

ENDOTOXIN FEVER IN GRANULOCYTOPENIC ANIMALS*

By JOHN C. HERION, M.D., RICHARD I. WALKER,† M.D.,
AND JEFFRESS G. PALMER, M.D.

(From the Department of Medicine, University of North Carolina
School of Medicine, Chapel Hill)

(Received for publication, February 27, 1961)

The febrile response produced by intravenous administration of bacterial endotoxin is thought to be mediated by a substance present in polymorphonuclear leukocytes (1, 2).

Extracts of such cells have been shown to contain a pyrogenic substance which differs from endotoxin (3-5), and a similar substance has been found in plasma during the period of endotoxin fever (6, 7). This material has a more direct effect than endotoxin on the central nervous system in producing fever (8). Endotoxin also affects the number of circulating granulocytes; the decrease which regularly follows endotoxin administration is temporally related to the fever response (9). *In vitro* interaction between polymorphonuclear leukocytes and endotoxin liberates leukocyte pyrogen while endotoxin is inactivated (10). These studies have led to the postulate that endotoxin fever results from the central action of endogenous pyrogen released by injured polymorphonuclear leukocytes (1, 2, 11).

A major objection to this hypothesis has been the normal febrile response to endotoxin in animals made leukopenic by mechlorethamine (HN₂) administration (12-14). This finding has been explained by postulating a direct action of endotoxin on the central nervous system heat-regulating centers (15) or the presence of a pyrogenic substance in cells other than leukocytes (16). The former view is possibly supported by the demonstration of a febrile response to intrathecally administered endotoxin and by failure to demonstrate endogenous pyrogen in the serum of leukopenic animals after endotoxin injection (15). Tissues other than leukocytes have been found to contain no pyrogenic substances with properties resembling leukocyte pyrogen (16, 17).

This paper presents data concerning the response of leukopenic animals to endotoxin and indicates that the febrile response in these animals is approximately proportional to the number of polymorphonuclear leukocytes remaining in the circulation. Agranulocytic animals show no significant increase in temperature.

* This study was supported by Grants C-1774 and CY 3015 from the National Cancer Institute, United States Public Health Service, and by the Richard Kerner Leukemia Fund.

† Research Fellow, National Cancer Institute.

Materials and Methods

Male, albino rabbits weighing 2.5 to 3.0 kg. were studied. They were housed in individual cages in an air-conditioned room where the studies were performed. For several hours on 3 to 4 successive days prior to study, and during the period of study, they were placed in specially constructed boxes; food and water were withheld. Rectal temperatures were measured for 45 to 60 minutes prior to endotoxin injection and for 6 hours afterward with a thermistor device (telethermometer, Yellow Springs Instrument Co., Yellow Springs, Ohio). The methods for counting leukocytes and preparing blood smears for differential cell counts have been previously described (9). The endotoxin was *Escherichia coli* lipopolysaccharide (bacto lipopolysaccharide, *E. coli* 026:B6, Difco Laboratories, Detroit) suspended in pyrogen-free, sterile, physiologic saline. This was given intravenously. Nitrogen mustard, (mustargen® hydrochloride, Merck, Sharp & Dohme, Philadelphia), freshly mixed in sterile, endotoxin-free, physiologic saline in a concentration of 1.0 mg. per ml. just prior to injection, was administered into a marginal ear vein. The initial dose of HN₂ was 2.5 mg./kg. A second injection of 0.5 mg./kg. was given 24 to 48 hours later to sustain the leukopenia. The febrile response to endotoxin was measured 4 days after the initial HN₂ injection, the time of maximum leukopenia.

Serum endogenous pyrogen was obtained from donor rabbits injected with 10 µg. of *E. coli* endotoxin. They were bled by cardiac puncture 120 minutes later using sterile, endotoxin-free syringes and needles. The blood remained for 1 hour at room temperature while clotting, was stored overnight at 4°C., and the serum collected by centrifugation. Sterile, endotoxin-free glassware was used for these procedures. The collected serum was pooled, stored at 4°C., and administered to recipients within 5 days. Serum, warmed to room temperature, was injected into a marginal ear vein of the recipient.

RESULTS

Production of Granulocytopenia:

Nitrogen mustard was used in doses larger than those of other investigators studying endotoxin fever (12, 14, 15); this produced maximum granulocytopenia 4 days after the initial injection. The initial injection of 2.5 mg./kg. was followed 48 hours later by a second injection of 0.5 mg./kg. With this regimen 3 of 37 animals died within 4 days. A high mortality resulted when the second HN₂ injection was given 24 hours following the first; 3 of 4 animals died within 4 days. 29 animals had circulating granulocyte counts less than 50 per mm.³ of blood 4 days after the initial HN₂ injection (Table I). 5 had no detectable granulocytes in the circulating blood and 1 had 6 basophils per mm.³ as the only granulocytes.

Lethality of Endotoxin in Leukopenic Animals:

Ten rabbits, made leukopenic with HN₂, received 0.5 ml. of commercial typhoid vaccine intravenously at the time of maximal leukopenia. All died within 6 hours. 5 additional leukopenic animals died within 6 hours following intravenous injection of 20 µg. of *E. coli* lipopolysaccharide. Numerous control animals have been given doses of equivalent and greater magnitude without significant mortality. None of the leukopenic animals given 5 to 12 µg. of *E. coli* lipopolysaccharide (approximately 2 to 4 µg./kg.) died within 6 hours (Table I). Some of these animals died within 24 hours but the majority survived a second and third daily injection of the same dose of endotoxin. The

animals that died within 24 hours after receiving endotoxin had either no detectable circulating granulocytes or only basophilic granulocytes (Table I). Recovery from granulocytopenia was apparent in all animals surviving two or three daily injections of endotoxin. Animals with agranulocytosis or with basophils as the only kind of granulocyte appear to have an increased susceptibility to the lethal effects of endotoxin.

Febrile Response of Agranulocytic Animals to Endotoxin:

Six rabbits with less than 1000 leukocytes per mm.³ of blood, and no circulating granulocytes other than basophils, failed to develop significant fever

TABLE I
Lethality of Endotoxin in Granulocytopenic Animals

Granulocytes circulating per mm. ³ blood	1st injection		2nd injection		3rd injection	
	No. injected/No. dead in 24 hrs.		No. injected/No. dead in 24 hrs.		No. injected/ No. dead in 24 hrs.	
	12 μ g.	5 μ g.	12 μ g.	5 μ g.	12 μ g.	5 μ g.
0	2/1	3/3	2/2	1/1	0	0
1-49	4/1*	20/0	0	2/2*	0	0
50-99	0	2/0	2/0	2/0	0	0
100-500	0	2/0	0	2/0	0	0
> 500	0	1/0	0	15/0	2/0	19/0
Totals	6/2	28/3	4/2	22/3	2/0	19/0

* Only basophils circulating.

after the first injection of endotoxin (Table II). 4 other animals, with circulating granulocytes and a febrile response to the first endotoxin injection, failed to develop fever when given a second injection of endotoxin 24 hours later. At this time only basophilic granulocytes were present in the blood. 1 animal exhibited agranulocytosis on 2 successive days; no fever was produced on either day by 5 μ g. of endotoxin. Eleven injections of endotoxin, representing either the first or second exposure, were given to animals with no circulating granulocytes or only basophils, but none developed significant fever during the 6 hour test period. No difference in the response to endotoxin injection has been observed between animals without circulating granulocytes and those with only basophils. Neither developed significant fever.

Febrile Response of Granulocytopenic Animals to Endotoxin:

Table III shows the febrile response to endotoxin of severely leukopenic animals with a few granulocytes other than basophils in the circulation. 4

TABLE II
Maximum Fever Response in the Absence of Circulating Granulocytes

No. of animals	Administration of endotoxin		Mean baseline temp. °C.	Maximum fever, °C.	
	Dose	Quantity		Min. 40-100	Min. 180-240
4*	1st	5 μg.	39.90	0.23	0
3‡	2nd	5	40.48	0.27	0
2	1st	12	40.65	0.13	0
2	2nd	12	38.90	0.10	0
5§	0	0	39.54	0.24	0

* 1 animal with 6 basophils only per mm.³ of blood.

‡ 2 animals with basophils only; one with 33 and one with 37 per mm.³ of blood.

§ Control animals received no endotoxin; temperature recorded for 6 hours.

TABLE III
Endotoxin Fever in HN₂-Treated Animals

Circulating granulocytes per mm. ³	Endotoxin dose	No. of animals	Baseline temp. °C Mean ± s.e.	Maximum Fever °C.	
				60-120 min. Mean ± s.e.	160-200 min. Mean ± s.e.
Group I 10-50	5 μg.	20	39.70 ± 0.15	1.00 ± 0.18	1.24 ± 0.10
Group II (Controls) 3226 ± 1893	5	15	39.21 ± 0.14	1.59 ± 0.12	2.33 ± 0.17
Group III (24 hrs. after HN ₂) 3973 ± 1063	5	4	39.89 ± 0.33	1.33 ± 0.19	1.79 ± 0.17
Group I A 10-50	12	4	38.96 ± 0.23	1.09 ± 0.19	1.88 ± 0.11
Group II A (Controls)	12	2	39.00 ± 0.50	1.65 ± 0.05	2.55 ± 0.25

days after the initial injection some animals treated with HN₂ were obviously ill, showing lassitude, an unkempt appearance, and, occasionally, diarrhea. In these characteristics, animals with no circulating granulocytes (or only basophils) showed no difference from animals with a few circulating granulocytes other than basophils. The results in granulocytopenic animals (group I) are compared with results in normal animals (group II) and with results in animals treated with HN₂ 24 hours prior to endotoxin administration (group III). Each of these animals received 5 μg. of endotoxin. Group III was included

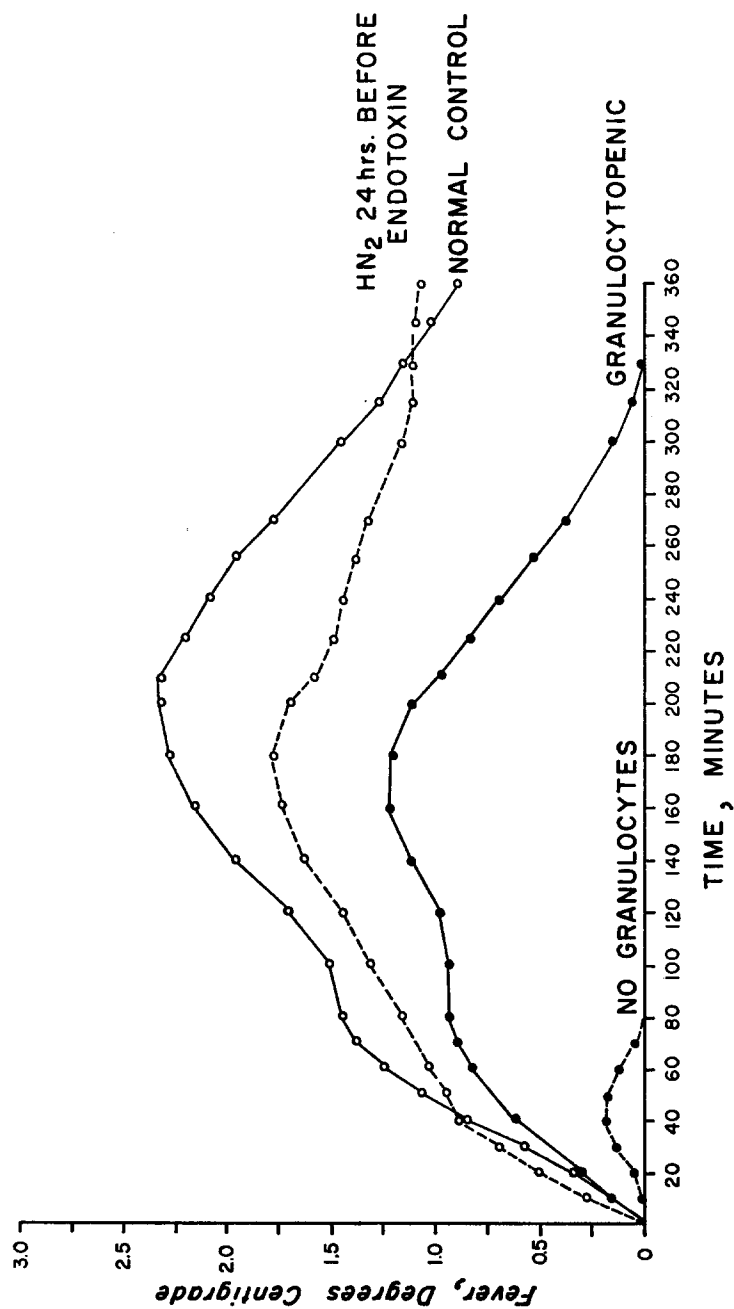


FIG. 1. Mean febrile response of rabbits to 5 μ g. intravenous *E. coli* lipopolysaccharide. Controls comprise 15 animals, granuloctopenics, 20 animals, and non-granuloctopenics receiving HN₂ 24 hours before endotoxin, 4 animals. Of 4 animals with "no granulocytes," 1 had a few basophils.

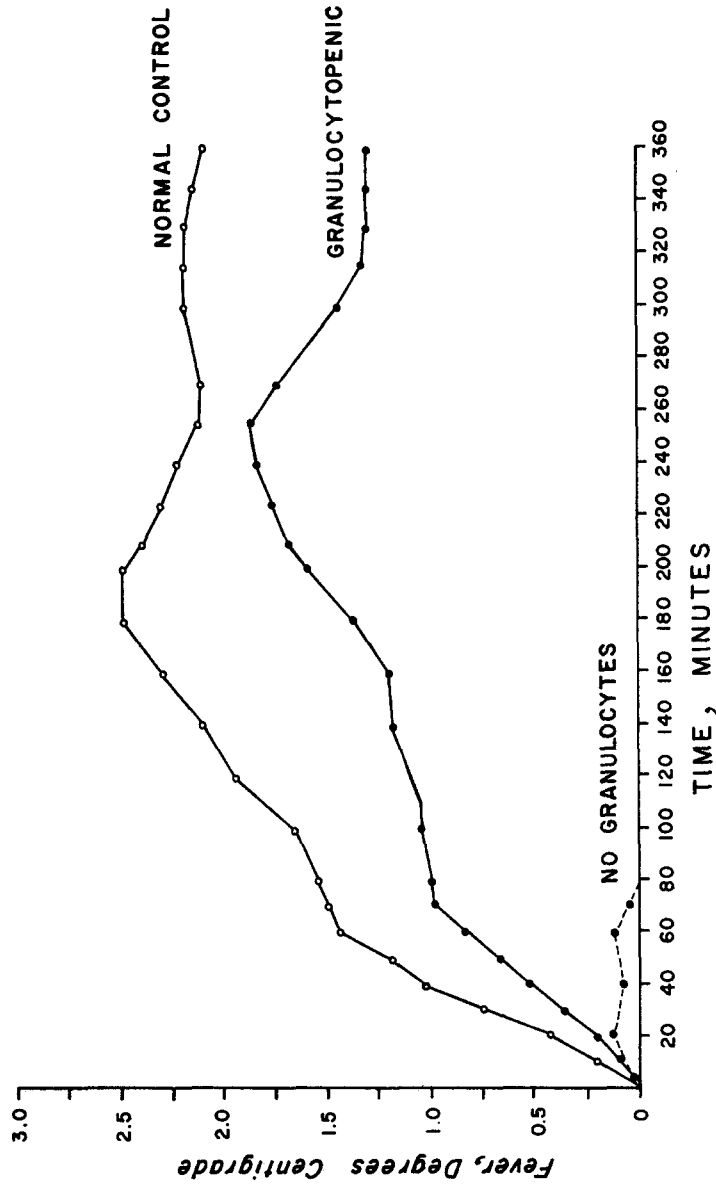


FIG. 2. Mean febrile response of rabbits to 12 μ g. intravenous *E. coli* lipopolysaccharide. Controls comprise 2 animals, granulocytopenics, 4 animals, and those with no granulocytes, 2 animals.

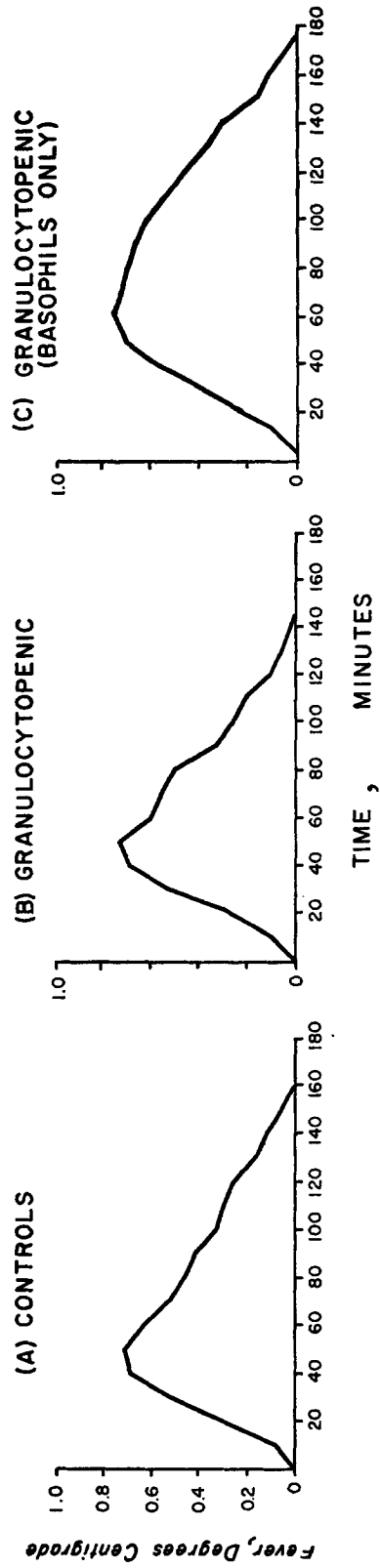


FIG. 3. Mean febrile response of rabbits to 10 ml. of serum containing endogenous pyrogen. *A* Normal animals; *B* Granuloctypenic animals (50 to 250 granuloctyes per mm.³); *C* Granuloctypenic animals with basophils only (20 to 26 per mm.³).

to determine whether HN_2 alters the febrile response to endotoxin prior to the appearance of significant granulocytopenia. The mean fever curves of these three groups are shown in Fig. 1. The insignificant febrile response of animals with no circulating granulocytes is shown for comparison. 4 other granulocytopenic animals (group I *A*) and 2 other normal animals (group II *A*) were each given 12 μg . of endotoxin. The mean fever curves of these two groups are shown in Fig. 2; the response of 2 animals with no circulating granulocytes to 12 μg . of endotoxin is included for comparison.

5 μg . of endotoxin produced an earlier maximum elevation of temperature in the granulocytopenic group (160 minutes) than in the HN_2 -treated, non-granulocytopenic group (180 minutes); both of these peaked earlier than the control group (200 minutes). The difference between the maximum fever developed by the granulocytopenic group and by the control group is highly significant ($p = < 0.01$). The difference between the maximum fever of the HN_2 -treated, non-granulocytopenic group and the maximum fever of the control group is not significant. An analysis of the fever curves of these three groups by the non-parametric sign test, however, reveals a significant difference between each of the curves. This suggests that the febrile response to endotoxin is modified by HN_2 prior to the onset of granulocytopenia. The febrile response at the time of maximum granulocytopenia is less than the febrile response of normal animals or HN_2 -treated, non-granulocytopenic animals, each given the same dose of endotoxin.

Response of Granulocytopenic Animals to Endogenous Pyrogen:

The ability of severely granulocytopenic animals to develop fever from a stimulus other than endotoxin was tested with endogenous pyrogen contained in 10 ml. of 120 minute serum. Fig. 3 *A* shows the mean febrile response of 5 control animals, Fig. 3 *B* the mean response of 5 animals with 50 to 250 granulocytes per mm^3 of blood, and Fig. 3 *C* the mean response of 4 animals with no circulating granulocytes other than basophils to the same dose of endogenous pyrogen. It is apparent that granulocytopenic animals and animals with only basophils develop as much fever as control animals from endogenous pyrogen.

DISCUSSION

The failure, in previous studies, to detect differences in the febrile response to endotoxin between normal and leukopenic animals may have resulted from the use of small doses of endotoxin; a submaximal fever was produced in the normal animals (14). We have observed a submaximal febrile response in normal rabbits with doses of *E. coli* lipopolysaccharide less than 1.5 μg . per kg. With equivalent doses of endotoxin there may be produced quantitatively less endogenous serum pyrogen in leukopenic than in normal animals. This could explain the failure of normal animals to develop fever following injection

of 120 minute serum from these leukopenic animals. The results of our study suggest such a quantitative difference; the amount of fever developed by leukopenic animals is roughly proportional to the degree of persistence of granulocytes. When a few circulating granulocytes remain, a biphasic febrile response to endotoxin occurs but this is significantly less in magnitude than in control animals. Agranulocytic animals develop no significant fever after receiving endotoxin. When the number of circulating granulocytes is reduced, the amount of endogenous pyrogen released is diminished; in severely granulocytopenic animals it may be sufficiently reduced to be undetectable by assay in another animal, although sufficient to produce a modified febrile response in the leukopenic animal. The agranulocytic animals and those with only basophilic granulocytes, which develop no fever after endotoxin injection, exhibit a febrile response to endogenous pyrogen equal to that of normal control animals. These findings support the hypothesis that leukocyte endogenous pyrogen is a necessary intermediate in endotoxin fever.

Nitrogen mustard may modify endotoxin fever prior to development of granulocytopenia. This might result from a reduction in total available granulocytes not reflected by the number in the circulation. The possibility exists, however, that mechanisms not concerned with leukocytes are responsible for this effect of HN_2 .

Previous studies (20, 21) suggest that of the granulocytes the basophil is most resistant to suppression by HN_2 . Our observations support this. Animals in which granulocytes comprised only basophils developed no fever following endotoxin injection; this response was similar to that of agranulocytic animals. With even a few circulating granulocytes other than basophils definite, although modified, fever regularly occurred. This suggests that basophils may be excluded as a source of endogenous pyrogen.

The enhanced lethality of endotoxin in leukopenic animals has been previously noted in irradiated (18) and HN_2 -treated (12) animals. Labeled endotoxin given intravenously has been found to concentrate promptly in the buffy coat (19). *In vitro* incubation with granulocytes results in the inactivation of endotoxin (10). Thus granulocytes may play a major role in the *in vivo* inactivation of endotoxin; in their absence a given dose of endotoxin may produce more intense biological effects.

SUMMARY AND CONCLUSIONS

The febrile response to bacterial endotoxin was measured in rabbits made leukopenic with nitrogen mustard. A striking increase in susceptibility to the lethal effects of endotoxin occurred in severely leukopenic animals. Animals without circulating granulocytes, or with only basophils, developed no significant fever after endotoxin injection. Animals with circulating granulocytes other than basophils exhibited a biphasic febrile response to endotoxin; this

response was significantly less in magnitude than that of control animals. Control animals, severely granulocytopenic animals, and animals with no circulating granulocytes other than basophils showed comparable febrile responses to serum pyrogen.

These results suggest that granulocytes inactivate endotoxin *in vivo* and support the hypothesis that leukocyte pyrogen is a necessary intermediate in endotoxin fever. Basophils do not appear to participate in this process. These observations also contradict previous studies that were taken to indicate a normal febrile response to endotoxin in leukopenic animals and suggest that those results are related to the persistence of granulocytes.

BIBLIOGRAPHY

1. Wood, W. B., Jr., The pathogenesis of fever, *Am. J. Med.* 1955, **18**, 351.
2. Wood, W. B., Jr., The role of endogenous pyrogen in the genesis of fever, *Lancet*, 1958, **2**, 53.
3. Beeson, P. B., Temperature-elevating effect of a substance obtained from polymorphonuclear leukocytes, *J. Clin. Inv.*, 1948, **27**, 524.
4. Bennett, I. L., Jr., and Beeson, P. B., Studies on the pathogenesis of fever. II. Characterization of fever-producing substances from polymorphonuclear leukocytes and from the fluid of sterile exudates, *J. Exp. Med.*, 1953, **98**, 493.
5. King, M. K., and Wood, W. B., Jr., Studies on the pathogenesis of fever. III. The leukocytic origin of endogenous pyrogen in acute inflammatory exudates, *J. Exp. Med.*, 1958, **107**, 279.
6. Atkins, E., and Wood, W. B., Jr., Studies on the pathogenesis of fever. I. Presence of transferrable pyrogen in the blood stream following the injection of typhoid vaccine, *J. Exp. Med.*, 1955, **101**, 519.
7. Atkins, E., and Wood, W. B., Jr., Studies on the pathogenesis of fever. II. Identification of an endogenous pyrogen in the blood stream following the injection of typhoid vaccine, *J. Exp. Med.*, 1955, **102**, 499.
8. King, M. K., and Wood, W. B., Jr., Studies on the pathogenesis of fever. IV. The site of action of leukocytic and circulating endogenous pyrogen, *J. Exp. Med.*, 1958, **107**, 291.
9. Herion, J. C., Walker, R. I., and Palmer, J. G., Relation of leukocyte and fever responses to bacterial endotoxin, *Am. J. Physiol.*, 1960, **199**, 809.
10. Collins, R. D., and Wood, W. B., Jr., Studies on the pathogenesis of fever. VI. The interaction of leukocytes and endotoxin *in vitro*, *J. Exp. Med.*, 1959, **110**, 1005.
11. Wood, W. B., Jr., Studies on the cause of fever, *New England J. Med.*, 1958, **258**, 1023.
12. Bennett, I. L., Jr., and Cluff, L. E., Influence of nitrogen mustard upon reactions to bacterial endotoxin: Schwartzman phenomenon and fever, *Proc. Soc. Exp. Biol. and Med.* 1952, **81**, 304.
13. Petersdorf, R. G., and Bennett, I. L., Jr., Studies on the pathogenesis of fever. VIII. Fever-producing substances in the serum of dogs, *J. Exp. Med.*, 1957, **106**, 293.

14. Freedman, H. H., Further studies on the passive transfer of tolerance to pyrogenicity of bacterial endotoxin. The febrile and leukopenic responses, *J. Exp. Med.*, 1960, **112**, 619.
15. Bennett, I. L., Jr., Petersdorf, R. G., and Keene, W. R., Pathogenesis of fever: evidence for direct cerebral action of bacterial endotoxins, *Tr. Assn. Am. Physn.*, 1957, **60**, 64.
16. Bennett, I. L., Jr., and Beeson, P. B., Studies on the pathogenesis of fever. I. The effect of injection of extracts and suspensions of uninfected rabbit tissues upon the body temperature of normal rabbits, *J. Exp. Med.*, 1953, **98**, 477.
17. Bennett, I. L., Jr., Studies on the pathogenesis of fever. III. Failure to demonstrate fever-producing substances in the tissues of leukopenic rabbits, *Bull. Johns Hopkins Hosp.*, 1956, **98**, 1.
18. Smith, W. W., Alderman, I. M., and Gillespie, R. E., Resistance to experimental infection and mobilization of granulocytes in irradiated mice treated with bacterial endotoxin, *Am. J. Physiol.*, 1958, **192**, 263.
19. Braude, A. I., Carey, F. J., and Zalesky, M., Studies with radioactive endotoxin. II. Correlation of physiologic effects with distribution of radioactivity in rabbits injected with lethal doses of *E. coli* endotoxin labelled with radioactive sodium chromate, *J. Clin. Inv.*, 1955, **34**, 858.
20. Block, M., Spurr, C. L., Jacobson, L. O., and Smith, T. R., Histopathologic effects of nitrogen mustard therapy upon normal and neoplastic hematopoietic tissues, *Am. J. Clin. Path.*, 1948, **18**, 671.
21. Karnofsky, D. A., Graef, I., and Smith, H. W., Studies on the mechanism of action of the nitrogen and sulfur mustards *in vivo*, *Am. J. Path.*, 1948, **24**, 275.