STUDIES ON TUBERCULIN FEVER*

III. Mechanisms Involved in the Release of Endogenous Pyrogen in Vitro

By ELISHA ATKINS,‡ M.D., and CORNELIS HEIJN, Jr.,§ M.D.

(From the Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut)

(Received for publication, March 26, 1965)

Although fever is a well known and common manifestation of hypersensitivity, little has been learned about the sequence of events by which antigen disturbs temperature regulation in sensitized individuals. Using tuberculin-induced fever in rabbits infected with BCG as a model, we have shown in previous papers that an endogenous pyrogen (EP) appears in the circulation of such animals during the febrile response (1, 2). When injected intravenously this agent causes immediate fevers in normal rabbits, and is, therefore, clearly distinct from tuberculin itself which produces fever after a latency of 45 to 60 minutes and is pyrogenic only in animals previously sensitized by BCG. The tissue source of EP in this form of hypersensitivity fever has remained uncertain. In several systems of hypersensitivity, including tuberculin, Johanovský has reported that when antigen is added *in vitro* to mononuclear cells from spleen or lymph nodes of specifically sensitized animals, a pyrogen is formed which he has called 'hypersensitivity pyrogen' to distinguish it from serum endogenous pyrogen present in other experimental fevers (3–8).

In the following experiments, tuberculin was added *in vitro* to various tissues of BCG-sensitized rabbits in an attempt to repeat Johanovský's findings and to determine the origin of the pyrogen which appears in the serum in tuberculin-induced fever. Johanovský's observations were not confirmed, and evidence is presented instead that sensitized blood cells release EP *in vitro* when incubated with tuberculin and hence may be a source of the circulating pyrogen in this form of hypersensitivity fever.

In additional experiments, it was found that normal blood cells, briefly incubated in plasma of sensitized animals, were similarly activated *in vitro* by tuberculin, an observation which suggests that humoral antibodies play a role in the genesis of tuberculin-induced fever. On the other hand, "transfer fac-

* Supported by a grant (E-1564) from the United States Public Health Service.

‡ Associate Professor of Medicine.

§ Present address: Department of Psychiatry, Pratt Diagnostic Clinic, Boston.

tors", presumably released when antigen is incubated with lymphocytes of hosts with delayed hypersensitivity, appeared to be ineffective in sensitizing blood cells to react with tuberculin *in vitro*.

Materials and Methods

General.—Glassware, needles, instruments, and other equipment used to process tissues were sterilized by dry heat at 170°C for 2 hours to inactivate pyrogens. Both physiologic saline (made from doubly distilled water) and commercial pyrogen-free saline[1] were used. Hanks' solution and phosphate-buffered saline (pH 7.2) were also prepared with doubly distilled water. All solutions were tested at intervals for pyrogenicity by injection of 20 ml in normal rabbits.

Male and female albino rabbits weighing 3 to 5.5 kg were used both as donors and to assay pyrogenicity. They were housed in single cages in an air-conditioned room. Temperatures were recorded in an adjacent room maintained at 68 to 72°F.

Temperature Recording.—Rectal temperatures were measured with Foxboro rabbit scanning equipment (1, 2). Rabbits were restrained either in stalls or in metal boxes with openings for the head and tail. Only trained animals which had been previously boxed for one or more 5-hour periods, or animals which had been used in other experiments and found reliable, were used. Rabbits with initial temperatures over 40.5°C or whose temperature varied more than 0.3°C during the hour before injection were not used. Temperatures were recorded every 15 minutes for 5 hours after injection or until they had returned to the baseline and remained at that level for 1 hour. Some animals which rapidly regained normal temperature after a brief monophasic fever were injected again on the same day with a different solution after their temperatures had been stable for at least 1 hour. In view of the normal variation of recipients to any one pyrogenic dose, individual recipients were occasionally given both experimental and control solutions at different times, either on the same or successive days. In such instances, the order of injections was reversed in an equal number of recipients to offset the possible effects of the first injection on the second.

In several experiments it was necessary to study responses of unrestrained recipients. These animals were left free in their cages except for the brief intervals necessary to make injections and record temperatures, when they were placed in boxes. They were removed from their cages every 15 minutes for an average duration of 3 minutes. Rectal mercury thermometers were used to record temperatures.

Techniques for plotting temperature responses and fever indices of injected rabbits have been presented earlier (1, 2).

Sensitization of Rabbits.—The strain of BCG used, techniques for its culture and harvesting, and methods of inoculating donor rabbits were identical, except where indicated, to those already presented (1, 2). Sensitization to products of this bacterial strain was achieved by intravenous injection of 2 mg BCG in fine suspension in saline. Recipients infected by this technique regularly develop marked cutaneous reactions of delayed hypersensitivity (2). In earlier experiments, in which attempts were made to duplicate Johanovský's techniques (see section 1 of Results) donor rabbits were sensitized with larger doses (3 to 10 mg) BCG intravenously. Donor rabbits were routinely tested for sensitivity by intravenous inoculation of 100 mg old tuberculin 2 to 6 weeks after infection. Only rabbits manifesting fevers of > 1.5°C. were used as donors, generally within 1 week after having been tested with tuberculin. Occasionally, animals that had been resensitized were used as donors. Since results with their blood cells and sera appeared identical with those of the majority of animals, which were sensitized only once, they have been combined without specific mention, except in section 8 of Results.

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[1] Baxter Laboratories, Morton Grove, Illinois.

Old Tuberculin.—Old tuberculin (OT) obtained from the Massachusetts Department of Public Health (Lots 50 and 50C) was used both to determine hypersensitivity of animals and in the *in vitro* experiments. This material contained 2000 mg OT per ml and was diluted (1:20) in saline for intravenous injection, or added (either in same dilution or undiluted) to suspensions of cells. Sensitivity of rabbits was determined by measuring the febrile response to injection of 100 mg OT (in 1 ml saline). Five times the testing dose of OT was nonpyrogenic in normal recipients (see Table I).

Spleen and Lymph Nodes.—Animals were exsanguinated by cardiac puncture. The abdomen was shaved, washed with acetone and opened with forceps and scissors. The spleen was grasped with forceps and separated from its attachments. It was divided several times and then

TABLE I

Pyrogenicity of Supernates of Spleen Cell Preparations from BCG-Sensitized Rabbits Incubated 18 Hours with Tuberculin in Vitro

Materials	Incubation	Recipients		Mean response
		No.	Condition	
	°C			°C*
Cells + serum/plasma + OT	37	31	Restrained	0.15
Cells + serum/plasma	37	9	"	0.10
Washed cells + HS‡ + OT	37	3	Unrestrained	0.10
Washed cells + HS + OT	37	3	Restrained	0.10
Cell extracts + HS + OT	37	3	Unrestrained	0.10
Cell extracts + HS + OT	37	1	Restrained	0
Cell extracts + HS + OT	4	3	Unrestrained	0
Cell extracts + HS + OT	4	1	Restrained	0
OT (500 mg) in serum/saline	—	12	"	<0.10

* Maximal temperature elevation within 1 hour.

‡ Hanks' solution.

pressed with a spatula through a No. 40 metal screen into a small glass funnel and washed down into a 50 ml centrifuge tube with serum, Hanks' solution, or phosphate-buffered saline. The suspension was drawn several times into a pipette or a syringe with a No. 17 needle, and a small amount was taken for culture and counting. Most cells were individually suspended after processing, with an occasional clump of two to four cells. The average yield of cells from a spleen varied from 9.6×10^8 cells (experiments in section 1 of Results) to 2.5×10^9 in later experiments.

Lymph nodes were dissected from the root of the mesentery and processed in the same manner with an average of 2.3×10^9 cells per donor.

In other experiments (see Figs. 1 and 2, and sections 9 and 10 of Results) suspensions of both spleen and lymph node cells were washed once in saline, resuspended in fresh saline and incubated at 37°C with tuberculin.[2] Recipients were generally injected with supernates of this mixture, obtained after centrifugation (2000 RPM) for 30 minutes. In some experiments the final suspension itself was injected. As materials from both spleen and lymph nodes occasionally caused immediate death on inoculation, heparin (500 U.S.P. units in 0.5 ml) was routinely given (as a separate injection or mixed with the material).

[2] All procedures after removal of tissues and before incubation with tuberculin were carried out in an ice water bath or at 4°C (during centrifugation).

Collection and Processing of Blood.—Donor rabbits were lightly anesthetized with intravenous pentobarbital sodium[3] (78 to 90 mg contained in 1.3 to 1.5 ml) and then given 2000 U.S.P. units heparin[4] (in 2 ml) intravenously. The chest (and abdomen, in cases where spleen and lymph nodes were obtained) were shaved and donors exsanguinated by cardiac puncture. After death, the chest was opened aseptically and an additional 10 to 20 ml of blood removed from the right side of the heart by an additional puncture. The heparinized blood (in volumes of generally 100 to 150 ml and containing usually 8×10^8 to 1.2×10^9 total leukocytes) was placed in a 250 ml centrifuge bottle and samples obtained for both culture and cell counts.[5] Tuberculin was usually added either to whole blood or to cells that had been centrifuged at 2000 RPM for 30 minutes at 4°C and were resuspended in a volume of saline equivalent or slightly less than the volume of plasma removed. For some experiments, blood cells were washed once in an equal volume of saline before being resuspended in additional saline. After incubation at 37°C for various periods, the blood was generally stored at 4°C overnight, cleared by centrifugation the following morning, and the supernate removed for injection. In a number of instances, second "harvests" were obtained by resuspension of the cells in the same volume of saline as previously used and by reincubation of this mixture for an additional 1½ hours, at the end of which the mixture was again centrifuged and the supernate removed.

Cells were always used immediately after the donor animal was bled. Plasma was stored at 4°C and generally used within several weeks of collection. All stored material was regularly cultured immediately before use and (in the case of plasma) was tested for lack of pyrogenic properties by injection of 5 to 10 ml intravenously and by incubation for 5 hours with an aliquot of normal blood cells.

In some cases where blood was incubated for prolonged periods with tuberculin, antibiotics (penicillin, 5000 units and streptomycin, 10 mg) were added.

Cultures.—One ml aliquots of blood and cell suspensions of other tissues, both before and after incubation, were cultured in thioglycollate broth. After 48 hours incubation at 37°C, the broth was subcultured to blood agar plates and incubated aerobically for another 48 hours before examination. Positive cultures were only rarely obtained and results of experiments with these tissues were discarded.

RESULTS

1. Incubation of Tuberculin in Vitro with Blood, Spleen, and Lymph Nodes of BCG-Sensitized Rabbits.—Fig. 1 shows the results of incubating OT with various tissues from the same sensitized donor rabbits. Cells from each tissue were resuspended in saline, incubated for 4 to 5 hours with OT at 37°C and the supernates subsequently injected into normal recipient rabbits (see Materials and Methods). Brisk fevers that reached a peak at 45 minutes were induced only with supernates from blood cells incubated with OT. By contrast, neither the spleen nor lymph node cells, in dosages 2 to 5 times that of blood leukocytes, released detectable amounts of pyrogen when incubated with OT.

In other experiments, various techniques employed by Johanovský (3–8)

[3] Diabutal™ (60 mg per ml). Diamond Laboratories, Inc., Des Moines, Iowa. Dispensed in 100 ml bottles (for veterinary use only). Lot 762-3576.

[4] Liquaemin® sodium "10" (1 cc, 1000 U.S.P. units). Organon, Inc., West Orange, New Jersey.

[5] Differential counts were not performed, but in studies employing similar techniques (9) granulocytes usually made up 45 to 55 per cent of the total.

for producing so-called hypersensitivity pyrogen were followed as closely as possible according to the following protocols.

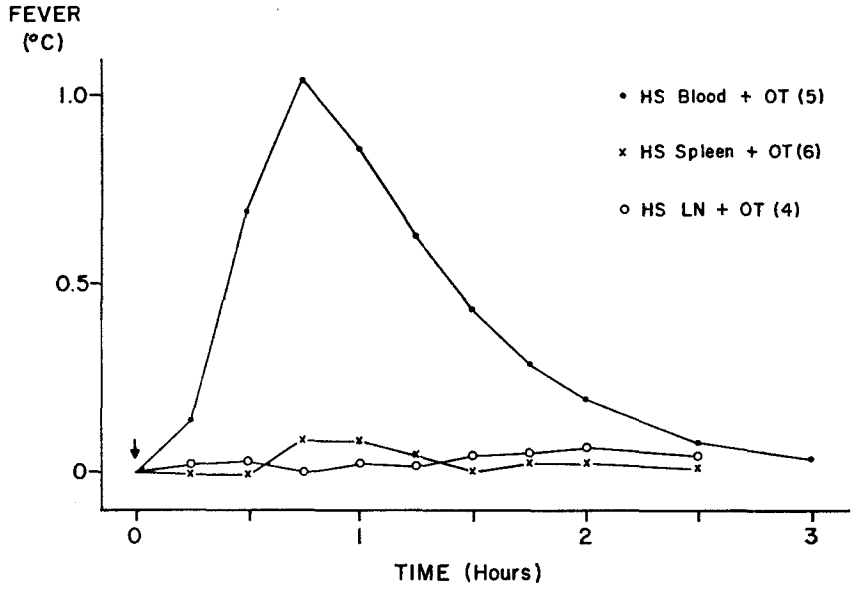


FIG. 1. Mean febrile responses of normal rabbits to supernatant fluids of saline suspensions of various BCG-sensitized rabbit tissues incubated for 5 hours with tuberculin. Individual injections were equivalent to the following dosages:

Blood:	2–3 × 10^8	leukocytes +	100 mg OT	
Spleen:	5 × 10^8	"	"	
Lymph Node (LN):	5 × 10^8	"	"	(2 recipients)
	1 × 10^9	"	"	"

In this and succeeding figures, numbers in parentheses indicate number of animals in each group. HS, hypersensitive; OT, old tuberculin.

Spleen cells: Spleen cells from hypersensitive rabbits were incubated with antigen under a variety of conditions. 2 × 10^8 to 1 × 10^9 mononuclear cells were present in individual preparations, incubated with 200 to 500 mg OT.

(*a*) Cells were suspended in fresh serum from hypersensitive rabbits and incubated with tuberculin at 37°C for 18 hours. Supernates were injected into normal recipients, restrained in boxes.

(*b*) Cells were washed four times in Hanks' solution at room temperature or 4°C. They were resuspended in Hanks' solution and incubated with tuberculin for 18 hours at 37°C. Supernates were injected into normal, restrained or unrestrained recipients.

(*c*) Cells were washed four times in Hanks' solution and incubated in Hanks' solution with tuberculin at 4°C for 16 hours. Supernates were injected into unrestrained recipients.

(*d*) Cells were washed four times in Hanks' solution, resuspended in Hanks' solution, and then disrupted by repeated freezing and thawing by dry ice in acetone and warm water. The "cell extract" was incubated with tuberculin at 4°C or 37°C for 18 hours and after centrifugation the supernate was injected into restrained or unrestrained recipients.

Maximum temperature elevations within 1 hour are tabulated in Table I. Only febrile responses over 0.5°C were considered positive by Johanovský in similar experiments using fewer cells. By this criterion only 2 of 54 recipients in the above experiment had positive responses.

TABLE II

Pyrogenicity of Supernates of Lymph Node Preparations from BCG-Sensitized Rabbits Incubated 18 Hours with Tuberculin in Vitro

Materials		Incubation	Recipients		Mean response
			No.	Condition	
		°C			°C*
Cells + plasma	+ OT	37	2	Restrained	<0.10
Cells + HS‡	+ OT	37	2	Unrestrained	0
Cell extracts + HS	+ OT	37	2	"	0.15

* Maximal temperature elevation within 1 hour.

‡ Hanks' solution.

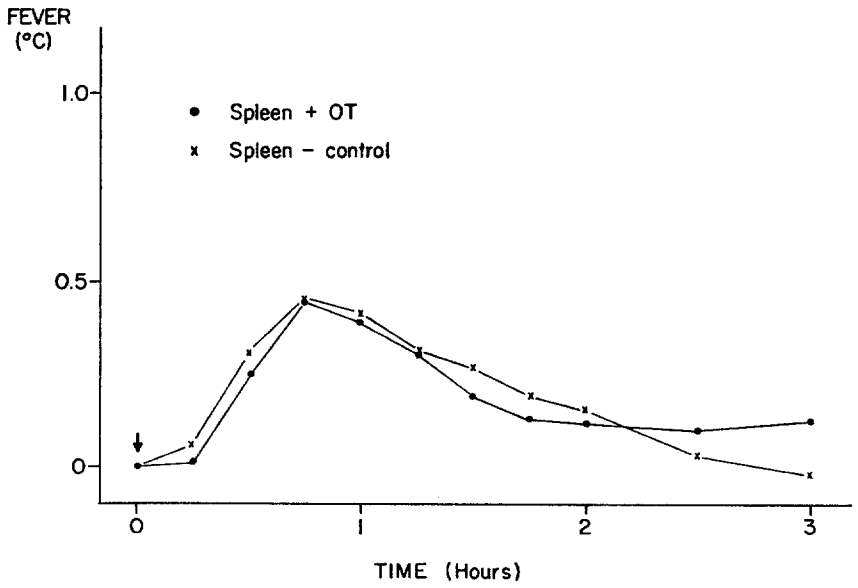


FIG. 2. Mean febrile responses to supernates of spleen cell suspensions in saline (from 4 BCG-sensitized donor rabbits) incubated 5 hours with tuberculin. Controls received supernates of the same suspensions incubated without tuberculin. Each dosage was equivalent to 5 × 10^8 spleen cells and (in the experimental group) 100 mg OT. There were 6 recipients in each group.

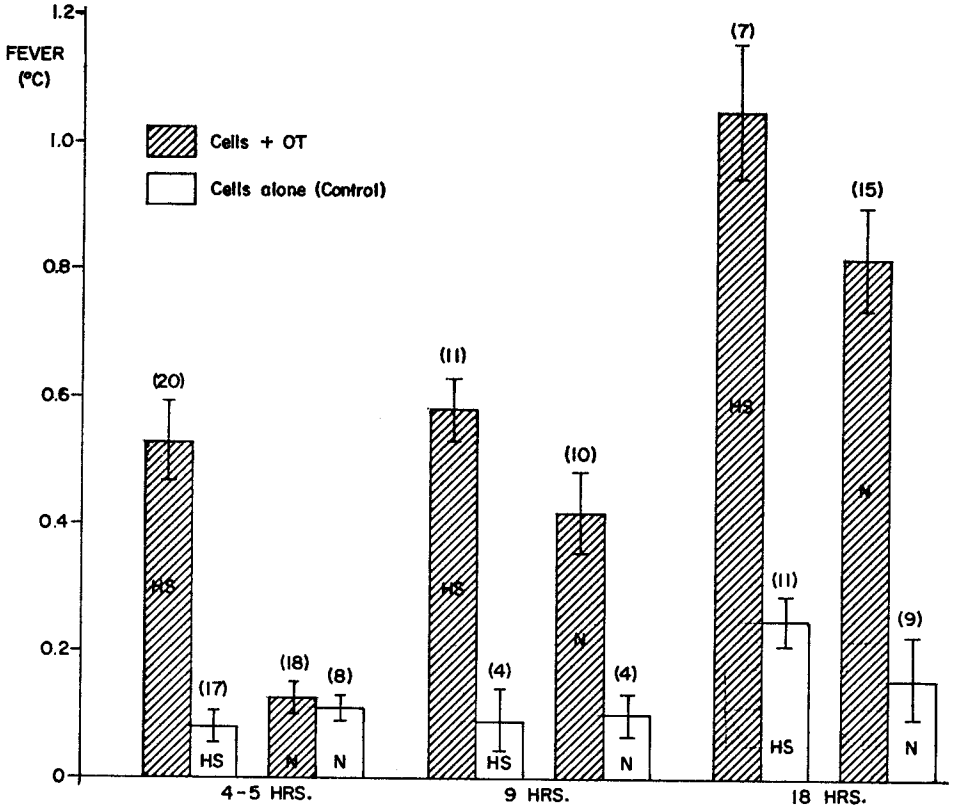


FIG. 3. Average maximal fevers (±SE) of recipients given supernatant fluids of blood cells, from either BCG-sensitized (HS) or normal (N) donor rabbits, resuspended in saline and incubated with tuberculin for the 3 intervals shown (hatched bars). Each dosage in this and subsequent figures, except where mentioned, was equivalent to 2 to 3 × 10^8 leukocytes + 100 mg tuberculin. Control responses to the same dosages of cells incubated without tuberculin are shown in open bars.

Lymph node cells:

(*a*) Aliquots of 2.5 × 10^8 cells were suspended in plasma taken from the same donor and incubated with 200 mg OT for 18 hours at 37°C. Supernates were injected into normal boxed recipients.

(*b*) Aliquots of 1.8 × 10^8 cells were washed four times in Hanks' solution and incubated in Hanks' solution with 300 mg OT for 18 hours at 37°C. Supernates were injected into unrestrained recipients.

(*c*) Aliquots of 2.5 × 10^8 cells were washed four times in Hanks' solution, resuspended in in Hanks' solution, and disrupted by repeated immersion in acetone-dry ice and warm water prior to incubation with 300 mg OT for 18 hours at 37°C. Seventy per cent of cells were dis-

rupted when freezing and thawing was repeated five times. Supernates were injected into unrestrained recipients.

Results are tabulated in Table II. No significant amount of pyrogen was demonstrated in the supernates.

Occasionally, supernates of spleen cells that had been washed once, resuspended in saline, and incubated for 4 to 5 hours with or without tuberculin caused prompt, brief fevers similar to those evoked by sensitized blood cells activated by tuberculin (see Fig. 1) or to the fevers produced by leukocyte

TABLE III

Febrile Responses Induced by Different Dosages of Tuberculin and Blood Leukocytes of BCG-Sensitized Rabbits Incubated at 37°C for Varying Periods in Vitro

OT	No. cells	Incubation	No. recipients	Mean Δ T
mg		*hrs.*		°C
300	3×10^8	12	4	0.83
100	4×10^8	5	3	0.77
100	2×10^{8}*	5	21	0.75
100	2×10^8	18	7	1.05
100	5×10^7	5	22	0.51
100	5×10^7	18	12	0.60
100	1×10^7	5	5	0.02
100	1×10^7	18	7	0.04
25	1×10^8	12	8	0.27
25	1×10^8	22	7	0.70
10	3×10^8	12	18	0.55
10	3×10^{8}‡	12	18	0.30

* Only donors with active cells (mean response 0.5°C or greater) are included in this category. Responses to lower cell dosages ($< 1 \times 10^8$) are to cells from the same (or comparable) donors.

‡ Cells from normal donors.

$P = < 0.01$ that mean responses of recipients in last 2 categories are identical.

pyrogen from sterile exudates. As shown in Fig. 2, supernates of such spleen cell suspensions were equally pyrogenic regardless of whether the cells had been incubated with tuberculin, and similar fevers were sometimes obtained with supernates of normal spleen cells under the same circumstances. Blood cell suspensions from sensitized donors, on the other hand, produced pyrogen only after incubation with tuberculin and there was no correlation between activity of blood and spleen cells from the same donor: some sensitized donors having active spleen cells but blood cells which did not release detectable amounts of EP after incubation with tuberculin and *vice-versa*. By contrast, in over 50 instances, supernates of mesenteric lymph node cell suspensions, in dosages of 5×10^8 to 1×10^9, were never pyrogenic, after being incubated with or without tuberculin.

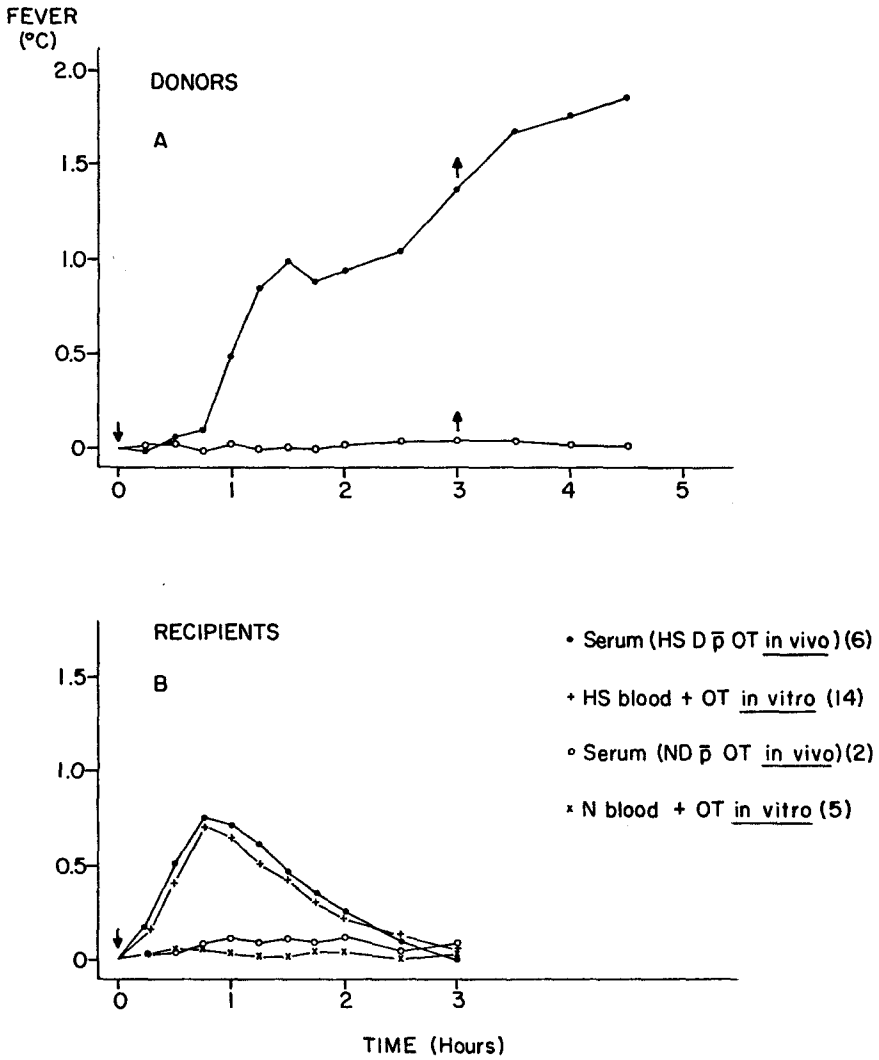


FIG. 4 *a*. Average responses of 5 BCG-sensitized donor rabbits (closed circles) and 5 unsensitized donor rabbits (open circles), each given 100 mg OT intravenously. Upright arrows indicate time of bleeding for passive transfer of serum to recipients Fig. 4 *b*.

FIG. 4 *b*. Mean responses of normal recipient rabbits to: 1. Serum (18 to 30 ml dosages) from donor rabbits shown above (adapted from material in reference 1). 2. Supernatant fluids from BCG-sensitized (HSD) or normal (ND) rabbit blood cells resuspended in saline and incubated for 5 hours with tuberculin. Supernates were injected in aliquots equivalent to 2 to 3 $\times$ 10^8 leukocytes + 100 mg OT. HSD = hypersensitive donor; ND = normal donor.

2. Comparison of Pyrogen Release from Sensitized and Normal Blood Cells Incubated in Vitro with Tuberculin.—The effect of tuberculin in mobilizing EP *in vitro* from blood cells of BCG-sensitized and normal rabbits was next compared. The bar graphs in Fig. 3 show the mean maximal fevers (± SE) produced by supernates of blood cells resuspended in saline and incubated with tuberculin in a ratio to give individual dosages equivalent to 100 mg OT per 2 to 3 × 10^8 blood leukocytes. Two points are of interest. First, it is apparent that at 4 to 5 hours, the shortest interval tested, only blood cells of sensitized donors liberated significant amounts of EP. Second, at the two longer intervals, tuberculin also activated normal blood cells to release EP, though the average amount liberated by normal cells was less than that produced by sensitized cells for the same period. The results obtained at 4 to 5 hours suggest a specific effect of tuberculin on sensitized cells, consistent with the selective pyrogenicity of tuberculin given intravenously to BCG-sensitized rabbits.

Table III shows the mean febrile responses produced when varying dosages of tuberculin and sensitized blood cells were incubated for periods of 5 to 22 hours. From these results it is apparent that cell counts of less than 5 × 10^7 released negligible amounts of EP, despite adequate dosages of tuberculin and prolonged incubation. On the other hand, as little as 10 mg OT mobilized small amounts of EP when incubated for 12 hours with a larger number of cells (3 × 10^8). No significant increase in pyrogenicity was observed with cell counts above 2 × 10^8 or with more than 100 mg OT for short or moderate periods of incubation (5 to 12 hours), though after prolonged incubation (18 hours) slightly higher fevers were produced with the "standard" dose of tuberculin (100 mg) and 2 × 10^8 cells (*cf.* also bar graphs in Fig. 3).

3. Comparison of Febrile Responses to Endogenous Pyrogen Released in Vivo and in Vitro.—Since tuberculin specifically activates the blood cells of BCG-sensitized rabbits after short periods of incubation *in vitro*, it seems quite possible that these cells are the source of the pyrogen which appears in the circulation of similarly sensitized rabbits injected intravenously with tuberculin.

Fig. 4 *a* depicts the mean changes in body temperature induced in both BCG-sensitized and normal rabbits given 100 mg OT intravenously. As previously reported, sera transferred from sensitized donors during fever produced immediate transient fevers in normal recipients indicating the presence of a circulating EP (1, 10). Normal animals, on the other hand, remained afebrile after a similar injection of OT and their sera were nonpyrogenic when given intravenously to recipient rabbits.

In Fig. 4 *b* the mean febrile response of normal rabbits to serum EP (obtained from sensitized donors 3 hours after an intravenous inoculation of 100 mg tuberculin) is compared with the mean response of another group of rabbits to saline supernates of resuspended sensitized blood cells incubated for 5

hours, in individual dosages of 2 to 3 $\times$ 10^8 cells per 100 mg tuberculin. The fever curves induced by the pyrogens obtained by these two different methods are nearly identical and are typical of the responses produced by leukocyte pyrogen (LP) released by granulocytes from peritoneal exudates (11–13).

By contrast, as shown in Fig. 4 *b*, both the sera of normal donors inoculated with OT and supernatant fluids of normal blood cells incubated for 5 hours with tuberculin were nonpyrogenic.

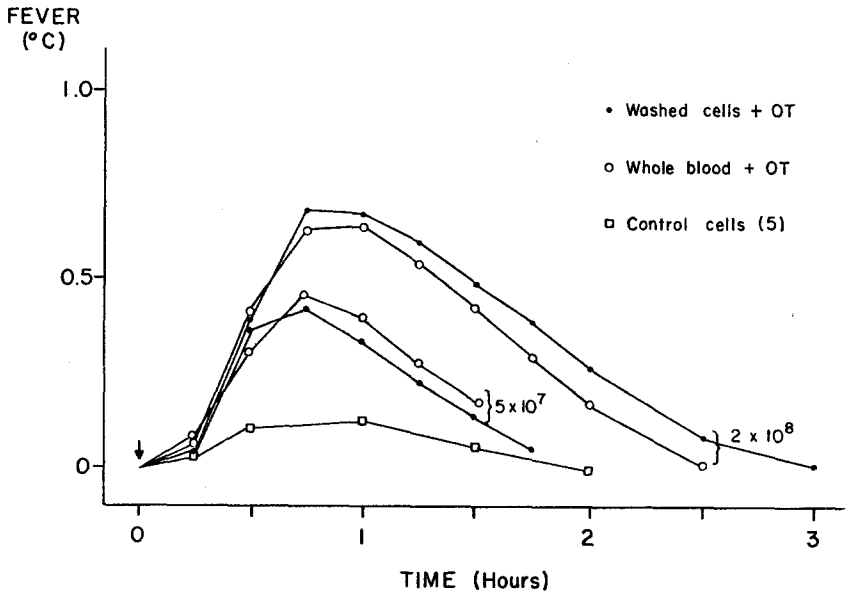


FIG. 5. Mean fevers induced by supernatant fluids from BCG-sensitized rabbit blood incubated for 5 hours with tuberculin. Blood from each donor was divided into (*a*) whole blood, and (*b*) cells washed and resuspended in saline before addition of tuberculin. Supernates were injected in dosages equivalent to 2 to 3 $\times$ 10^8 or 5 $\times$ 10^7 leukocytes with 100 mg OT. Control inoculations were supernates (of either whole blood or washed cells) incubated for the same time without tuberculin and injected in the larger equivalent dosage (2 $\times$ 10^8 leukocytes). Each curve (2 to 3 $\times$ 10^8 dosage) is mean of 8 recipients; curves for 5 $\times$ 10^7 dosage are means of 3 recipients each.

4. Relative Release of Pyrogen in Vitro by Cells in Whole Blood and by Cells in Saline.—Wood and his colleagues have presented evidence that pyrogen production from leukocytes is an active process, which may be modified by a number of factors in the extracellular environment (14, 15). Using peritoneal leukocytes as a source of EP, they have found that the normal release of pyrogen from these cells in a saline medium was nearly completely inhibited by physiological concentrations of potassium and calcium. In view of these observations, it seemed of interest to compare release of pyrogen when tuberculin

was incubated with blood cells in plasma or with cells washed and resuspended in saline. Blood obtained by cardiac puncture from each of 4 sensitized donor rabbits was divided in half and the cells from one-half washed once and resuspended in an approximately equal volume of saline. The other half was left as whole blood, and after removal of a sample of cells for control incubation, tuberculin was added to each half in individual dosages of 100 mg per 2×10^8 leukocytes. Blood of an additional sensitized donor was similarly processed and incubated with tuberculin in dosages of 100 mg per 5×10^7 leukocytes.

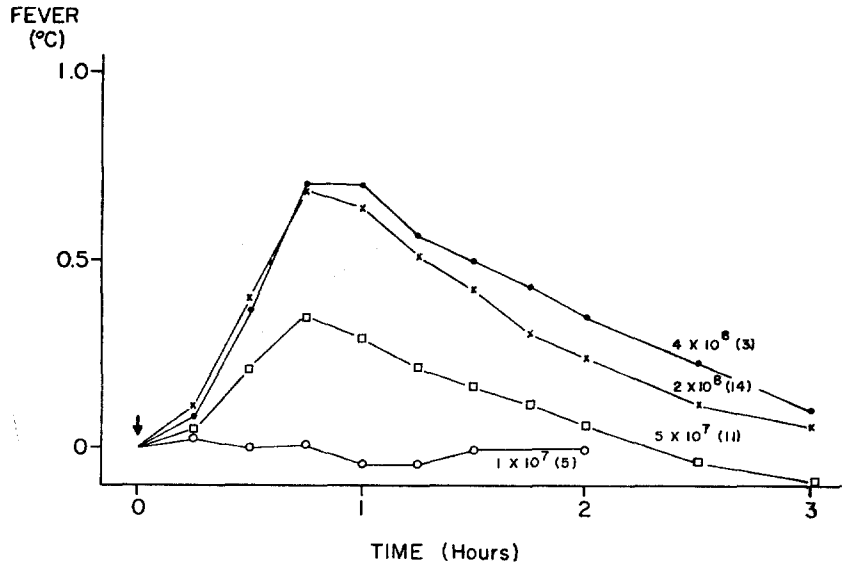


FIG. 6. Average fevers induced by supernates of varying dosages of BCG-sensitized donor blood leukocytes resuspended in saline and incubated 5 hours with tuberculin (see text and Table IV for details).

As shown in Fig. 5, supernates from both whole blood and resuspended cell portions incubated for 5 hours with OT produced almost identical fevers at each of the two dosages tested. Since the responses fall on the steep part of the dose-response curve defined by Bornstein *et al.* for leukocyte pyrogen (13), it seems safe to assume that there is no significant reduction in pyrogen release from sensitized blood cells incubated with OT in plasma containing physiological concentrations of potassium and calcium. Similar results have been obtained with whole blood incubated *in vitro* with other activators such as endotoxin (9) and parainfluenza virus (16).

5. Correlation of Cell Dosage with Febrile Responses Induced by Endogenous Pyrogen.—Fig. 6 shows the average febrile responses of normal recipients to supernates from varying doses of sensitized blood leukocytes resuspended in

saline and incubated for 5 hours with 100 mg aliquots of tuberculin. In order to minimize the variation in reactivity of cells from different donors, cells from each donor were tested in at least two successive dosages (see Table IV), and care was taken to select only those cells which gave comparable responses

TABLE IV

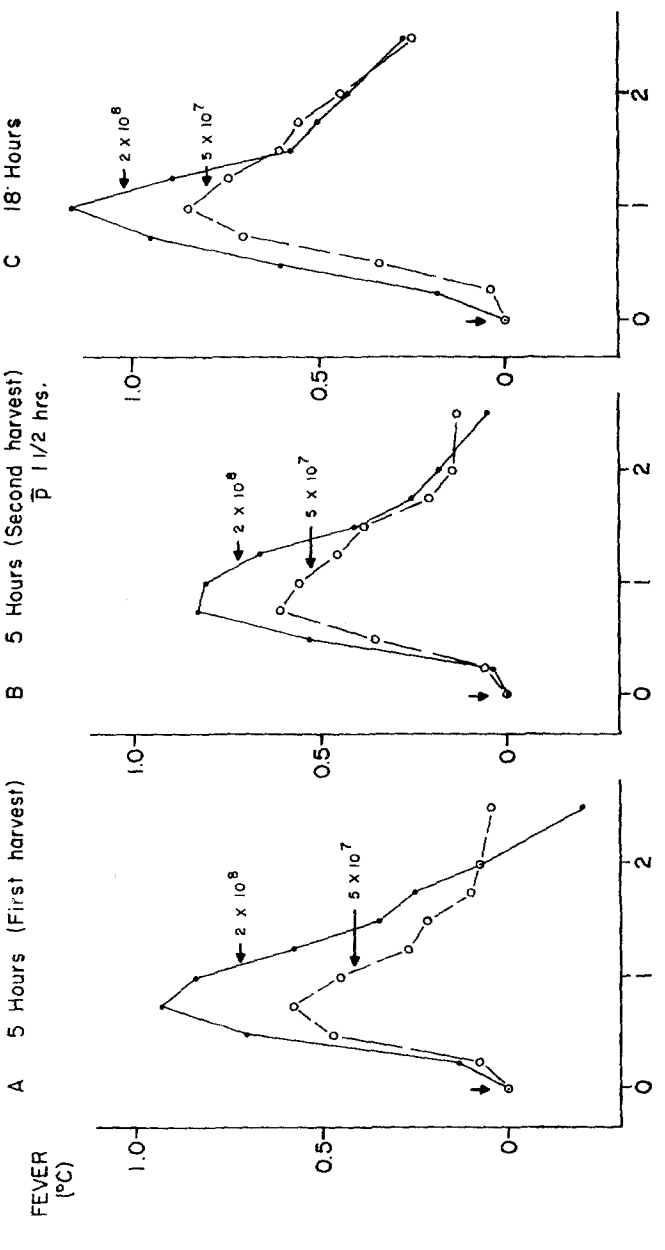
Fevers Induced by Supernates of Varied Dosages of BCG-Sensitized Donor Blood Leukocytes Incubated (37°C for 5 Hours) with 100 mg Tuberculin in Vitro

Donor	WBC dosage	No. Recipients	Endogenous pyrogen*	
			Δ T	F.I.
			°C	*3 hrs.*
4159	1×10^7	5	0.02	0.2
Average		5	0.02	0.2
4159	5×10^7	4	0.33	1.7
4198	5×10^7	4	0.49	3.1
4260	5×10^7	3	0.25	1.8
Average		11	0.36	2.2
4159	2×10^8	3	0.50	3.8
4198	2×10^8	3	0.90	4.9
4260	2×10^8	3	0.63	4.5
4013	2×10^8	2	0.60	2.6
4040	2×10^8	3	0.98	7.1
Average		14	0.73	4.6
4013	4×10^8	2	0.70	4.2
4040	4×10^8	1	0.85	9.0
Average		3	0.78	6.6

* Figures in both columns represent average values for indicated number of recipients. Summed averages are shown for each cell dosage.

‡ Fever index (see Materials and Methods).

for the same dose. In addition, to obviate differences in response of individual recipients, all injections were given to a single group of recipients. Minimal responses (0.36°C) were obtained with 5×10^7 leukocytes; fevers of double this height (0.73°C) were produced by 2×10^8 cells. The response was not significantly altered with double this number of leukocytes in a small group of recipients. With other individual donors, pyrogen release from a comparable number of leukocytes was considerably greater (see Figs. 7 *a* to 7 *c*).



FIGS. 7 *a* to 7 *c*. Mean fevers induced by supernates of blood cells (from a single BCG-sensitized donor) resuspended in saline and incubated with tuberculin. Aliquots of 2×10^8 and 5×10^7 leukocytes were incubated for either 5 or 18 hours with 100 mg OT. Responses to supernates of the 5-hour cells, reincubated for 1½ hours in freshly added saline (second harvest) are also shown. Supernates of the 2×10^8 dosage were given to the same 3 recipients; those of the 5×10^7 dosage were injected in another group of 4 recipients.

6. Effect of Varied and Repeated Incubation Periods on Release of Pyrogen in Vitro.—Wood and his colleagues have shown in a series of studies that release of EP from granulocytes is an active process which proceeds optimally at 37°C and appears to require S-H–dependent enzymes (17, 18). In view of the evidence that both blood and exudate granulocytes generate EP rather than simply release preformed pyrogen, the following experiment was designed to determine whether BCG-sensitized blood cells behaved similarly when exposed *in vitro* to tuberculin.

Heparinized blood cells from a single donor were resuspended in saline and divided into aliquots that were incubated for either 5 or 18 hours with tuberculin in dosages of 5×10^7 and 2×10^8 leukocytes per 100 mg OT. At the conclusion of the 5 hour period of incubation the supernates were removed and the blood cells resuspended in freshly added saline and reincubated for an additional 1½ hours. The cells were then again centrifuged and the supernates containing the "second harvest" removed. Supernates from each of the 2 cell dosages incubated for 5 hours (first and second harvests) as well as supernates from the single incubation at 18 hours were injected into a single group of rabbits; the same 3 recipients received all 3 incubations at the 2×10^8 dosage, while another group of 4 recipients were given the 3 incubations with 5×10^7 cells.

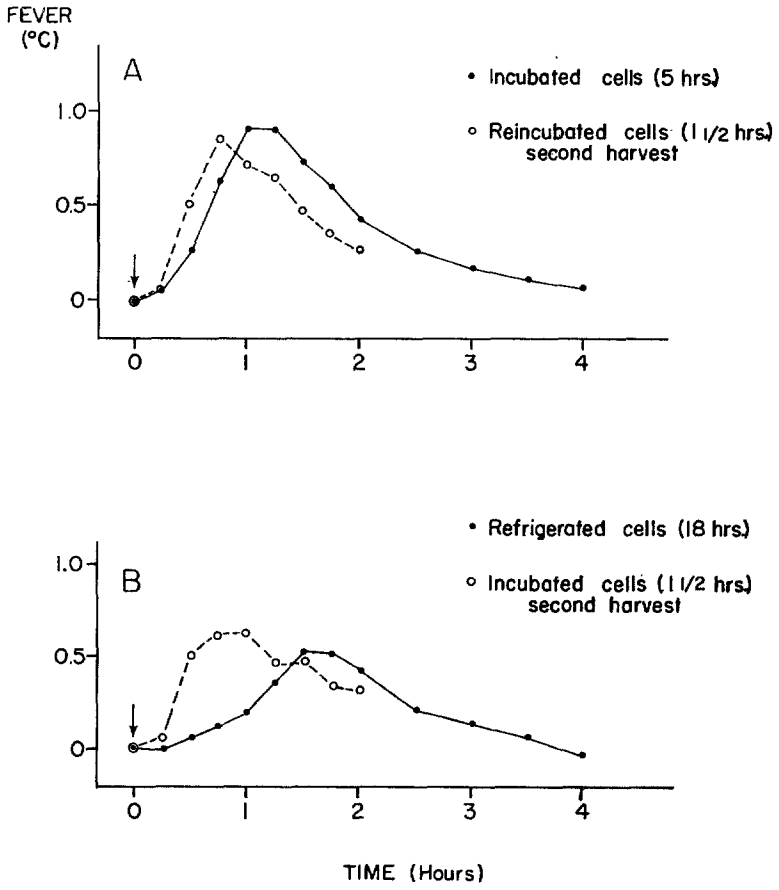
The results are shown in Figs. 7 *a* to 7 *c*. It is apparent that reincubation of the cells for this brief period produced a second almost equally potent release of EP from both the 5×10^7 and 2×10^8 dosages of leukocytes. Furthermore, consistent with the concept that EP is generated by activated leukocytes, blood cells incubated for the longer interval (18 hours) released considerably more pyrogen than did those incubated for only 5 hours. In Fig. 7 *c* the mean response induced by supernates of 5×10^7 cells incubated for 18 hours equalled that produced by 2×10^8 cells after 5 hour incubation. Since these responses fall on the steep part of the dose-response curve for leukocyte pyrogen, it is apparent that approximately 4 times as much pyrogen was released by cells incubated for the longer period. These findings were repeatedly confirmed in a number of other similar experiments.

7. Comparison of Pyrogen Release in Vitro from Refrigerated and Incubated Blood.—Release of leukocyte pyrogen almost ceases at 4°C (17) whereas Johanovský has reported that his so-called hypersensitivity pyrogen is produced when sensitized cells (or cell extracts) are mixed with antigen *in vitro* at either 4°C or 37°C (5, 6). In the following experiment, the effect of temperature was determined on the release of pyrogen from BCG-sensitized rabbit blood to which tuberculin was added *in vitro*.

Blood cells from a single donor were resuspended in saline and then divided in half. White counts were obtained, and tuberculin added to give aliquots of 2×10^8 cells per 100 mg OT. Half the blood was then left at 4°C for 18 hours

while the other half was incubated at 37°C for 5 hours, as in previous experiments. At the end of these intervals, both samples were centrifuged, the supernates removed for injection, and the cells resuspended in fresh saline. Both the previously refrigerated and incubated samples were then incubated at 37°C for an additional 1½ hours to give a second harvest, as in the preceding experiment.

The results are shown in Figs. 8 *a* and 8 *b*. As in previous work with leuko-



FIGS. 8 *a* and 8 *b*. Mean responses induced by supernates of blood cells (from a single BCG-sensitized donor) resuspended in saline with added tuberculin. Fig. 8 *a*. Supernates of blood cells incubated 5 hours with tuberculin (closed circles); supernate of the same cells resuspended in saline and reincubated an additional 1½ hours, (second harvest, open circles). Fig. 8 *b*. Supernates of blood cells refrigerated for 18 hours with tuberculin (closed circles); supernates of the same cells resuspended in saline and incubated for 1½ hours (second harvest, open circles). All dosages equivalent to 2×10^8 leukocytes and 100 mg tuberculin. Each curve represents average of 4 recipients.

cyte pyrogen, refrigerated cells failed to release EP; the small delayed fevers produced by supernates of these cells were presumably caused by the tuberculin which had been added to the cell suspension, as the recipients were subsequently found to be weakly sensitive to tuberculin. On the other hand, the second harvest from these cells, obtained after a subsequent 1½ hour incubation at 37°C, produced prompt fevers characteristic of EP, as did both first and second harvests of the cells initially incubated at 37°C (see Fig. 8 *a*).

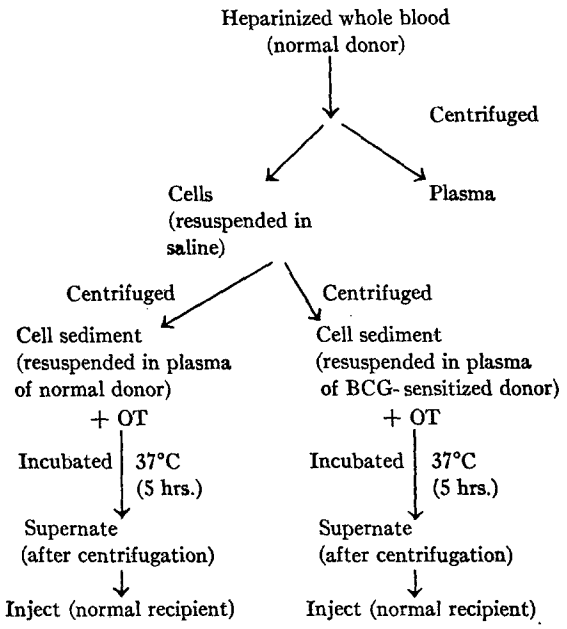


FIG. 9. Flow diagram. See text for details.

8. Release of Endogenous Pyrogen by Normal Blood Cells Incubated with Tuberculin in Plasma of BCG-Sensitized Rabbits.—Since cells of BCG-infected rabbits appeared to be selectively activated to produce EP after brief incubation with tuberculin *in vitro*, it seems likely that such cells have been specifically sensitized to the antigens present in this material. The following experiment was designed to determine the possible role of circulating antibody in sensitizing blood cells. Though blood of BCG-infected rabbits contains HA antibodies to extracts of BCG (19), circulating antibody is generally thought to play little or no role in producing the characteristic reactions of tuberculin hypersensitivity.

Blood from normal donor rabbits was centrifuged and resuspended in saline. After white blood cell counts were performed, the resuspended cells were divided

in half, recentrifuged, and the supernatant saline removed. One aliquot of cells was then resuspended in plasma of a BCG-sensitized donor rabbit; the other, in plasma from another normal donor. Tuberculin was added in a dosage of 100 mg per 2 to 3 × 10^8 leukocytes and the mixtures incubated for 5 hours. At the end of this period, the cells were recentrifuged and the supernate injected into normal recipients (see Fig. 9).

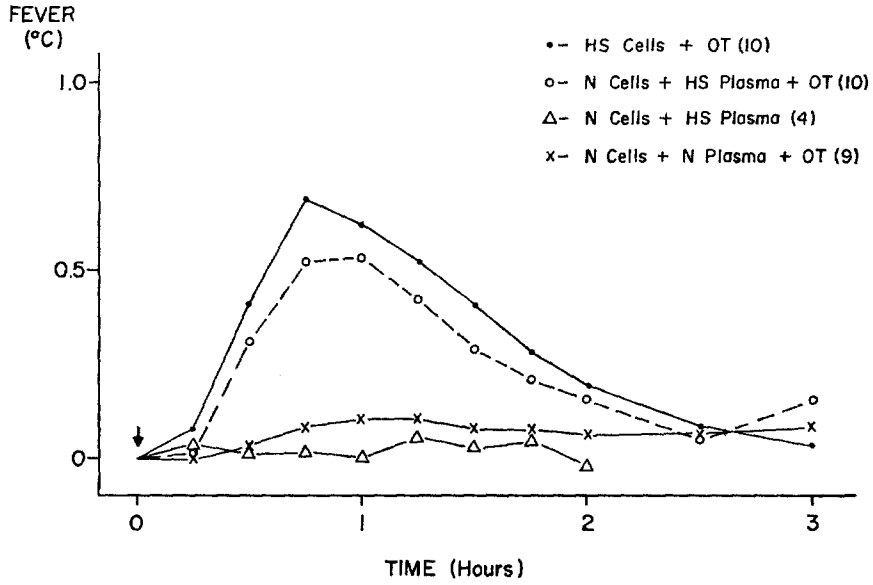


FIG. 10. Mean febrile responses induced by supernatant fluids from normal rabbit blood cells washed and resuspended in plasma of either BCG-sensitized (HS) or other normal (N) rabbits and incubated 5 hours with tuberculin. Controls received supernates from an equal number of cells of the same donor incubated in sensitized plasma alone. Also shown are responses to supernates of tuberculin-incubated blood cells from the sensitized donor rabbits whose plasma was incubated with normal blood cells. All dosages were equivalent to 2 to 3 × 10^8 leukocytes incubated (except controls) with 100 mg tuberculin.

In 2 experiments, cells from the same normal donor were divided as indicated in the protocol above. In additional experiments (2 with plasma of sensitized animals; 2 with normal plasma) the donor cells were incubated in the plasma of sensitized or normal donors alone. An aliquot of cells, treated in the same manner but without added tuberculin, served as a control in each experiment.

The results are shown in Fig. 10. Normal blood cells, incubated with plasma of sensitized donors,[6] were activated by tuberculin and released nearly as

[6] One of the 4 donors had been resensitized with BCG; the other 3 were sensitized for the first time. There were no significant differences in activation of blood cells by any of these sera.

much EP as did cells of the sensitized donors from whom the plasma was obtained. By contrast, little or no pyrogen was released by cells suspended in sensitized plasma alone or in normal plasma to which tuberculin had been added.

These results suggest that plasma of BCG-sensitized rabbits contains factors (presumably antibodies) that are capable of activating blood cells exposed to the specific antigen(s) contained in tuberculin.

9. Failure to Activate Normal Blood Cells with Supernates of Sensitized Lymphocytes Previously Exposed to Tuberculin in Vitro.—There are many reports that mononuclear cells of delayed hypersensitive animals contain factors (independent of circulating antibody) which are released by disruption or by specific antigen and which are capable of passively transferring this form of sensitivity (20–24). Accordingly, an attempt was made to determine whether such cellular factors, like those in plasma, would sensitize normal cells to generate EP when exposed to tuberculin *in vitro*.

The design of these experiments was similar to the last, in which normal blood cells were resuspended in plasma. Mesenteric lymph nodes were obtained from both BCG-sensitized[7] and normal donor rabbits, dissected and gently pressed through a No. 40 wire screen. The cell suspension from 1 or 2 donors was washed once and resuspended in saline usually with antibiotics (penicillin, 5000 units and streptomycin, 10 mg). Tuberculin was then added in dosages of 100 mg per 5×10^8 cells and the mixture incubated at 37°C for 5 hours. Following this, the cells were centrifuged (generally after remaining at 4°C overnight) and the supernatant fluids (presumably containing transfer factors released from sensitized cells by tuberculin) (20) were harvested for later use as indicated below.

Blood cells from single normal donors were next resuspended in saline, divided in half, centrifuged, and one aliquot resuspended in the supernatant fluid obtained from the suspension of sensitized lymph node cells which had been incubated 5 hours with tuberculin as indicated above; to the other aliquot was added the supernate from a suspension of normal lymph node cells that had been similarly incubated with tuberculin. Supernates were added in dosages equivalent to 5×10^8 lymphocytes per 2 to 3×10^8 blood leukocytes. An additional dose of tuberculin was added to this mixture (100 mg per 2 to 3×10^8 blood cells), and after a 5 hour incubation the blood cells were centrifuged and the supernatant fluid assayed in normal recipients for EP (in dosages equivalent to 100 mg OT and 2 to 3×10^8 blood cells). See Fig. 11.

[7] Though all donor rabbits had vigorous febrile responses to tuberculin, some had blood cells which were unreactive when incubated for 5 hours *in vitro* with OT, whereas cells of others were highly reactive. Lymph nodes from both types of donors were used because of the evidence that serum antibodies mediated the release of pyrogen from blood cells *in vitro* and that the responses of delayed hypersensitivity may be suppressed in the presence of circulating antibody (25).

In certain experiments, the blood cells were resuspended in the lymph node cell suspensions themselves (previously incubated with OT), rather than in supernates of these suspensions.

A total of 14 experiments was performed; in most of these, supernates of lymph node cell suspensions from 1 sensitized and 1 normal donor were incubated with the divided blood cells of a single rabbit as described in the above protocol; in others, the blood cells were incubated in supernates from a single sensitized or normal donor only.

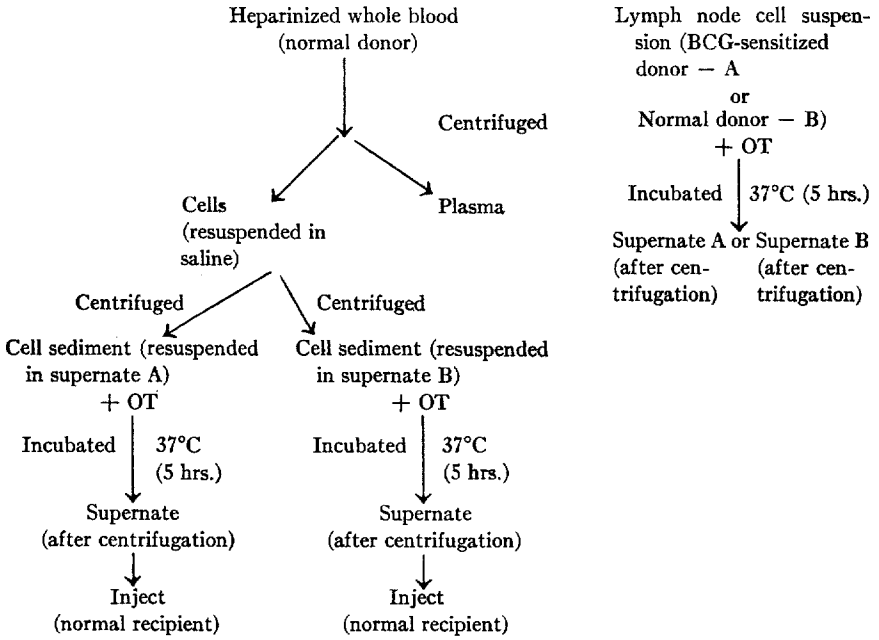


FIG. 11. Flow diagram. See text for details.

The results of these experiments showed no significant release of pyrogen. A total of 31 recipients, given supernates of blood cells incubated with tuberculin in the sensitized lymphocyte preparation, had a mean response of 0.24°C, with only 3 recipients developing fevers of 0.5°C or over (a single recipient had a rise of 0.7°C). Of 20 recipients receiving supernates of blood cells and tuberculin incubated in the preparation from normal lymphocytes, the mean temperature rise was 0.20°C. Similar results were obtained in several experiments of the same design, but in which supernates of sensitized and unsensitized spleen cells were substituted for those of lymph node cells.[8]

Under these conditions, therefore, no evidence was obtained that lympho-

[8] Experiments with spleen cells were more difficult to interpret, however, than were those with lymph node cells, because supernates of such cells alone often induced fever (see Fig. 2).

cytes, obtained from either spleen or lymph nodes of sensitized rabbits and incubated with tuberculin, release factors that sensitize normal blood cells to react with tuberculin and produce pyrogen *in vitro*.

10. Response of Normal Rabbits Injected Intravenously with Mixtures of Tuberculin and Plasma or Blood Cells of Sensitized Donors.—Additional experiments were performed in which tuberculin was added *in vitro* to plasma of sensitized donors and incubated for 15 to 20 minutes at 37°C. The mixture was then injected intravenously (in dosages of 100 mg per 30 to 50 ml plasma) into normal recipients. In 10 animals (about one-third of those injected) this material caused small but characteristic tuberculin fevers, mean elevation of 0.55°C (0.35 to 1.25°C) at 2 hours after latencies of 30 to 45 minutes. The resulting delay in fever indicates that such mixtures, like tuberculin itself in sensitized animals, act indirectly, and it seems clear that combination of antigen with serum antibodies does not in itself constitute EP. Control injections with normal plasma, to which tuberculin had been added, were regularly nonpyrogenic.

In comparable experiments, OT was similarly incubated *in vitro* with the blood cell sediments (resuspended in an equal volume of saline) from the same sensitized donor rabbits. This mixture was then given to unsensitized recipients in dosages of 100 mg tuberculin per 2.5 to 7.5 $\times$ 10^8 total leukocytes. In 10 rabbits (about one-third of the total number injected), delayed fevers were again produced, with a mean maximal rise of 0.65°C (0.35 to 1.25°C) at 1¾ hours. Although these experiments do not define the mechanism or site of pyrogen release, it seems evident that sensitized blood cells to which antigen is added *in vitro* are capable of mobilizing EP *in vivo* as well as when incubated *in vitro*. In experiments of similar design, tuberculin was added to normal blood cell sediments, resuspended in saline, or to washed cell suspensions of either spleen or lymph nodes of normal or sensitized rabbits. These suspensions consistently failed to produce fever in normal recipients.

DISCUSSION

Fever has long been recognized as perhaps the most frequent and subtle sign of tuberculosis (26, 27). In the absence of conclusive evidence that tubercle bacilli contain a primary toxic factor, most of the manifestations of this disease, including fever, have been attributed to the effects of antigen in an allergic host. Although circulating antibodies have been found in tuberculosis (19, 28), hypersensitivity of the delayed or cellular type is believed to play the predominant role. Numerous studies (reviewed in references 29–33 *a*) have demonstrated the cytotoxic effects of tuberculin on hypersensitive tissues *in vitro*.

During fever induced by intravenous inoculation of tuberculin in BCG-sensitized rabbits, a circulating pyrogen has been demonstrated by passive transfer techniques (1, 10). This pyrogen is active in normal recipients, and is,

therefore, clearly distinct from tuberculin. Its presence raises a number of questions about the role of hypersensitivity in the pathogenesis of this form of fever.

1. With what type of antibodies, circulating or cellular, do the antigens present in tuberculin react to initiate the febrile response?

2. Is the antigen-antibody complex itself directly pyrogenic, or does it produce fever indirectly by releasing from the host's tissues an endogenous pyrogen (EP) which is the immediate cause of fever?

3. If fever is caused by an EP, from what tissue is it derived?

In a series of publications, Johanovský has presented evidence that tuberculin (or PPD) reacts *in vitro* with specifically sensitized mononuclear cells from spleen or lymph nodes to produce a rapidly acting "hypersensitivity pyrogen." This pyrogen differed in several respects from both leukocytic and circulating endogenous pyrogens present in other experimental fevers (3–8). Hypersensitivity pyrogen was produced when antigen was added to disrupted as well as to viable cells. Its appearance, unlike that of leukocyte pyrogen, was not critically dependent upon temperature, since equal amounts were present in samples kept at 4° or at 37°C.[9]

The observations reported here do not confirm Johanovský's findings. Addition of tuberculin to either whole or disrupted cells from the spleens or mesenteric lymph nodes of sensitized donor rabbits failed to produce a rapidly acting pyrogen. Although supernates from occasional samples of both normal and sensitized spleen cell suspensions were mildly pyrogenic, addition of tuberculin did not increase the pyrogenicity of these samples. No evidence was obtained that a pyrogen is produced by the reaction of antigen with sensitized mononuclear cells.

On the other hand, the data presented here suggest that tuberculin reacts specifically with blood cells of BCG-sensitized donor rabbits to produce an endogenous pyrogen after short periods of incubation *in vitro*.[10] It seems clear that EP is not derived from the reaction of tuberculin with factors in the plasma alone. Sensitized rabbit plasma, to which tuberculin had been added *in vitro*, failed to cause immediate fevers when injected intravenously into normal

[9] Johanovský has since been unable to repeat these findings (personal communication).

[10] It is not clear what factors are responsible for activating normal leukocytes that are exposed for longer periods (9 to 18 hours) to tuberculin *in vitro*. Since tuberculin is a crude culture filtrate, it may contain impurities, (such as contaminating Gram-negative bacterial endotoxins) present in quantities undetectable by injection of tuberculin *in vivo*; alternatively, small amounts of other antigens, to which the cells of normal donor rabbits are naturally sensitized, may be present. Finally, tuberculin is readily adsorbed to normal, as well as to sensitized leukocytes, (34, 35) and thus may be directly toxic to such cells. In regard to several of these alternatives, neither precipitation of OT with trichloroacetic acid to form PPD, nor preincubation of OT with sera of pyrogen-tolerant animals (to inactivate contaminating endotoxins) diminished its capacity to liberate pyrogen from normal blood cells.

recipients. Moreover, washed blood cells, incubated *in vitro* with tuberculin, were as potent a source of pyrogen as was whole blood (see Fig. 5). The cellular source of tuberculin-induced EP has not been established by these studies. Red blood cells (11) and platelets (36) apparently do not contain a pyrogen, however, and the granulocyte has been implicated in several other experimental fevers produced by microbial agents (37). When endotoxins of Gram-negative bacteria are incubated *in vitro* with either human (36) or rabbit blood (9) an EP is mobilized which has the same pyrogenic properties as tuberculin-induced EP. This response has been shown to be critically dependent upon leukocytes, presumably granulocytes (36). Endotoxins similarly activate rabbit granulocytes obtained from sterile peritoneal exudates and resuspended in fresh plasma (38). Furthermore, granulocytes are the only known cell in the blood from which an EP may be obtained, although studies with pure samples of other blood leukocytes have not been reported. Freshly obtained normal blood leukocytes contain little or no preformed EP. Large amounts of EP may be generated, however, when such cells are incubated with certain activators of which three are known: Gram-negative bacterial endotoxin (9), phagocytosis (under certain circumstances) (39), and an agent obtained from sterile peritoneal exudates (14). There is also recent evidence that myxoviruses (40) as well as EP itself (41) can liberate pyrogen from blood leukocytes *in vitro*.

A number of the factors in the extracellular environment which modify the release of EP from granulocytes have been identified by Wood and his coworkers. Prominent among these are K^+ and Ca^{++}, which in physiological concentrations almost completely suppress EP production by "activated" granulocytes from peritoneal exudates (15). However, as in other situations in which activators of leukocytes such as endotoxins are added, release of EP from sensitized cells incubated with tuberculin was not affected by the physiological concentrations of these cations present in normal plasma (see Fig. 5).

The studies reported here suggest, as do those of Wood, that pyrogen is actually generated by activated cells. After exposure to tuberculin, cells released a second, equally potent "harvest" of pyrogen when reincubated at 37°C for a short period in freshly added saline. Cells kept at 4°C, on the other hand, failed to liberate detectable amounts of pyrogen. Similar results have been obtained when purified endotoxin was employed as the stimulus (9).

On the assumption that granulocytes form 50 per cent of blood leukocytes, dose-response curves with leukocytes from most sensitized donors indicate considerably less release of pyrogen after exposure to tuberculin *in vitro* than occurs with an equivalent number of exudate granulocytes resuspended in saline. Average fevers of only 0.35°C were obtained with 5×10^7 total sensitized blood leukocytes from one group of donors (approximately 2.5×10^7 granulocytes). Fevers of this height are produced by only one-fifth as many exudate granulocytes (13). With occasional donors, apparently highly sensi-

tized, the febrile responses obtained from equivalent numbers of blood and exudate granulocytes are more nearly equal (see Fig. 7). These findings suggest, perhaps, that a larger per cent of granulocytes in the blood have been activated by tuberculin *in vitro*.

It is of interest that soluble antigens (present in tuberculin) mobilized EP from sensitized blood cells *in vitro*. This did not occur when a particulate (Rh) antigen (present on the surface of Type D erythrocytes) was incubated *in vitro* with whole blood of a D-sensitized donor (42). When transfused into a sensitized subject, however, D cells were sequestered at sites in the RES and produced both leukopenia and fever. The fever appeared after a delay of about 1 hour and was presumably caused by release of EP *in vivo*.[11] Admixture of tuberculin with either serum or cells of sensitized donor rabbits also produced fevers with a delayed onset in some normal recipient rabbits, a response that similarly suggests that EP was released *in vivo*. Unlike D cells, however, tuberculin appeared to mobilize EP as effectively *in vitro* as it did in these *in vivo* experiments (compare, Results, sections 2 and 10, respectively).

Since pyrogen may be recovered from homogenates of normal rabbit tissues other than granulocytes (43, 43*a*) the question may be raised whether such tissues are an alternative or major source of the EP present in the circulation of animals with tuberculin-induced fever. Further studies will be necessary to explore this possibility. It seems significant, however, that spleen and lymph nodes, which would seem most likely to be implicated (in view of their capacity to produce antibodies and to passively transfer reactivity to tuberculin) did not elaborate a pyrogen on contact with OT *in vitro*.

These studies lend no support for the existence of a separate "hypersensitivity pyrogen", produced by combination of antigen with specifically sensitized mononuclear cells. On the other hand, they do suggest that immunologically competent tissues such as spleen and lymph nodes play an indirect role in tuberculin fever by supplying antibodies which sensitize blood cells to release EP on contact with tuberculin *in vivo* or *in vitro*. When suspended in plasma of BCG-sensitized rabbits, normal blood cells mobilized EP after a short incubation with tuberculin *in vitro*. Since washed blood cells of sensitized rabbits were fully capable of reacting with tuberculin *in vitro*, the antibody that presumably mediates the release of pyrogen by tuberculin appears to be closely bound to the surface of the involved cells, as has been described for cytophilic antibody (44–46). Whether the antibody is bound to leukocytes, erythrocytes, or both is conjectural. Tuberculin causes destruction of erythrocytes of sensitized animals *in vivo* (47) and hemolysis was often noted in these experiments

[11] Since the febrile reaction seemed to be dependent upon immune hemolysis, Jandl has hypothesized that immune hemolysis liberates antigen-antibody complexes which activate the recipient's blood leukocytes to produce EP *in vivo*.

after prolonged incubation of sensitized blood cells with tuberculin *in vitro*. This reaction may, therefore, release antigen-antibody complexes which, in turn, activate granulocytes, as suggested by Jandl's studies (42).[12] Tuberculin also produces *in vitro* lysis of both lymphocytes and granulocytes of sensitized animals (29) so that the mechanism of leukocyte activation may be a more direct one. Attempts to determine both the type of blood cell and antibody involved in this process are underway, since patients with active tuberculosis have both 19S and 7S antibodies to tuberculin (28). Circulating antibodies have long been known to be associated with tuberculous infection. Evidence that such antibodies have a role in mediating tuberculin fever was unexpected, however, as they have not usually been thought to be responsible for the characteristic features of this disease.

The manifestations of tuberculin and other forms of delayed hypersensitivity have been widely attributed to the reaction of antigen with factors present in specifically sensitized mononuclear cells. Passive transfer of delayed hypersensitivity to various antigens has been reported both with extracts of sensitized mononuclear cells (21–24) and with the supernatant fluids from suspensions of similar cells to which the specific antigen was added (20). It was of interest, therefore, that supernates, presumably containing "transfer factors" released by tuberculin from the cells of either spleen or lymph nodes of BCG-infected donor rabbits, failed to sensitize normal blood cells to react with added tuberculin *in vitro*. This finding seems consistent with the view that viable (and hence, potentially antibody-producing) mononuclear cells are necessary to effect passive transfer of delayed as well as immediate forms of hypersensitivity in animals other than man (48, 49). Whether delayed hypersensitive cells can be shown by other techniques to contain factors, separate from antibodies, which play a role in producing tuberculin fever remains to be determined, although none was demonstrable in these studies.

On the basis of the observations reported here, the following series of events is possibly one that may be initiated when tuberculin is given to specifically sensitized animals.

Intravenously injected tuberculin reacts directly with granulocytes in the blood (or at other sites) which have already been sensitized by prior infection of the host with BCG. Since these cells appear to be immunologically incompetent, tuberculin presumably reacts with antibody passively adsorbed to their surface from other, antibody-producing cells (44, 45). As a result of this reaction, the blood cells are activated and release an endogenous pyrogen. This agent, in

[12] Supernates of 4×10^9 pure red cells incubated with 500 mg OT in serum failed to cause either immediate or delayed fevers in 2 recipients. No evidence was obtained from these data, therefore, to implicate this cell directly or indirectly in the genesis of tuberculin-induced fever.

turn, acts directly on the thermoregulatory centers of the brain to cause the increased temperature. These postulated events are presented diagrammatically in Fig. 12.[18]

Finally, these observations on tuberculin-induced fever suggest that the release of EP from specifically sensitized blood cells, incubated *in vitro* with antigen, may be a sensitive technique for the investigation of other allergic conditions associated with fever (50).

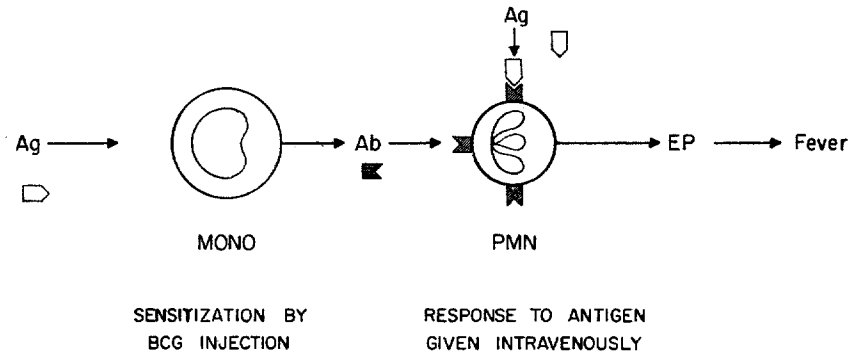


FIG. 12. Postulated mechanism of tuberculin-induced fever. Ag, antigen; Ab, antibody Mono, mononuclear cell; PMN, polymorphonuclear leukocyte.

CONCLUSION

In a search for the source of the circulating endogenous pyrogen (EP) that mediates tuberculin-induced fever, tuberculin was incubated *in vitro* with various tissues of rabbits sensitized by intravenous infection with BCG.

Evidence was obtained that tuberculin specifically stimulates cells in the blood of sensitized rabbits to generate pyrogen *in vitro*, whereas both lymph node and spleen cells from the same donors were inactive.

Since normal blood cells, incubated in plasma of sensitized donors, were similarly activated, it is postulated that circulating antibodies play a role in sensitizing cells (presumably granulocytes) to release pyrogen on contact with tuberculin, both *in vitro* and *in vivo*.

Release of endogenous pyrogen *in vitro* may be a sensitive means of detecting immunologic reactions between antigen and specifically sensitized blood cells in other allergic states accompanied by fever.

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[18] Allen has recently reported (Allen, I. V., Immunol., 1965, 8, 396) that agranulocytic rabbits sensitized by BCG react with fever and have circulating EP when given tuberculin intravenously, unlike similarly agranulocytic animals given endotoxin. These data suggest that cells other than circulating granulocytes must also be implicated in tuberculin-induced fever.

The technical assistance of Mrs. Lorraine Francis is gratefully acknowledged.

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