# The Fate of Circulating Leukocytic Pyrogen in the Rabbit[1,2] (35689)

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Human and rabbit polymorphonuclear leukocytes, when properly activated, are able to release a pyrogenic substance (leukocytic pyrogen) which then acts on the central nervous system, probably the hypothalamus, to initiate the febrile process (Fig. 1) (1-4). Leukocytic pyrogen (L.P.) is a nondialyzable protein which is present in the circulation in amounts proportional to the febrile response (5-8).

Although considerable information is available concerning the events shown in Fig. 1, virtually nothing is known of the fate of the circulating L.P. The experiments reported herein were designed to determine possible ways by which the body removes or inactivates L.P. after it has been released into the circulation. Three possible ways were examined: excretion in or inactivation by the urine, plasma inactivation, and removal by the liver.

*Materials and Methods.* Male and female New Zealand rabbits weighing between 2 and 3 kg were obtained from a single dealer. Rectal temperatures were measured with a multichannel temperature recorder[3] in rabbits previously shown to give uniform responses to standard doses of L.P. Fever indices were calculated by measuring areas under 2-hr fever curves with a planimeter by the method of Bornstein *et al.* (9). The L.P. used in these experiments was prepared from rabbit peritoneal exudate cells as previously described (5, 9). Several lots were pooled to provide a uniform preparation, diluted, if necessary, and stored in sealed glass ampoules at —60°. The bacterial endotoxin[4] was prepared from *Salmonella abortus equi.* All glassware was sterilized and made pyrogen-free by heating at 180° for 2 hr in a hot air oven. Prior to surgery the perfusion apparatus was made pyrogen-free by pumping 10% HCl through the system for 20 min, then flushing with pyrogen-free water until the water was free of HCl. All surgical procedures were carried out under sterile conditions. Animals were anesthetized prior to surgery with Diabutal.[5] All fluids and samples of pyrogen were cultured on blood agar plates to rule out bacterial contamination.

*Rate of clearance of L.P. from plasma.* The rate of clearance of L.P. from the plasma was determined to help predict the most likely sites of its removal or inactivation. Several investigators (7, 8) have shown that the concentration of circulating endogenous pyrogen is proportional to the intensity of the animal's fever. The half-time of plasma L.P. was used as a measure of the clearance rate of L.P. For this measurement two assumptions must be made: first, that endogenous pyrogen and L.P. either are identical or at least act in a similar fashion; second, that as soon as a rabbit's fever begins to fall after receiving L.P., the degree of temperature elevation ($\Delta T$) is proportional to the amount of L.P. remaining in the plasma. This proportionality allows the relative concentration of L.P. in the plasma to be calculated for any given time after the rectal temperature be-

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[2] Presented in part before the International Association of Pathologists, St. Louis, Missouri, 1969.

[3] Yellow Springs Instruments, Inc., Yellow Springs, Ohio.

[4] Obtained from Difco Laboratories, Detroit, Michigan.

[5] Obtained from Diamond Laboratories, Inc., Des Moines, Iowa.

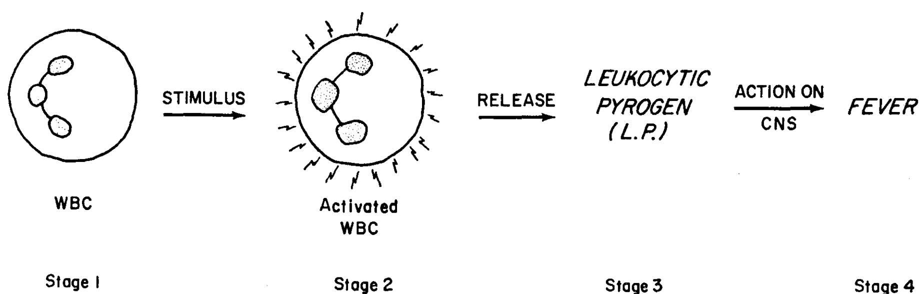


FIG. 1. A normal leukocyte (left) apparently may be activated by any number of stimuli, among them endotoxin, viruses, phagocytosis of bacterial or inert particles. The activated leukocyte then releases its pyrogen (L.P.) as has been demonstrated to occur *in vitro* and *in vivo* in a number of animals including man. The leukocytic pyrogen circulating in the blood then apparently acts on the hypothalmus to cause an increase in the amount of heat retained and produced by the animal with a resultant fever.

gins to fall by substituting $\Delta T$ for L.P. concentration.

From 3–5 values of $\Delta T$ and their corresponding times ($t$) after injection were taken from the descending slope of 30 L.P. fever curves obtained from rabbits which had received 2–4 ml of L.P. The log $\Delta T$ was plotted against the time after injection of L.P. and the resulting regression line is shown in Fig. 2. Using the numerical value of the slope ($K$) of the regression line in the equation $t_{½} = 2.303 \log 0.5/K$, we calculated the half-time to be 38 min.

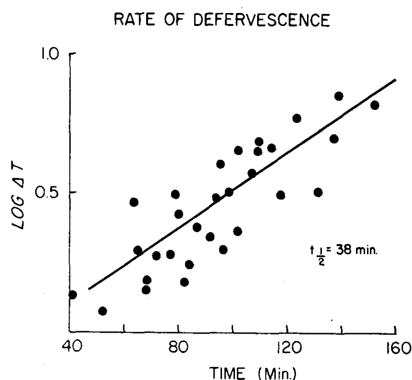


FIG. 2. The defervescence curve obtained after giving rabbits leukocytic pyrogen. The log of the temperature elevations ($\Delta T$), for times after peak rectal temperature, were plotted against time in minutes. The data points are taken from 10 individual rabbit fever curves. The straight line represents the best fit to data points from 30 rabbit fever curves using the least-squares method of fitting a regression line.

*Urinary excretion of L.P.* The rapid removal of L.P. from the plasma as indicated by a half-time of 38 min and the low molecular weight reported for L.P. (10) both suggested the possibility of direct excretion of L.P. by the kidney. The following experiment was performed to test this hypothesis. Rabbits were injected with 0.005 $\mu g$ of endotoxin and their febrile courses carefully followed by measurement of rectal temperatures. The animals were then killed by an overdose of Diabutal at 1, 2, or 3 hr after endotoxin injection. As shown in Table I most animals were killed at 2 hr because the half-time values for circulating L.P. indicated that by this time most of the L.P. would have disappeared from the circulation. The bladder was exposed surgically; the urine was removed by needle and syringe, then centrifuged at 400$g$ for 30 min at 5° to remove cells. The volume of urine recovered varied from 50–200 ml (Table I). The urine was dialyzed against 0.9% saline for 2 days and concentrated by overnight dialysis against polyethylene glycol compound 20-M[6]. The concentrate was diluted to 10 ml and injected intravenously, and the fever indices shown in Table I were calculated from the resulting rectal temperature curves.

*The effect of urine on L.P.* To see if there was an inhibitor to L.P in the urine, the following experiment was done. Urine from

[6] Obtained from Union Carbide Corp., 270 Park Ave., New York, N.Y. 10017.

TABLE I. Pyrogenic Activity of Urine from Febrile Rabbits.

| | Fever index ± SEM[a] (cm²) | | |
|---|---|---|---|
| Urine volume collected (ml) | 1 hr | 2 hr[b] | 3 hr |
| 15–50 | — | 0.23 ± 0.3(5)[c] | — |
| 75–125 | 0 (2) | 0.68 ± 0.4(4) | — |
| 175–200 | — | 0.78 ± 0.4(3) | 0.91 ± 0.3(2) |

[a] None of these fever indices represent a positive febrile response.
[b] Most samples were taken at 2 hr after endotoxin injection because most of the L.P. has been removed from the circulation (8).
[c] The number in parentheses indicates number of urine samples collected.

TABLE II. The Effect of Rabbit Urine on L.P. Activity.

| Sample[a] | | | | |
|---|---|---|---|---|
| L.P. (ml) | Urine (ml) | No. | Incubation time (hr) | Fever index ± SEM (cm²) |
| 1 | 8 | 12 | 3 | 1.9 ± 0.26 |
| 1 | 8 | 10 | 0 | 1.8 ± 0.27 |
| 1 | 0 | 15 | 0 | 2.1 ± 0.34 |

[a] Urine was collected under sterile conditions from four normal rabbits, pooled and mixed with pooled phosphate-buffered (pH 7.2) L.P. as indicated.

normal nonfebrile rabbits was collected as described above and the supernatant fluid stored until used in pyrogen-free glassware at 4°. Samples for intravenous injection were prepared as follows: (a) 8 ml of urine and 1 ml of L.P. were mixed and incubated at 37° for 4 hr prior to injection; (b) 8 ml of urine and 1 ml of 0.9% saline were mixed and incubated at 37°, for 4 hr and then injected followed by the immediate injection of 1 ml of L.P.; and (c) 1 ml of L.P. alone was incubated at 37° for 4 hr, then injected. Fever indices were then calculated (Table II).

*The effect of plasma on L.P.* Rabbit blood obtained by cardiac puncture was drawn into syringes containing 1 ml heparin[7]/100 ml of blood, then centrifuged at 400*g* for 30 min at 5°. The plasma was aspirated and pooled. Samples for intravenous injection were prepared as follows: (a) samples containing 1 ml of L.P. and 9 ml plasma were mixed and incubated at 37° for 3 hr; (b) 1-ml samples of L.P. were incubated at 37° for 3 hr. At the end of the incubation period 9 ml of plasma, which had been stored at 4° during the incubation period, were added to the samples containing L.P. alone; and (c) 1 ml of L.P. was used as a control. All samples were then injected intravenously into rabbits, and rectal temperatures recorded over a 2-hr period (Table III).

*The effect of the liver on L.P.* Rabbit livers were perfused *in situ* by a method adapted from techniques described by Deykin *et al.* (11) and Spaet (12). The perfusion system is shown diagrammatically in Fig. 3. A midline abdominal incision was made and the portal vein and the inferior vena cava were dissected free. The superior mesenteric, the right renal, and right adrenal veins were ligated, and the portal vein was catheterized using polyethylene tubing (PE280).[8] The inferior vena cava was catheterized just above the left renal and adrenal veins. The diaphragm was then cut and the inferior vena cava ligated above the diaphragm.

The perfusate, which consisted of 30 ml of

[7] Obtained from Organon, Inc. West Orange, N.J.

[8] Obtained from Clay-Adams, Inc., 141 East 25th Street, New York, N.Y.

TABLE III. The Effect of Incubation with Plasma on L.P. Activity.

| Sample composition[a] | | | | |
|---|---|---|---|---|
| L.P. (ml) | Plasma (ml) | No. | Incubation time (hr) | Fever index ± SEM ($cm^2$) |
| 1 | 9 | 12 | 3 | 3.0 ± 0.26 |
| 1 | 9 | 12 | 0 | 2.4 ± 0.22 |
| 1 | 0 | 11 | 0 | 2.3 ± 0.34 |

[a] Blood was collected from 10 rabbits and after centrifugation the plasma was pooled and used throughout these experiments.

TABLE IV. The Disappearance of Pyrogenic Activity from Liver Perfusates.

| | Fever index[a] ($cm^2$) | | | BSP clearances (% remaining)[b] | |
|---|---|---|---|---|---|
| Experiment no. | 5 min | 20 min | 40 min | 5 min | 15 min |
| 1 | 3.6 | 1.5 | 0 | ND[c] | ND |
| 2 | 3.0 | 0 | 0 | ND | 84 |
| 3 | 3.3 | ND | 0 | ND | 12 |
| 4 | 1.2 | 0.80 | 0.64 | 71 | 53 |
| 5 | 1.0 | 0 | 0 | ND | 46 |
| 6 | 2.1 | 1.8 | 1.2 | ND | 71 |
| 7 | 0.70 | 1.3 | 0.52 | ND | ND |
| 8 | 4.3 | 4.2 | 1.1 | 79 | 25 |
| 9 | 5.6 | 2.3 | 2.4 | 70 | 23 |
| 10 | 6.5 | 0 | 2.7 | 100 | 65 |
| 11 | 2.4 | 2.1 | 0.81 | 78 | 31 |

[a] Fever indices were determined on an aliquot of the perfusate 5, 20, and 40 min after start of the perfusion.

[b] BSP clearances were determined 5 and 15 min after injection of the BSP. A sample was withdrawn 2 min after injection of BSP for estimation of BSP concentration at 0 time.

[c] Not done.

0.9% saline, 20 ml of pooled L.P., and 1 ml of heparin, was pumped from the collection bottle into the portal vein at a rate of 20 ml/min by a peristaltic pump. The effluent flow was returned by the catheter in the inferior vena cava to the collection bottle, thus maintaining continuous flow in a closed system.

At 5, 20, and 40 min after starting the perfusion, 4-ml samples were withdrawn from the collection bottle to be assayed for L.P. activity by injection into rabbits as previously described. The 5-min sample was used as a control because it was assumed that the perfusate would be thoroughly mixed with the blood in the liver by this time. Four perfusions (Table V, Experiments 1–4) were done using saline alone to check for possible hepatic pyrogens. To establish normal values 0.15 ml of Bromsulphalein[9] (BSP) were injected 5 min after the onset of perfusion (Table V, Experiments 5–7) of saline, samples of perfusate were removed 5 and 15 min later, and the amount of BSP present was determined colorimetrically. After 40 min of perfusion in either control or experimental animals, 0.15 ml BSP was injected into the system, and samples of perfusate were collected 5 and 15 min later for BSP determination. Immediately after collection of samples for BSP determinations, tissue slices from several hepatic lobes of each animal were removed, fixed in 10% buffered formalin for at least 72 hr, sec-

[9] Sodium sulfobromophthalein (BSP) was obtained from Hynson, Wescott and Dunnington, Inc. Baltimore, Maryland 21201.

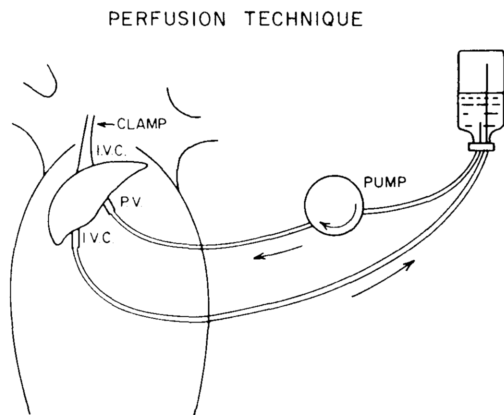


FIG. 3. The perfusion system was connected as shown. The perfusate was pumped from a 50-ml serum bottle into the portal vein (P.V.) and returned to the serum bottle through the inferior vena cava (I.V.C.). Reagents were added and samples were withdrawn from the serum bottle.

tioned, and stained with hematoxylin and eosin, oil red O, Gomori's reticulin, periodic acid–Schiff before and after treatment with diastase, and Masson's trichrome.

*Results.* Although the 38-min half-time of circulating L.P., as seen in Fig. 2, shows that L.P. is rapidly removed from the plasma, it is not removed by the kidneys because no pyrogenic activity was detected in the urine (Table I) and because no inhibitor to L.P. was found in the urine (Table II).

Since L.P. is not removed from the plasma into the urine, it seemed possible that an inhibitor to L.P. existed in the plasma itself. However, when L.P. was incubated with plasma no significant decrease in pyrogenic activity could be demonstrated (Table III).

Because of the liver's known ability to catabolize endogenous proteins (11, 12), this organ seemed the next most likely site of destruction of L.P. The results of 11 liver perfusions are shown in Table IV. In three perfusions (4, 6, and 10) in which BSP determinations indicated liver damage, significant amounts of L.P. remained in the perfusate at the end of 40 min although in Experiment 9 a significant amount of L.P. remained at 40 min even though the BSP clearance was normal. In all the other perfusions except Experiment 7 there was a highly significant decrease in the concentration of L.P. between the 5- , the 20- , and the 40-min samples. In some of the experiments all of the L.P. had disappeared from the perfusate by the time the 20-min sample was taken.

Examination of the liver sections by light microscopy failed to reveal any significant changes other than slight edema and a mild decrease in glycogen in the liver cells of the experimentally perfused livers.

*Discussion.* Leukocytic pyrogen is not excreted in the urine of febrile or postfebrile rabbits in an active form, nor is there an active inhibitor to L.P. in the urine or in the plasma. However, L.P. is actively removed

TABLE V. Clearance of BSP from Rabbit Livers Perfused with Saline.

| | Fever index[a] ($cm^2$) | | | BSP clearances (% remaining)[b] | |
|---|---|---|---|---|---|
| Experiment no. | 5 min | 20 min | 40 min | 5 min | 15 min |
| 1 | 0 | 0 | 0 | ND[c] | ND |
| 2 | 0.35 | 0 | 0 | 54 | 19 |
| 3 | 0 | 0 | 0 | 66 | 23 |
| 4 | 0 | ND | 0 | ND | 27 |
| 5[d] | | | | 61 | 16 |
| 6[d] | | | | 100 | 41 |
| 7[d] | | | | 64 | 21 |

[a] Fever indices were determined on an aliquot of the perfusate 5, 20, and 40 min after start of the perfusion.

[b] BSP clearances were determined 5 and 15 min after injection of the BSP. A sample was withdrawn 2 min after injection of BSP for estimation of BSP concentration at 0 time.

[c] Not done.

[d] BSP clearances were determined 5 and 15 min after the start of the perfusion.

from the liver perfusate under our experimental conditions. The role of the liver in the removal of L.P. is further strengthened by the impaired uptake of L.P. by those livers in which extensive damage had occurred as indicated by the BSP test. The rate of removal of L.P. by the perfused liver is, as expected, considerably faster ($t_{1/2} \approx 15$ min) than the removal of L.P. by the liver ($t_{1/2} = 38$ min) in the intact animal because the L.P. is presented to the liver by perfusion more rapidly than is possible by the normal circulation.

Although at this point we can only speculate on the mechanism of removal of L.P. by the liver, the most likely possibility is the direct uptake by hepatic cells, as occurs with other endogenous proteins (11, 12), followed by either degradation or direct excretion into the bile. It is also possible that L.P. is taken up by Kupffer cells, although participation of the entire reticuloendothelial system seems unlikely because no alteration in response to L.P. is seen in animals whose reticuloendothelial system has been blocked (13). Whether the plasma clearance of L.P. is restricted to the liver or whether other organs or tissues also take part in the clearance process is not yet known. The rapid, almost complete, disappearance of L.P. from the liver perfusate strongly suggests that the liver is the principal, perhaps only, site of clearance. Studies to determine the mechanism of uptake of L.P. by the liver, to determine if other organs are involved, and to determine whether the same mechanism applies in man are now in progress.

*Summary.* Leukocytic pyrogen (L.P.) is not actively inhibited by urine or by plasma nor is it excreted in an active form by the kidney. L.P. is promptly and almost completely cleared by the perfused liver within 20 min, suggesting that the liver is the organ primarily responsible for this action in the febrile rabbit.

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