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FROM ENDOTOXIN TO SNAKE VENOM**

The host-parasite relationship in many infectious diseases of animals and of man continues to challenge the knowledge and imagination, not only of microbiologists and those in other basic disciplines, but that of clinicians as well. There remain many unexplained features of this relationship bearing on the problem as to why invading microorganisms cause illness. One aspect of this relationship which has attracted considerable attention in recent years involves Gram-negative bacteria. The metabolic and morphological alterations induced in the host by bacterial somatic preparations known as endotoxins have been studied intensively in many laboratories and clinics. The purpose of this report is not to review the extensive literature on the subject but rather to summarize the results of investigations which have been carried out by my associates and myself. I shall refer largely to various communications from our group in the Department of Medicine and from those of Dr. Maurice Visscher and his associates in the Department of Physiology, which in turn document the related work of others.

WHY INVESTIGATE THE ACTION OF ENDOTOXINS?

My interest in endotoxin was aroused almost 25 years ago while working on the problem of gonococcal arthritis with Dr. Chester S. Keefer. During the course of those investigations it was observed that gonococci, in common with many other species of Gram-negative bacteria, were killed by human blood without the intervention of phagocytes.[29] In other words, fresh serum lysed the organisms. Further studies revealed that a suspension of lysed gonococci was extremely toxic when injected into the tissues of animals. In a discussion on the pathogenesis of gonococcal infections at the time, it was postulated that the inflammatory reaction induced by gonococci was due, in part at least, to the autolyzed organisms.[25]

With the advent of the sulfonamides and antibiotics the problem of gonococcal disease offered less of a clinical challenge, and interest in endotoxin subsided until attention was attracted to the possible role of endotoxin

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in the pathogenesis of illness caused by brucellae. While investigating the therapy of brucellosis in Mexico City with my associate, Dr. A. I. Braude, and with Dr. M. Ruiz-Castaneda, an interesting sequence of clinical events aroused a further interest in endotoxin.[27] Patients having severe and prolonged illness due to *Brucella melitensis* were given chlortetracycline. Shortly after the initial dose of the drug, the condition of several of the patients became worse. They exhibited a rise in temperature, tachycardia, and an alarming decline in blood pressure. Subsequently, they showed marked improvement. These manifestations suggested that the antibiotic shared in the destruction of large numbers of brucellae in the host with the liberation of endotoxin from the lysed organisms. It was postulated that the condition of the patients became worse not only because of the inherent toxicity of the endotoxin, but because they had acquired hypersensitivity to brucella antigen. This concept was to occupy our thoughts for several years.*

Further general interest in endotoxin was stimulated by clinical observations in patients having systemic infections due to several species of Gram-negative bacteria commonly associated with urinary tract infections. These microbes are not highly pathogenic but they do threaten life when they invade the blood stream, probably largely due to the endotoxin of the organisms. The endotoxin frequently participates in the syndrome of shock.[87] These clinical observations stimulated an extensive study on the hemodynamic changes induced by endotoxin in cooperation with the Department of Physiology.

INTRODUCTORY STUDIES WITH BRUCELLA ENDOTOXIN

Endotoxin has been described as a lipoprotein-carbohydrate complex localized on or near the surface of Gram-negative bacterial cells. Endotoxins have been prepared according to a variety of procedures. Elaborate techniques have been detailed for making "purified" material. Some have concluded that the toxic component in endotoxin is a macromolecular polysaccharide fraction. We believe that this concept of endotoxin is too narrow, although the carbohydrate moiety is an important toxic factor. It is not unlikely that the disintegration of bacterial cells in the host results in the liberation of several undefined, but active substances. The sum total

* In this presentation, I shall not develop this concept any further. This has been done elsewhere (Spink, W. W., *The nature of brucellosis,* University of Minnesota Press, 1956, Chap. 6; Spink, W. W., "The significance of bacterial hypersensitivity in human brucellosis: Studies on infection due to strain 19 *Brucella abortus,*" *Ann. int. Med.*, 1957, *47*, 861; Abernathy, R. S. and Spink, W. W., "Studies with brucella endotoxin in humans: The significance of susceptibility to endotoxin in the pathogenesis of brucellosis," *J. clin. Invest.*, 1958, *37*, 219.

effect of these somatic components on the host also remains undefined. At the present time, when purified somatic bacterial material is presented to the host, a reproducible train of events ensues, but this does not necessarily reflect the course of changes under natural conditions, in which the host is challenged with many unknown bacterial factors.

Nevertheless, in order to reduce the number of variables to a minimum, we have also tried to employ a standardized and partially purified preparation of endotoxin. With but few exceptions we have used a Boivin-type of endotoxin, which involves extraction of the bacterial cells with trichloracetic acid. The method for making the endotoxin has been given in detail elsewhere.[12]

Many of our investigations with endotoxin have been carried out in mice. Again, this selection was carried out in an attempt to lend uniform conditions to the many experiments. Mice are quite suitable for comparative studies because a genetically homogeneous population was available, and, also, because relatively small amounts of endotoxin were necessary for experimental purposes. Our initial studies on brucella endotoxin in a population of male ABC mice compared the toxicity of endotoxin prepared from strains of brucellae having varying degrees of virulence. The toxicity of endotoxin had no relationship to the virulence of the living culture. Endotoxin prepared from a culture of Strain 19 of *Br. abortus,* having attenuated virulence, was just as lethal for mice as endotoxin made from a highly invasive strain of *Br. melitensis.*[26] Death of the mice uniformly occurred between 12 and 24 hours. More recent studies have shown that endotoxins prepared from *Escherichia coli* and *Salmonella typhosus* were weight for weight more toxic for mice than brucella endotoxin.

STUDIES IN THE RELATIONSHIP BETWEEN ADRENOCORTICAL ACTIVITY AND ENDOTOXIN

Having established that endotoxin from brucellae, as well as endotoxin prepared from other species of Gram-negative bacilli, was lethal for mice, a series of studies was made on the role of the adrenal cortex in the defense mechanism of the mouse against brucella endotoxin as well as against viable brucellae. Interest in this aspect of the endotoxin studies was stimulated by the report of others[30] stating that the generalized Shwartzman reaction induced by endotoxin in rabbits was greatly accelerated by administering cortisone. It was anticipated that cortisone would abet the lethal action of endotoxin in mice. On the contrary, it was demonstrated repeatedly that administering cortisone protected mice against lethal doses of endotoxin.[26]

The influence of endotoxin on the normal temperature rhythm of the mouse was also studied in relation to adrenal activity. First, the 24-hour rhythm of temperature in the normal mouse was determined. The rectal temperatures varied between 36° and 39.5° C., with the lowest temperatures occurring during midday and the highest readings in the early morning hours. The peak of the temperature corresponded with the nocturnal activities of mice. This activity, in turn, can be directly correlated with adrenal function. It was observed that endotoxin caused a rise in temperature within seven to nine hours after endotoxin was administered, and then a profound drop in temperature to 30° C. occurred shortly before death.[12] Very small quantities of endotoxin altered the physiological rhythm of the temperature without causing death. Adrenalectomized mice were particularly susceptible to the action of endotoxin, both with respect to lethality and to alterations in temperature. However, administered corticosteroid stabilized the temperature in intact and adrenalectomized mice given endotoxin, as well as protecting them against a lethal outcome.[11, 13]

Though not directly related to the problem of endotoxin activity, observations were made in mice which were *simultaneously* infected with brucellae and given cortisone.[8] Under these circumstances a relatively benign disease was converted into a fulminating infection with a marked proliferation of the brucellae in the tissues. Adrenocorticosteroid administration had little adverse effect on more chronically infected mice. These observations in mice could not be correlated with subsequent investigations in human patients. The differences observed between acute brucellosis in mice and in humans, and the response to steroids, could be ascribed to the fact that brucellae and steroids were given to the mice at the same time, whereas in man, invasion of the tissues by brucellae had occurred before the steroid was given. This permitted stimulation of the defense mechanism in the human host and the establishment of an immune response before exposure to exogenous steroid. Another possible explanation for the difference is the relatively large dose of steroid used in mice. Patients seriously ill with acute brucellosis were dramatically improved within a short period of time when given steroid.[20] These studies have resulted in the recommendation that steroid therapy over a brief period of time should be employed along with the antibiotics in the management of the more severely ill patients.

A series of studies has been directed by Melby and Egdahl[21] on the effect of endotoxin on activity of the adrenal gland in the dog. By direct cannulation of the adrenal vein the secretion of hydrocortisone was measured under different circumstances. Brucella or *E. coli* endotoxin administered intravenously to dogs resulted in a prompt rise in the output of hydrocortisone.

This effect was mediated through the pituitary gland, since administered endotoxin was without effect in hypophysectomized animals, although a rise in the level of hydrocortisone could be provoked in these animals with ACTH. This pointed out quite clearly that in the dog the effect of endotoxin on adrenal activity was mediated through the pituitary. Similar observations have been recorded by others[88] for the rat. Following the initial response of the adrenal to endotoxin in intact animals, a further response could be elicited by ACTH but not as marked as that obtained prior to the administration of endotoxin. It was concluded that although endotoxin caused an increased secretion of cortical hormone, the functional capacity of the cortex was temporarily diminished by the endotoxin.

These studies were extended to human patients having shock due to bacterial infections.[22] It was observed that the blood levels of hydrocortisone were considerably higher in the patients with shock than in normal controls. Of further interest was the finding that administered hydrocortisone disappeared more rapidly from the blood of the patients with shock than from the blood of normal persons. But this rapid metabolism of exogenous hydrocortisone uniformly took place only in the patients who recovered. In the patients dying of shock the half-life of administered hydrocortisone was more prolonged than in normal individuals and considerably more so than in patients with shock who recovered.

STUDIES ON THE HEMODYNAMIC CHANGES INDUCED BY ENDOTOXIN

In an attempt to acquire more precise information concerning the nature of shock induced by endotoxin a group of experimental studies was designed in cooperation with members of the Department of Physiology. When large doses of endotoxin prepared from either *E. coli* or *Br. melitensis* were injected intravenously into dogs, a two-stage decline in arterial blood pressure was observed.[19] Within 30 seconds following the administration of endotoxin there was a precipitous drop in the systemic arterial pressure, and a simultaneous rise in the pressure of the portal vein. As the portal vein pressure returned to the pre-injection level, a rise in the femoral arterial pressure took place. This was then followed by a gradual decline in the systemic pressure over a period of several hours ending in death of the animals. It was demonstrated quantitatively that the *immediate* drop in arterial pressure was due to the pooling of the blood in the liver and intestine, and a reduction in venous return to the heart.[20] This immediate effect on pressure could be abolished by eviscerating the animal. However, this did not prevent the secondary decline in blood pressure. A progressive fall in blood pressure occurred in the eviscerated dog when cardiac filling

was maintained at a constant level, which indicated a decline in total peripheral resistance. It is unlikely that in the dog the shock induced by endotoxin is mediated through the central nervous system.[33] It was concluded that the critical problem in the dog with endotoxin shock was the initial pooling of blood in venous beds. This results in a failure of venous blood to return to the heart, causing in turn a diminution in cardiac output.[34] However, it should be emphasized that a species difference has been observed in the response to endotoxin by Dr. L. Hinshaw and his group working in Dr. Visscher's laboratory. Immediate engorgement of the liver was not noted in the cat, rabbit and monkey, possibly because constriction of the hepatic vein does not occur in these species as in the dog. But a progressive fall in systemic blood pressure was seen similar to that in the eviscerated dog. Thus, there is no evidence in the cat, rabbit and monkey for pooling of blood in the splanchnic bed, but a gradual decline in peripheral resistance does occur.

The foregoing observations in the dog implied that if the arterial pressure was to be stabilized in endotoxin shock, the cardiac output must be increased. This could possibly be accomplished by decreasing the pooling of blood peripherally. Vasopressor agents were evaluated experimentally, and it was observed that metaraminol (Aramine) did diminish the venous pooling in the dog and increase the return of venous blood to the heart.[32] These observations were further extended to human patients in the treatment of shock.[31, 35]

The rapidity with which endotoxin altered the hemodynamics in the experimental animal and the apparent primary vascular effect of endotoxin prompted further quantitative studies employing different techniques. Using the method of MacLean *et al.*[20] for weighing the liver, direct observations on the dog's kidney were made in a similar way in the eviscerated dog.[14] As quickly as 15 seconds after the injection of *E. coli* endotoxin into the femoral vein there was a marked decrease in the weight of the kidney. In fact, the size of the kidney diminished under direct observation. The arterial pressure fell *after* the change in kidney weight, indicating that the vasoconstriction occurring in the kidney was not initially due to the decline in blood pressure. The effect of *E. coli* endotoxin on renal function was studied on the isolated and perfused kidney of the dog.[15] When the kidney was perfused with heparinized blood at a constant pressure from a heart-lung preparation, impairment of renal tubular function occurred. There was a prompt and progressive deterioration in the rate of para-amino hippurate clearance, and the specific gravity of the urine decreased markedly. Recent unpublished data by Hinshaw and others have shown that the changes in

renal function are probably related to the effect of endotoxin on the renal blood flow. The Tm PAH is not appreciably altered after endotoxin. When the influence of *E. coli* endotoxin on the pulmonary circulation was studied in the isolated heart-lung preparation of the dog and cat, the outstanding feature was an increase in small vein resistance with a gain in the weight of the lung.[18]

ACQUIRED RESISTANCE TO ENDOTOXIN

Endotoxin rapidly affects changes in the tonicity of the vascular system, which in turn contributes to further physiological and morphological alterations in the host. Many of these resultant abnormal factors can, and have been measured. A phenomenon of endotoxin activity that has been well confirmed in many laboratories is the development of tolerance or resistance of animals to endotoxin following the injection of repeated doses of the material. But, here again, the problem is complicated. Tolerance to the fever-promoting property of endotoxin is not synonymous with the development of tolerance against the lethal action of endotoxin. Recently, dogs have been made tolerant to endotoxin by Drs. Egdahl and Melby so that no fever was induced by endotoxin, but the same animals continued to manifest increased adrenocortical activity.

That aspect of the problem of resistance that has engaged our group more prominently has been related to the lethal activity of endotoxin. This interest was an outgrowth of the observation that ACTH and the adrenocorticosteroids protected animals against lethal doses of endotoxin. It was also observed that totally unrelated substances such as chlorpromazine, and the polymer, polyvinylpyrrolidone (PVP), protected mice against the lethal action of brucella endotoxin.[2,6] Protection has also been afforded by antihistaminics (Benadryl), yeast extracts, barbiturates (Nembutal), and an environment of lowered temperature.

One of the perplexing problems of endotoxin resistance that has been explored is the duration of resistance against a lethal dose of endotoxin following the injection of sublethal amounts.[1] In mice it was found that a single sublethal dose of brucella endotoxin offered homologous protection for as long as four months, and after three weekly injections of sublethal amounts, protection was extended as long as ten months. Heterologous protection against other endotoxin was of shorter duration. The precise nature of this form of immune response is not clearly understood. It was of further interest to demonstrate that mice challenged with a lethal dose of endotoxin but simultaneously given either adrenocorticosteroid or chlorpromazine were protected against subsequent lethal doses of endotoxin.[5]

Brucella endotoxin administered to mice also resulted in increased resistance to living brucella organisms.

ACQUIRED SENSITIVITY TO ENDOTOXIN

The preceding discussion has been concerned with the acquisition of increased resistance of mice to endotoxin. It was possible to alter the response of this species so that they became more susceptible to the lethal action of endotoxin. It has been generally confirmed that mice are relatively resistant to histamine, but an increased susceptibility, in some strains of mice at least, develops following injection with *Hemophilus pertussis* vaccine. Since the administration of endotoxin to the dog is followed by the liberation of histamine into the blood stream, it was of interest to see if pertussis vaccine increased the susceptibility of mice not only to histamine, but also to brucella endotoxin. A series of experiments in this direction has revealed further the complicated nature of the problem.[4] It was observed that pertussis vaccine increased the susceptibility of Swiss-Webster, but not ABC mice, to histamine. The injection of brucella endotoxin was not followed by increased susceptibility to histamine in either strain of mice. Both Swiss-Webster mice and ABC mice became more susceptible to brucella and to typhoid endotoxin after the administration of *H. pertussis* vaccine. And finally, the protective action of chlorpromazine and Benadryl against endotoxin was abolished after pertussis vaccination, but the protection offered by adrenocorticosteroids remained unaltered.

An increase in the susceptibility of mice to endotoxin was demonstrated in another way. Mice were infected with a small inoculum of *Br. melitensis*. Approximately seven days to four weeks later a dose of brucella endotoxin that was not lethal for uninfected mice was injected intraperitoneally into the infected animals, and death ensued within 24 hours. Repeated minimal injections of the endotoxin in the infected animals was associated with a marked inflammatory reaction in the visceral peritoneum. This was at first interpreted as a manifestation of specific brucella hypersensitivity. However, subsequent experiments showed that mice infected with brucella were also more susceptible to the endotoxin of *E. coli* than normal controls. This sensitizing effect of an infection to endotoxin has been noted by others,[8] who have concluded that an explosive multiplication of bacteria induced by the endotoxin was responsible for the accelerated lethal effect. Perhaps this affords an explanation for our own results, since we have been able to protect mice infected with brucellae against endotoxin by the simultaneous administration of chlortetracycline.

AND NOW — TO SNAKE VENOM

It became known to us that the train of events induced in animals by endotoxin could be produced by many other agents. Furthermore, the action of endotoxin simulates the manifestations associated with anaphylactic shock, although the latter involves a specific immunological mechanism. The term "anaphylactoid shock" has been employed in the past for the nonspecific reactions caused not only by endotoxin but by many colloidal preparations.[36]

Peptone is one of many substances that have been studied intensively in an attempt to elucidate the mechanism of anaphylactoid shock. We observed that a population of ABC mice were resistant to an injection of peptone, but peptone proved to be lethal to mice six days after the administration of *H. pertussis* vaccine.[17] In addition, mice sensitized to pertussis vaccine could be protected against the lethal action of peptone with cortisone, chlorpromazine, and an antihistaminic agent.

In comparing other substances with endotoxin we became particularly interested in snake venom because of the observations reported by Essex and his associates.[9] They stated that the administration of rattlesnake venom to the dog was followed by a prompt drop in systemic blood pressure, similar to that which we had observed following the injection of endotoxin. But instead of noting a rise in the weight of the liver, Essex and his group recorded a drop in the weight of this and other organs. Dr. Essex kindly supplied us with the rattlesnake venoms, *Crotalus terrificus* and *Cortalus atrox*. Studies in mice have been made with these and also with moccasin venom, *Agkistrodon piscivorus piscivorus*. Some preliminary studies have also been made with a tropical rattlesnake venom, *Crotalus terrificus terrificus* and also with a venom from *Bothrops jararaca,* supplied to us by the Butatan Institute in Brazil. We have observed in the dog that rattlesnake venom caused a prompt drop in blood pressure, but the pressure eventually became restored at normal levels. The gradual secondary decline in pressure seen with endotoxin did not take place. It has become quite apparent to us from observations made on mice that rattlesnake venom obtained from different snakes of the same species will produce varying results. This is attributable to the fact that snake venom is such a complicated biologic material.[7] In this respect the complexity of endotoxin simulates snake venom. It should also be appreciated by the investigator that venom obtained from the same snake at different periods of time will vary in biologic activity.

Direct observations on the vascular effects of snake venom have been made by Fulton *et al.*,[10] employing the everted cheek pouch of the golden hamster. Moccasin venom injected beneath the epithelium results shortly thereafter in a marked arteriolar constriction, stasis of the blood, and then the appearance

of petechiae, with a diapedesis of erythrocytes through the vessel walls at venous junctions.

Possible immunological relationships between snake venom and endotoxin have been explored just enough to invite further investigations. It has been observed in studies on the Shwartzman reaction that the administration of moccasin venom made animals refractory to endotoxin.[23] In the experiments cited rattlesnake venom was not effective. Others[39] noted some cross protection in animals between moccasin venom and the endotoxin of *Salmonella typhimurium*. We have found that populations of ABC mice can be protected against lethal doses of rattlesnake venom *(Crotalus terrificus)* following three weekly injections of brucella endotoxin. This resistance has endured for as long as 6 weeks after the last injection of endotoxin. Protection against such venom has also been demonstrated after the administration of *E. coli* and *S. typhi* endotoxins. The three endotoxins also gave good protection to mice against moccasin venom and the venom of *Bothrops jararaca*.

Further investigations are under way with the precipitin test, which has included the gel-diffusion technique, in an attempt to elucidate further the immunological relationship between endotoxin and snake venom. To date, with this technique it has not been possible to demonstrate cross precipitins in the sera of mice immunized with endotoxin or snake venom. Several well-defined precipitin bands could be demonstrated when antivenom horse serum and venoms were employed in the gel-diffusion method.

THE CRUX OF THE ENDOTOXIN PROBLEM

In attempting to formulate a basic concept concerning the mode of action of endotoxin we have looked upon endotoxin as promptly "triggering" alterations in vascular activity. Vasoconstriction, vasodilatation, and increased permeability of the vessels are rapidly followed by profound metabolic and morphological changes in the tissues of the host. Various substances such as norepinephrine, serotonin, histamine, heparin, and proteolytic enzymes are very likely liberated into the blood stream, and these in turn alter the physiological and metabolic equilibrium of the host. Endotoxin sets in motion a chain reaction within the tissues and body fluids of the host that may result in death.

The most outstanding feature of endotoxin activity is the rapidity with which endotoxin acts when introduced into either the circulation of the intact host or into the perfusate of isolated organs. Changes in vascular activity can be measured within 10 to 15 seconds after the administration of endotoxin. This would indicate that either endotoxin acts directly upon the

endothelial wall or that it causes an alteration in the blood, which in turn affects the function of the vessel. If the primary action of endotoxin occurs in the blood, the changes may involve enzymatic activity because of the almost immediate response induced by relatively small amounts of material.

There is some suggestive evidence that the primary action of endotoxin operates through some factor or factors in the blood, and then secondarily upon the tissues of the host, including the vascular endothelium. Rosenfeld[24] found that endotoxin prepared from *Pseudomonas aeruginosa, E. coli,* or *Serratia marcescens* inhibited the *in vitro* adrenocortical activity of the calf adrenal only when the perfusate consisted of homologous whole blood. No effect of endotoxin was elicited when artificial physiological perfusates were employed. Likewise, Hinshaw and his associates[16] working with the isolated and perfused lung of the dog, observed a pulmonary vascular response when heparinized blood was used as a perfusate, but not when dextran was employed. Also, there was little effect from endotoxin when defibrinated blood was used in the heart-lung-kidney preparation in place of heparinized blood.

The accumulating evidence emphasizes that the action of endotoxin is complex. Effects of endotoxin action must not be mistakenly offered as basic causes for the alterations induced in the host. Endotoxin "triggers off" a chain reaction of vascular and metabolic changes. The crux of the endotoxin problem in the host-parasite relationship resides largely in the few seconds that elapse after endotoxin appears in the blood stream of the host.

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