

PASSIVE TRANSFER OF TOLERANCE TO PYROGENICITY OF BACTERIAL ENDOTOXIN

BY HENRY H. FREEDMAN, Ph.D.

(From the Princeton Laboratories, Inc., Princeton)

(Received for publication, November 24, 1959)

The typical biphasic febrile response of the normal rabbit given endotoxin intravenously is modified by continued daily administration of the bacterial lipopolysaccharide. This development of tolerance (1) to endotoxin pyrogenicity is characterized by disappearance of the second, major, phase of fever and by gradual diminution of the initial phase (2). Attempts at passive transfer of tolerance to pyrogenicity have yielded negative results (1, 3). Therefore it is generally considered that tolerance cannot be so conferred upon a normal animal (2, 4). In a previous paper (5), we have shown that passive transfer of another aspect of endotoxin tolerance, substantial resistance to lethality of homologous and heterologous endotoxin, could be achieved with serum of rabbits given a brief series of injections of bacterial lipopolysaccharide.

The present report describes the decrease in magnitude and duration, with disappearance of the second phase, of the biphasic fever pattern of the normal rabbit given endotoxin subsequent to the administration of plasma or serum of tolerant donor rabbits. Studies on reticuloendothelial system function in recipient animals, tolerant by passive transfer, will be reported separately (6).

Materials and Methods

The endotoxin used throughout was the lipopolysaccharide of *S. typhosa* O 901 (Difco). Temperatures were taken with clinical mercury thermometers prior to and at 30, then 60, minute intervals after giving endotoxin. A "fever index" for each rabbit was determined by plotting its fever curve on standard graph paper, cutting out the area under the curve, and weighing the cut-out. The weight is directly proportional to the area, and the latter summates magnitude and duration of fever. The area was enclosed by dropping a vertical from the last measured temperature to the time axis. Donor blood was collected aseptically either by heart puncture or by bleeding from the carotid with precautions to avoid pyrogen contamination. In control experiments it was noted that hundredths of a microgram of the endotoxin given shortly before the 2.5 μ g. test dose resulted in substantial increase in the febrile response, making mandatory avoidance of contamination of the donor blood. All glassware, syringes, and needles were kept at 175°C. for 2 to 3 hours before use. Pyrogen-free distilled water was used for preparing solutions and these, as well as representative samples of donor serum or plasma, were proven non-pyrogenic in normal rabbits. Plasma was obtained by using one volume of 3.8 per cent sodium citrate for five volumes of whole blood and centrifuging the same day. For serum, blood was allowed to clot and was kept at room temperature for 4 to

6 hours, then refrigerated overnight. Plasma and serum were vialled in single-dose volumes and frozen until used, usually the same week. The rabbits, weighing 2 to 2½ kg., were of mixed breed and both sexes. In all experiments, normal controls or controls given plasma or serum of normal donor rabbits were observed along with the rabbits pretreated with plasma or serum of tolerant donors.

Tolerant Donor Rabbits.—Varied schedules for inducing tolerance with endotoxin were investigated. Two are described here to illustrate the importance of this factor. *Procedure A*: days 1 and 2, 2.5 µg.; days 3 and 4, 5 µg.; days 5 and 6, 10 µg. *Procedure B*: day 1, 1 µg.; day 2, 2 µg.; day 3, 3 µg.; day 4, 5 µg.; day 5, 10 µg.; day 6, 20 µg. In all instances the marginal ear vein was used for the injections and the donor blood was taken on day 7, 20 to 24 hours after the last dose of endotoxin. Temperatures of these donor rabbits were recorded and the typical modifications of the fever curve with developing tolerance were observed. Differences in development of tolerance, reflecting the two procedures, are discussed below in relation to differences in effect observed in the recipient rabbits.

Tolerant RES-Blocked Donor Rabbits.—These animals were prepared by procedure A, but on day 7, 20 hours after the last injection, were given, intravenously, 10 cc. of a suspension of carbon (Gunther Wagner, C11/1431a) containing 45 mg./cc. in 2 per cent neutral gelatin to block the reticuloendothelial system and thus destroy their endotoxin tolerance (7, 8). When the blood was clear of carbon, about 4 hours later, a test dose of 2.5 µg. of endotoxin was given one RES-blocked rabbit to prove the loss of tolerance. At the same time plasma or serum was collected from the other rabbits. The pyrogen-injected control exhibited loss of tolerance by this procedure by responding not only with a greater fever than that elicited by 10 µg. the day before, but greater than the original response to the test dose on day 1.

Normal Donor Rabbits.—These animals, from the same stock, provided plasma or serum as controls for the tolerant donors described above.

Recipient Test Rabbits.—The test dose of endotoxin for quantitating the febrile response was 2.5 µg., given intravenously. This effected a substantial fever, highly consistent in both magnitude and time-course. There was the added advantage of having a large group of normal control responses from day 1 of procedure A for inducing tolerance. The rabbits were acclimatized to the stocks in which they were kept during the experiment by prior training, during which time their temperatures were also taken. Base line temperatures were obtained during the 1 to 2 hours before starting the experiment. An occasional rabbit having a temperature over 103.4°F. was excluded. The febrile response was followed for 5 or 6 hours after giving the endotoxin so that the rabbits were kept in the stocks a total of 7 or 8 hours. Food and water were withheld during this period. Test animals were used only once, except that in some experiments they were retested with twice the dose of endotoxin (5 µg.) the following day. In all instances plasma or serum was given intravenously in 10 cc. volume 30 minutes before the endotoxin.

EXPERIMENTAL

Effect of Passive Transfer on Time-Course of Febrile Response.—The nature of the biphasic febrile response of the normal rabbit to intravenous endotoxin and the alteration in time-course of this response with the development of tolerance by continued daily administration make it convenient (2) to consider whether an animal exhibits its maximum fever during the second phase (normal response), or during the first phase (tolerant response) with defervescence continuing from about the 2nd hour. The animals used in these studies have been classified in this way in Table I. It may be seen that only 3 of 37 control rab-

bits failed to show maximum fever at 2.5 to 4 hours. The mean increase for the 34 that did was 3.3°F. In contrast, 21 of 25 rabbits pretreated with plasma or serum of donors rendered tolerant by all three methods described above

TABLE I
No. of Recipient Rabbits Developing Maximum Fever at 1st or 2nd Phase and Mean Temperature Increase for the Animals Exhibiting Maxima at These Times

Group	No. of animals	No. at 0.5-1.5 hrs.	No. at 2.5-4.0 hrs.
Control	25	1 (2.4°F.)	24 (3.3°F.)
Normal donor plasma or serum	12	2 (2.1°F.)	10 (3.2°F.)
Tolerant donor plasma or serum	25	21 (2.4°F.)	4 (2.3°F.)

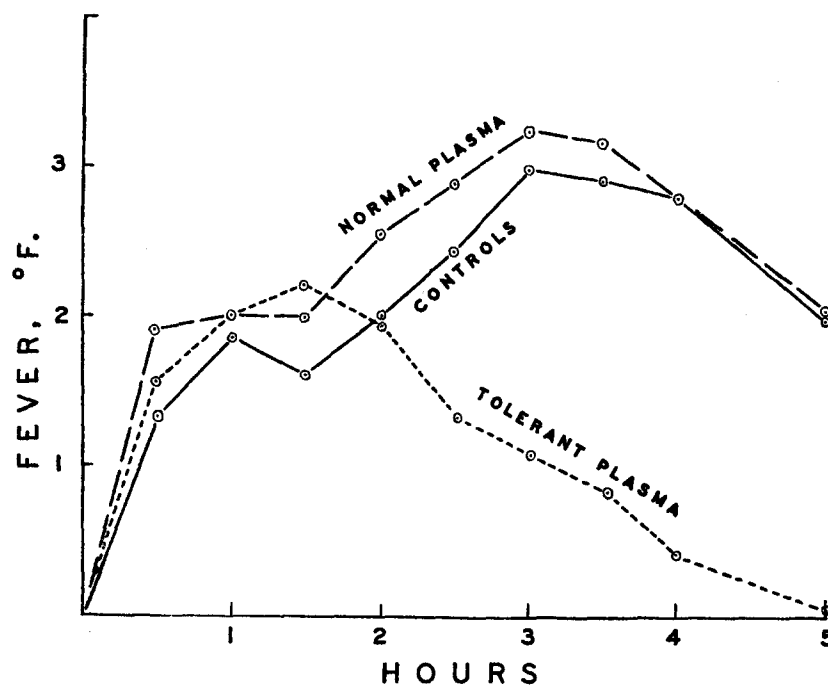


FIG. 1. Effect of pretreatment with plasma of tolerant donor rabbits upon febrile response of normal recipient rabbits given 2.5 μ g. endotoxin.

exhibited maximum fever during the first 1½ hours, with a mean rise for these animals at this time of 2.4°F. The 4 which reached maximum temperature at about 3 hours differed from the controls in that their fever was less (2.3°F.) and they had maintained essentially a plateau of response from the 1st hour. Thus the time-course of the febrile response of test rabbits given plasma or

serum of tolerant donors resembled that of animals having had one or more previous daily injections of endotoxin.

Passive Transfer by Plasma of Donors Prepared by Procedure A.—A total of 10 rabbits was treated with plasma from donors rendered tolerant by this method. Results of a representative experiment comprising 4 rabbits given this "tolerant" plasma and 4 given normal plasma are shown in Fig. 1. For comparison the febrile response of 7 rabbits given only the endotoxin is included. These 7 were donors of "tolerant" plasma for this experiment, and the

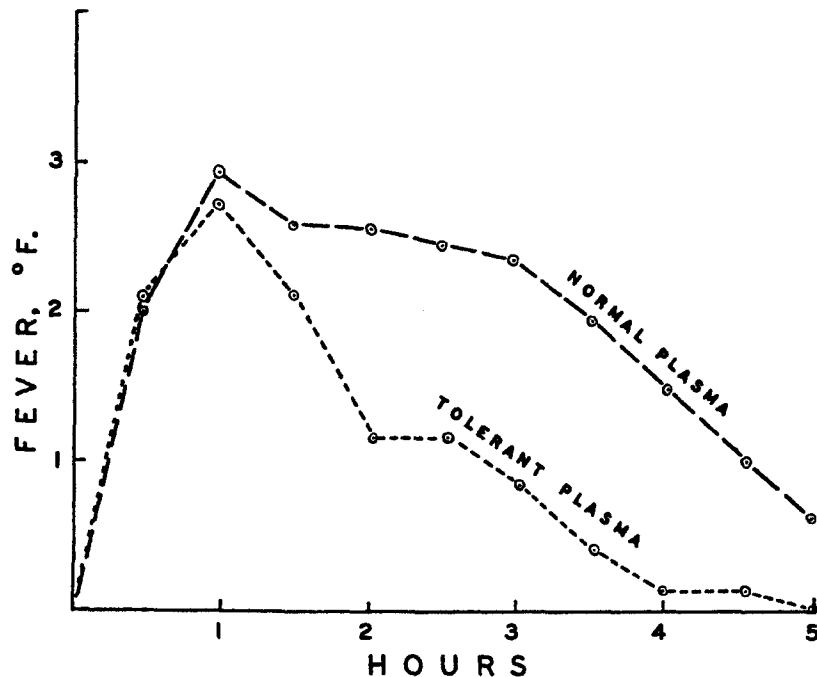


FIG. 2. Second day response of recipient rabbits to 5 μ g. endotoxin. Same experimental groups as Fig. 1.

fever curve was obtained on day 1 following the same 2.5 μ g. dose of endotoxin used for the test groups. It is clear that 10 cc. of normal plasma given 30 minutes before the endotoxin did not significantly modify the ensuing fever whereas the pretreatment with tolerant donor plasma did. The disappearance of the second major phase of fever is striking.

In view of the absence of the normal full course of fever in the experimental animals just described, it was decided to rechallenge the following day, using twice the dose (5 μ g.) of endotoxin to overcome the degree of tolerance known to follow a single injection. It was thought that perhaps the lesser response on the 1st day might be reflected in a lesser degree of tolerance from the test dose. The opposite proved to be the case, as shown in Fig. 2 for the same two groups

of 4 rabbits each described in Fig. 1. The rabbits given normal plasma the day before did not respond with the typical biphasic curve of day 1, but did suffer substantial fever. Those given the tolerant rabbit plasma before endotoxin the previous day responded this 2nd day with fever of lesser magnitude and shorter duration than the controls. The relative nature of endotoxin tolerance, depending as it does upon dosage and time schedules, precludes meaningful comparisons of degree of tolerance, but it may be of interest to note that this 2nd day response of the experimental group was almost identical with that of the 7 donor rabbits for this experiment when given the same 5 μ g. dose on day 3 of *Procedure A*, after 2 (instead of 1) daily injections of 2.5 μ g. each.

Comparison of Efficacy of Serum and Plasma.—Neither serum nor plasma of

TABLE II
Comparison of Effectiveness of Tolerant Donor Serum and Plasma: Means of Maximum Temperature Increases of Individual Recipient Rabbits at 1st and 2nd Phases, with Total Fever Indices

Group	No. of animals	Mean increase at		Fever index*
		0.5-1.5 hrs.	2.5-4.0 hrs.	
Controls.....	25	2.2°F.	3.3°F.	584 \pm 34
Tolerant donor plasma.....	10	2.0°F.	1.2°F.	284 \pm 45†
Tolerant donor serum.....	6	2.5°F.	2.3°F.	440 \pm 42§

* Mean \pm standard error.

† $p < 0.001$.

§ $p < 0.01$.

normal donor rabbits altered the response of recipient rabbits to subsequent endotoxin. From tolerant donors, citrated plasma proved to be more effective than serum. In Table II are presented the data demonstrating this difference. The plasma or serum was from donors prepared by procedure A. The mean temperature increases at the first and second phases are given here for *all* rabbits of each group. The means were obtained from the maximum increase of each individual rabbit during each phase. A plot of the 10 rabbits given "tolerant" plasma yielded a curve like that shown in Fig. 1. The serum-treated rabbits differed in that their response was biphasic, but with a slightly lower temperature at the second rise. They differed from the controls, too, in total magnitude of fever. It should be emphasized that the differences in fever index are greater than indicated in the table since at the time temperatures were last taken (usually 5 hours) the experimental animals showed little or no fever whereas the control rabbits had temperatures about 2°F. above their base lines. With the test dose used the fever of the controls lasted about 6½ hours.

Comparison of Effect of Procedures A and B for Tolerant Donors.—The fever

curves for 4 rabbits each, pretreated with plasma of donors prepared by procedures A and B are given in Fig. 3. Plasma obtained by procedure B modified the time-course of response to endotoxin of the recipients, but not the total magnitude of fever. Included in the figure are the responses of the donor rabbits for this experiment (3 for A and 4 for B) recorded on day 4 of treatment with endotoxin when both groups received 5 $\mu\text{g.}$, but differed in the amounts given the 3 previous days (*vide supra*). It is well recognized that the febrile

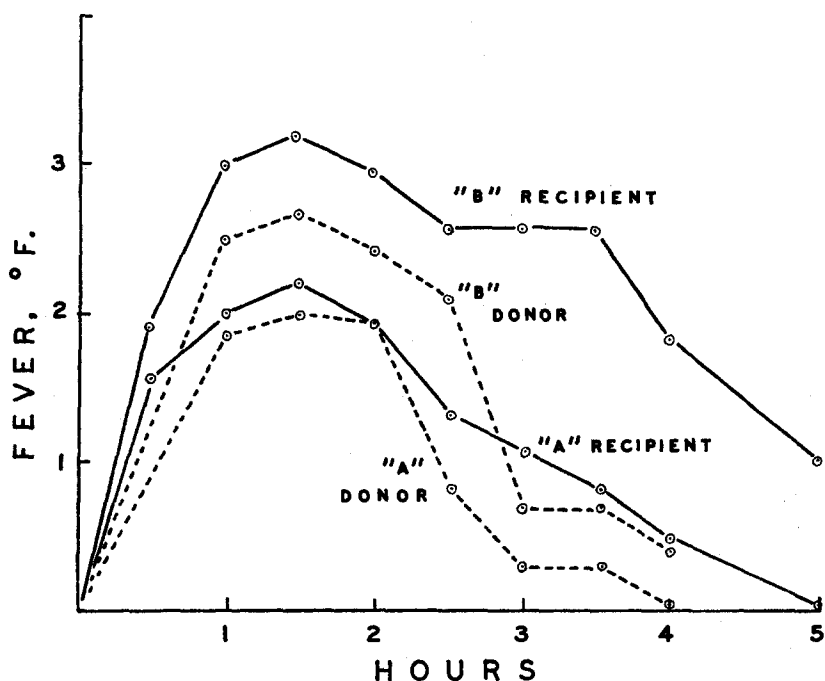


FIG. 3. Comparison of effectiveness of plasma obtained by procedures A and B for developing tolerant donors upon modification of febrile response of recipient rabbits. Responses of donor groups to 5 $\mu\text{g.}$ endotoxin on day 4 of schedule for inducing tolerance are also shown.

response of a tolerant rabbit to a particular dose of endotoxin depends upon the relative magnitude of the preceding doses. The constant daily increase in endotoxin dose in procedure B appears to be the only basis for the relative inefficacy of this donor plasma to lessen the fever of the recipient animals by passive transfer.

Passive Transfer from Tolerant RES-Blocked Donors.—Since the reticulo-endothelial system (RES) appears to be more than casually related to the state of endotoxin tolerance (7-10), it was decided to determine whether blood taken from animals prepared by procedure A but then given a large dose of

carbon before bleeding would modify the febrile response to endotoxin in recipient rabbits. It is to be noted that at the time of bleeding, the RES of these donors was thoroughly incapacitated and the endotoxin tolerance present 4 hours before was completely lost; these donors were, in fact, exquisitely sensitive to endotoxin. Five recipient rabbits were tested, 3 with serum and 2 with plasma. An experiment in which 3 recipient rabbits received this serum before endotoxin and 2 rabbits were given only the endotoxin is illustrated

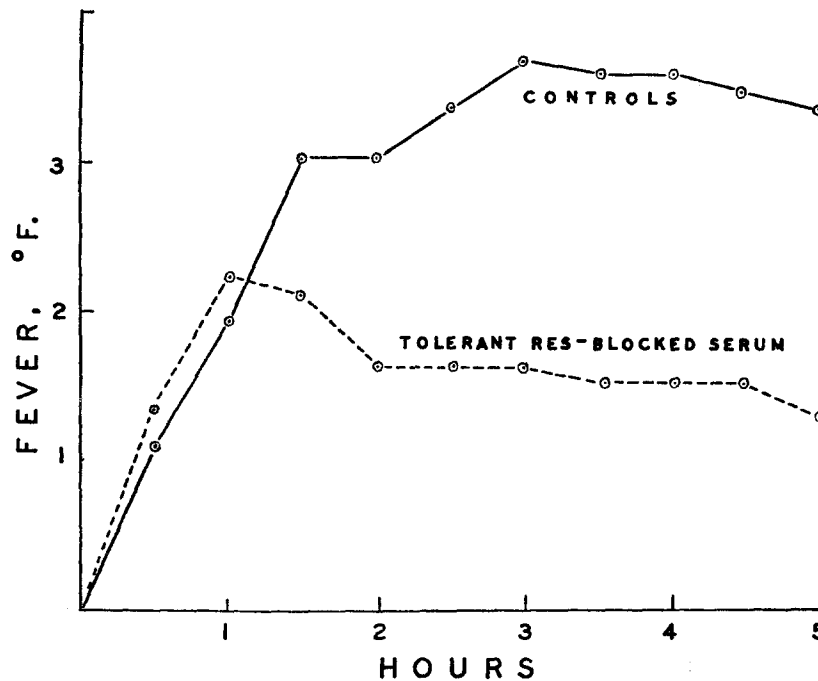


FIG. 4. Modification of febrile response of normal recipient rabbits to 2.5 μ g. endotoxin by prior injection of serum of tolerant RES-blocked donors.

in Fig. 4. The mean fever index for the 5 test rabbits for the blocked donors was 390 ± 26 , which, compared to the control index of 584 ± 34 , differs significantly ($p < 0.001$). Thus, although the donors in this experiment were no longer tolerant themselves, passive transfer of their former tolerance to pyrogenicity was still demonstrable.

DISCUSSION

It is clear that the plasma or serum of rabbits made tolerant to bacterial endotoxin may confer tolerance to the pyrogenic action of the endotoxin upon normal rabbits. It is equally clear that the manner in which tolerance is induced

in the donors is crucial. In the present report the two schedules for producing tolerant donors consisted of equal numbers of daily injections, and were done at the same time. The significant difference, then, is solely the relative magnitude of the daily challenges. An analogous finding was made by Zweifach *et al.* (11) who demonstrated tolerance to tumbling trauma in endotoxin-tolerant rats prepared by constant dose schedules. This cross-tolerance was not found in other rats given daily doses of endotoxin by a regimen involving sharp dose increments.

Both Beeson (1) and Cluff (3) used serum of donor rabbits made highly tolerant to endotoxin by longer periods of treatment than the period reported here. In the first instance gradually increasing doses of toxin were used and in Cluff's experiment a constant dose was employed. As in Beeson's experiment, and unlike Cluff's, we challenged the recipient rabbits with a dose of endotoxin less than the last dose given the donors. The difference in time interval between injections of plasma and endotoxin (30 minutes here compared to 1 hour in the experiments of Beeson and of Cluff) is hardly likely to be of consequence in view of the enhanced tolerance of the experimental rabbits when rechallenged 24 hours later. The 30 minute interval was adopted to minimize the total time the rabbits spent in the stocks.

Why a different schedule of doses of endotoxin over a given period of time,¹ or why long-term treatment with endotoxin to produce a high degree of tolerance, should adversely affect the suitability of the donor for passive transfer cannot be answered. Considering the well recognized relative nature of endotoxin tolerance (4) it is not unreasonable to postulate that the presence of the substance found in the blood in these experiments (and present in the no longer tolerant RES-blocked donors) is stimulated by prior injections of endotoxin and that the amount present at a given time depends upon the relative magnitude of the immediately preceding dose, reflecting utilization in the course of developing tolerance. This hypothesis suggests that sharply increased doses result in increased utilization of previously elaborated factor leaving little in the blood 24 hours later. The absolute length of time over which endotoxin is given may be of importance; the exquisitely tolerant animal may elaborate little "endotoxin-tolerant factor" in response to what has become a minor disturbance. Experiments to test these hypotheses are in progress.

Although plasma obtained by procedure B did not substantially alter the febrile response of recipient rabbits, it did protect mice against an LD₇₅ dose of endotoxin to a degree indistinguishable from that achieved with plasma of procedure A (unpublished experiments).

¹ The sera of rabbits prepared by procedures A and B did not differ in precipitin titer to the homologous endotoxin. Both showed titers of from 1:16 to 1:32 using dilutions of the endotoxin (1 mg./ml.) in saline and undiluted serum.

It should be noted that Farr *et al.* (12) described an alteration of the febrile response to toxin in normal rabbits previously infused with 55 ml. of plasma from donors given *S. typhi* organisms daily for 3 months. This modification consisted of a delay in development of the second rise in fever, without reduction in total magnitude. Its pertinence derived from its resemblance to the particular course of development of tolerance to pyrogenicity in their donors.

Landy and associates (13) found a greater inactivation of endotoxin by citrated plasma than by serum during incubation *in vitro*. They were able to relate this effect to an inhibitory action of free calcium on their "endotoxin detoxifying component." The lesser effectiveness of serum in our *in vivo* experiments can hardly be explained by this *in vitro* mechanism since the recipient rabbit has a wealth of available calcium. Grant and Whalen (14) described an opposite effect of citrate: enhancement of formation of "endogenous pyrogen" from plasma and endotoxin *in vitro*. They also obtained a decreased latency in onset of fever with possible slight augmentation *in vivo* when endotoxin was given with citrate. The possible role of anticoagulant in our test system is being examined further.

The continued presence of activity in the blood of rabbits no longer tolerant after an RES-blocking dose of colloid has several implications. There is abundant evidence that serologically demonstrable specific antibody plays no role in endotoxin tolerance (4). The possible involvement of non-demonstrable protective (15) or neutralizing antibody is disproved by the susceptibility to endotoxin of the blocked donors. The experiment offers further evidence that tolerance to endotoxin is based upon an altered state in the tissues of the animal and not upon inhibitory factors (12) in the blood. Exchange transfusion of tolerant rabbits with normal rabbit blood was done by Cluff and Bennett (16) and this procedure did not restore the normal febrile response to subsequently injected endotoxin.

That a response at the tissue level is necessary for tolerance is consistent with our investigations on recipient animals (6). The protection against lethality of endotoxin in the mouse (5) by pre-administration of "tolerant" plasma is not obtained if the plasma and the endotoxin challenge are mixed extemporaneously and given as a single injection. Most important, the blood of the tolerant donor stimulates the RES of the recipient as measured by subsequent carbon clearance in the latter.

SUMMARY

The typical febrile response of normal rabbits given bacterial endotoxin intravenously may be modified by prior administration of plasma or, less effectively, serum of endotoxin-tolerant donors. This altered response is characterized by disappearance of the second rise in fever and by a striking reduction in fever index. It thus resembles the course of fever shown by rabbits made

tolerant to endotoxin by one or more previous daily doses. This transfer of tolerance by plasma or serum depends critically upon the manner in which tolerance is induced in the donors.

The plasma of donor rabbits made tolerant, then given an RES-blocking dose of carbon, still confers tolerance upon normal recipient rabbits. Such donors have lost their tolerance and are highly sensitive to endotoxin at the time their blood is taken.

The implications of these findings for endotoxin tolerance and for transfer of this phenomenon are discussed. The evidence is consistent with the hypothesis that both tolerance and its transfer are based upon RES function and are independent of antibody.

The author is indebted to Prof. Albert S. Gordon of New York University for directing his attention to the bacterial endotoxins.

The skilled technical assistance of Miriam Graff is gratefully acknowledged.

BIBLIOGRAPHY

1. Beeson, P. B., Tolerance to bacterial pyrogens. I. Factors influencing its development, *J. Exp. Med.*, 1947, **86**, 29.
2. Grant, R., Refractoriness to pyrogens. Effects of incubation of pyrogen with plasma from normal and refractory donors in the response of refractory recipients, *Am. J. Physiol.*, 1953, **173**, 246.
3. Cluff, L. E., Studies of the effect of bacterial endotoxins on rabbit leukocytes. II. Development of acquired resistance, *J. Exp. Med.*, 1953, **98**, 349.
4. Bennett, I. L., Jr., and Cluff, L. E., Bacterial pyrogens, *Pharmacol. Rev.*, 1957, **9**, 427.
5. Freedman, H. H., Passive transfer of protection against lethality of homologous and heterologous endotoxins, *Proc. Soc. Exp. Biol. and Med.*, 1959, **102**, 504.
6. Freedman, H. H., RES and passive transfer of endotoxin tolerance, Conference on the Reticuloendothelial System (RES), *Ann. New York Acad. Sc.*, in press.
7. Beeson, P. B., Effect of reticulo-endothelial blockade on immunity to Shwartzman phenomenon, *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 146.
8. Beeson, P. B., Tolerance to bacterial pyrogens. II. Role of the reticulo-endothelial system, *J. Exp. Med.*, 1947, **86**, 39.
9. Good, R. A., and Thomas, L., Studies on the generalized Shwartzman reaction. II. The production of bilateral cortical necrosis of the kidneys by a single injection of bacterial toxin in rabbits previously treated with thorotrast or trypan blue, *J. Exp. Med.*, 1952, **96**, 625.
10. Zweifach, B. W., Benacerraf, B., and Thomas, L., The relationship between the vascular manifestations of shock produced by endotoxin, trauma, and hemorrhage. II. The possible role of the reticulo-endothelial system in resistance to each type of shock, *J. Exp. Med.*, 1957, **106**, 403.
11. Zweifach, B. W., and Thomas, L., The relationship between the vascular manifestations of shock produced by endotoxin, trauma, and hemorrhage. I. Certain similarities between the reactions in normal and endotoxin-tolerant rats, *J. Exp. Med.*, 1957, **106**, 385.

12. Farr, R. S., Clark, S. L., Jr., Proffitt, J. E., and Campbell, D. H., Some humoral aspects of the development of tolerance to bacterial pyrogens in rabbits, *Am. J. Physiol.*, 1954, **177**, 269.
13. Rosen, F. S., Skarnes, R. C., Landy, M., and Shear, M. J., Inactivation of endotoxin by a humoral component. III. Role of divalent cation and a dialyzable component, *J. Exp. Med.*, 1958, **108**, 701.
14. Grant, R., and Whalen, W. J., Latency of pyrogen fever. Appearance of a fast-acting pyrogen in the blood of febrile animals and in plasma incubated with bacterial pyrogen, *Am. J. Physiol.*, 1953, **173**, 47.
15. Wharton, D. R. A., and Creech, H. J., Further studies of the immunological properties of polysaccharides from *Serratia marcescens* (*Bacillus prodigiosus*). II. Nature of the antigenic action and the antibody responses in mice, *J. Immunol.*, 1949, **62**, 135.
16. Cluff, L. E., and Bennett, I. L., Jr., Factors influencing the alteration of the pyrogenic action of endotoxin by serum, *Bull. Johns Hopkins Hosp.*, 1957, **101**, 281.