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FRANK J. DIXON HENRY G. KUNKEL

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PREFACE

The articles in the present volume illustrate the pervasiveness of current immunology. This discipline extends from the specialities of genetics, both fundamental and applied, to structural chemistry, particularly of antigen receptors and cellular antigens—the molecules responsible for individuality and for the integration of the immunologic network—and finally to those biological sciences that deal with the production and action of effector molecules which carry out immunologic design. Just as immunologists have contributed much to the understanding of gene structure and function, cell–cell interactions, and the process of inflammation, so have the talented investigators in these separate fields contributed much to immunology. The present volume is the product of such a diverse group of researchers, whose quite separate interests come together to advance our knowledge of immunology.

The first article, by Drs. Tony Hugli and Hans Müller-Eberhard, deals with the structural and functional characterization of anaphylatoxins, spasmogénic substances released during complement activation. These low molecular weight peptide fragments of C3 and C5 elicit a variety of cellular responses, which implies that they play a significant role in inflammation and acute allergic reactions. Their stimulation of multiple cell types at pico- and femtomolar concentrations suggests a hormone-like action via specific cell surface receptors. By using the information now available defining the amino acid sequences of these molecules, much of which has come from the authors' laboratory, it is now possible to synthesize oligopeptides with the properties of anaphylatoxins and these compounds should serve as effective tools for analyzing further the mechanism(s) by which these potent substances function.

The use of *H-2* mutations to study functions of the murine major histocompatibility complex began only a few years ago, but already has yielded much information. For example, this line of endeavor has shown that (1) a single *H-2* locus can control a variety of immunologic phenomena, (2) strong MLC reactions can occur to *H-2K* and *D* antigens as well as to *I* region antigens, (3) histogenetically and serologically detectable antigens are of comparable complexity, and (4) small changes in the H-2 molecule, perhaps a single amino acid substitution, can have drastic effects on phenotype. Moreover, this work has provided numerous leads for further research on the molecular determinants of immune function. In the second contribution, Dr. Jan Klein, an outstanding authority in the field of immunogenetics, sum-

marizes this progress and puts this experimental approach in appropriate perspective. Since this field will undoubtedly increase in activity and importance, his review serves well to set the stage and orient the reader for what is to come.

Another approach to the study of the MHC, viz., via the analysis of the protein products of the murine 17th chromosome, is treated in the third article by Drs. Ellen Vitetta and Donald Capra, both of whom are important contributors to this field. Many of the gene complexes that map between the centromere and *TLa* appear to play an essential role in cell–cell interactions and the control of immune responsiveness, embryogenesis, and differentiation. All the products of these genes are glycoproteins, most of which are expressed on cell surfaces presumably as receptors and often in association with β-microglobulin. This review analyzes the current state of biochemical knowledge about these glycoproteins (including their primary structure), emphasizes their common features and interrelationships, and explores the implications of these qualities for the evolutionary origins of the associated genes. It is suggested that this segment of chromosome 17 acts in some ways as a "super gene" in which the genes and gene complexes coding for a number of functionally interrelated molecules are closely linked, presumably as a result of selective pressures.

One of the most significant of recent developments in immunology has been the recognition of idiotypes of antibodies and lymphocyte receptor molecules and of the formation of anti-idiotypic antibodies, which may serve as regulators of immune function. Since the individual antigenic specificity of an antibody, i.e., its idiotype, must depend upon its unique variable region sequences, the idiotype is an expression of antibody V region genes and as such has been successfully exploited as a probe in a variety of fundamental immunologic and genetic studies. The article by Dr. K. Eichmann provides an excellent background for this subject and emphasizes two new areas of research to which the author is a major contributor. The first concerns the molecular nature and identity of cell surface receptors and the structure of their idiotypic determinants. The second is the possible function of these determinants as key elements of regulation within a network of immunocytes that are interlocked to one another through idiotype–anti-idiotype interactions.

Implicit in the clonal selection hypothesis is the existence of a repertoire of genetically determined B-cell clonotypes. The genetic determination, the development, and the regulation of this library of immunologic responses are the subjects of the last article, by Drs. Nolan Sigal and Norman Klinman. These authors draw on their exten-

sive experience in this field to put into perspective the clonal selection scheme with its more than 10^7 clonotypes and multiple forms and levels of control. In keeping with the genetic basis of the scheme, all members of an inbred strain express the same repertoire of major clonotypes, but it is not yet possible to prove that every clonotype is shared, an observation that would rule out a role for random somatic events in immunologic development. The authors also trace the maturation of the repertoire from its restricted state in the neonate to its full development in the adult and note the many levels for control of this expression.

We wish to thank the authors for their long and painstaking efforts in the preparation of these excellent reviews, and the publishers, who present this product with great skill.

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Anaphylatoxins: C3a and C5a[1]

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I. Introduction

Activation of the coagulation, fibrinolytic, and complement systems leads to the formation of primary and secondary reaction products, both of which play important biological roles. While the primary products enter the main pathway of their respective systems, the secondary products constitute activation peptides such as the kinins, fibrinopeptides and anaphylatoxins. Anaphylatoxins are low molecular weight, biologically active polypeptides that are released during complement activation from C3[3] and C5 and are commonly denoted as C3a and C5a. For the purpose of this review, anaphylatoxins will be defined as spasmogenic factors derived from serum complement components. They are functionally defined by their actions on the vascu-

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[2] Dr. Müller-Eberhard is the Cecil H. and Ida M. Green Investigator in Medical Research, the Research Institute of Scripps Clinic.

[3] The terminology for the complement components conforms to the recommendations of the World Health Organization Committee on Complement Nomenclature (1968).

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lature, smooth muscle, mast cells, and certain types of peripheral blood cells.

C3 and C5 are each composed of two polypeptide chains, the alpha chain (α) having a molecular weight of 100,000–120,000 and the beta chain (β) a molecular weight of 70,000–80,000. Anaphylatoxin formation results from a highly selective, proteolytic scission of the α-chain of the parent molecules. Current evidence indicates that C3a and C5a originate from the NH_2-terminus of the α-chains of C3 and C5, respectively.

Human blood and the blood of other mammals have two distinct mechanisms of complement activation. These mechanisms can be set into motion by immune complexes, by microbial or fungal polysaccharides, and, presumably, by certain virus particles (Cooper *et al.*, 1976). The two mechanisms are called the classical and the properdin, or alternative, pathways of complement. The classical pathway involves complement components C1, C2, and C4, which form specific enzymes capable of cleaving C3 and C5 and liberating anaphylatoxins (Müller-Eberhard, 1975). The corresponding alternative pathway involves enzymes that are constituted from C3, C3 proactivator (Factor B), C3PA convertase (Factor D), and properdin (Medicus *et al.*, 1976) and produce an analogous selective cleavage of components C3 and C5. Current evidence suggests that, whether activation of complement proceeds via the classical or the alternative mechanism, the resulting C3 or C5 fragments are identical both biologically and chemically. The C3a and C5a anaphylatoxins have been isolated and characterized in recent years, permitting their detailed molecular definition. Their multiple activities imply that anaphylatoxins play a significant role in inflammation and possibly in some acute allergic reactions. A recent comprehensive review written by Ryan and Majno (1977) examines the action of numerous plasma mediators involved in the acute inflammatory response. The remarkable potency of the anaphylatoxins makes them likely candidates for figuring prominently in the various processes of inflammation.

II. Historical

Sixty-seven years ago at the University of Berlin, Friedberger (1910) observed that a potent toxin was formed when fresh serum was treated with aggregated immunoglobulin. He demonstrated that serum taken from a guinea pig and incubated with an immune precipitate was toxic, often fatal, when re-injected into the donor animal. Armed with the knowledge that the principal in serum producing this toxin was

thermolabile and could be generated in serum without adding foreign protein (Novy and deKruif, 1917) it was concluded that the toxic factor might be derived from the complement system, at that time poorly understood. Since the physiological effects of the toxic substance in guinea pigs mimicked the symptoms of anaphylaxis or anaphylactic shock as defined by Portier and Richet (1902), Friedberger termed the factor an "anaphylatoxin."

Subsequent studies showed that anaphylatoxin formation could be initiated by substances other than immune precipitates. Among these additional activators were complex polysaccharides, such as agar (Bordet, 1913a), inulin (Bordet and Zunz, 1915), starch (Nathan, 1913a,b), and dextran (Hahn *et al.*, 1954). All the activating agents mentioned are capable of generating anaphylatoxin activity in sera from rats, rabbits, guinea pigs, or pigs. Conceptualizing his observations, Friedberger proposed the "humoral anaphylatoxin theory," which claimed that humoral factors were directly responsible for the shock-inducing behavior of activated serum. Later, when histamine was found to be released from circulating cells by the action of anaphylatoxins, a "cellular histamine theory" became accepted. Proposal of a cellular release mechanism proved to be particularly attractive for explaining the shock phenomenon, since the anaphylatoxin mediates cellular vasoamine release and thereby may act indirectly to promote its spasmogenic effect. However, rats did not express the typical shock syndrome when activated rat serum was injected, even though rat serum proved to be considerably more toxic in guinea pigs than did activated guinea pig serum. This phenomenon was attributed to the fact that anaphylatoxin causes very little histamine liberation in rat serum (Hahn *et al.*, 1954), but excites liberal histamine release in both guinea pig serum and lung (Hahn and Oberdorf, 1950; Hahn, 1954). Although it is now well established that the anaphylatoxins can release histamine from mast cells and basophils, the issue of whether the systemic and tissue responses to these factors are entirely direct or indirect effects still remains unresolved.

A review by Hahn, which appeared in 1960, accurately summarized the accumulated knowledge concerning the anaphylatoxins at that time. It contained fewer than 45 references that covered more than 50 years of investigating the biological actions of anaphylatoxin. Near the end of his treatise, Hahn addressed himself to a discussion of the differential role that anaphylatoxins might play in the phenomena of anaphylactic and Forssman shock. It had been established that the ability to form anaphylatoxin did not diminish in the serum of an animal experiencing anaphylactic shock, whereas it diminished by about 50%

in the serum of animals with Forssman shock (Giertz *et al.*, 1958). Hahn concluded from the difference between the two types of systemic shock that Forssman shock is mediated by humoral mechanisms, probably involving anaphylatoxin, and that anaphylactic shock is probably mediated by cellular events.

Hahn's conclusion has been borne out by investigations of the past 10 years. The acute allergic reaction is now attributed to antibody of the IgE class, which in conjunction with allergens effects mediator release from mast cells or basophils via a complex cellular process that is independent of complement and hence of anaphylatoxin (Ishizaka *et al.*, 1970; Kaliner and Austen, 1973). However, there are acute allergic reactions caused by antibody classes other than IgE that are capable of activating complement. Thus, the extent of participation of the anaphylatoxins in allergic reactions remains to be defined.

As mentioned, in 1917 Novy and deKruif had already speculated that anaphylatoxin activity was a function of a protein arising from serum, not a direct interaction of immune complexes with the tissues. Osler *et al.* in 1959 furnished the first experimental evidence indicating that anaphylatoxin is indeed derived from complement, specifically from its "third component." The classical "third component" has since been shown to consist of six proteins, namely C3, C5, C6, C7, C8, and C9. Vogt and Schmidt (1966) provided further evidence demonstrating that the classical rat anaphylatoxin was generated by a serum protease activated by contact with Sephadex and that protease seemed similar to the anaphylatoxin-forming activity of cobra venom. Toward the end of the 1960s Lepow and associates (1969), working with human complement proteins, showed anaphylatoxin to be a low molecular weight product of C3, and Jensen (1966), working with guinea pig complement, showed it to be a derivative of C5. The discrepancy was resolved by the demonstration that a low molecular weight fragment with anaphylatoxin activity can be enzymtically cleaved both from human C3 and from human C5 (Cochrane and Müller-Eberhard, 1968). These two anaphylatoxins were unique and were shown to have distinct biological specificities. At about the same time, similar fragments were cleaved from analogous complement proteins of guinea pig serum (Jensen, 1967), and of pig serum (Vogt *et al.*, 1971b), and the corresponding C3 and C5 fragments were subsequently isolated directly from activated pig and human serum (Vallota and Müller-Eberhard, 1973; Hugli *et al.*, 1975a; Corbin and Hugli, 1976).

As realistic amounts of highly purified C3a and C5a were made available, it became possible to delineate similarities and differences

of their effects on cells, including mast cells, basophils, neutrophils, monocytes, macrophages, and smooth muscle cells. It also became possible to tackle the comparative structural analysis of the two peptides, a feat that led to the complete elucidation of their respective covalent structures (Hugli, 1975a; Fernandez and Hugli, 1977b) and to the realization that they share a measure of sequence homology (Fernandez and Hugli, 1977a,b). Thus, the similarity in biologic function was reduced to a similarity in chemical structure. It is anticipated that their functional differences will eventually be attributed to discrete structural differences. The use of synthetic oligopeptides with anaphylatoxin-like activities (Hugli and Erickson, 1977) will aid in identifying the chemical basis of anaphylatoxin specificity and activity.

The remarkable biological potency of the anaphylatoxins emphasized the probable existence of very strict control mechanisms. A major regulatory principle was found in serum; it appeared to be an enzyme with carboxypeptidase B specificity (Bokisch *et al.*, 1969). Removal of the COOH-terminal arginine residue from either C3a or C5a totally abrogates anaphylatoxic activity (Bokisch and Müller-Eberhard, 1970; Fernandez and Hugli, 1976). Discovery of the serum enzyme and, subsequently, its effective inhibition allowed both anaphylatoxins (e.g., C3a and C5a) to be produced in whole serum without being inactivated (Vallota and Müller-Eberhard, 1973).

With a growing understanding of the classical complement pathway in the 1960s and the alternative pathway in the 1970s, the enzymes directly responsible for anaphylatoxin liberation became definable. Although the convertases of the classical pathway differ from those of the alternative pathway in subunit composition, their overall structures are similar and their substrate specificities are identical. The essential zymogen of the alternative pathway [i.e., C3PA (Factor B)] was detected because it was required for cleavage of C3 or C5 (Jensen, 1967) by cobra venom factor, a process later described almost without reference to anaphylatoxin formation (Götze and Müller-Eberhard, 1971). On the other hand, Stegemann *et al.* (1964, 1965) and Vogt and associates (1966, 1969a) employed cobra venom factor extensively as an activator of anaphylatoxin in serum without further reference to complement activation. Today it is clear that the anaphylatoxin-forming enzymic site of the alternative pathway resides in Factor B, as that of the classical pathway resides in component C2.

The present view deals primarily with the studies of the past 15 years, which permits us the advantage of hindsight in explaining many of the questions posed during the first 50 years of anaphylatoxin research. That is not to say that the early contributions go unnoticed or

unappreciated, for they have indeed provided valuable insights and have stimulated many of the studies in progress today.

III. Formation and Control of Anaphylatoxins

Investigations following the discovery of anaphylatoxin activity in serum led to the description of two well characterized polypeptides, the C3a and C5a anaphylatoxins. However, our present knowledge of their chemical nature and their mechanisms of formation in serum did not come easily. For example, we now know that serum contains an indigenous, enzymic control mechanism capable of rapidly inactivating both anaphylatoxins. The resulting inactive products are little changed chemically and are immunologically indistinguishable from their active forms. Until recently this anaphylatoxin inactivator remained unrecognized. Ignorance of the existence of an inactivator caused uncertainties that lasted for more than 50 years. During that time, investigators puzzled over the presumed number of different anaphylatoxins and the circumstances prompting the differential expression of anaphylatoxin activity in sera taken from various animals. The anaphylatoxin inactivator is now known to be an exopeptidase that functions in the serum of all higher mammals studied to date. Since the apparent efficiency of the inactivator varies with the source of the serum, it must be concluded that the inactivator constitutes a major factor in the expression of anaphylatoxin activity *in vitro* and *in vivo*.

Earlier implication of complement in the generation of anaphylatoxin activity had been based on circumstantial evidence. Then Osler and his colleagues (1959) performed experiments designed to answer this very question. By demonstrating that anaphylatoxin formation occurred concurrently with complement fixation and C3 consumption but failed to appear when complement activation was prevented by heating, by metal chelation, or by phlorizin, an inhibitor of C3 activation, they concluded that anaphylatoxin forms as a direct consequence of complement activation. Later, observations by Ratnoff and Lepow (1963) and others (Smink *et al.*, 1964; Dias da Silva and Lepow, 1965) showed that isolated human C1 esterase can induce a response characteristic of anaphylatoxin when injected into guinea pig skin, proving that an established complement enzyme was indeed capable of generating anaphylatoxin activity. In addition, these studies provided some of the earliest evidence that anaphylatoxin formation might be an enzymic process, a concept questioned at the time. In experiments that followed, the active factor induced by C1 esterase was compared with that generated in serum by the introduction of enzymically inert

substances such as complex polysaccharides. Both activities were biologically identical (Dias da Silva and Lepow, 1965). Such comparison is possible because smooth muscle is specifically desensitized by repeated applications of the anaphylatoxin, a phenomenon known as tachyphylaxis. Stimulation of smooth muscle by C3a is followed by a state of unresponsiveness to a subsequent stimulation by C3a regardless of the animal origin of the C3a. The same phenomenon applies to C5a. However, C3a will stimulate smooth muscle rendered unresponsive to C5a, and vice versa. These investigators also showed that formation of anaphylatoxin by C1 esterase was inhibited either by EDTA or by salicylaldoxime and phlorizin, all of which prevent C3 activation, a finding that implicated additional complement proteins in the process of anaphylatoxin formation.

In Germany, Vogt and Schmidt (1966) independently concluded that anaphylatoxin formation was an enzymic process. Their conclusion was based on their ability to separate the contact-activation event from the step that actually produced a functional anaphylatoxin. They succeeded in isolating a plasma fraction, reportedly not C1 esterase, that was activated either by Sephadex or Bio-Gel. The activated plasma fraction could then be mixed with a fraction containing the anaphylatoxin precursor "anaphylatoxinogen," in the complete absence of contact activators, with the resultant formation of anaphylatoxin. Although Vogt and Schmidt correctly concluded that the activation was enzymic, they erroneously surmised that the enzymes involved were unrelated to complement. Unknowingly, they had demonstrated that anaphylatoxin had formed via the alternative (properdin) pathway of complement activation. This realization would come later, when the C3 activator system was described and the process was shown to activate C3 without involving components C1, C2, and C4 (Brade and Vogt, 1971a; Götze and Müller-Eberhard, 1971). Regardless, the impact of the observations of the 1960s was far-reaching, since thinking at that time was divided between ascribing anaphylatoxin formation to an enzymic process or simply to physical aggregation of selected serum components. Giertz and Hahn (1961) addressed this very problem. In their discussion they considered the formation of toxic aggregates, action of denatured toxic proteins and assorted physical interactions which may lead to enzyme activation as possible modes of anaphylatoxin formation. However, by late 1966 complement was convincingly implicated in the formation of anaphylatoxin and the process was understood to be enzymic.

Definitive studies identified C3 and C5 as the anaphylatoxinogens after these proteins were recognized as complement components and

became available in purified form. Dias da Silva *et al.* (1967) detected anaphylatoxin activity in a mixture containing purified $C\overline{1s}$, C2, C3, and C4. Since they found that the activity resided in a small fragment derived from the C3 molecule, they defined C3 as the precursor of human anaphylatoxin. Jensen (1967) reached a different conclusion working with functionally purified components from guinea pig serum. He found the anaphylatoxinogen to be associated with a serum fraction containing C5. The activity generated from this fraction corresponded in specificity to classical anaphylatoxin activity, as evidenced by desensitization of the guinea pig ileum. [Jensen's serum fraction contained C3, and presumably C3a was formed; however, this activity might well have been overshawdowed by the more potent C5a.] In contrast, the C3-derived fragment did not cause cross-desensitization to classical anaphylatoxin, and therefore Dias da Silva and co-workers (1967) interpreted this behavior as evidence for a "new" anaphylatoxin. Their results with isolated human C5 were inconclusive since no classical anaphylatoxin activity could be demonstrated when they added C5 to the reaction mixture containing $C\overline{1s}$, C2, C3, and C4. In retrospect, the disparate results obtained by Jensen and Lepow and his group are entirely explicable. While C3 convertase functions efficiently in both a particle-bound and soluble form, C5 convertase is an efficient enzyme only in its bound form. Jensen used the cell-bound enzyme, while Lepow's group relied on the soluble system.

It was Jensen (1967) who first demonstrated that anaphylatoxin activity could be generated by treating complement components with trypsin. Cochrane and Müller-Eberhard (1968) finally identified both human C3a and C5a after isolation directly from C3 and C5 that had been treated previously with either the appropriate complement enzyme or with trypsin. They showed that C5-derived anaphylatoxin (C5a) was a low molecular weight polypeptide (approximately M_r 10,000) that did not desensitize ileal strips to C3a.

Thus, for the first time it was demonstrated that there are two biologically and chemically distinct anaphylatoxins. The current view on the properties of their precursors and the mechanisms of their formation may be summarized as follows. C3 is an M_r 180,000 β_2-globulin that is present in serum with the greatest abundance of all complement proteins, approximately 1500 μg/ml. In man, the plasma disappearance half-time of the protein, $t_{1/2}$, is 50 hours, its fractional catabolic rate is 2.12% per hour, and its calculated rate of synthesis is 1.16 mg/kg. The protein contains 2.5% carbohydrate, including neutral hexose, hexosamine, and neuraminic acid. It consists of an M_r 110,000 α-chain and a 75,000 β-chain, both linked by disulfide bonds