BACTERIAL ENDOTOXINS

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Preface

Although current interest in bacterial endotoxins cuts across numerous areas of specialization in medicine and experimental biology, there is no complete documentation of the manifold advances in this field during recent years. There exists a voluminous literature in this field but despite the ever-increasing interest in the fundamental and practical aspects of endotoxin research, there has been only poor integration of existing knowledge. Some of this integration, when attempted, was for the benefit of a few key workers only. Thus a number of these met in Freiburg, Germany, in 1959, at Bryn Mawr, Pennsylvania, in 1961, and at Wakulla Springs, Florida, in 1963. However, it was as a result of these workshops that the idea was conceived to organize a larger meeting which would not only represent the major facets of endotoxin research but, more importantly, would form the basis for the printed compilation of an assessment of our present knowledge as well as a projection of future research needs. The time seemed to be appropriate for such an attempt, since the last few years had seen substantial progress in our understanding of some of the mechanisms by which endotoxins act, and a better delineation of their manifold biological effects.

The meeting which formed the basis for this volume took place at the Institute of Microbiology, Rutgers University, New Brunswick, New Jersey, on September 4, 5, and 6, 1963. It was patterned after the format used so successfully by our British colleagues: the principal papers were submitted well before the meeting, reproduced, and copies were distributed to the 212 participants. At the meeting these contributions were summarized in 10-minute statements by the authors and the major time of the meeting was then devoted to discussion. This volume contains the original papers and an edited version of the discussion. (The undersigned are principally responsible for the editing of the discussions. We trust that in seeking to compress and to polish some of the remarks, we did not alter their meaning.) In addition to those who served as the original nucleus of the major discussion panels, a number of additional contributors were invited to submit brief reports.

In the planning and conduct of the meeting, as well as in the subsequent editing, we had the invaluable help of the chairmen of the various sessions, Drs. Ribi, Zweifach, Berry, Suter, Kass and Stetson. Miss Kathleen Moore served as Technical Editor and aided substantially in the production of the present volume. Mr. Edward Isaacs, Executive Secretary of the Institute of Microbiology, carried the responsibility for numerous and complex organizational matters with unique success;

without his efficient help our plans for this kind of meeting could not have materialized. The National Science Foundation made possible both the meeting and the publication of this volume through grants GE-716 and GN-207. In addition, the Wallace Laboratories, Cranbury, N.J., a Division of Carter Products, and The Education and Research Foundation of the American Medical Association made generous contributions to defray some of the cost of publication. Also, The Difco Laboratories, Detroit, Michigan, provided funds to help cover the costs of rapid transcription of the discussions. To them, as well as the many others who helped us overtly and behind the scenes, we express our deepest gratitude.

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Introduction

Approaches to the Mechanisms of Endotoxin Action

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The almost overwhelming expansion of research on endotoxins during the past decade can be amply documented by mere inspection of the titles of the papers that make up this volume. Realizing that we are witnessing what can be called, in modern terminology, an "endotoxin explosion," I think it may be useful to commence by considering a few generalizations. In almost any field, the rapid accumulation of data derived from diverse investigative studies makes it more and more difficult to interpret and correlate experimental findings and to assess their significance. While complexity is a complication by no means unique to the study of bacterial endotoxins, there are peculiar circumstances that aggravate the problem in this field. Therefore, I propose to examine the forest instead of the trees by reviewing briefly several obvious but nonetheless important attributes and characteristics of endotoxins and endotoxin research and by expressing some personal ideas on the ultimate objectives of the study of endotoxins.

Endotoxins possess an intrinsic fascination that is nothing less than fabulous. They seem to have been endowed by Nature with virtues and vices in the exact and glamorous proportions needed to render them irresistible to any investigator who comes to know them.

They intrigue the chemist. The molecular basis for their biological action seems always on the verge of discovery but somehow just eludes detection. Furthermore, arguments over artifact versus the real thing and fractionation versus contamination are resurrected and reresurrected so regularly and vigorously when analytic methodology is discussed that they are achieving a kind of cyclic immortality which, if only there were more variation in their form, would amount to reincarnation.

Being antigenic, endotoxins are susceptible to immunological manipulations. They can be labeled internally or externally with radioactive isotopes and they can be tritiated. They can be precipitated, hemagglutinated, fluoresced, and radioautographed. For what more could one wish?

Along with these convenient properties, endotoxins possess a range of biological activity that seems specifically designed to cut across all of the categories into which research and researchers are presently classified and indexed. They can affect structure and function of numerous organs and cells, change tissue and blood levels of many (perhaps too many) enzymes, modify carbohydrate, fat, and protein metabolism, raise or lower body temperature, increase or decrease resistance to bacterial and viral infections and other noxious stimuli (including themselves), cause hemorrhage and increase coagulation of blood, modify hemodynamics in every accessible anatomical site, cause or prevent shock, modify gastric secretion, destroy tumors, and affect the function of several endocrine glands. This spectrum of activity makes possible at least one prediction: an investigator in almost any biological field is likely to obtain a "positive" result if he tries endotoxin in the experimental system he is using. A corollary is the well-known situation in which dramatic physiological alterations have been elicited by inadvertent contamination of other materials by endotoxin. Indeed, so strikingly active are endotoxins that, through the years, many an investigator has decided to abandon the problem upon which he had been working to turn full attention and energy directly to the study of these bacterial products.

As products of different bacterial species, endotoxins are distinctly specific and yet, in most of their activities, they are delightfully and frustratingly nonspecific. They have been suggested repeatedly as a possible key to understanding the peculiar and distinguishing features of the diseases produced by gram-negative bacteria, but the uniformity of the physiological responses to heterologous endotoxins makes it clear that the answer is not one of simple cause and effect alone.

Perhaps of even more interest in relation to human disease is the fact that the stigmata of endotoxemia bear close resemblances to many disorders not presently considered to be primarily infectious in origin. Furthermore, many reactions to endotoxin imitate but seem never to duplicate those evoked by various hypersensitivity states, chemical intoxications, dietary injuries, etc. Until now, however, this mimicry has never been exact, and the reactions to endotoxin retain a distinctive and inexplicable character of their own. The fact that endotoxemia so closely resembles the reactions elicited by other agents has given rise to numerous comparative studies, and one can now be almost certain that any given investigator will conclude that the similarity of this reaction to endotoxin and that elicited by another stimulus is probably attributable to some "final common pathway" of response.

Few areas of research exceed the field of endotoxins in evoking the overwhelming desire of an investigator to take his findings and mould a hypothesis by analogy.

Not only do endotoxins elicit striking effects by themselves but they possess almost limitless ability to potentiate or antagonize or to be potentiated or antagonized by the action of other agents and states including adrenal steroid hormones, catecholamines, ionizing radiation, radiomimetic drugs, denervation, pregnancy, hemorrhage, dietary manipulation, high environmental temperatures, and so-called reticuloendothelial blockade.

Endotoxins are highly toxic but resistance or tolerance is easily induced. So spectacular is this tolerance that it is almost impossible to imagine that it is not of great significance in naturally occurring disease. Similarly, the Shwartzman reaction seems bound to be something more important than a laboratory artifact. It has been said in jest that endotoxins will probably turn out to be the cause of most of the human diseases now classified as idiopathic and that they may also prove to be the cause of human health. As unlikely as this may seem to even the most enthusiastic formulator of hypotheses concerning endotoxin, these are possibilities that have yet to be excluded.

No small part of the temptation to assign endotoxins an important role in the etiology of various disorders and diseases is attributable to their ready availability. The generous supply of endotoxin in most mammalian intestinal tracts is an invitation to theorize and to postulate freely, in contrast, for example, to the difficulties of coming up with an antigen or an antibody in some of the diseases presumed to be of autoimmune origin.

Finally, in addition to their other attractive features, endotoxins possess definite therapeutic properties. Their usefulness in the production of artificial fever is well known and they are still widely used for this purpose in many parts of the world. The systemic discomfort which accompanies the pyrogenic reaction and the development of tolerance, the main problems in employing this form of treatment, have stimulated many attempts to develop methods of fractionation and purification in the hope that these drawbacks can be minimized and that a marketable product will result. Results of these efforts have been, at best, equivocal, although they seem to have been commercially successful in some instances. Interest in endotoxin therapy of malignant hypertension and malignant tumors waxes and wanes but remains viable. Past claims for the efficacy of these materials include acceleration of healing, alleviation of atopic hypersensitivity, stimulation of central nervous system regeneration, and many others now abandoned. The hope that some remarkable curative property of endotoxin will be uncovered persists, however, and it is certain that we have not yet heard the last of the matter.

What are the ultimate objectives of research on endotoxins? What are we trying to find out? It certainly cannot be said that the immediate aim of every study discussed in this symposium has been the elucidation of the mechanisms of the biological action of endotoxin in man. Yet, many of us, perhaps most of us, look forward to the achievement of this anthropocentric goal at some future time and, of course, every scrap of information about endotoxin is a step in this direction. The exact understanding of endotoxic action in man, when it comes, will be welcome, worthwhile, and intrinsically a good thing. Furthermore, it is abundantly clear that this detailed knowledge of endotoxin that is eventually to be ours will bring with it an enormously greater understanding of other physiological, immunological, and biochemical phenomena gained by the deliberate use of the endotoxin model in the laboratory or by serendipity. This, too, will be welcome, worthwhile, and intrinsically a good thing.

Even when we have accomplished the difficult task of defining the mode of action of endotoxin in man, there will remain for many of us

an additional problem. This will be to determine the actual significance of endotoxins in human health and disease, not in the controlled environment of the research laboratory but in daily life, in the field, in nature as we have modified it. Endotoxin can cause fever but how many human fevers are endotoxic? Endotoxin can cause shock but how often is shock in man endotoxic? Endotoxin can modify resistance to infection but how often does endotoxin influence the susceptibility of man? To me, it seems that this last aspect of the "endotoxin problem" poses the greatest difficulty when we attempt to generalize a tree into a forest in discussing the significance of our experiments.

Contents

Part I

Chemistry

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Recent Investigations on the Polysaccharide Component of Enterobacterial Endotoxins (S and R Forms)

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It is known that enterobacterial somatic O antigens are part of the endotoxic complex that is a main constituent of the bacterial cell wall. The whole somatic antigen is composed of protein, lipid, and polysaccharide, the last functioning as the carrier of determinant groups. It is also known that the lipopolysaccharide component of the endotoxic complex, with a molecular weight in the order of 10^6, can be extracted directly from enterobacterial cells or from purified cell walls, and exerts endotoxic as well as O-antigenic properties.

Preparation of Nontoxic O Antigens

Many attempts have been made to detoxify bacterial O antigens (1–3). Under conditions of mild acid hydrolysis, lipopolysaccharides are split into lipid (lipid A), free fatty acids, and lipid-free degraded polysaccharide. The degraded polysaccharide can also be obtained by direct extraction of bacteria with acetic acid [Freemann procedure (4)]. Its molecular weight is about 20,000; it is no longer toxic and no longer antigenic, but it still carries all known O-antigenic specificities, as long

as the determinant structures are not acid-labile. Prolonged treatment of lipopolysaccharides with diluted alkali or hydroxylamine leads to disaggregation and de-esterification; fatty acids are set free. The final alkali-stable product has a sedimentation constant S_{20} of 8 to 10S (5, 6), corresponding to a molecular weight of about 200,000. During alkali treatment, toxicity and pyrogenicity decrease, whereas affinity for the surface of erythrocytes increases (5). Even after prolonged alkali treatment, various lipopolysaccharides may still retain considerable pyrogenicity and toxicity. O-specific groups remain fully active. Only alkali-labile structures, such as the determinant of *Salmonella* O factor 5, which, according to Staub *et al.* (7), contains terminal *O*-acetyl-D-galactose, are destroyed.

After very short alkali treatment (5), similar to short incubation with serum (8), lipopolysaccharides often show increased biological activity. This indicates that the exposure of active groups or a certain particle size may be important for optimal activity, such as pyrogenicity or stimulation of the bone marrow.

The lipid A component of lipopolysaccharides is a long-chain fatty acid derivative of a poly-(phospho-D-glucosamine). Various lipopolysaccharides do not show any release of glucosamine during alkali treatment, indicating that lipid A is *firmly bound* to the O-specific polysaccharide component. After hydrolysis of alkali-treated lipopolysaccharides, part of the glucosamine is found as *N*-β-hydroxymyristinoyl glucosamine, which is a main constituent of lipid A in many enterobacterial lipopolysaccharides.

Grabar and Oudin (9) have tried to produce nontoxic O antigens by coupling the degraded polysaccharide of *S. typhi* to carrier proteins, according to the method of Goebel and Avery. Together with Staub *et al.* (10), we have shown that, in certain cases, coupling of monosaccharidic determinant end-groups of O antigens, such as colitose (3-deoxy-L-fucose), to proteins may lead to artificial antigens which, in suitable animals (goats), elicit antibodies with specific O cross-reactivity. But the monosaccharidic end-groups of the highly branched polysaccharides do not represent the whole O-specific structure which, as is well known from the work of Kabat, Morgan, and others, is oligosaccharidic in nature.

We, therefore, reinvestigated procedures to couple O-specific degraded polysaccharides to carrier proteins (11). *p*-Aminobenzyl ethers of the polysaccharides from *Escherichia coli* O 111, *S. paratyphi* B, and *S. gallinarum* of varying degree of substitution were prepared, diazotized, and coupled to proteins. The synthetic complexes were nontoxic. In rabbits and mice some of these artificial antigens elicited agglutinating (and precipitating) O antibodies after the injection of doses in the order of 10–100 μg of antigen (Table I). Studies were also undertaken to cross-link units of degraded polysaccharide(s) to higher-molecular complexes, because it is well known that the molecular weight of polysaccharides (for instance, of dextrans) can be of great significance with respect to their antigenicity. These antigens may also be of some value for investigations on the anti-infectious properties of O antibodies, a problem which needs further clarification.

TABLE I

HEMAGGLUTINATION TITERS OF RABBIT SERA AFTER IMMUNIZATION WITH *Escherichia coli* O 111 POLYSACCHARIDE-BENZYL-AZO-EDESTIN COMPLEX [a]

Antigen dose (μg)	Rabbit No.	Titer [b]		
		(A)	(B)	(C)
10	907a	1:<10	1:640	1:1280
	908a	1:<10	1:160	1:640
	909a	1:<10	1:640	1:1280
	918a	1:<10	1:1280	1:1280
100	919b	1:<10	1:80	1:1280
	924b	1:<10	1:640	1:5120
	925b	1:<10	1:320	1:1280
	926b	1:<10	1:160	1:1280

[a] From Westphal *et al.* (11).

[b] Blood samples before immunization (A). On 1, 4, and 18, 10 or 100 μg, respectively, of antigen were given iv and sc. On day 25 blood samples were taken (B). On day 40, 40 and 400 μg, respectively, were given iv and samples (C) were taken on day 46.

Lipopolysaccharides from S and R Forms

Hundreds of enterobacterial lipopolysaccharides have been extracted in which the respective polysaccharide components show a widely varying sugar composition, which is also reflected in the species-specific character of the many different oligosaccharidic side chains (see 12, 13). Nevertheless, many of these lipopolysaccharides exert endotoxic activities, such as pyrogenicity in rabbits or lethality in mice, in the same order of dosage. Of the sugar constituents, only D-glucosamine and heptose can occur in a structure common to all endotoxically active lipopolysaccharides. Since the discovery of a ketodeoxyoctolonic acid (KDO) by Heath *et al.* (14), this constituent, found in many enterobacterial lipopolysaccharides, could also be taken into consideration. All other sugar constituents can be excluded as part of any endotoxically specific structure, because highly active lipopolysaccharides are known in which one or the other of these monosaccharide constituents is completely lacking. For instance, two *E. coli* strains were found (13), *E. coli* O 17 and *E. coli* O 73, in which galactose is absent in the purified lipopolysaccharide. The endotoxin of *Shigella dysenteriae* does not contain any glucose. Besides the monosaccharides mentioned, all other sugars (galactosamine, mannose, pentoses, deoxy- and dideoxyhexoses) are absent in the lipopolysaccharides extracted from *R* forms (15). It is known, however, that many R forms contain lipopolysaccharides in their cell walls that are endotoxically as potent as those of their parent S form.

From many smooth forms (S forms) of enterobacterial species, rough