THE PRESENCE OF ENDOGENOUS PYROGEN IN NORMAL RABBIT TISSUES*

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The presence of an endogenous pyrogen within rabbit polymorphonuclear leucocytes was first reported by Beeson (1) and later confirmed in more detailed studies by Bennett and Beeson (2, 3). Although endogenous pyrogen was not found in other rabbit tissues, suspicions have persisted that there may be alternative sources of this material.

Since there are clinical and experimental fevers in which the granulocyte does not appear to be implicated, we have reexamined uninfected rabbit tissues for pyrogen. In sufficient dosage, all tested tissues yielded pyrogen and this paper describes the methods used and some properties of the pyrogen. Evidence is presented that tissue pyrogen is not derived from Gram-negative bacteria or from polymorphonuclear leucocytes. Some of these findings have been reported briefly elsewhere (4, 5).

Methods

Rabbits of New Zealand white, chinchilla, brown, and albino strains, 2.5 to 3.5 kg in weight, were used both as donors of tissue and as recipients. Pyrogen assay was conducted in a quiet, temperature-controlled room with trained rabbits which responded consistently to pyrogen. Animals were restrained in wooden stalls and their temperatures were automatically recorded every 8 minutes from an indwelling rectal thermistor. Only rabbits whose temperature varied by less than 0.2°C for 1 hour before the start of the experiment were injected. All injections were made into the marginal ear vein.

Because of the occasional deaths or adverse reactions following inoculation of extracts, most animals were given 2500 to 5000 units heparin immediately prior to or mixed with the injection. Where possible, doses were adjusted to give a temperature rise of 0.5–1.0°C. Response was measured both as the maximum rise in 2 hours after injection and, by planimetry, as the area under the fever curve in °C hours without arbitrary limitation of its duration (fever area).

All glassware and apparatus had been rendered pyrogen-free by heating to 170°C for 3 hours or by sterilizing with 10 per cent benzalkonium chloride (roccal, Bayer Products Ltd., England) and rinsing many times, the final washings being tested for pyrogenicity. Sodium chloride in all instances was a 0.15 M pyrogen-free solution which is referred to as "saline."

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Bacterial endotoxin was a purified preparation from *Proteus vulgaris* kindly supplied by Organon Laboratories Ltd., England (E pyrogen). The dose of this pyrogen inducing fever of 0.5–1.0°C was about 0.03 μg when injected in a saline medium or 0.003 μg when mixed with plasma or serum.

Tissue extracts were prepared from healthy donor rabbits some of which had been treated

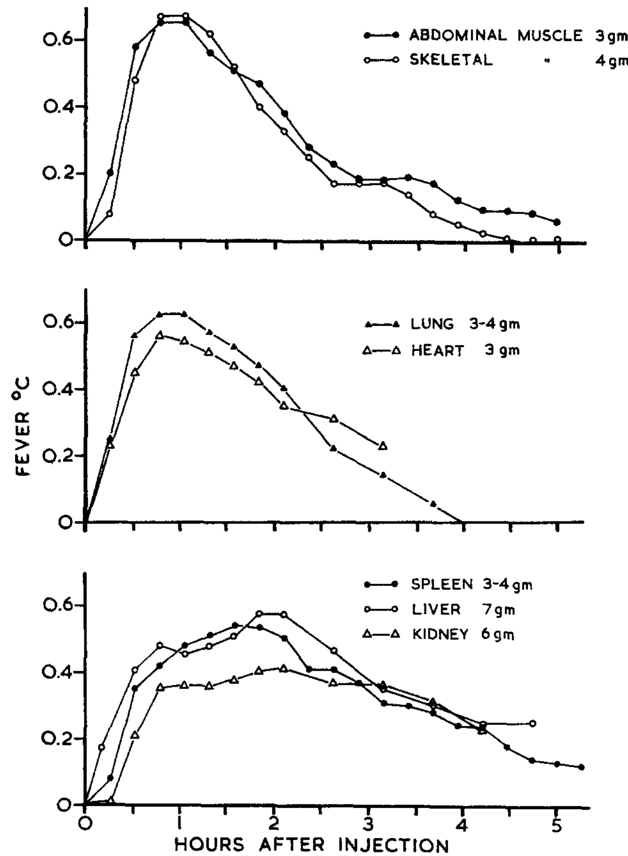

Fig. 1. Mean fever curves of 5 or 6 recipients to saline extracts of various tissues. Dose (expressed as the wet weight of tissue from which the extract was derived) is shown together with the type of tissue on the individual charts.

with oral tetracycline. White blood cell counts were regularly done immediately before the experiment and animals with abnormal values suggesting infection were eliminated. The rabbit was then anesthetized with intravenous nembutal, shaved, and washed in a warm detergent solution and heparin (10,000 units) administered intravenously. Most animals were then bled out by cardiac puncture, with a simultaneous infusion of 100 to 200 ml saline into an ear vein to ensure maximal removal of blood.

After death, the animal was dissected with sterile techniques and the organs or tissues placed in sterile vessels immersed in ice. Homogenates of tissues were prepared with a Waring blendor using either a 800 ml glass jar or a 250 ml stainless steeel jar. In the former, 100 to

300 gm tissue wet weight were mixed with 125 to 375 ml saline and in the latter 5 to 80 gm tissue wet weight were mixed with 100 ml saline. In most cases, saline mixed with tissues contained streptomycin 50 mg and penicillin 20,000 units per 100 ml. Blending was carried out for various periods both at 4°C and room temperature and tissues kept on ice except when being homogenized. Optimum pyrogen yields were obtained with a 2 minute period of blending which was usually done at room temperature, although environmental temperature at the two levels employed did not appear to be critical. Histological examinations of homogenates showed only some recognizable fibrous tissue cells and collagen. After homogenization, the material was transferred to steel centrifuge tubes of 100 ml capacity and centrifuged at about 10°C at 20,000 *g* for 30 minutes. After this treatment, the deposit of tissue weighed about the same as the original wet weight of tissue and the volume of supernatant fluid was similar to the amount of saline added initially. Supernates were either injected immediately or stored at 4°C. Bacterial cultures were performed in glucose broth and meat broth on blood and portions of tissue removed both at the time of dissection and after their homogenization. After 48 hours incubation, subcultures were made onto blood agar which was incubated aerobically and anaerobically. Culture for virus was unsuccessful since tissue extracts were toxic to tissue culture cells. The only organ to show a high proportion of positive cultures (about 40 per cent) was lung, the organism consisting of a spore-bearing Gram-positive rod (possibly emanating from hay in the diet) or a pleomorphic Gram-negative rod. These and the rare instances of blood or other tissue infections have been excluded from the results. Occasional cultures (about 10 per cent) produced a low concentration of Gram-positive cocci, almost certainly a contaminant from the atmosphere; extracts of these tissues differed in no way from those with negative cultures and so they have not been excluded.

Blood removed from the donor animals was tested for pyrogenicity in several ways. Whole blood, or packed cells resuspended in saline, was incubated at 37°C for 3 to 16 hours and the supernatant injected into recipient animals in volumes equivalent to 8×10^8 or less original total white blood cells. In most instances plasma, separated immediately from blood, was also injected in amounts of up to one-half the total obtained from the donor. From 4 animals, whole blood, or packed cells resuspended in saline, were blended for 2 minutes in the Waring blendor and subsequently centrifuged at 20,000 *g* for 30 minutes; the supernatant was injected in doses equivalent to 3.2×10^8 or less total white cells. Blended blood occasionally evoked a delayed temperature rise; otherwise, none of these preparations of blood produced fever. None of them produced the rapid, transient fevers characteristic of leucocyte or tissue pyrogen. The results with blood are presented in full in another paper (6).

RESULTS

Characteristics of Fever Produced by Tissue Pyrogens.—Mean febrile responses to intravenous injections of extracts from 7 tissues are shown in Fig. 1. Fever curves produced by extracts of abdominal muscle, skeletal muscle, heart, and lung were similar in shape; temperature rise was well established within 30 minutes of injection and had largely subsided by 3 hours. In these respects, the curves resemble responses to leucocyte pyrogen and differ from those to endotoxin. Extracts of spleen, liver, and kidney produced fevers which were lower and broader and, with the exception of the response to liver extract, were slower in onset. Since larger doses sometimes produced an initial fall in temperature with temporary weakness or even death of the animal, noxious agents in extracts of these tissues may have contributed to the altered patterns of fever produced by smaller doses. The other extracts were administered in doses up

to 30 gm without noticeable ill-effects although an occasional animal died in convulsions immediately after injection. With all extracts it was necessary to add heparin to avoid occasional fatal reactions to quite small doses.

Variation in responses of different animals to the same material appeared to be no greater than that seen with endotoxin or leucocyte pyrogen and responses were not affected by previous injections of the same or other tissue extracts.

Comparison of Dose-Response Curves of Tissue Pyrogen and Endotoxin in Serum.—The dose-response curve of tissue pyrogen from a single pool of skeletal

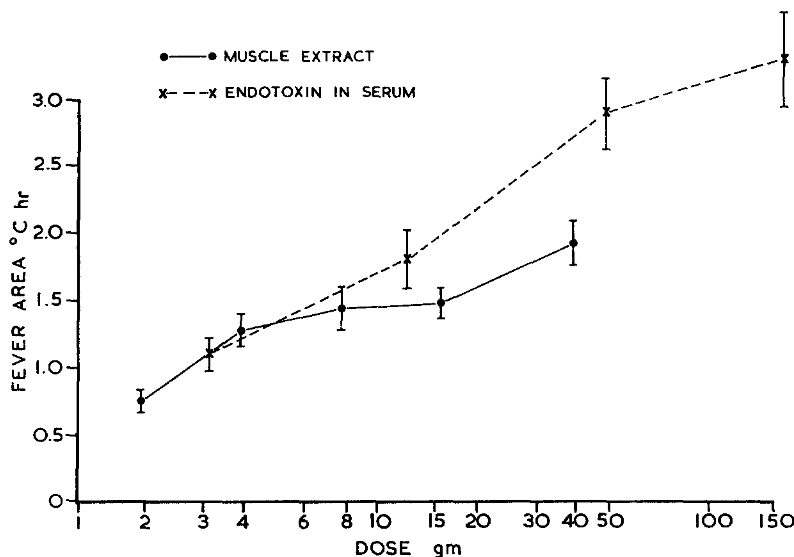

FIG. 2 *a*. Response (mean ± SE) measured as area of fever to graded doses of a pool of skeletal muscle extract derived from 2 donors. Four doses were injected into the same 10 recipients and the fifth (largest) dose into 5 of them. Also shown for comparison is the response (mean ± SE) of a different group of 8 rabbits to 4 graded doses of endotoxin in serum (each four times the preceding dose); the smallest dose contained 0.00075 μg endotoxin in 0.25 ml serum. This curve has been arbitrarily fitted to coincide with the muscle extract curve at a response of 1.10°C hour fever area, corresponding to a tissue dose of 3 gm.

muscle extract was next investigated and compared with similar curves obtained with endotoxin in serum and with leucocyte pyrogen. The results are shown in Figs. 2 *a* and 2 *b* where the mean responses (± SE's) of both fever height and area plotted against dose of muscle extract on a logarithmic scale.[1]

[1] When dose is expressed linearly against response the correlation coefficient was 0.48 ($0.01 > p > 0.001$) for height of fever and 0.56 ($p < 0.001$) for area; with dose expressed in logarithmic form the correlation coefficients were 0.63 ($p < 0.001$) and 0.64 ($p < 0.001$) respectively. This indicates a superiority of correlation of response with the logarithm of the dose (as might have been expected on general grounds) although comparisons of correlation coefficients with the *z* transformation test showed that significance of the differences did not attain the 5 per cent level.

Fever height increased linearly and significantly with the lowest three doses but failed to increase above a dose of 7.6 gm. Fever area increased significantly with the lowest two doses but then failed to increase significantly until the largest dose. Fever height is more conveniently measured than area and is more accurate because it is often difficult to determine when the base line has been resumed with some tissue fevers (Fig. 1). As will be seen below, dose of other tissues was usually correlated better with height than area of fever. These results show that assays for these pyrogens should be conducted with doses giving fevers less than 0.8°C in height or 1.4°C hour in area (that is of less than 8 gm

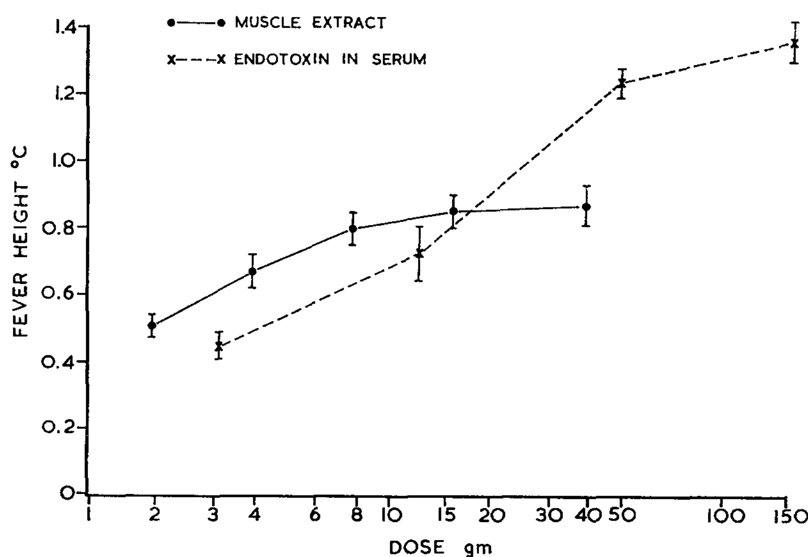

FIG. 2 *b*. The same experiment shown in Fig. 2 *a* but with responses expressed as fever height (mean ± SE).

tissue). As an index of response within this dose range, fever height appears to be at least as satisfactory as area.

A similar dose-response curve for endotoxin in serum (with means and SE) is shown for comparison with the curve obtained with tissue extract in Figs. 2 *a* and 2 *b*. Since the two types of pyrogen have different units of dose, the two curves have been arbitrarily fitted to coincide at a response of 1.10°C hour area of fever (corresponding with a tissue dose of 3 gm). The exact point of coincidence, however, does not affect the two important differences between the curves. Firstly, the endotoxin curve lies below the tissue curve when response is expressed as height of fever because endotoxin fevers are lower than tissue fevers of the same area. Secondly, taken as a whole, the slope of the endotoxin dose-response curve (expressed as either height or area of fever) is significantly steeper than that of muscle pyrogen though a plateau of response is reached

with each pyrogen. Bornstein, Bredenburg, and Wood (7) have shown that dose-response curves for leucocyte pyrogen attain a similar "ceiling." Their curve obtained with leucocyte pyrogen is steeper than our tissue pyrogen curves and lies parallel to our endotoxin curve, but above it when response is measured as height of fever. The relationship between fever height and area for the 3 types of pyrogen is illustrated in Fig. 3 which shows mean fever curves of similar height induced by leucocyte pyrogen, endotoxin in serum and muscle extract.

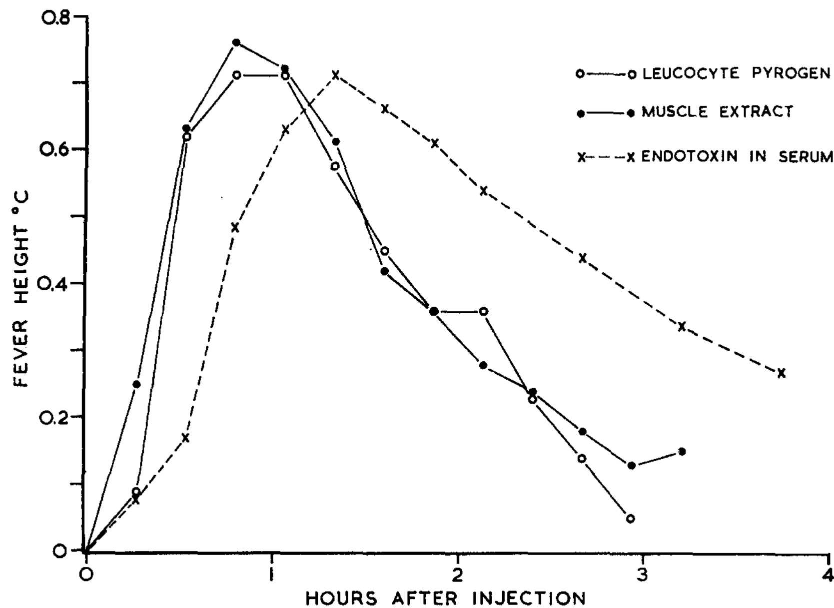

Fig. 3. Mean fever curves produced by three pyrogens, each injected into 8 rabbits. Leucocyte pyrogen was prepared from a mixture of whole blood with purified endotoxin incubated at 37°C for 4 hours and then centrifuged; each dose was a supernate equivalent to 0.003 μg endotoxin and 1.6×10^8 leucocytes. Endotoxin in serum was injected in a dose of 0.003 μg endotoxin with 1 ml serum after the mixture had been incubated at 37°C for 15 minutes. Muscle extract was a preparation of skeletal muscle injected in a dose obtained from 7.6 gm wet weight of tissue.

The curves for muscle extract and leucocyte pyrogen coincide closely except for the more rapid response of the tissue curve. The curve for endotoxin in serum is appreciably delayed in onset and broader than the other curves. The fever pattern and dose-response curve to tissue pyrogen appear to distinguish this agent from Gram-negative bacterial endotoxins; other means of differentiating these substances are examined below.

Since extracts of the various tissues were not injected in a range of very low doses, a minimal pyrogenic dose was not established. However, over 90 per cent of recipients responded to the following dosages with significant fever (>0.3°C):

spleen and abdominal muscle, 1.5 gm; skeletal muscle, 2.0 gm; liver 2.5 gm; heart, lung, and kidney 3.0 gm.

Correlation of Pyrogen Content of Similar Tissues from Different Donor Animals.—Extracts of the same organs from different donors were compared for variation in content of tissue pyrogen. Such a comparison is only valid at low dosage, because of the dose-response relationship previously described, and with large groups of recipients, because of the variation in response of recipients.

TABLE I

Comparison of Pyrogenic Responses (Means and 95 Per Cent Confidence Limits) to Similar Doses of Tissue Extracts Obtained from Different Donors

Tissue	No. donors	Dose	Fever responses		
			Height	Area	No.
		gm	*°C*	*°C hr.*	
Skeletal muscle	2	2.0	0.51 ± 0.07	0.75 ± 0.19	10
	1	2.5	0.49 ± 0.08	*0.91 ± 0.19	15
	1	2.4	0.45 ± 0.10	*0.61 ± 0.25	15
	1	2.2	0.46 ± 0.07	0.72 ± 0.17	13
Abdominal muscle	3	4.6	0.79 ± 0.23	1.71 ± 0.65	7
	2	4.7	0.76 ± 0.17	1.41 ± 0.37	8
	3	6.4	‡0.56 ± 0.15	1.57 ± 0.40	7
	1	5.0	‡0.80 ± 0.17	1.38 ± 0.37	6
Liver	2	7.5	0.55 ± 0.13	1.48 ± 0.52	10
	3	7.5	0.62 ± 0.11	1.73 ± 0.48	7

* $t = 2.02, 0.10 > p > 0.05$.
‡ $t = 2.67, 0.05 > p > 0.02$.

Data for extracts of skeletal muscle, abdominal muscle, and liver are shown in Table I. Extracts from pools of 2 or 3 donors are also included since tissues of small organs must often be pooled in studies of this kind. Mean fever responses to the same tissue from different donors or donor pools usually lay within the 95 per cent confidence limits of other means in the group; in the two cases where they do not, "Student" t test has been applied and the results are shown at the foot of Table I. A significant difference in fever height was found in only one instance, between two extracts of abdominal muscle. On the basis of these findings it seems justifiable to group together data on tissues obtained from different donors as is shown in Figs. 4 *a* and 4 *b*. Individual responses (as fever area) to extracts of lung, spleen, liver, and abdominal muscle are plotted against the logarithm of the dose (less than 8 gm in all cases) and the regression line for response on dose is given. Also shown for comparison are responses to the lowest 3 doses of the dose-response curve for skeletal muscle extract taken from Figs. 2 *a* and 2 *b*. Correlation coefficients and regression equations for these data

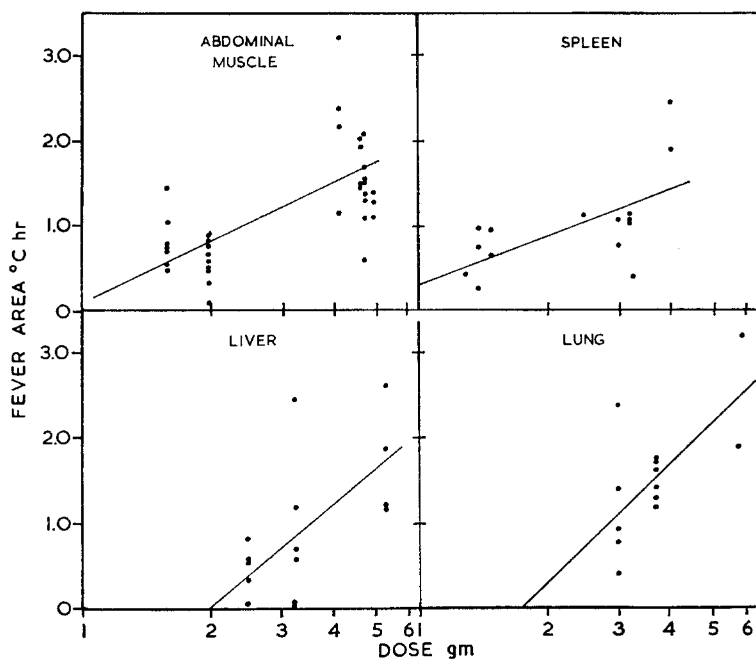

FIG. 4 *a*

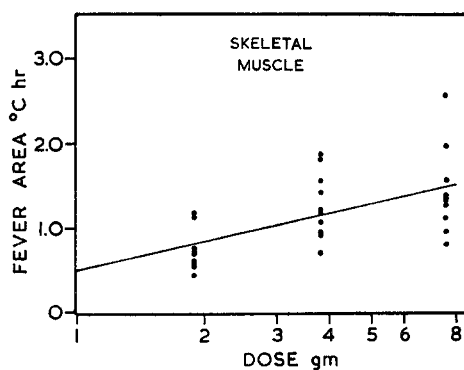

FIG. 4 *b*

FIGS. 4 *a* and 4 *b*. Individual responses to extracts of tissues from different donors with regression lines (regression equations shown in Table II). Dose of tissue is represented logarithmically. Skeletal muscle responses were taken from the experiment shown in Fig. 2 *a*. Responses are shown as fever areas since, for spleen extract, the correlation coefficient between log dose and fever height was not significant; with this exception correlation coefficients between log dose and fever height were higher than those between log dose and fever areas (Table II).

are given in Table II together with similar data for the lowest three doses of skeletal muscle extract taken from the dose-response curve shown in Figs. 2 *a* and 2 *b*.

Comparison of Tissue Pyrogens with Bacterial Endotoxin.—

Rapidity of response: At present, the most readily available criteria for distinguishing endogenous pyrogens from bacterial endotoxin are their biological properties. The distinction becomes more difficult if endotoxin is in a protein medium because it then more closely resembles endogenous pyrogens. This problem has been examined briefly elsewhere (5). In this paper, endotoxin has always been administered after incubation for at least 10 minutes in stored plasma or serum, when the modifying effect is fully achieved.

The rapidity of fever response to tissue extracts is illustrated by Figs. 1 and 3. In a number of individual pyrogenic responses, latency was measured in

TABLE II

Correlation Coefficients and Regression Equations (Response on Logarithm of Dose in gm) for Tissue Extracts Injected in Doses of Less Than 8 gm tissue

Tissue	No.	Fever response					
		Height °C			Area °C hr.		
		µg	*p*	Equation	µg	*p*	Equation
Skeletal muscle	30	0.65	<0.001	0.462 log dose + 0.396	0.58	<0.001	1.132 log dose + 0.502
Abdominal muscle	35	0.73	<0.001	1.135 log dose + 0.117	0.65	<0.001	2.323 log dose + 0.125
Liver	16	0.75	<0.001	2.210 log dose − 0.642	0.65	0.01 > p > 0.001	4.11 log dose − 1.252
Lung	13	0.72	0.01 > p > 0.001	1.985 log dose − 0.412	0.66	0.01 > p > 0.001	4.760 log x − 1.138
Spleen	15	0.40	>0.1	—	0.63	0.01 > p > 0.001	1.859 log x + 0.309

minutes as the interval between injection and the first progressive temperature rise exceeding base line by 0.15°C. Fever latency varies with the dose given, as well as with the type of pyrogen, and so for both endotoxin and tissue pyrogens mean latencies have been calculated in three groups, according to the magnitude of response. The results are shown in Table III. Latency of response to endotoxin is more dependent on dose than is the case with tissue pyrogens which in this respect resemble leucocyte pyrogen (6). Hence differences in latencies are more apparent with small responses. In the two groups of smaller responses, extracts of all tissues, except kidney, showed delays that were significantly shorter than those from endotoxin. Kidney extracts, as well as larger doses of spleen and liver extracts, produced a great variability in fever latency and for these tissues there were many individual examples of delays less than any of the endotoxin responses of the same size. The longer delays were probably related to noxious effects produced in the recipients by these 3 extracts which

TABLE III

Comparison of Delay in Onset of Fever after Injection of Various Pyrogens. Mean Delays and 95 Per Cent Confidence Limits in Minutes are Shown for Fevers of Three Orders of Magnitude

Source of pyrogen	Fever height		
	0.25–	0.65–	1.05+
	°C	°C	°C
Endotoxin in plasma or serum	40.0 ± 1.9 (36)	30.8 ± 1.7 (32)	24.2 ± 2.1 (28)
Skeletal muscle	24.2 ± 2.3 (30)	19.2 ± 1.9 (30)	16.0 ± 2.6 (14)
Abdominal muscle	21.6 ± 1.7 (20)	18.7 ± 1.4 (30)	16.7 ± 1.6 (11)
Liver	24.0 ± 3.0 (35)	24.0 ± 5.6 (17)	23.0 ± 9.8 (8)
Kidney	31.2 ± 9.9 (10)	36.6 ± 14.6 (11)	
Heart	16.0 ± 5.3 (6)	20.0 ± 3.5 (8)	16.0 (1)
Lung	16.0 ± 0.0 (5)	19.1 ± 2.9 (9)	16.0 (1)
Spleen	27.1 ± 4.6 (13)	21.3 ± 8.6 (3)	32.0 (1)

The figures in parentheses show the number of responses.

TABLE IV

Response (Mean ± 95 Per Cent Confidence Limits) to Various Pyrogens Before and the Day Following Challenge with 1.0 μg Purified Endotoxin (Proteus vulgaris) Intravenously

Pyrogen	Dose	No. Responses	Fever height	
			Before challenge	After challenge
			°C	°C
Purified endotoxin (*Proteus vulgaris*)	0.003 μg in 2 ml serum	7	0.81 ± 0.17	0.13 ± 0.05
Typhoid-paratyphoid A and B vaccine*	2 ml of 1 in 8000 in diluted serum	6	1.00 ± 0.24	0.08 ± 0.11
Muscle extract	4.5 gm	6	0.73 ± 0.19	0.82 ± 0.16
Liver "	7.5 gm	8	0.55 ± 0.08	0.65 ± 0.14
Kidney "	5.5 gm	8	0.48 ± 0.13	0.54 ± 0.14
Lung "	3 to 4 gm	6	0.68 ± 0.18	0.61 ± 0.25
Heart "	3 gm	5	0.60 ± 0.16	0.59 ± 0.08
Spleen "	1.5 to 3 gm	6	0.36 ± 0.08	0.34 ± 0.13

* Burroughs-Wellcome and Co., London. This vaccine is stated to contain 2×10^9 organisms per ml. (1×10^9 *S. typhi*; 5×10^8 *S. paratyphi* A and 5×10^8 *S. paratyphi* B).

depressed temperature for periods of up to 30 minutes, thus probably masking the true rapidity of action of tissue pyrogen. It may be concluded that mean fever latency distinguishes endotoxin from tissue pyrogens, if doses are adjusted to give responses of less than 1°C and adverse reactions are not produced.

Response in endotoxin-refractory recipients: Response to endotoxin is greatly reduced if it is incubated in serum obtained from animals rendered tolerant to endotoxin and then injected into other endotoxin-tolerant recipients (8). Re-

sponse to skeletal muscle extract was tested in this way and was found not to be diminished, in common with circulating endogenous pyrogen and leucocyte pyrogen (8, 9). However, this test is tedious to perform and did not completely abolish the response to our preparation of purified endotoxin (5). Responses to endotoxin may be suppressed more effectively and easily if the recipient rabbit is injected the day previously with a single large dose of endotoxin. The day following such challenge the animal does not respond to moderate doses of endotoxin but responds to leucocyte and circulating endogenous pyrogen (5). Responses to endotoxin and to various tissue pyrogens injected before, and the day following, administration of 1.0 μg purified endotoxin from *Proteus vulgaris* are

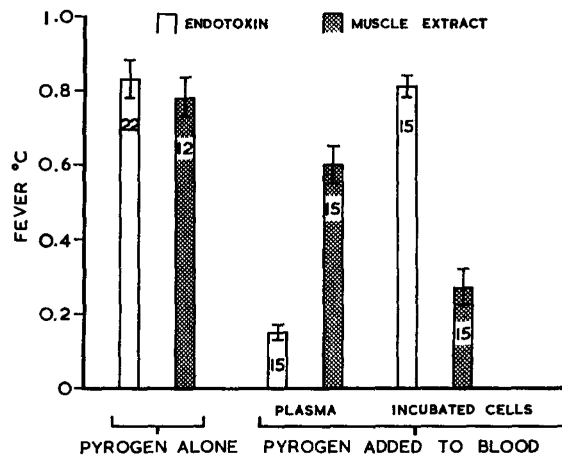

FIG. 5. On the left: Febrile responses (mean ± SE) to control injections of 0.003 μg endo toxin in 2 ml plasma and to 4.5 gm abdominal muscle extract.

On the right: Responses to supernates obtained from mixtures of the pyrogens with 2 similar aliquots of whole blood (see text). Results are means of 3 similar experiments and the number of individual responses are shown in the columns.

shown in Table IV. Response to tissue pyrogen was not modified by this treatment whereas comparable endotoxin fevers were almost completely suppressed, whether they were induced by endotoxin from the same or a different bacterial source (*e.g.* typhoid vaccine) as that used for challenge (see Table IV).

Reaction with whole blood in vitro: Endogenous pyrogens may be further differentiated from endotoxin by their reactions with whole blood *in vitro.* A mixture of whole blood and endotoxin after 30 to 60 minutes shows little or no pyrogenicity in the cell-free plasma, but the pyrogen can be demonstrated in the cellular deposit; further, if the cells are resuspended in saline and incubated, endogenous pyrogen, presumably of leucocyte origin, appears in the supernatant fluid (6). The reactions to muscle extract and endotoxin in this system were compared in this way in the following experiment. Blood obtained from 2 or 3 donors

was divided into 2 pools; abdominal muscle extract was added to one pool in a dose of 4.5 gm per 1.6×10^8 leucocytes, and endotoxin to the other in a dose of 0.003 μg per 1.6×10^8 leucocytes. The mixtures were kept at room temperature for not more than 30 minutes and then centrifuged at 1000 *g* for 30 minutes at 4°C. After removal of plasma, the cells were resuspended in saline and incubated at 37°C for 4 hours. The mixture was then again centrifuged and the

TABLE V

Febrile Responses (Mean ± SE) to Injections of Various Tissue Extracts of Rabbits Treated with Nitrogen Mustard Intravenously (see text)

Tissue extract	Donor*	Dose	No. Responses	Fever height
		gm		*°C*
Abdominal muscle	A	2	4	0.41 ± 0.15
	"	4	6‡	0.60 ± 0.03
	"	8	4	0.74 ± 0.08
	B	6	4	0.52 ± 0.12
	D	5	4	0.54 ± 0.12
Skeletal muscle	A	2	4	0.41 ± 0.05
	"	4	6‡	0.53 ± 0.06
	"	8	4	0.65 ± 0.05
	C	4	8‡	0.46 ± 0.08
	"	5	8‡	0.68 ± 0.12
	D	5	4	0.48 ± 0.10
Liver	B	6	6	0.51 ± 0.06
	D	5	4	0.36 ± 0.05
Heart	D	2	3	1.05 ± 0.31

* Peripheral white blood cell count on day of dissection: A, 550 per mm³; B, 1350 per mm³; C, 900 per mm³; D, 1000 per mm³.

‡ Responses, half of which were obtained before, and half after, recipients had been rendered refractory to endotoxin by the intravenous injection of 1.0 μg purified endotoxin.

supernatant fluid was removed. Plasma and the supernates from incubated cells were assayed for pyrogenicity in doses equivalent to the dose of pyrogen added initially,—4.5 gm of muscle extract or 0.003 μg endotoxin. Three similar experiments were performed each with assays in 5 recipients and the mean results of pooled data are shown in Fig. 5. Control responses to the pyrogens alone were similar. Plasma from the endotoxin-treated mixture contained very little pyrogen, whereas that from the muscle extract mixture contained most of the pyrogen added originally (some probably remained trapped within the blood cell deposit). The supernates from incubated cells also differed in that the endotoxin mixture was highly pyrogenic due to production of leucocyte pyrogen

while little pyrogen was present in the supernate of the muscle extract mixture. Under these conditions, therefore, muscle extract pyrogen added to whole blood neither attaches to cells nor activates leucocytes into a pyrogen-producing state; in these respects it differs from endotoxin and resembles leucocyte pyrogen (6).

From all these data it is apparent that pyrogens present in tissue extracts are distinct from endotoxin and must be considered endogenous agents.

Comparison of Tissue Pyrogens with Leucocyte Pyrogen.—Tissue pyrogens resemble leucocyte pyrogen in all the properties so far examined except for the shapes of some tissue fevers and the slopes of dose-response curves. However,

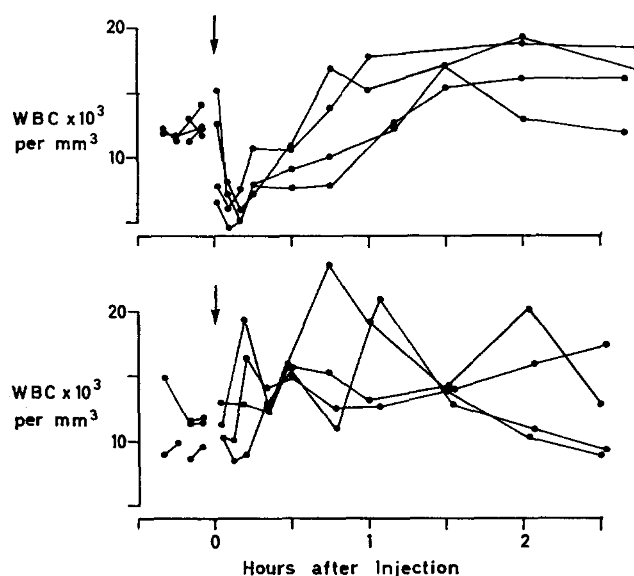

FIG. 6. Peripheral blood total white blood cell count (WBC) of individual rabbits in response to intravenous injections of skeletal muscle extract in a dose equivalent to 8 gm tissue (upper chart) and 0.003 μg endotoxin in serum (lower chart).

other evidence suggests that tissue pyrogens are not derived from leucocytes. Blood removed from these donor animals neither yielded pyrogen when extracted in the same manner as tissues, nor released pyrogen when incubated (6). Further, there was no difference in the pyrogenicity of tissue extracts whether or not the donor animal was bled out before dissection. It might be held that sequestered leucocytes are widely distributed in tissues and that, unlike blood leucocytes, they contain pyrogen. To test this possibility, tissue extracts were prepared from animals whose leucocyte population had been depressed by treatment with nitrogen mustard. Four rabbits were given an initial intravenous dose of nitrogen mustard 2.5 mg per kg body weight and 0.5 mg per kg at one or two intervals thereafter. Donors were protected against infection by treatment with

daily penicillin 200,000 units and streptomycin 200 mg intramuscularly. Five to 12 days later, when white blood cell counts had been reduced to <2000 per mm^3, for a period of 2 to 5 days, polymorphonuclear leucocytes formed only a small fraction of the total but accurate differential counts were not possible because of the paucity of cells. These animals were then bled out and the tissues extracted in the usual manner. Table V shows the peripheral leucocyte counts on day of exsanguination together with the mean responses obtained from these extracts. That the responses were not due to contaminating endotoxin was shown by the short latent periods before fever and the unaltered responses in recipients rendered refractory to endotoxin. Hence, the induction of profound leucopenia for periods of 2 to 5 days before dissection did not abolish the pyrogenicity of tissue extracts so that a leucocyte origin for tissue pyrogen seems unlikely.

Other Properties of Tissue Pyrogens.—

Response of circulating leucocytes: The response of the peripheral blood total leucocyte count of 4 rabbits to injections of skeletal muscle extracts (equivalent to 8 gm tissue) is shown in Fig. 6; for comparison, response to similarly pyrogenic doses (mean fever 0.8°C) of serum-augmented endotoxin is also shown. In all animals, injections of tissue extract caused an abrupt fall in the white blood cell count, predominantly in the granulocyte series, with a gradual return to base line or slightly higher levels after 45 to 60 minutes. In this small dose, endotoxin produced variable changes in the level of circulating leucocytes, but in no recipient was there an abrupt leucopenia. The response to tissue extract resembles that obtained by King (10) with injections of either leucocyte pyrogen or serum endogenous pyrogen, but the leucocytic response cannot be definitely ascribed to tissue pyrogen because of the many other agents present in the crude extract.

Physical properties: When an extract of skeletal muscle was ultrafiltered through a cellophane membrane at a negative pressure of 700 mm Hg, all the pyrogen was retained in the residual fluid; this indicates a large size for the pyrogen molecule. Injections of the ultrafiltrate did not induce either fever or leucopenia. Tissue pyrogen was not retained by a sintered glass filter of porosity of 0.5 to 1.0 μ and was not sedimented by centrifugation at 20,000 *g* for 1 hour so that the pyrogenic property is presumably not associated with particles of the size of most viruses or larger.

Heat and acid stability: There was no loss of activity at 4°C after storage for one month or after storage at -20°C for 24 hours. Incubation of relatively large doses of tissue extract at 37°C for 2 to 3 hours produced no obvious change in pyrogenicity, but when incubated for 15 hours or more, tissue extracts were appreciably less pyrogenic. Heat stability of the pyrogen in these crude extracts of tissue could not be tested at higher temperatures as material heated to 56°C or higher invariably caused immediate death upon intravenous injection.

The acid stability of tissue pyrogenic factor was tested by the slow addition (5 to 10 minutes) of N HCl into stirred tissue extract immersed in ice. Aliquots were removed for estimation of pH electrically and after 10 to 20 minutes, the mixture was brought back to the original pH by adding N NaOH in the same manner. A precipitate formed on acidification and heavier precipitation occurred on neutralization; the precipitate was removed by centrifugation and

TABLE VI

Pyrogenic Responses (Means and 95 Per Cent Confidence Limits) of Skeletal Muscle Extracts Treated with Acid or Alkali (see text)

pH	Dose*	Fever height	Number responses
	gm	°*C*	
5.1	6✠	0.76 ± 0.34	4
4.1	6✠ (3)	0.51 ± 0.08	12
	12✠ (2)	0.59 ± 0.22	5
3.8	4★	0.35 ± 0.21	3
	8★	0.52 ± 0.16	4
3.0	4★	0.42 ± 0.10	4
	6✠	0.56 ± 0.35	4
	8★	0.57 ± 0.09	6
2.1	8★	0.45 ± 0.27	4
1.5	12✠	0.22 ± 0.15	4
9.7	8★	0.37 ± 0.18	5

Where they exceed one, the number of titrations performed is shown in parentheses after the dose.

* Preparations derived from the same pool of tissue extract are denoted by identical symbols.

the supernatant was assayed. One sample was treated initially with alkali and then neutralized with acid. Table VI shows the pyrogenic activity of extracts after exposure to varying levels of pH. Initial pH of all extracts was about 6.0. Significant pyrogenic activity was retained with pH as low as 3.0 at which level about 75 per cent of the protein had been removed. In particular should be noted responses to the same extracts (marked by symbol in Table VI) treated to different levels of pH; there was some reduction in pyrogenic activity after treatment to pH 4.1 compared with pH 5.1 but no further detectable reduction after treatment to pH 3.0 The heavy precipitation during treatment may have led to loss of pyrogen by coprecipitation rather than, or in addition to, loss by

acidification. Pyrogenic activity did not survive a pH of 1.5 and was markedly reduced by pH 9.7.

Pyrogenicity of Tissues Incubated with Endotoxin.—The endotoxins of Gram-negative bacteria are among the most potent agents known that stimulate the release of leucocyte pyrogen. In an attempt to determine whether these agents could stimulate other body tissues to produce increased amounts of pyrogen the following experiment was devised.

From a single donor, samples of liver and abdominal muscle were aseptically chopped into pieces of about 1 inch length and placed in separate sterile bottles;

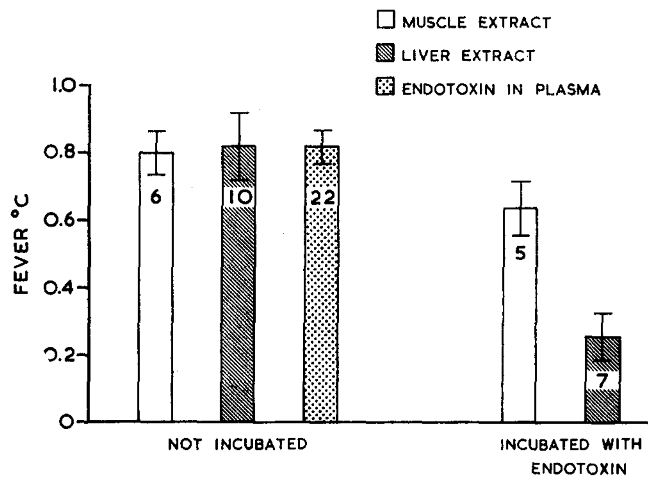

FIG. 7. On the left: Febrile responses (mean ± SE) to injections of 5 gm skeletal muscle extract, 5 gm liver extract and 0.003 μg endotoxin in plasma.

On the right: Responses to the same doses of tissues which were extracted after they had been incubated 20 hours as chopped portions together with endotoxin 0.003 μg per 5 gm of tissue (see text). Figures in the columns show the number of individual responses.

saline solution with antibiotics was added to each tissue together with endotoxin 0.003 μg per 5 gm of tissue. The mixtures were incubated at 37°C for 20 hours and then blended and centrifuged in the usual way. For comparison, samples of the two tissues were extracted in the standard manner without added endotoxin or incubation. These samples were assayed in doses equivalent to 5 gm of tissue, and the mean responses and SE's are shown in Fig.7. Response to the same dose of endotoxin in plasma was obtained as a control for the augmentation in fever to be expected from the admixture of endotoxin with tissue protein. Febrile responses to both tissue extracts, especially that of liver, were significantly reduced after incubation with endotoxin. From this experiment there is no evidence that endotoxin generates additional endogenous pyrogen from these tissues, as has been demonstrated to occur when endotoxin is incubated with

rabbit leucocytes. However, the results must be considered inconclusive in establishing this point as different periods of incubation were not tested. Since much of the initial pyrogenic activity, both of tissues and of endotoxin, was lost during incubation (see Fig. 7) it seems possible that any pyrogen produced by the effect of endotoxin on these tissues could have been similarly inactivated.

DISCUSSION

Disease or damage of almost any organ in the body may be accompanied by fever. This manifestation of illness has long been associated clinically with tissue injury and, in the latter part of the 18th century, fever was widely attributed to a product of tissue "putrefaction." With the discovery of bacterial pyrogens (subsequently identified as the endotoxins of Gram-negative bacteria) at the turn of the 20th century, tissue factors were temporarily forgotten as a cause of fever, while recognition grew that bacterial pyrogens were pervasive contaminants responsible for the fevers that followed injection of various biologic materials.

More recently, attention has again been focused on substances liberated by cells of the host as the immediate cause of various experimental fevers, including those produced by bacterial endotoxins.

Considerable evidence now exists that a number of microbes and their products produce fever indirectly by releasing from host cells a circulating endogenous pyrogen similar to, or perhaps identical with, the pyrogen present in sterile granulocytic exudates (11). Moreover, it is clear that circulating leucocytes, though containing little or no pyrogen, mobilize large amounts of this substance after exposure *in vitro* to a number of activators, including Gram-negative bacterial endotoxin (6, 9, 12), virus (13), and antigen in allergic hosts (14). In addition, both phagocytosis of certain particles (15) and an agent present in acute peritoneal exudates (16) are capable of activating blood granulocytes to release pyrogen.

The present studies reopen the possibility of alternative tissue origins of endogenous pyrogen(s) in febrile diseases, such as crush injuries of the extremities or acute hepatic necrosis, where the granulocyte may not be implicated. Several investigators have previously claimed that pyrogenic substances may be extracted from various tissues. The first of these agents, "pyrexin" (17), derived from inflammatory exudates, appears almost certainly to have been contaminated by pyrogens of bacterial origin (3). Similarly, the endotoxic activity of the "tissue polysaccharides" of Landy and Shear has been traced to incidental contamination of these substances with Gram-negative bacteria during purification procedures (18). On the other hand, Bennett and Beeson failed to find pyrogen in saline extracts or suspensions of tissues other than granulocytes from sterile exudates or those containing granulocytes, such as blood, bone marrow, and sites of Shwartzman or Arthus reactions (2, 19). The discrepancy between their

careful studies and the work reported here may be partly due to the somewhat larger doses employed here or to different methods of extraction. Many of Bennett and Beeson's preparations were injected after incubation for 6 hours, a procedure which we have shown to diminish tissue pyrogen activity. Also the authors state that intravenous injections of extracts or suspensions of tissue did not *consistently* produce fever. From our experience with such materials, it seems possible that pyrogenic reactions to larger doses in their recipient rabbits may have been masked in some instances by shock.

Special precautions were taken to avoid endotoxin contamination in the experiments reported here, and by many tests tissue pyrogen behaved similarly to pyrogen extracted from leucocytes and differed from bacterial endotoxin, even when this agent was incubated briefly in serum to potentiate its biologic action.

Circulating blood leucocytes cannot be the source of tissue pyrogen since blood did not yield pyrogen either when homogenized or incubated. It also seems unlikely that the pyrogen is derived from leucocytes present in the tissues and subsequently activated by factors in the tissue extracts. Production of leucocyte pyrogen requires intact cells incubated at temperatures well above 4°C, the temperature at which these tissues were kept before and after the short period required for homogenization (20). Furthermore, as already shown, blood leucocytes are not activated after brief contact with tissue extracts *in vitro*. Similarly, leucocytes sequestered and perhaps already activated in the tissues do not appear to be responsible for the pyrogenicity of these tissue extracts. Dose-response curves with tissue pyrogen differ from those reported with leucocyte pyrogen (7). Most significantly, typical tissue pyrogen was present in extracts of tissues derived from rabbits rendered profoundly granulopenic for periods of 2 to 5 days by nitrogen mustard.

One of the most important questions arising from these studies is whether there are specific stimuli capable of activating these tissues to produce more pyrogen, as is the case with granulocytes. The existence of such activators would greatly increase the likelihood that tissue pyrogens play a significant role in producing fever in disease. Dosages of up to 40×10^7 blood leucocytes are inactive as a source of pyrogen whether incubated in saline or disrupted, whereas as few as 1×10^7 produce significant fevers after activation by endotoxin, a difference representing at least a fortyfold increase in activity (6). Endotoxin in this study appeared to be ineffective, however, in increasing the pyrogen content of either skeletal muscle or liver when incubated with these tissues *in vitro* although variation in the conditions of incubation are required to establish this point.

With further biochemical characterization of tissue and leucocyte pyrogens, the relation of these agents to each other, as well as their role in disease, should be clarified. Since tissue injury and inflammation (with consequent activation

of granulocytes) are often intimately associated, it is virtually impossible with current techniques to determine the source of endogenous pyrogen in many diseases. At present, therefore, no conclusions can be drawn on the possible role of tissue pyrogen in fevers induced either experimentally or by natural disease.

SUMMARY

Saline extracts of homogenized, uninfected, rabbit tissues produced febrile responses when injected intravenously into rabbits. Extracts of muscle, lung, and heart evoked fevers that were similar to those induced by leucocyte pyrogen; extracts of spleen, liver, and kidney caused more sustained fevers. The minimal pyrogenic dose appeared to be between 1.5 and 3 gm wet weight of tissue.

Evidence is presented that neither Gram-negative bacterial endotoxin nor polymorphonuclear leucocytes (circulating or sequestered in the tissues) can be implicated as the source of pyrogen in tissue extracts. It seems likely, therefore, that a pyrogenic material of truly endogenous origin is widely distributed in tissues.

Tissue pyrogen appears to be a large molecule which is relatively resistant to treatment with acid but not with alkali. Possible pathological roles for this endogenous agent (or agents) are briefly indicated.

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