DELAYED HYPERSENSITIVITY

IV. Systemic Reactivity of Guinea Pigs Sensitized to Protein Antigens*

By JONATHAN W. UHR,‡ M.D., and M. W. BRANDRISS,§ M.D.

(From the Department of Microbiology, New York University College of Medicine, New York)

(Received for publication, June 26, 1958)

In the preceding paper, studies were reported in which guinea pigs with delayed hypersensitivity to protein antigens were injected by various routes with a desensitizing dose of specific antigen (1). Although such amounts of antigen sufficed to prevent subsequent delayed skin reactivity for several days, none of the highly sensitive animals showed obvious signs of distress following these injections. In particular, there were no signs suggestive of the type of delayed shock seen in tuberculin-sensitive guinea pigs injected with a large amount of tuberculin (2), nor were there signs either of immediate or of protracted anaphylaxis.

In this paper a characteristic febrile response and several other features of the systemic reaction that follow specific challenge of guinea pigs with delayed hypersensitivity are described. This systemic reactivity has been compared to that which follows specific challenge of guinea pigs with passively acquired circulating antibody and the reactivity of animals with *both* delayed hypersensitivity and circulating antibody, passively or actively acquired.

Materials and Methods

Antigens.—Ovalbumin (Ea), diphtheria toxoid (To), and horse gamma globulin (HGG) were the same materials used in the preceding study (1). Crystalline bovine serum albumin (BSA) and bovine gamma globulin (BGG) were obtained from Armour, Inc. Antigens were dissolved in saline (0.85 per cent NaCl in distilled water) except for BGG which was dissolved in saline buffered at pH 7.4. The protein content of the antigen solutions was calculated from the absorption at 277 mμ of aliquots diluted in 0.25 N acetic acid.

Antisera.—Rabbit antitoxin, anti-ovalbumin, and anti-horse gamma globulin were the same as described in the preceding paper (1). The anti-bovine serum albumin was a pooled serum from two rabbits, each of which received a 4 week course of several intradermal and

* Aided by a grant from the National Institutes of Health, United States Public Health Service.

This work was conducted in part under the sponsorship of the Commission on Immunization, Armed Forces Epidemiological Board, and was supported in part by the Office of the Surgeon General, Department of the Army, Washington, D. C.

‡ Senior Research Fellow, United States Public Health Service.

§ Present address: Johns Hopkins Hospital, Baltimore.

intravenous injections totalling 52 mg. BSA. They were exsanguinated 2 weeks after the last injection and the pooled serum was passed through a Millipore filter and then stored at −20°C. The serum contained 2.08 mg./ml. antibody protein specifically precipitable by BSA (3). Examination by the gel diffusion technique of Ouchterlony (4) revealed a single line of precipitate with BSA (25 mg./ml). Rabbit anti-bovine gamma globulin serum was obtained and measured in a similar manner. It contained 2.40 mg./ml. antibody protein and in an Ouchterlony plate multiple precipitin lines were obtained with BGG. The guinea pig antitoxin was a pooled serum from 36 animals each of which had received a single intraperitoneal injection of 42 Lf of diphtheria toxoid in complete Freund's adjuvant 1 month before exsanguination. The serum was absorbed with a suspension of non-toxigenic *C. diphtheriae* to remove antibodies against non-toxigenic diphtherial proteins. The serum contained 1.35 mg. specifically precipitable antitoxin protein per ml. equivalent to 80 *in vitro* units per ml. Analysis of the pooled serum on Ouchterlony plates using purified and crude toxoids revealed single coalescent lines of precipitation. The most concentrated toxoid used contained 1 mg. toxoid protein/ml. and was only 65 per cent specifically precipitable by antitoxin. This indicated that precipitating antibodies against non-toxigenic diphtherial proteins had been removed or that they were directed against antigens not represented in sufficient amounts in the toxoids used.

Endotoxin.—*E. coli* endotoxin (Difco) was dissolved in saline and heated to 80°C. for 10 minutes before use.

Sensitization.—Specific precipitates prepared as previously described were usually suspended in complete adjuvant which contained 1 mg./ml. of killed *Mycobacterium butyricum* (Difco) (5). Each animal was injected, usually intramuscularly, with 0.5 ml. containing 3 μg. protein antigen.

Challenge.—Ten days after the sensitizing injection, when the animals were highly sensitive (1), they were challenged, usually intraperitoneally, with 4 mg. of antigen. Protein antigens were passed through a Millipore filter just prior to use. Intravenous injections were made using one of the veins in the ear or hind foot.

Sensitization and Challenge of Tuberculous Animals.—BCG organisms were grown in Long's media at 32°C. with continual shaking (6). Three weeks later, they were harvested, washed with saline, and 1 mg. wet weight of living bacilli suspended in saline were injected intraperitoneally into each animal. In order to produce shock, the infected animals were injected intraperitoneally 4 weeks later with 0.5 to 1.0 ml. of undiluted Old Tuberculin (New York City Health Department).

Recording of Temperatures.—Animals were kept in their cages unrestrained and had access to water and food during the experiments. Rectal temperatures were taken with an electronic thermometer (Tri-R), inserted 4 cm. and allowed to remain in place 30 seconds for equilibration. Temperatures were usually determined two or three times over a 30 minute period just before an experiment and the average taken as the base line. Animals whose base line temperatures were above 103°F. were not used.

Leucocyte Counts.—Animals were bled from the retro-orbital space with a capillary pipette. The white count was determined in a Levy hemocytometer. A smear was made with each count and one hundred cells were classified for differential counts.

RESULTS

The Febrile Response of Normal and Sensitized Animals Challenged with Non-Specific Proteins.—It was noted early in this work that hourly rectal temperatures of individual normal guinea pigs frequently vary by as much as 1°F., and that even greater variations may result from pronounced changes in environmental temperature. However, the average temperatures of *groups*

of three or more animals remained essentially unchanged under the conditions of the experiments to be reported. Fig. 1 shows that injection of proteins or saline into normal guinea pigs produces a slight febrile response. Three of the animals included in this group were injected with 1 ml. of "pyrogen-free" saline (Cutter) and each showed a temperature elevation of 0.5°–1°F. The animals previously injected with mycobacteria (Fig. 1) showed a greater febrile

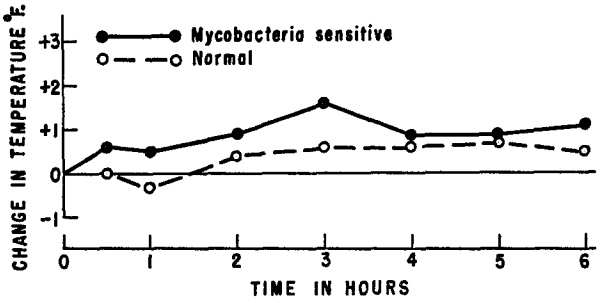

FIG. 1. Non-specific febrile responses in guinea pigs. Nine normal animals were injected intraperitoneally, in groups of three with 4 mg. Ea, 4 mg. To, or 1 ml. "pyrogen-free" saline. The four mycobacteria-sensitive animals, injected 10 days previously with specific precipitates in *complete* adjuvant, were challenged with 4 mg. of the non-specific protein (To or Ea).

TABLE I

Plan of Experiments to Demonstrate Specificity of Febrile Response in Delayed Hypersensitive Guinea Pigs after Specific Challenge

Sensitization	Group	1st day challenge	2nd day challenge	
			Plan I	Plan II
Protein A*	1	A‡	A‡	B
	2	B	B	A‡
Protein B*	3	B‡	B‡	A
	4	A	A	B‡

* Proteins A and B are immunologically distinct from each other.

‡ Specifically challenged.

response to the protein solutions than did normal animals. The cause of this difference is not known but may be due to traces of contaminating endotoxin in the challenging solutions, since animals sensitized to mycobacteria are known to be particularly susceptible to the toxic effects of bacterial pyrogens (7, 8) as well as to certain irritants (9).

As will be seen in the following sections, this type of non-specific response could be readily distinguished from the specifically elicited fever. However, it was necessary to carefully control the experiments as shown in Table I.

The animals in an experiment were divided into two (or more) groups, and each group was sensitized to a different protein antigen (A and B in Table I). Ten days later, one-half the animals in each group were injected with 4 mg. of the antigen to which they were sensitive, and the others with 4 mg. of the unrelated antigen. On the following day, one of two plans was employed: either (Plan I) the animals were injected for a second time with the same antigen they had received 24 hours earlier; or (Plan II) the groups previously challenged specifically now received the non-specific antigen; and the animals previously challenged non-specifically were now injected with the antigen to which they were sensitive. Temperatures were recorded on both days at hourly intervals for 5 to 6 hours after the injections.

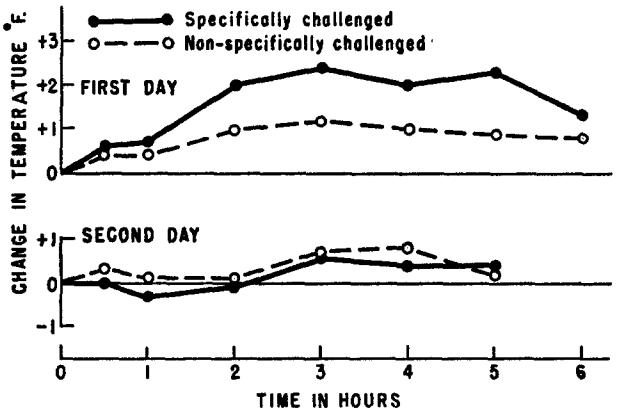

FIG. 2. Specific febrile response in sensitized guinea pigs. Groups of four animals were sensitized either to To, Ea, or HGG 10 days before intraperitoneal injection with 4 mg. of antigen according to Plan I in Table I.

Febrile Response to Specific Antigen.—

Groups of four guinea pigs were sensitized to Ea, HGG, or To. Ten days later one-half the animals in each group were injected with 4 mg. of the specific antigen. Each of the other two animals in each group were injected with 4 mg. of a different non-specific antigen. The following day each animal received a second injection of the same antigen it had received 24 hours earlier (Plan I, Table I).

Fig. 2 shows that on the first day, the specifically elicited febrile response was considerably greater than that which followed non-specific challenge. Each of the six specifically challenged animals developed a febrile response of at least 2°F. On second challenge, however, the febrile patterns of both groups were similar. These observations show that the pronounced febrile response only follows *specific* challenge and therefore *cannot* be due solely to injection of pyrogens contaminating the protein solutions used for challenge. Nevertheless, it seemed possible that the smaller *non-specific* response might have resulted from injection of traces of pyrogens and might be eliminated by inducing partial tolerance to bacterial endotoxins (10). Accordingly, fourteen animals sensitized with either Ea- or To-immune precipitates received six intravenous injections

each containing 5 μg. of bacterial endotoxin during the following 10 days. The average maximal febrile response to these injections declined from an initial 3.8°-2.4°F. by the 10th day. On the 11th and 12th day they were challenged with To and Ea according to Plan II in Table I. The temperature patterns shown in Fig. 3 confirm those of the previous experiment in which a specific febrile response was obtained which was markedly greater than the non-specific. Thirteen of the fourteen animals showed a febrile response of at least 2°F. after specific challenge. While the non-specific response had not been completely eliminated in the control animals by pretreatment with bacterial endotoxin, its magnitude was reduced compared to that seen in the first experiment (Fig. 2).

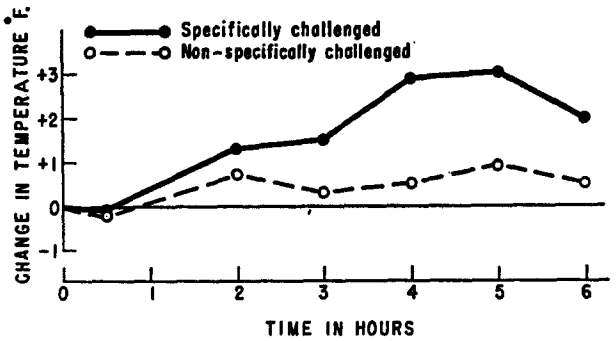

FIG. 3. Specific febrile response in sensitized animals pretreated with endotoxin. Seven To- and seven Ea- sensitive animals each received six intravenous injections of 5 μg. bacterial endotoxin/injection during the 10 days following sensitization. All were then challenged intraperitoneally with 4 mg. of antigen according to Plan II in Table I, and the febrile responses of both days were plotted together.

In other experiments, sensitive guinea pigs injected with 4 mg. of specific antigen intravenously or intradermally showed specific febrile responses lasting 6 to 10 hours analogous to that seen following intraperitoneal challenge.

Specific Febrile Responses Elicited on 2 Successive Days.—The preceding experiments demonstrated that injection of a desensitizing dose of the same specific antigen on 2 successive days into sensitized guinea pigs only produced a specific febrile response on the 1st day. Apparently the animals had been specifically desensitized to systemic reactivity as well as to skin reactivity by the first injection of antigen (1).

To investigate the specificity of systemic desensitization, animals were sensitized simultaneously to two different protein antigens (Ea and To) and challenged successively with each of them at 24 hour intervals. Six doubly sensitized guinea pigs were challenged with Ea and six with To. On the following day three animals in each group were again injected with the same antigen. The other three in each group received the "second" antigen to which they were still sensitive. Fig. 4 shows the fever patterns on each day of the six animals challenged with a

different antigen on 2 successive days; and the six injected on the 2nd successive day with the same antigen.

As can be seen from Fig. 4, specific challenge on the 1st day elicited the usual febrile response. On the next day, those challenged with the "second" antigen showed more fever than those injected again with the same antigen, thus confirming the specificity of systemic desensitization. However, the febrile response to a "second" specific challenge was less than that seen following the first specific challenge 24 hours previously. It will be recalled that a somewhat analogous situation exists in regard to skin reactivity. In doubly sensi-

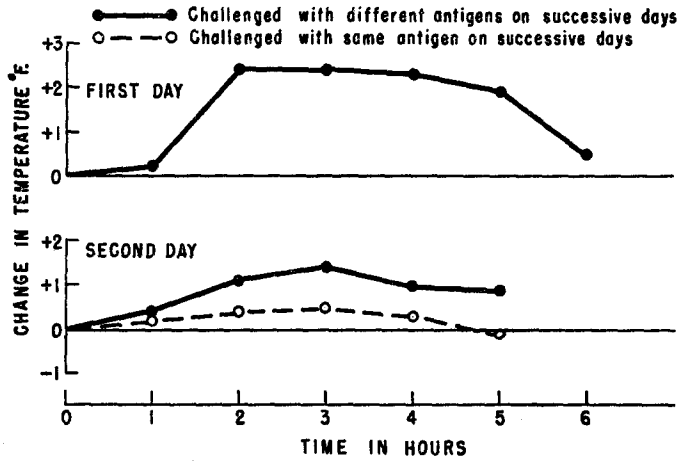

FIG. 4. Specific febrile response on 2 successive days. Twelve animals were sensitized to *both* Ea and To and 10 days later six were injected intraperitoneally with 4 mg. To, and the other six with 4 mg. Ea. The following day three of each group were rechallenged with the same antigen and the others with the "second" antigen to which they were still sensitive.

tized animals specific desensitization to one antigen causes a transient decrease in skin reactivity to intradermal challenge with the "second" antigen (1).

Role of Killed Mycobacterium butyricum in the Sensitizing Injection.—The inclusion of killed mycobacteria in the sensitizing injection is known to enhance, but is not essential for induction of delayed skin reactivity (5). The effect of omitting mycobacteria from the sensitizing injection upon the temperature response to subsequent specific challenge was investigated. Five guinea pigs sensitized to Ea and three to To with *incomplete* adjuvant were challenged with Ea on 2 successive days. Fig. 5 shows that while the febrile response of these animals was less than that seen in mycobacteria-sensitized animals, nevertheless, the specifically challenged group again showed more fever than the nonspecifically challenged. As was seen in earlier experiments, the desensitizing dose of antigen on the 1st day abolished the specific febrile response on the succeeding day.

Specific Febrile Response to a Small Dose of Antigen.—In the experiments described thus far the amount of antigen in the challenging injection sufficed to abolish skin reactivity (1).

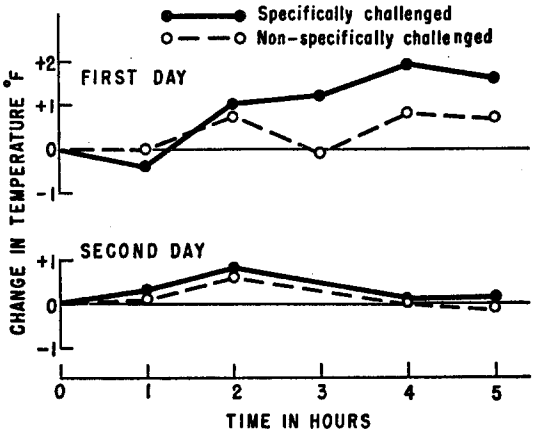

FIG. 5. Specific febrile response in guinea pigs sensitized with specific complexes suspended in incomplete adjuvant. Five animals were sensitized to Ea and three to To 10 days before intraperitoneal injection of 4 mg. Ea on 2 successive days.

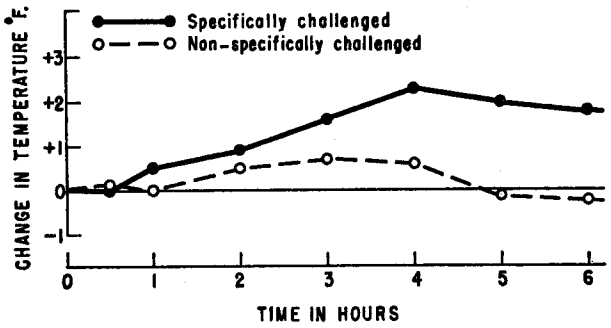

FIG. 6. Specific febrile response to a small dose of antigen. Six guinea pigs were sensitized to To and three to BSA 10 days before intraperitoneal injection of 4 mg. To.

In the following experiment guinea pigs sensitized to To or BSA were challenged according to Plan II, Table I, with 100 μg. of specific antigen, an amount which is insufficient to completely abolish subsequent skin reactivity.

Only the results of injecting To are shown in Fig. 6 which indicates that injection of only 100 μg. elicited a specific febrile response of approximately the same magnitude as a desensitizing dose of To. The animals that were injected with 100 μg. BSA (not shown in Fig. 6) did not show a specific response. This may be explained by the fact that BSA is a relatively poor antigen in guinea pigs (11, 12). Skin testing at the conclusion of the experiments confirmed

that sensitivity had not been abolished since all the animals showed delayed skin reactions between 10 and 20 mm. in diameter to 3 μg. of the specific antigen.

Specific Febrile Response in Animals Rendered Tolerant to Bacterial Endotoxin. —Daily injections of bacterial endotoxin eventually elicit resistance to its toxic and fever-producing actions (10). This appears to be associated with functional alterations of the reticulo-endothelial cells (13). It was possible that these alterations might affect the specific febrile response of sensitized animals.

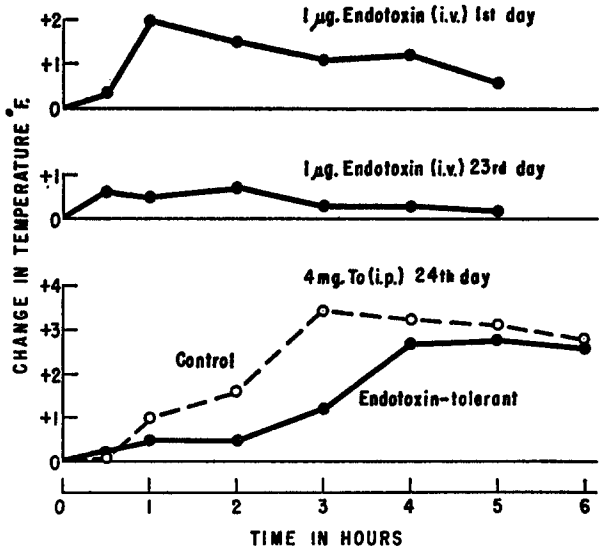

FIG. 7. Specific febrile response in endotoxin-tolerant guinea pigs. The responses of five To-sensitive animals to 1 μg. bacterial endotoxin, before and after induction of tolerance, and to injection of 4 mg. To are shown. The latter febrile response is compared to that seen in two control sensitive animals.

Thirty-six guinea pigs were sensitized to To and 4 days later a course of daily injections, usually intravenous, of increasing amounts of bacterial endotoxin was begun. Three weeks later the animals could tolerate 500 μg. of endotoxin. Only ten animals, however, survived this severe course of treatment and these were emaciated. Twenty-three days after the first injection of endotoxin, the surviving guinea pigs were again injected intravenously with 1 μg. of endotoxin and the temperature responses measured.

Fig. 7 shows the fever patterns on the 1st and 23rd day of the five animals which showed the least fever after the final endotoxin challenge. On the following day, these five selected animals and two control sensitive animals were challenged intraperitoneally with To. As seen from Fig. 7, the endotoxin-tolerant animals still showed fever after specific challenge. Although the febrile response was somewhat delayed in onset, by 4 to 6 hours the degree of fever reached was equivalent to that seen in the control-sensitive group (see also Fig. 3). This was an unexpected finding, since in previous studies of experi-

mental fever only endogenous pyrogen has been shown to produce a full febrile response in endotoxin-tolerant *rabbits*; viruses of the influenza group and bacterial endotoxins do not (14, 10). It was also of interest that both the onset and peak of endotoxin-induced fever occur earlier than in the specific febrile response following intravenous challenge of sensitive animals.

Effect of Cortisone upon the Specific Febrile Response.—ACTH and corticosteroids are known to possess significant antipyretic activity in several laboratory animals and in man (15, 16). For example, ACTH may significantly decrease the febrile response following injection of killed typhoid bacilli and influenza virus in rabbits (17). Furthermore, these hormones have been shown

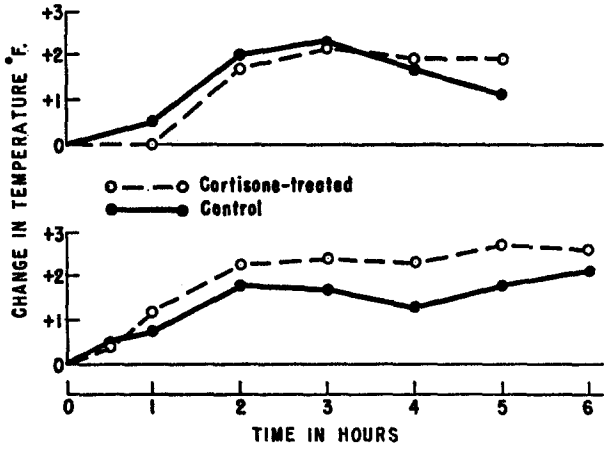

FIG. 8. Specific febrile response in sensitized guinea pigs pretreated with cortisone. The upper figure represents ten sensitive animals (To or BGG) six of which were treated with 10 mg. cortisone 30 minutes before intraperitoneal injection with 4 mg. of specific antigen. In the lower figure three out of five To-sensitive animals received 5 mg. cortisone daily for 3 days before all were challenged with 4 mg. To.

to exert suppressive effects on delayed skin reactivity in sensitized animals and man to tuberculin and to streptococcal vaccine (18–20).

The effect of injecting cortisone just prior to the specific challenge of sensitized animals was studied.

To- and BSA-sensitive animals were injected with 5 mg. cortisone acetate, intramuscularly, and, also, 5 mg. of hydrocortisone sodium succinate, intravenously, 30 minutes before challenge. The combination of the rapidly acting intravenous preparation and the more slowly absorbed saline suspension was felt to insure immediate high blood levels of corticosteroids which would persist for the duration of the "expected" febrile response.

As shown in Fig. 8, there was no significant difference between the febrile responses of the cortisone-treated and control-sensitive groups. Three normal animals injected only with cortisone showed no febrile response. Because of

this unexpected result, a second experiment was performed in which pretreatment with cortisone was prolonged to ensure saturation of the tissues with the hormone. Three To-sensitive animals were injected intramuscularly with 5 mg. cortisone acetate on 3 successive days. One hour after the last cortisone injection they and two control-sensitive animals were injected with To. Fig. 8 shows that the results of this experiment were analogous to the preceding one.

Temperature Responses of Specifically Challenged Normal and Delayed Hypersensitive Guinea Pigs with Circulating Antibody.—Small amounts of circulating antibody are capable of sensitizing normal or delayed hypersensitive guinea pigs to respond to intradermal challenge with an Arthus reaction (21, 5). This reaction differs from that which follows skin testing of guinea pigs with delayed hypersensitivity to proteins without detectable circulating antibody (5). Since it is known that hypothermia frequently accompanies anaphylactic shock in the guinea pig (22), it was probable that circulating antibody would alter the systemic reactivity of both normal and delayed hypersensitive animals to specific intraperitoneal challenge. The temperature patterns of groups of guinea pigs, normal or To-sensitive, injected 24 hours previously with either 5, 35, or 110 μg. guinea pig antitoxin N were determined after specific challenge. Fig. 9 shows that 5 μg. of antibody N sufficed to sensitize normal or delayed hypersensitive animals to a brief mild *hypothermic* response. After 1 hour, the delayed hypersensitive animals showed fever, the normals did not. These results were confirmed with a second antigen-antibody system. Four normal animals sensitized with 5 μg. rabbit anti-Ea showed a similar temperature depression after specific challenge. All animals, normal and delayed hypersensitive, that received 35 or more μg. of antibody N showed severe hypothermia after specific challenge. In order to see whether delayed hypersensitive guinea pigs with actively acquired circulating antibody showed a similar response as did those with passively acquired antibody, To-sensitive animals were stimulated intradermally with small amounts of free To. At the time that their total circulating antitoxin N ranged between 5 to 50 μg.[1] they were specifically challenged. All responded with a fall in temperature. Also shown in Fig. 9 is the progressive hypothermia of specifically challenged tuberculous animals.

It has been shown that only 5 μg. of antibody nitrogen is sufficient to sensitize guinea pigs to hypothermia after specific challenge. It was possible that smaller amounts, even quantities insufficient to be later detected in the circulation, might sensitize to a febrile response and mimic the systemic reactivity of delayed hypersensitive animals. Accordingly, 28 guinea pigs, in groups of

[1] Calculated from the antitoxin N/milliliter assuming a serum volume of 20 ml. These figures represent minimal values since antibodies against non-toxigenic diphtherial proteins were not measured.

three or four were injected intravenously with either 1, 0.1, 0.01, or 0.001 μg. antibody N. As shown in Fig. 10 specific challenge 24 hours later with 4 mg. antigen did not produce a specific febrile response in these animals.

Leucocyte Counts.—As can be seen from Table II, injection of a desensitizing dose of specific antigen into delayed hypersensitive guinea pigs causes the development of a striking lymphopenia that persists for many hours. Thus, the

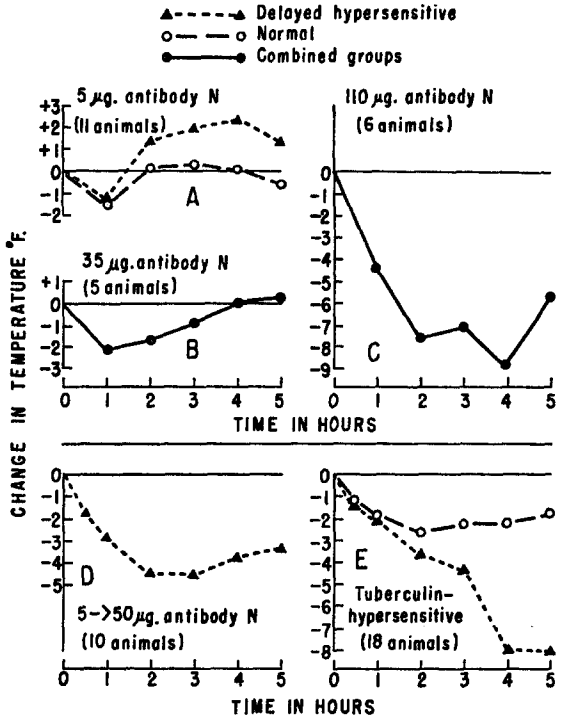

FIG. 9. Temperature responses following specific intraperitoneal challenge.

A, *B*, and *C* represent normal (including non-specifically sensitive) and To-sensitive animals injected 24 hours previously with homologous antitoxin.

D represents To-sensitive animals with actively acquired antitoxin.

E represents normal and BCG-infected animals injected with 0.5 ml. Old Tuberculin.

average lymphocyte count of twelve such animals fell 70 per cent 6 hours after specific challenge. Lymphopenia is not seen in either non-specifically challenged delayed hypersensitive animals or after specific challenge of animals with passively acquired circulating antibody, indeed, the latter group shows a lymphocytosis. Granulocytosis occurs in delayed hypersensitive animals whether challenged with the specific or non-specific protein. Normal animals injected with 10 μg. endotoxin show a prompt and profound granulocytopenia followed by a granulocytosis and lymphopenia. These latter findings are in

agreement with leucocyte changes reported in rabbits after injection of endotoxin (23).

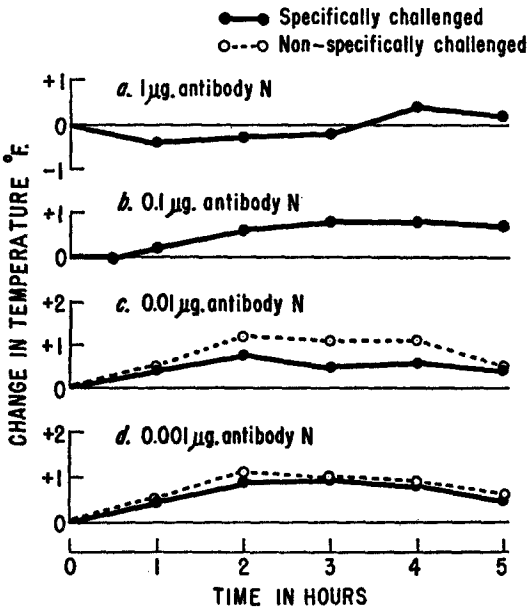

FIG. 10. Temperature responses after intraperitoneal challenge of guinea pigs previously sensitized with small amounts of circulating antibody. Groups of 3 to 4 animals were injected intravenously with antisera 24 hours before challenge with 4 mg. antigen. Temperature curves (*a*) and (*b*) represent the response of normal animals previously sensitized with guinea pig antitoxin (AT) to challenge with To; (*c*) and (*d*) the response of mycobacteria-sensitive[2] animals previously injected with rabbit AT or anti-ovalbumin (anti-Ea) to challenge with Ea. Mycobacteria-sensitive animals previously injected with guinea pig AT or anti-Ea are also included in (*d*), since their temperature responses were strictly analagous to those seen in the animals sensitized with rabbit antisera.

Relation of Specific Delayed Systemic Reactivity to Tuberculin Shock.—Tuberculin-sensitive guinea pigs after injection of a large amount of Old Tuberculin may show progressive hypothermia, weight loss, muscular weakness, prostration, and frequently death 6 to 36 hours after challenge. Examination of their organs reveals severe congestion of the liver, spleen, and mesenteric blood vessels as well as acute inflammatory changes surrounding pre-existing tuberculous foci and the needle tract of the tuberculin injection. There may be transudation of fluid into the body cavities (2, 24).

It has been postulated that the acute inflammatory changes associated with tuberculin shock are the result of a specific response of the host's tissues to the parenteral injection of a large amount of tuberculin. The nature and extent of the acute inflammatory reactions were felt sufficient to account for shock and death. Tuberculin shock occurs not only in experimentally sensitized animals but also in tuberculous man. "Flareup" of tuberculous lesions and violent systemic reactions with death have been reported in humans following injection of

[2] Injected with 0.5 ml. complete adjuvant 10 days before.

minute and large amounts of Old Tuberculin respectively (2). A shock-like syndrome also follows injection of large numbers of killed streptococci in rabbits previously infected with streptococci (25). Examination of the organs of such rabbits that do *not* succumb within the first 24 hours following the specific challenge shows extensive perivascular infiltrations with lymphoid cells. This histologic alteration is the characteristic lesion of delayed skin reactions

TABLE II

Leucocyte Counts

No. of animals		Sensitization*	Challenge	Time after challenge	Circulating leucocytes per mm.³ (average)		
Total	Tested				Total	Polymorphonuclear	Lymphocytes
				hrs.			
12	12	Delayed hypersensitivity	Specific antigen	0	10,170	3,560	6,610
	3‡			¼	11,770	4,385	7,180
	8			1	10,100	5,656	4,343
	12			6	10,260	7,900	2,155
	5			24	11,680	6,891	4,672
8	8	Delayed hypersensitivity	Non-specific antigen	0	8,340	2,419	5,755
	8			6	15,800	8,690	6,952
	6			24	8,800	3,872	4,752
5	5	Passive antibody	Specific antigen	0	5,140	2,416	2,724
	5			6	7,840	2,900	4,625
5	5‡	None	10 μg. endotoxin	0	8,880	2,131	6,028
	3			¼	4,500	270	4,005
	3			3	4,470	1,118	2,995
	5			6	8,030	4,898	2,730

* Delayed hypersensitive animals were sensitized to To or Ea 10 days before challenge. Passive antibody was 100 μg. guinea pig antitoxin N or 50 μg. rabbit anti-Ea N injected intravenously 24 hours before challenge.

‡ Challenged intravenously. All other challenging injections were given intraperitoneally.

to tuberculin and other protein antigens (26, 27). A similar delayed shock occurs after specific challenge of mice previously infected with *Histoplasma* (28).

Since tuberculin and streptococcal shock occurred in sensitized animals with delayed skin reactivity to the specific antigen and were "delayed" in onset in comparison to classical anaphylactic shock, it was not surprising that these delayed shock states were considered by some to be the prototypic systemic reactions of animals with delayed hypersensitivity (25, 29). There are, however, alternative interpretations possible. Experimentally infected animals usually develop circulating antibodies, and protracted shock and death may follow parenteral injection (usually *not* intravenous) of antigen into animals with circulating antibody (21, 30,

31). In addition, culture filtrates of both tubercle bacilli and streptococci may contain pyrogens (32, 33). Stetson *et al.* have presented strong evidence that bacterial endotoxins present in Old Tuberculin may be responsible for initiating shock and death in tuberculous guinea pigs after specific challenge (32).

It is evident from the preceding experiments that guinea pigs with delayed hypersensitivity to any one of five different protein antigens do not develop delayed shock or death after injection of a desensitizing dose of the specific antigen. It was conceivable that either lengthening the interval between sensitization and challenge, or increasing the challenge dose might alter the systemic reactivity after specific challenge of these animals. Eight To- or BSA-sensitive animals were specifically challenged 1 month after sensitization;

TABLE III

Deaths in Specifically Challenged Normal and To-Sensitive Guinea Pigs Previously Injected with Homologous Antitoxin

Antitoxin N*	Fatalities	
	Normal‡	To-sensitive
μg.		
1	0/2	0/2
5	1/8	0/4
35	2/5	0/2
110	4/8	4/5
Total.....	6/22 (27%)	4/13 (31%)

* Guinea pig antitoxin was injected intravenously 24 hours before intraperitoneal challenge with 4 mg. To.

‡ Normals include six animals sensitive to Ea.

and seven Ea-sensitive guinea pigs were injected 10 days later with 60 to 100 mg. Ea. None showed shock after specific challenge.

It was also possible that circulating antibody by itself, or in conjunction with delayed hypersensitivity might render guinea pigs susceptible to a condition resembling tuberculin shock after specific challenge. Twenty-six normal and thirteen To-sensitive animals were passively provided with 1 to 110 μg. guinea pig antitoxin N, 24 hours before specific challenge. Table III shows that the percentage of fatalities of these animals did not appear to be influenced by the presence of the delayed hypersensitive state. Furthermore, the severity of the outward signs of distress appeared to correlate solely with the amount of antibody N each animal had previously received. The systemic reactivity and gross anatomical findings of these animals were compared to those seen in two dozen BCG-infected guinea pigs injected, intraperitoneally, 1 month later with 0.5 ml. Old Tuberculin. The shock state in the group passively sensi-

tized with circulating antibody differed in several ways from that seen in specifically challenged tuberculous animals: (*a*) Signs of anaphylaxis were prominent. (*b*) Deaths usually occurred within 5 hours. (*c*) Deceased animals showed emphysema of the lungs and not the severe congestive changes seen in guinea pigs that succumb to tuberculin shock.

Observations were also made of the reactions to specific challenge of eighteen To-sensitive animals with actively acquired circulating antibody. (Fig. 9D shows the temperature response of the survivors.) The responses of these animals were generally analogous to those previously described in animals passively sensitized with circulating antibody. The only exceptions were several animals that died more than 5 hours after the specific challenge. These animals had moderate congestion of the viscera and only slight emphysema of the lungs.

With these exceptions, specific challenge of delayed hypersensitive guinea pigs with circulating antibody did not produce a condition which closely resembled tuberculin shock. Furthermore, there was no evidence that delayed hypersensitivity rendered animals with circulating antibody more susceptible to shock after specific challenge.

DISCUSSION

It is over a half century since von Pirquet suggested that fever and other systemic manifestations of certain infectious diseases might be due to hypersensitivity of the host to one or more products of the infective agent (34). Since then it has been demonstrated that animals or man previously infected with tubercle bacilli, streptococci, *Brucella*, and other organisms may develop febrile reactions after injection of the corresponding killed bacteria or their products (35–39). In addition, rabbits sensitive to proteins may show fever after challenge with the corresponding antigen(s) (40, 41).

While these febrile responses may have been initiated by systemic absorption of specific antigen into a sensitized host, there are certain objections against an unqualified acceptance of such a conclusion. For example, some of the febrile responses that were observed may have been due to injection of bacterial endotoxins. Culture filtrates of streptococci and tubercle bacilli as well as Gram-negative organisms themselves are pyrogenic for normal animals (32, 33, 42). Furthermore, animals during sensitization may develop heightened reactivity to endotoxins and perhaps other non-specific challenging materials, as in this study, in which guinea pigs sensitized with specific precipitates in complete adjuvant showed elevation of temperature following injection of *non-specific* protein solutions. The specificity of the response can only be definitely established if control experiments are included in which there is comparison of the responses of sensitized animals to injections of specific and strictly analogous but non-specific antigens. Finally, there is the problem of differentiating systemic reactivity due to absorption of antigen into the circulation from a systemic response to a focal area of tissue damage at the site

of injection. For example, Pappenheimer *et al.* (43) in a study of immunization reactions in adults noted a correlation between delayed skin reactivity to diphtheria toxoid and a febrile response following a later immunizing injection of toxoid. Since the febrile response was invariably accompanied by a severe local reaction at the subcutaneous injection site, it was possible that the systemic reaction was a response to the local inflammation and not to circulating antigen.

In the experiments reported here the specificity of the febrile response has been firmly established. That the response is not due to injection of contaminating bacterial pyrogens has been demonstrated not only by its specificity, but also by the persistence of a febrile response in endotoxin-tolerant animals. The experiments have also demonstrated that fever can result from intravenous injection of specific antigen into delayed hypersensitive guinea pigs. Furthermore, the febrile response occurs several hours *before* any visible local inflammation could occur from an intradermal challenge. Indeed, intradermal challenge with 4 mg. of specific antigen causes systemic reactivity *without* macroscopic local inflammation (1). This difference between systemic and skin reactivity is probably related to the relatively slow development of the local reaction. Sensitized cells which might have participated in the skin reaction interact with antigen absorbed into the circulation from the injection site, resulting in initiation of a systemic response and specific desensitization of the cells. These studies have also shown, however, striking similarities between the systemic and local skin response. (*a*) Both are enhanced if mycobacteria are included in the sensitizing injection. (*b*) Injection of 4 mg. of specific antigen specifically prevents elicitation of either response on the following day. (*c*) Injection of 4 mg. of specific antigen transiently depresses systemic as well as skin reactivity to a second antigen in animals sensitive to both. (*d*) The presence of small amounts of circulating antibody alters the character of both responses.

Although there are many different forms of experimental fever, Atkins (36) and Wood (44) have postulated that release into the circulation of an endogenous pyrogen as a product of cellular injury may be a common pathogenetic mechanism. They have suggested that this pyrogen may be the factor which acts upon the hypothalamus to cause fever. The granulocyte has been incriminated as a principal source of this pyrogen (45–47), however, granulocytopenic animals still show fever after injection of bacterial endotoxins (48) or influenza viruses (49). Furthermore, fever occurs in granulomatous and viral infections whose characteristic pathologic lesions have few polymorphonuclear leucocytes.

When discussing the relation of the febrile responses described in this paper to other experiments reported in the literature, it must be constantly borne in mind that we have used only guinea pigs, in contrast to other studies of experimental fever which have usually employed rabbits, dogs, or man. The febrile response in specifically challenged delayed hypersensitive guinea pigs may also be mediated by an endogenous pyrogen released from sensitized cells but this

has not yet been proved. There is, however, a striking resemblance between the observed fever with its accompanying lymphopenia and the response seen in rabbits injected with viruses of the influenza group in which an endogenous pyrogen has been demonstrated (50, 51). There is no further support from our experiments to indicate that the granulocyte would be a principal source of such a pyrogen. On the contrary, it is cells of the lymphoid series that respond to specific intradermal challenge in delayed hypersensitive guinea pigs (27). Since as little as 100 μg. of antigen can elicit a febrile response in specifically sensitized guinea pigs it is suggested that induction of the delayed hypersensitive state may be an additional factor in the pathogenesis of fever seen in certain infectious diseases.

If 5 or more micrograms of antibody nitrogen are injected into normal or delayed hypersensitive animals, specific challenge is followed by a brief period of hypothermia. Only in the delayed hypersensitive animals is it soon replaced with a febrile response. If more than 30 μg. antibody nitrogen are injected, delayed hypersensitive as well as normal guinea pigs show severe hypothermia which in the former instance masks the thermogenic effects of the specific challenge.

These studies indicate that systemic reactivity as well as skin reactivity in delayed hypersensitive guinea pigs differ *biologically* from those reactions resulting from the interaction of antigen with detectable circulating gamma globulin antibody either in the circulation or skin. Adding to the weight of evidence that they are probably *mechanistically* different is the inability to sensitize to a specific febrile response with minute amounts of passively administered circulating antibody.

SUMMARY

Guinea pigs with delayed hypersensitivity to protein antigens show a specific febrile response accompanied by a lymphopenia following injection of a desensitizing dose of specific antigen. No signs of shock are observed in highly sensitive animals following this injection. The response is not prevented in sensitive guinea pigs by inducing endotoxin tolerance or by pretreating with cortisone before specific challenge. Using a suitable antigen in sufficiently sensitive animals as little as 100 μg. can elicit a pronounced febrile response. Injection of a desensitizing dose of antigen specifically abolishes systemic as well as skin reactivity for several days. Normal or hypersensitive (delayed-type) animals passively sensitized with sufficient amounts of serum antibody show hypothermia after specific challenge and may show a delayed type of fatal shock. Differences were noted between their systemic reactivities, however, and the reactivity seen in specifically challenged tuberculous animals.

BIBLIOGRAPHY

1. Uhr, J. W., and Pappenheimer, A. M., Jr., Delayed hypersensitivity. III. Specific desensitization of guinea pigs sensitized to protein antigens, *J. Exp. Med.*, 1958, **108**, 891.

2. Rich, A. R., The pathogenesis of tuberculosis, Springfield, Illinois, Charles C. Thomas, 2nd edition, 1951.
3. Gitlin, D., Use of ultraviolet adsorption spectroscopy in the quantitative precipitin reaction, *J. Immunol.*, 1949, **62,** 437.
4. Ouchterlony, O., Diffusion-in-gel methods for immunological analysis, *Progr. Allergy*, 1958, **5,** 1.
5. Uhr, J. W., Salvin, S., and Pappenheimer, A. M., Jr., Delayed hypersensitivity. II. Induction of hypersensitivity in guinea pigs by means of antigen-antibody complexes, *J. Exp. Med.* 1957, **105,** 11.
6. Long, E. R., A study in fundamentals of the nutrition of the tubercle bacillus: The utilization of some amino acids and ammonium salts, *Am. Rev. Tuberc.*, 1919, **3,** 86.
7. Freund, J., The effect of heterologous bacterial products upon tuberculous animals, *J. Immunol.*, 1936, **30,** 241.
8. Bordet, P., Contribution a l'etude de l'allergie. L'Allergie non specifique, *Ann. Inst. Pasteur*, 1936, **56,** 325.
9. Chase, M., *Rockefeller Institute Bull.*, 1956–57, **2,** 71.
10. Beeson, P. B., Tolerance to bacterial pyrogens. I. Factors influencing its development, *J. Exp. Med.*, 1947, **86,** 29.
11. Pappenheimer, A. M., Jr., Scharff, M., and Uhr, J. W., Delayed hypersensitivity and its possible relation to antibody formation, *in* Mechanisms of Hypersensitivity, (J. H. Shaffer, editor), Boston, Little Brown and Co., in press.
12. Weigle, W. O., and Dixon, F. J., The elimination of heterologous serum proteins and associated antibody responses in guinea pigs and rats, *J. Immunol.*, 1957, **79,** 24.
13. Beeson, P. B., Tolerance to bacterial pyrogens. II. Role of the reticuloendothelial system, *J. Exp. Med.*, 1947, **86,** 39.
14. Atkins, E., and Huang, W. C., Studies on the pathogenesis of fever with influenzal viruses. III. The relation of tolerance to the production of endogenous pyrogen, *J. Exp. Med.*, 1958, **107,** 415.
15. Bennett, I. L., Jr., and Beeson, P. B., The effect of cortisone upon reactions of rabbits to bacterial endotoxins with particular reference to acquired resistance, *Bull. Johns Hopkins Hosp.*, 1953, **93,** 290.
16. Atkins, E., Allison, F., Jr., Smith, M. R., Wood, W. B., Jr., Studies on the antipyretic action of cortisone in pyrogen-induced fever, *J. Exp. Med.*, 1954, **101,** 353.
17. Kass, E. H., and Finland, M., Effect of ACTH on induced fever, *New England J. Med.*, 1950, **243,** 693.
18. Long, D. A., and Miles, A. A., Opposite actions of thyroid and adrenal hormones in allergic hypersensitivity, *Lancet*, 1950, **1,** 492.
19. Osgood, C. K., and Favour, C. B., The effect of adrenocorticotropic hormone on inflammation due to tuberculin hypersensitivity and turpentine and on circulating antibody levels, *J. Exp. Med.*, 1951, **94,** 415.
20. Fischel, E., The relationship of adrenocortical activity to immune response, *Bull. New York Acad. Med.*, 1950, **26,** 255.

21. Benacerraf, B., and Kabat, E. A., A quantitative study of the Arthus phenomenon induced passively in the guinea pig, *J. Immunol.*, 1950, **64,** 1.
22. Seegal, B. C., *in* Agents of Disease and Host Resistance, (F. P. Gay, editor), Springfield, Illinois, Charles C. Thomas, 1935.
23. Bennett, I. L., Jr., and Beeson, P. B., The properties and biologic effects of bacterial pyrogens, *Medicine*, 1950, **29,** 365.
24. Boyden, S. V., The immunologic response to antigens of the tubercle bacillus, *Progr. Allergy*, 1958, **5,** 149.
25. Hitchcock, C. H., Camero, A. R., and Swift, H. F., Perivascular reactions in lung and liver following intravenous injection of streptococci into previously sensitized animals, *J. Exp. Med.*, 1934, **59,** 283.
26. Gell, P. G. H., and Hinde, I. T., The histology of the tuberculin reaction and its modification by cortisone, *Brit. J. Exp. Path.*, 1951, **32,** 516.
27. Kaplan, M. H., The cellular response in forms of delayed and immediate-type skin reactions in the guinea pig, *in* Mechanisms of Hypersensitivity, (J. H. Shaffer, editor), Boston, Little Brown and Co., in press.
28. Salvin, S. B., Hypersensitivity in mice with experimental histoplasmosis, *J. Immunol.*, 1955, **75,** 1.
29. Raffel, S., *in* Immunity, New York, Appleton-Century-Crofts, Inc., 1953, 249.
30. Dienes, L., Comparative study of the anaphylactic and tuberculin types of hypersensitiveness. I. General reactions similar to the tuberculin shock in tuberculous guinea pigs sensitized with various antigens, *J. Immunol.*, 1931, **20,** 221.
31. Stone, S. H., Protracted anaphylactic shock after subcutaneous eliciting injection, *Fed. Proc.*, 1958, **17,** 536.
32. Stetson, C. A., Schlossman, S., and Benacerraf, B., Endotoxin-like effects of Old Tuberculin, *Fed. Proc.*, 1958, **17,** 536.
33. Stetson, C. A., The endotoxic properties of lysates of Group A hemolytic streptococci, *J. Exp. Med.*, 1956, **104,** 921.
34. von Pirquet, C. E., Allergy, *Allergy, Arch. Int. Med.*, 1911, **7,** 383.
35. Stetson, C. A., Jr., Studies on the mechanism of the Schwartzman phenomenon. Similarities between reactions to endotoxins and certain reactions of bacterial allergy, *J. Exp. Med.*, 1955, **101,** 421.
36. Atkins, E., The relation of a circulating pyrogen to the cause of experimental fever, *Bull. New York Acad. Med.*, 1956, **32,** 846.
37. Spink, W. W., The significance of endotoxin in Brucellosis: Experimental and clinical studies, *Tr. Assn. Am. Phyn.*, 1954, **67,** 283.
38. Bennett, I. L., Jr., Studies on the pathogenesis of fever. V. The fever accompanying pneumococcal infection in the rabbit. *Bull. Johns Hopkins Hosp.*, 1956, **98,** 216.
39. King, M. K., and Wood, W. B., Jr., Studies on the pathogenesis of fever. V. The relation of circulating endogenous pyrogen to the fever of acute bacterial infections, *J. Exp. Med.*, 1958, **107,** 305.
40. van Genderen, H., and Durville, H. H. M., Some aspects of the fever-producing action of purified bacterial pyrogens and non-bacterial antigens, *Acta Physiol. et Pharm. Neerl.*, 1957, **5,** 349.

41. Farr, R., Febrile response upon injection of bovine albumin into previously sensitized rabbits, (abstract), *J. Clin. Inv.*, 1958, in press.
42. Friis, T., Measurement of the body temperature of guinea pigs. I. Reaction of normal animals to administration of purified tuberculin and indifferent fluids, *Acta Path et Microbiol. Scand.*, 1955, **37**, 225.
43. Pappenheimer, A. M., Jr., Edsall, G., Lawrence, H. S., and Banton, H. J., A study of reactions following administration of crude and purified diphtheria toxoid in an adult population, *Am. J. Hyg.*, 1950, **52**, 323.
44. Wood, W. B., Jr., Studies on the cause of fever, *New Eng. J. Med.*, 1958, **258**, 1023.
45. 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.
46. 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.
47. 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, **70**, 64.
48. Bennett, I. L., and Cluff, L. E., Influence of nitrogen mustard upon reactions to bacterial endotoxins: Schwartzman phenomenon and fever, *Proc. Soc. Exp. Biol. and Med.*, 1952, **81**, 304.
49. Bennett, I. L., Jr., and Wagner, R. R., Production of fever by influenzal viruses. V. Effect of nitrogen mustard (HN_2), *Bull. Johns Hopkins Hosp.*, 1955, **97**, 43.
50. Wagner, R. R., Bennett, I. L., Jr., and LeQuire, V. S., The production of fever by influenzal viruses. I. Factors influencing the febrile response to single injections of virus, *J. Exp. Med.*, 1949, **90**, 321.
51. Atkins, E., and Huang, W. C., Studies on the pathogenesis of fever with influenzal viruses. I. The appearance of an endogenous pyrogen in the blood following intravenous injection of virus, *J. Exp. Med.*, 1958, **107**, 383.