STUDIES ON THE MECHANISM OF THE SHWARTZMAN PHENOMENON*

ACCELERATED CUTANEOUS REACTIVITY TO BACTERIAL ENDOTOXINS

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Intradermal injections of bacterial endotoxins in normal rabbits ordinarily yield delayed inflammatory reactions which are not manifest in the gross for several hours and reach maximal development only after a day or more. While the mechanism underlying these characteristic cutaneous reactions is not understood, it has been pointed out that the reactions resemble, in many respects, those of delayed hypersensitivity (1). The present study was prompted by recent chance observations which suggested that the dermal reactivity of rabbits to bacterial endotoxins could be much modified by previous exposure of the animals to endotoxins. It will be shown that prior injection of endotoxins leads to the development of Arthus-type reactivity and that this is correlated with the development of an immune response of unexpected qualitative and quantitative characteristics. The present report is concerned with a general description of the accelerated cutaneous reactivity, its passive transfer with serum and its possible significance.

Materials and Methods

Animals.—Albino hybrid rabbits of either sex weighing approximately 1.6 to 1.8 kilos were used. Commercial rabbit pellets and water were allowed *ad libitum.*

Endotoxins.—Lyophilized lipopolysaccharide endotoxins from several Gram-negative bacteria were obtained from the Difco Laboratories, Inc., Detroit, and were dissolved in physiologic saline solution for injection. Volumes of 2.0 ml. for intravenous injections and 0.2 ml. for intradermal injections were used throughout. Intradermal injections were made in the upper abdominal quadrants, from which hair had been removed with electric clippers.

Histologic Studies.—Samples of skin to be examined histologically were removed immediately following sacrifice of the animals, fixed in Bouin's solution and stained with hematoxylin-eosin.

Serologic Studies.—Qualitative precipitin reactions were carried out in capillary precipitin tubes, using bacterial endotoxins in concentrations of 10 to 50 mg./ml. mixed with equal

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volumes of serum. Antibody titers were determined by a hemolytic technique using complement and endotoxin-modified erythrocytes (2).

EXPERIMENTAL

The Response of Normal Rabbits to Intradermal Injections of Endotoxins.—

At intervals over a period of 12 months during the study, approximately fifty normal rabbits received single intradermal injections of *Escherichia coli* or *Salmonella typhosa* endotoxin, in doses ranging from 1 to 400 micrograms. The sites of injection were observed at frequent intervals during the first 10 hours and at daily intervals thereafter for a week. Representative injection sites were removed 1, 6, 12, or 24 hours after the injections for subsequent histologic examination.

In these normal rabbits, the intradermal injections of endotoxins consistently produced delayed inflammatory reactions. During the 1st hour, progressive flattening of the injection blebs was noted as the inoculated fluid was slowly absorbed. Microscopically, no inflammatory tissue reaction was evident at 1 hour. By the 6th hour signs of inflammation were usually still absent in the gross, but histologic examination of sections of skin removed at this time revealed the inception of an inflammatory process, with margination of leucocytes and early perivascular infiltration by polymorphonuclear leucocytes. The cutaneous reactions became demonstrable in the gross between the 6th and 12th hours and progressed to maximum development by the 20th or 24th hour. The inflammatory lesions were characteristically flat with poorly defined margins and exhibited only mild erythema and induration, histologic examination revealing a moderate degree of leucocytic infiltration in the dermis. Edema was never prominent in these lesions. After the 1st day, the cutaneous inflammation rapidly faded and resolution was complete within several days.

The intensity of the inflammatory response varied with the amount of endotoxin administered, doses of 50 micrograms or less ordinarily not producing macroscopically visible lesions. Considerable variation in the intensity of the inflammatory response from rabbit to rabbit was noted, but the time of appearance and development of the reactions was essentially similar in all cases.

Accelerated Dermal Reactivity in Rabbits Previously Exposed to Endotoxins.—

During the course of the study, approximately one hundred rabbits first received single intravenous injections of 100 to 400 micrograms of *E. coli* endotoxin and then received intradermal test injections of 1 to 400 micrograms of *E. coli* or *S. typhosa* endotoxin. Inspection and histologic examination of the resulting cutaneous lesions were performed as described in the preceding section.

The inflammatory reactions seen in these animals were not delayed and mild but accelerated and intense. During the 1st hour, the injection blebs,

instead of flattening, increased progressively in size, maintained their sharp margins and developed pronounced edema and central erythema. Microscopically, there was an outpouring of edema fluid and perivascular accumulations of polymorphonuclear leucocytes were demonstrable within an hour. By the 6th hour, at a time when no inflammatory reactions were visible in the normal controls, the cutaneous lesions in these experimental rabbits approached maximal intensity (Fig. 1). Some measured more than 3 cm. in diameter and were elevated 0.5 to 1 cm. above the surrounding skin surface; erythema and induration were pronounced, and leukocytic infiltration in the dermis was extensive. Most of the reactions reached their maximum development between the 6th and 12th hours, and by 24 hours some were beginning to wane. The histologic features of lesions removed at 24 hours consisted of severe edema, intense perivascular and interstitial infiltration by neutrophils and extravasation of erythrocytes in moderate numbers. Necrosis was rarely conspicuous in these lesions.

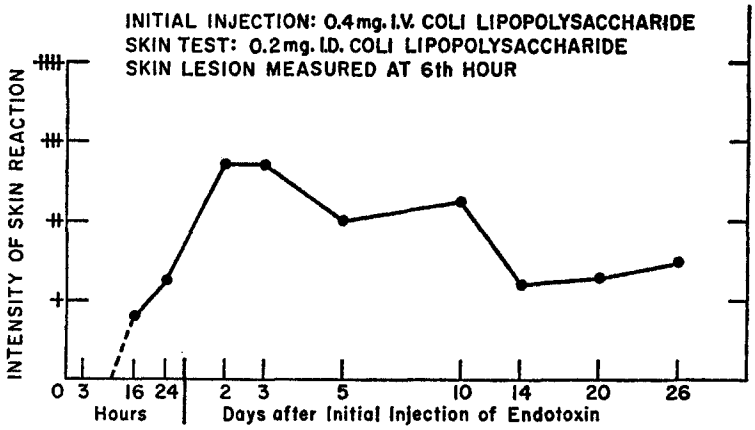
The accelerated reactions seen in these rabbits resembled Arthus reactions, both in general appearance and in being readily discernible within an hour and well developed by the 6th hour. The reactions clearly differed from those of the normal rabbits not only in the time of onset and rapid development, but in the character of the reactions, the accelerated reactions showing pronounced edema and intense acute inflammatory changes.

The Duration of the Accelerated Reactivity Following a Single Injection of Endotoxin.—

Ten groups, each consisting of 4 rabbits, received single intravenous injections of 0.4 mg. of *E. coli* lipopolysaccharide at time intervals varying from 26 days to 3 hours prior to skin testing. All animals were then skin-tested simultaneously with 0.2 mg. of the same endotoxin. A control group of 4 normal rabbits without prior exposure to endotoxin was similarly tested. The cutaneous inflammatory reactions were inspected at 1, 3, 6, and 24 hours after the intradermal injections. Average estimations of the intensity of the lesions at 6 hours are shown in Text-fig. 1. This time interval was selected for illustration because at this point the accelerated reactions were well developed whereas the primary delayed response in the control animals had yet to appear.

In normal rabbits and in those tested 3 hours after a prior injection of endotoxin, only the normally delayed inflammatory reaction ensued, as indicated in Text-fig. 1 by the absence of visible inflammation at the 6th hour after skin testing. However, skin tests performed as early as 16 hours after the intravenous injections resulted in a weak but definite accelerated response. Maximal intensity of the accelerated response was found when rabbits were skin-tested on the 2nd and 3rd days following the first injection; thereafter the reactivity declined slowly with time. In some animals, weak but definite accelerated reactivity was demonstrable for as long as a month.

Intravenous doses of as little as 0.1 microgram of endotoxin produced detectable accelerated reactivity, suggesting that this response is approximately as sensitive as the pyrogenic response to endotoxin (3). Maximal effects were produced with doses of the order of 100 micrograms of endotoxin given intravenously. The intramuscular and subcutaneous routes of injection of endotoxin also served, though less efficiently, to elicit this state of altered cutaneous reactivity.



TEXT-FIG. 1. Each point represents the average intensity of the skin reactions of four rabbits. All skin reactions were measured 6 hours after the skin test dose of *E. coli* endotoxin, and were graded + (5 to 10 mm. in greatest diameter), ++ (11 to 20 mm.), +++ (21 to 30 mm.) or ++++ (more than 30 mm. in greatest diameter). The larger lesions consistently showed higher degrees of erythema and induration, so that the data provide an expression of the intensity, as well as the size, of the reactions.

Specificity and Cross-Reactions.—

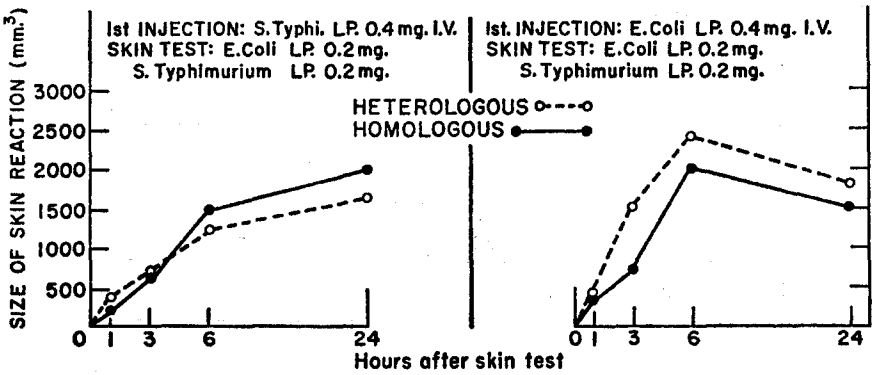
A number of substances were tested in an effort to determine whether the capacity to modify the reactivity to skin tests with endotoxin is limited to endotoxins. Subcutaneous injections of xylene and mineral oil which caused non-specific inflammatory reactions, injections of Freund's incomplete and complete adjuvants which stimulated the appearance of acute phase proteins and produced marked granulomatous inflammation, and intravenous injections of zymosan which are known to elevate the properdin titer were found not to modify the subsequent cutaneous reactivity to endotoxins.

Although the reaction was thus apparently specific for endotoxins as a group, specificity did not extend to individual endotoxins. That is, rabbits which received a modifying injection of one endotoxin responded with typical accelerated reactions to skin testing with other endotoxins.

In the experiment illustrated in Text-fig. 2, one rabbit received a modifying intravenous injection of 0.4 mg. of *S. typhimurium* lipopolysaccharide, and a second rabbit received a similar dose of *E. coli* lipopolysaccharide. Two days later each rabbit was skin-tested with

both endotoxins—the homologous lipopolysaccharide in the right upper quadrant and the heterologous one in the left upper quadrant of the abdomen. The cutaneous reactions were examined 1, 3, 6, and 24 hours after intradermal injections. The size of each lesion was expressed numerically as the product of: longitudinal diameter × transverse diameter × height above surrounding skin surface.

It can be seen that the responses of each animal to the homologous and heterologous endotoxins were essentially the same. In similar experments with lipopolysaccharide endotoxins derived from *Salmonella typhosa*, various sero-



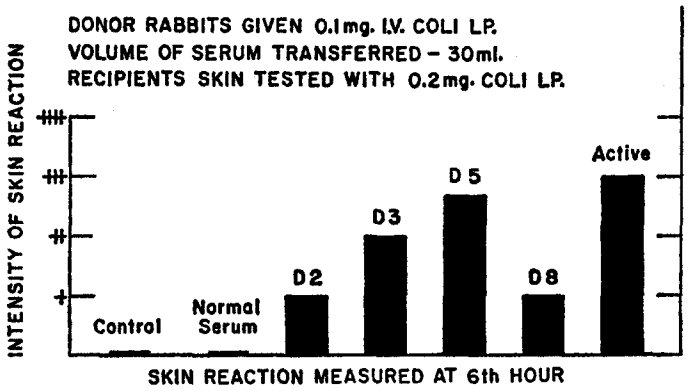
TEXT-FIG. 2. The greatest diameter of each skin lesion was measured in millimeters multiplied by the diameter at right angles to this, and then multiplied by the estimated elevation of the lesion above the surrounding skin surface. The resulting rough expression of the volume of each lesion is plotted against the time after injection of the skin test dose of endotoxin. The skin reactions to the homologous and heterologous endotoxins do not appear different when expressed in this way, and were also judged to be similar in size and intensity upon simple inspection.

groups of *Escherichia coli* and *Salmonella typhimurium*, it was found that prior injection of any one modified the subsequent response to intradermal test injections of each of the others.

Passive Transfer of Accelerated Reactivity with Serum.—In view of the resemblance of these accelerated reactions to Arthus phenomena, attempts were made to detect by passive transfer the existence of a humoral factor responsible for the altered reactivity. To this end, a number of experiments were performed in which serum was transferred to normal rabbits from rabbits which had previously received intravenous injections of endotoxin. A typical experiment is described below.

A group of 6 donor rabbits each received 0.1 mg. of *E. coli* lipopolysaccharide intravenously. Blood specimens of 10 to 15 ml. were collected by cardiac puncture from each donor 1 day before and 2, 3, 5, and 8 days after the modifying injection. Blood specimens were allowed to clot at room temperature for 1 hour, and serum was separated by centrifugation and stored

in the frozen state until shortly before use. Pooled sera, in 30 ml. amounts, obtained on each of the specified days before and after the injection of endotoxin were given intravenously at a slow rate to 5 normal recipient rabbits. There were no apparent untoward effects following the administration of these relatively large volumes of serum. Skin tests with the homologous endotoxin were performed on the recipient rabbits within 1 hour following serum transfer. For comparison, skin tests were similarly performed on a normal rabbit and on a rabbit which had been injected 2 days previously with 0.2 mg. of *E. coli* lipopolysaccharide given intravenously. The skin reactions recorded on the 6th hour are presented in Text-fig. 3.

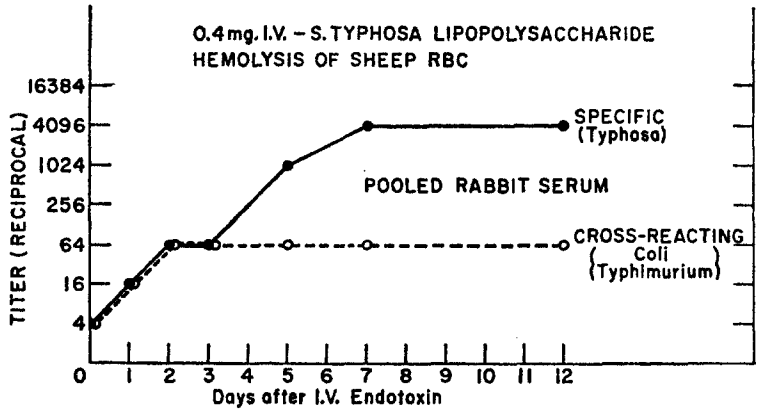


TEXT-FIG. 3. Each bar represents the magnitude of the skin reaction of a single rabbit. The grading of reactions from + to ++++ was done as described in the legend to Text-fig. 1. Rabbits receiving serum obtained from donor rabbits which had received intravenous injections of endotoxin 2 or 8 days previously showed small but definite skin reactions 6 hours after skin testing. Rabbits receiving serum drawn 3 or 5 days after intravenous injection of endotoxin showed skin reactions which approached in size and intensity the reactions of "actively" stimulated animals. The bar labeled "Control" and that labeled "Normal Serum" illustrate the reactions of, respectively, a normal rabbit and one which had received normal serum. The labels D2, D3, D5, and D8 refer to the day after endotoxin injection on which serum was obtained from the donor animals. The bar labeled "Active" shows the skin reaction of a rabbit which had itself been injected 2 days previously with 0.2 mg. of *E. coli* endotoxin.

It is evident that the injection of normal rabbit serum did not produce accelerated reactivity in the recipient which, like the normal rabbit, responded only with the usual delayed inflammation. Serum obtained 2 days after an injection of endotoxin transferred a weak but definite accelerated reaction. Serum obtained on the 5th day provided maximal transfer of accelerated reactivity, the recipient's response approximating in intensity the response of the actively modified animal.

It was possible in six of nine such experiments to passively transfer the accelerated reaction with serum. The prompt appearance of altered skin reactivity following serum transfer rules out the possibility that the recipient animals were being affected by pyrogenic contaminants in the serum, since more than 12 hours is required (Text-fig. 1) for the appearance of accelerated

reactivity even after large doses of endotoxin. The serum recipients exhibited the same cross-reactivity to other endotoxins as described in the preceding section. It was consistently found that while the highest degree of accelerated reactivity was demonstrable on the 2nd and 3rd day after stimulation with endotoxin (Text-fig. 1), serum drawn on the 5th day was more effective in transferring the reactivity than was that drawn on the 2nd day (Text-fig. 3), but the significance of this observation is not clear.



TEXT-FIG. 4. The specific antibody response (solid line) and non-specific or cross-reacting antibody response (dashed line) to a single injection of *S. typhosa* endotoxin are shown. During the first 3 days, the antibody response appears to be non-specific; that is, the sera showed an increasing titer to all three endotoxins tested. By the 5th day and thereafter, the sera showed appreciably higher titers to *S. typhosa* than to the other two endotoxins.

The Appearance of Cross-Reacting Antibodies in Serum Following Endotoxin Injections.—The character of the accelerated cutaneous reactions and the observations that the reactivity could be transferred with serum indicated that the reactions might, in fact, be Arthus reactions mediated by circulating antibody produced in response to the first injection of endotoxin. Since Arthus reactions are generally the result of interactions between antigen and precipitating antibody, qualitative precipitin reactions were performed by mixing the endotoxin solutions with rabbit sera obtained at intervals after single injections of endotoxin. While precipitating antibodies were demonstrable in the sera of these animals, they were not found earlier than the 7th day after the injection of endotoxin, and were strictly specific for the immunizing antigen. Furthermore, rabbits hyperimmunized by a series of weekly injections of endotoxin were found to have very high specific precipitating antibody titers, but on skin test showed either the normal delayed inflammatory reaction or weak accelerated reactions. For these reasons, it seemed unlikely that these antibodies were involved in the accelerated cutaneous reactivity under study.

The results of other serologic studies were of more interest, however. It has been shown by Neter *et al.* (2) that erythrocytes to which endotoxin has adsorbed are agglutinated by anti-endotoxin sera, and may be lysed by the addition of complement. This method was applied as illustrated in the following experiment.

Three normal rabbits each received intravenously 0.4 mg. of *S. typhosa* lipopolysaccharide. Blood specimens in volumes of 5 ml. were collected from each rabbit before the endotoxin injection and 1, 2, 3, 5, 7, and 12 days following it. Serum was separated from each clotted specimen, pooled, and stored in the frozen state. Antibodies to endotoxin in these sera were

TABLE I

Accelerated Reactivity and Cross-Reacting Antibody Titer

Rabbit No.	Day—0		Day—1		Day—3	
	Cross-reacting antibody titer	Accelerated reaction	Cross-reacting antibody titer	Accelerated reaction	Cross-reacting antibody titer	Accelerated reaction
59-91	1:4	0	1:8	+	1:16	+
59-83	1:16	0	1:32	+	1:32	++
59-79	1:16	0	1:32	+	1:64	+++
59-96	1:16	0	1:64	++	1:512	++++

Skin test: *E. coli* lipopolysaccharide 0.2 mg. Accelerated reaction measured at 6th hr. Cross-reacting antibody titer determined with *S. typhosa* Lp.

Skin reactions were graded as described in the legend for Text-fig. 1. Cross-reacting antibody titers were determined as in the experiment illustrated in Text-fig. 4. The size and intensity of the skin reaction, measured 6 hours after the skin test dose, showed in this experiment a good correlation with the cross-reacting antibody titer. These rabbits did not receive an initial intravenous injection of endotoxin; the first skin test dose of endotoxin served to stimulate the serologic response and the accelerated dermal reactivity.

determined employing the modified sheep erythrocyte hemolysis method (2). Batches of sheep erythrocytes were modified separately by the homologous antigen (*S. typhosa* lipopolysaccharide) and by heterologous antigens *E. coli* and *S. typhimurium* lipopolysaccharides in concentrations of 1 mg./ml. 0.2 ml of a 2.5 per cent suspension of modified red cells, 0.2 ml. of guinea pig complement (Carworth Farms) diluted 1/10, and 0.2 ml. of antiserum in varying dilutions were mixed in that order and incubated at 37°C. for one-half hour. The tubes were then read in the gross for hemolysis and the endpoint of the titration was taken as the last dilution of serum producing definite hemolysis.

The antibody response of rabbits to a single intravenous injection of *S. typhosa* endotoxin is illustrated in Text-fig. 4. Normal serum obtained before endotoxin stimulation revealed a hemolytic titer of $\frac{1}{4}$ to each of the endotoxins. One day after the modifying injection there was a 4-fold rise in titer to all three antigens, and a titer of $\frac{1}{64}$ was reached by the 2nd day. Thereafter, the antibody titers against the heterologus endotoxins of *E. coli* and *S.*

typhimurium remained at the same level, whereas the specific antibody titer to *S. typhosa* climbed sharply to a high level by the 7th day. In similar experiments, there was some variation in the titer of cross-reacting antibody from animal to animal, but in the majority of cases the titer rose to maximal values by the 3rd day. The data indicate that the antibody produced during the first 3 days was primarily of the cross-reacting type, and the appearance of this antibody coincided with the appearance of accelerated reactivity in the skin. Attempts to correlate the intensity of the accelerated skin reaction with the titer of cross-reacting antibody in individual rabbits were encouraging but were rendered difficult by the technical problems involved in obtaining accurate quantitative expressions of skin reactivity and antibody levels. The most suggestive of the experiments along this line is summarized in Table I.

DISCUSSION

These experiments clearly raise new questions concerning the mechanism by which endotoxins produce the Shwartzman phenomena and other effects. The elicitation of either the generalized or local Shwartzman phenomenon is usually accomplished by the administration of two doses of endotoxin, one given from a few hours to a day or so after the other. The occurrence of an immunologic response during this period may be of significance in view of the suggestive relationships between reactions to endotoxins and various hypersensitivity reactions (1, 4). The cross-reactivity observed in this study is of particular interest in view of the fact that one endotoxin can be used to prepare for, and another to provoke, the Shwartzman phenomenon.

These findings may also have bearing on the problem of "tolerance" to endotoxins (5). In a recent report of the successful transfer of tolerance by serum (6) the author concluded that antibodies were not involved, because of the cross-reactivity or lack of specificity of the transferred tolerance. The hypothesis that cross-reacting antibodies of the type found in the present study may be involved in endotoxin tolerance is currently under study.

It is planned to continue this investigation with a view to determining whether there exist separate antibody species, one being cross-reactive in nature and appearing early after stimulation with endotoxins, the other being specific for the particular endotoxin used and appearing later in the course of the immune response. It is tempting to consider the possibility that the cross-reacting activity of the early sera may be directed against that portion of the endotoxin molecule which determines its toxicity and which may be similar or identical from one endotoxin to another, with the specific precipitating antibody which appears later being directed against the somatic polysaccharide. Anamnesis of earlier experiences with other endotoxins may be involved in the rapid appearance of cross-reacting antibody as it may in some of the *in vivo* reactions to endotoxins (1).

It has recently been reported by Rowley (7) that injections of *E. coli* endotoxin in mice result in increased phagocytosis of *S. typhimurium* both *in vivo* and *in vitro*. Although the sera of these animals were reported not to contain antibodies, it is possible that cross-reacting antibodies might have been detectable by the sensitive method of Neter *et al.* (2). The possibility that such antibodies may be involved in the non-specific immunity elicited by endotoxin would seem to deserve investigation. The effect of previous exposure to endotoxins should also probably be considered in the interpretation of titrations of endotoxin by means of skin tests (8).

SUMMARY

Rabbits given single injections of endotoxin and then skin-tested with endotoxin from 1 day to 1 month later exhibit accelerated skin reactions resembling the Arthus phenomenon.

Injection of one endotoxin alters the subsequent reactivity of rabbits to other endotoxins as well.

The state of altered reactivity can be transferred with serum, and appears to be related to the presence of non-precipitating cross-reactive antibody, rather than to specific precipitating antibody.

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EXPLANATION OF PLATE

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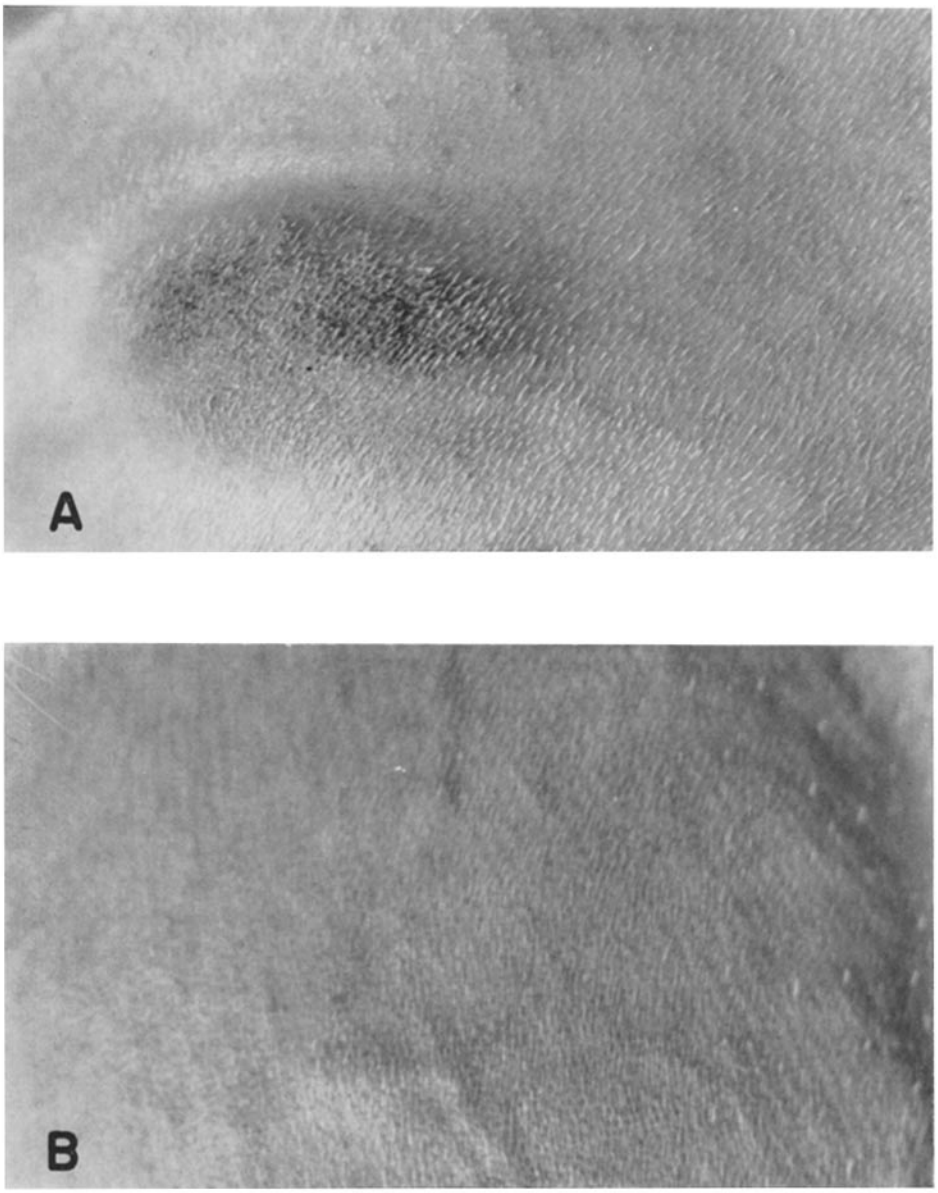
FIG. 1. Photographs of skin reactions in rabbits injected intradermally 6 hours previously with 0.2 mg. *E. coli* endotoxin in 0.2 ml. physiologic saline solution.

FIG. 1. A. Rabbit which had received, 2 days earlier, an intravenous injection of 0.2 mg. *E. coli* endotoxin. The skin test has elicited a large, erythematous, indurated lesion. Similar reactions, in various degrees of intensity, were seen in every rabbit so treated.

FIG. 1. B. Normal rabbit. The remains of the injection bleb can be distinguished, but no inflammatory reaction has occurred. None of the normal rabbits tested showed more than slight erythema at the injection site 6 hours after skin testing, and most showed no evidence whatever of a skin reaction during this period.



(Lee and Stetson: Accelerated reactivity to endotoxins)