Release of an endogenous pyrogen in vitro from rabbit mononuclear cells. *J. Exp. Med.* **126**:357.
9. Hall, C. H., Jr., and E. Atkins. 1959. Studies on tuberculin fever. I. The mechanism of fever in tuberculin hypersensitivity. *J. Exp. Med.* **109**:339.
10. Atkins, E. 1963. Studies in staphylococcal fever. II. Responses to culture filtrates. *Yale J. Biol. Med.* **35**:472.
11. Grey, H. M., W. Briggs, and R. S. Farr. 1961. The passive transfer of sensitivity to antigen-induced fever. *J. Clin. Invest.* **40**:703.
12. Root, R. K., and S. M. Wolff. 1968. Pathogenetic mechanisms in experimental immune fever. *J. Exp. Med.* **128**:309.
13. Dinarello, C. A., P. T. Bodel, and E. Atkins. 1968. The role of the liver in the production of fever and in pyrogenic tolerance. *Trans. Ass. Amer. Physicians Philadelphia*. **81**:334.
14. Harris, T. N., and W. E. Ehrich. 1946. The fate of injected particulate antigens in relation to the formation of antibodies. *J. Exp. Med.* **84**:157.
15. Cohen, S., P. Vassalli, B. Benacerraf, and R. T. McCluskey. 1966. The distribution of antigenic and nonantigenic compounds with draining lymph nodes. *Lab. Invest.* **15**:1143.
16. Lind, P. E. 1968. Cellular localization of ¹²⁵I-labelled *Salmonella adelaide* flagellin

- following injection into the rat in the presence of Freund's complete adjuvant. *Aust. J. Exp. Biol. Med. Sci.* **46**:189.
17. Han, S. S., I. H. Han, and A. G. Johnson. 1970. The fate of isologous, homologous and heterologous ferritin molecules in the rat. *Amer. J. Anat.* **129**:141.
 18. Bloom, B. R. 1970. Effector mechanisms activated by cellular recognition. *In* Immune Surveillance. R. T. Smith and M. Landy, editors. Academic Press, Inc., New York. 161.
 19. Bodel, P., and M. Dillard. 1968. Studies on steroid fever. I. Production of leukocyte pyrogen *in vitro* by etiocholanolone. *J. Clin. Invest.* **47**:107.
 20. Moore, D. M., S. F. Cheuk, J. D. Morton, R. D. Berlin, and W. B. Wood, Jr. 1970. Studies on the pathogenesis of fever. XVIII. Activation of leukocytes for pyrogen production. *J. Exp. Med.* **131**:179.