Mechanism of Cell and Tissue Damage Produced by Immune Reactions

IInd International Symposium on Immunopathology

Brook Lodge (Michigan, USA) 1961

Edited by

Prof. Dr. Pierre Grabar, Paris
Prof. Dr. Peter Miescher, New York

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The general purpose of the first Symposium on Immunopathology was to bring together in a mutually rewarding, integrative effort, investigators from different disciplines. This approach was so enthusiastically received that all participants expressed their desire to hold similar Symposia at regular intervals. Originally the International Committee of Immunopathology had planned a Second Symposium in 1963. However, since 1958 immunopathology has become such a major research area in medicine, and such rapid strides have been made, that it was deemed mandatory to hold a Second Symposium at an earlier date. The Upjohn Company very generously offered to sponsor this meeting, and put at our disposal superb facilities of the Brook Lodge Conference center.

In the first Symposium a rather broad spectrum of immunopathology was covered. This time, it seemed more desirable to limit the discussions to one of the most important problems, that is, mechanisms of tissue damage produced by immune reactions. This topic is of special relevance for basic research as well as for clinical immunopathology. Again, immunologists, immunochemists and clinicians were invited to discuss the recent progress in this field.

The first part of the symposium deals with basic aspects, the second with the clinical and pharmacological implications.

In the preparation of the second Symposium and in its actual organization, Dr. G. McMahon acted as an efficient secretary general. The committee gratefully acknowledges his invaluable help, and is further indebted to him for his willingness to continue as secretary general of the International Committee of Immunopathology.

All the papers presented at the second Symposium are published in this volume. The discussions in this Symposium have been edited with special care in order to give to the reader a more comprehensive view on multiple facets of current problems. The discussions have been grouped in logical sequence; repetitions have been avoided as much as possible. We are especially indebted to Drs. B. Benacerraf, E. Franklin, H. Müller-Eberhard, Z. Ovary, Noel Rose and Bela Strauss for their collaboration in editing the discussions. – We are grateful to all participants for discussing their current research in detail with such enthusiasm.

The publishers, Benno Schwabe & Co., again have obligingly undertaken the work of printing the present volume, a task on which they have spared neither trouble nor expense. In this connection, we owe our warmest thanks to Dr. h. c. Chr. Overstolz, and to Dr. H. G. Oeri for their most active cooperation. The distribution of the book in the United States of America has kindly been undertaken by the publishers Grune & Stratton, Inc., New York and London.

Paris and New York, May 1962 / *Pierre Grabar* *Peter A. Miescher*

WELCOME

As an organization of people vitally interested in medical science, we welcome this opportunity to be host to the second International Symposium on Immunopathology. It is our sincere hope that the papers and the discussion at this meeting of distinguished international scientists will generate ideas from which will come further contributions to basic research in medicine.

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President
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[1] Dr. *Ado* was unable to attend the Symposium, however kindly submitted his manuscript for publication.

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CONTENTS

I. The Role of Complement in Antibody-Mediated Tissue Damage

Mechanism of Action of Guinea Pig Complement

Tibor Borsos and Manfred M. Mayer[1]

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During the past 20 years the sequential action of the guinea pig complement components and the roles of Ca^{++} and Mg^{++} have been elucidated, experimental technics for isolation of the intermediate products have been devised, and progress has been made in separating and purifying the components of complement [7]. These advances have brought the study of complement to a stage where it offers new opportunities for probing into the mysteries which now occupy the center of immunologic inquiry.

In this brief review, we will seek to outline the subject's present status, to depict obstacles impeding progress, and to draw attention to problems awaiting solution.

The term complement was coined at the turn of the century. It was believed that it functions as an auxiliary to antibody. Since then, it has been learned that complement comprises at least six distinct factors or components, which act in a definite sequence to bring about cell injury. It appears to be the function of antibody to initiate the series of reactions which comprise the complement system, and to give specific direction to its cytotoxic action on certain bacteria and protozoa, as well as cells of higher organisms. Complement also participates in the neutralization of some viruses, enhances phagocytosis and plays a role in the complex events of immunologic tissue injury known collectively as allergic reactions.

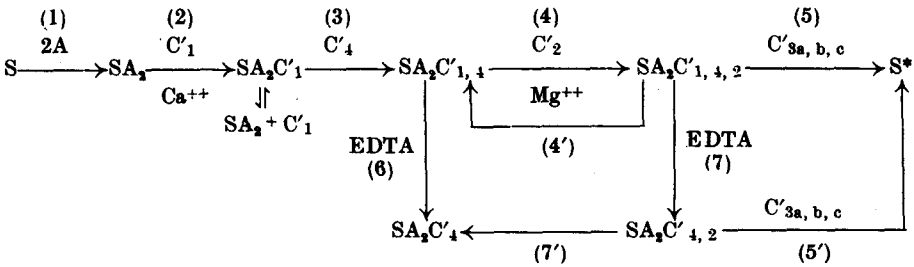
The ease with which the hemolytic reaction can be observed and measured accurately is responsible for its widespread use in studies of the complement system. Most of the information now available has come from studies of the hemolytic reaction and many of these basic investigations have been made with a model system comprising sheep erythrocytes, the corresponding rabbit antibody and fresh guinea pig serum as a source of complement. At present, the technical procedures developed in the study of this model system represent the most refined methods available for investigations of complement. However, the essential concepts and

[1] Supported in part by grants from the National Science Foundation, United States Public Health Service and contract with Office of Naval Research.

experimental tactics are amenable to general application and may serve as a guide to the study of the many immunologic phenomena involving complement.

Hemolytic Reaction Mechanism

Recent studies of immune hemolysis have been directed primarily toward elucidation of the successive reactions which comprise the complement system. The first step in the complex series of events to be described is the combination of possibly two molecules of antibody (A) with certain antigenic sites (S) on the erythrocyte surface, the antibody molecules being located in close proximity with respect to one another, creating a receptor SA_2, as postulated by *Weinrach* and *Talmage* [12], which then reacts with the complement (C′) factors C'_1, C'_4, C'_2, $C'_{3a,\,b,\,c}$ in a series of successive reactions according to the following scheme:



Ca^{++} is essential as a ligand in reaction (2) and Mg^{++} catalyzes reaction (4). Reactions (4′) and (7′) indicate that $SA_2C'_{1,\,4,\,2}$ and $SA_2C'_{4,\,2}$ sites are unstable and revert to $SA_2C'_{1,\,4}$ or $SA_2C'_4$, respectively, at a rate depending on temperature. The half-life of $SA_2C'_{1\,4,\,2}$ and $SA_2C'_{4,\,2}$ made with guinea pig complement, is about ten hours at 0° C, about 25 minutes at 30° C and 10 minutes at 37° C. Reaction (5) and (5′) comprises three steps involving C'_{3a}, C'_{3a} and C'_{3c}; their sequence of action has not yet been established definitively. Reaction (6) indicates that treatment of $SA_2C'_{1,\,4}$ with EDTA yields $SA_2C'_4$. Reaction (7) indicates that treatment of $SA_2C'_{1,\,4,\,2}$ with EDTA yields $SA_2C'_{4,\,2}$ which can be converted to S* by the action of the C'_3 complex. S* denotes a lesion in the erythrocyte membrane which impairs the osmotic balance of the cell.

The intermediate products, at a cellular level, are designated by the symbols EA, EAC'_1, EAC'_4, $EAC'_{1,\,4}$, $EAC'_{1,\,4,\,2}$, $EAC'_{4,\,2}$, and E*. The symbol EA designates cells with one or more SA_2 receptors, the number of these, of course, depending on the antibody multiplicity, i.e., the number of antibody molecules per cell. Each sheep erythrocyte can combine with as many as 5000 molecules of A, but usually experiments are performed with an antibody multiplicity of about 1000, and it has been estimated that this will yield approximately 125 SA_2 receptors per cell. The intermediate product EAC'_1 refers to cells with at least one $SA_2C'_1$ site. Similarly, $EAC'_{1,\,4}$ designates cells with one or more $SA_2C'_{1,\,4}$, the upper limit with respect to the number of such sites per cell being the number of SA_2 per cell. The same considerations apply also to the other intermediates. The symbol E* refers to damaged

cells which lyse spontaneously. One S* is sufficient to transform a cell to the state E*, as explained in a later section on the one-hit theory of immune hemolysis.

During the past seven years experimental methods have been developed by which sheep erythrocytes can be transformed into each of these intermediates by appropriate treatments with rabbit antibody and guinea pig complement or complement fractions. Furthermore, fractionation methods for separation of the complement factors from guinea pig and human serum have been devised, and with these technical advances it is now possible to study some of the complement reaction steps individually [7].

A major issue in studies of the action of C′ is the question whether the immune hemolytic reaction is a "single-hit" or "multiple-hit" process. This problem is of fundamental importance because it holds the key to development of a quantitative relationship between the molecular reactions of cell constituents with antibody and the complement components, and the final event at a cellular level, namely, hemolysis, on which experimental observation and measurement are based.

The concept of a one-hit reaction is familiar to virologists, because a single virus particle is usually sufficient to produce infection of one cell. In the case of hemolysis by antibody and complement, definition of this concept presents a more complex situation because of the multicomponent nature of complement. It is postulated that the six complement factors react at discrete loci upon the cell surface, each locus being created by the union of an antibody molecule, or perhaps two molecules as postulated by *Weinrach* and *Talmage* [12], with an antigenic site, S, on the surface of the cell. These sensitized sites, SA or SA_2, react with the complement factors in the sequence C'_1, C'_4, C'_2, $C'_{3a,\ b,\ c}$ resulting in damage of an unknown nature to the structure of the cell membrane at or near this locus. Such a damaged site is designated by the symbol S*. The one-hit theory postulates that the production of one S* upon a cell is sufficient for lysis. As defined in this way, the theory takes cognizance of the fact that production of one S* requires action of antibody and six complement components, and the possibility that more than one molecule of any of these factors may act at a single site is not necessarily excluded.

The development of the one-hit theory over the past twelve years has been reviewed recently [9]. The earlier experiments with whole C′ yielded only suggestive evidence; to obtain rigorous proof, two conditions had to be met for at least one of the complement reaction steps: 1. Kinetic evidence that the reaction starts without lag, and 2. titration data on this step showing direct proportionality between the amount of reagent (i.e., complement component) and the number of resultant product sites.

When chromatographically purified C'_2 became available for study of the conversion of $EAC'_{1,4}$ to $EAC'_{1,4,2}$ we found that this reaction starts without lag, thus satisfying the first of these two conditions [3]. Furthermore, a plot of the proportion of cells converted to $EAC'_{1,4,2}$ vs. the quantity of C'_2 yielded a curve

which was entirely concave to the abscissa, confirming the kinetic evidence that the conversion of $EAC'_{1,4}$ to $EAC'_{1,4,2}$ is a one-hit reaction. In terms of reactive sites upon the cell surface this means that formation of a single $SA_2C'_{1,4,2}$ suffices to transform a cell to the state $EAC'_{1,4,2}$.

A quantitative analysis of $SA_2C'_{1,4,2}$ formation as a function of the amount of C'_2 was then made by application of the Poisson distribution function.

If $y_{1,4,2}$ designates the proportion of cells in the state $EAC'_{1,4,2}$ and if z denotes the average number of $SA_2C'_{1,4,2}$ per cell, the Poisson function yields the following relationship for the case that one $SA_2C'_{1,4,2}$ suffices to transform a cell to the state $EAC'_{1,4,2}$:

$$z = -\ln(1-y_{1,4,2})$$

For experimental evaluation it is necessary to treat the cells with the C'_3 factors in high concentration so that practically all $SA_2C'_{1,4,2}$ will go to S*. Under these conditions, the degree of lysis, y, will approach $y_{1,4,2}$. With the C'_3 reagents available at present it has been possible to obtain 70% conversion of $SA_2C'_{1,4,2}$ to S*, but this value probably can be improved with further progress in the purification of the C'_3 factors.

On the basis of the proposed model in Ref. 3, the number of $SA_2C'_{1,4,2}$ generated should be directly proportional to the quantity of C'_2 used for reaction, provided the stipulated excess of $SA_2C'_{1,4}$ over C'_2 is sufficiently large. Due to experimental limitations, we are currently limited to a moderate excess and consequently, plots of the number of $SA_2C'_{1,4,2}$ generated vs. quantity of C'_2 tend to depart from linearity as the multiplicity of C'_2 is increased. In some experiments, strict linearity has been observed up to a multiplicity of 1.2, while at the other extreme, we have experiments deviating from linearity at multiplicities as low as 0.4. It is not known whether these departures from theoretically predicted behavior can be attributed entirely to inadequacy of $SA_2C'_{1,4}$ excess, or whether other currently unknown factors play a role.

Studies by *L. G. Hoffmann* [6] of the action of purified C'_1 on EA, as well as the conversion of the resulting EAC'_1 to $EAC'_{1,4}$ by reaction with C'_4, have yielded results conforming more closely to the direct linear relationship between the number of product sites and quantity of reagent. Thus, C'_1 and C'_4, as well as C'_2 appear to react in a stoichiometric fashion.

Studies by *Green* et al. [4] indicate that E* is a cell which has suffered impairment of osmotic regulation. Cells in this state experience rapid loss of intracellular K^+ and become permeable to the Na^+ of the medium. These changes can be explained by assuming the production of "holes" in the cell membrane large enough to permit rapid exchange of inorganic cations and small molecules, but not of macromolecules. The resulting disturbance of osmotic regulation leads to swelling, and consequently, macromolecules become able to pass the cell membrane. This picture of the mechanism of cell lysis would be compatible with the one-hit theory.

In this connection it is necessary to consider the fact that red cells differ in respect to their lytic susceptibility. Such variation could arise from occurrence of unfruitful reactions if it is assumed that with more resistant cells the proportion of un-

productive encounters is greater than with less resistant cells. Such a mechanism involves the possibility of multiple hits, but it does not constitute a cumulative process and in this sense the characteristics of the reaction would still be those of a one-hit reaction. The term "one-hit" then refers to a non-cumulative model, which means that successive hits constitute independent events. This formulations would recognize the possibility that some sites that have reacted with each of the complement components are not S*.

Current Status of Analytical Technics

While most of the intermediates can be made, current methods are not satisfactory from the standpoint of controlling the number of intermediate sites per cell. In the case of $EAC'_{1,4,2}$, this problem has been solved partially by use of a known number of C'_2 molecules for treatment of $EAC'_{1,4}$. However, the number of $SA_2C'_{1,4}$ sites on these cells cannot be controlled precisely, partly because of the present uncertainty surrounding the nature and action of C'_1, and also for lack of adequately purified preparations of C'_4. The importance of proper site control cannot be overemphasized, for it represents the foundation of quantitative evaluation, which, in turn, is the cornerstone of sound interpretation. Many of the uncertainties and apparent contradictions are likely due to lack of proper quantitation.

The new analytical technics are based on the simple principle that each factor of the C′ system should be measured in terms of the conversion of the appropriate precursor to its successor. While this approach necessitates preparation of the requisite intermediate product, as well as purification of the C′ factors, its theoretical and practical advantages over the classical technics, in which the complement factors are assayed with the reagents R_1, R_2, R_3 and R_4, are so great that we expect general adoption of the new technics within the next few years.

The most compelling reason for this assertion is the virtual impossibility of obtaining preparations of R_1, R_2, R_3 and R_4 which meet the essential requirements necessary for their use, namely, that they supply an adequate excess of all of the C′ factors except the one to be titrated, and conversely, that they be completely free of the latter. Moreover, they should be free of interfering factors ("not anti-complementary").

Thus, *Heidelberger* et al. [5] have shown that the component titers depend on the quantity of R reagent used. This observation, of course, violates the first essential requirement, i.e., that of supplying an excess of all the components other than the one to be titrated. Furthermore, the R reagents, at low dilution or undiluted, are often hemolytic when used alone. This invalidates the second requirement, namely, the complete absence of the factor to be titrated. This difficulty cannot be circumvented by use of a greater dilution because of conflict with the first requirement. In addition, "anti-complementary" action may be encountered in some cases.