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DEFINED IMMUNOFLUORESCENCE AND RELATED CYTOCHEMICAL METHODS

Editors
Ernst H. Beutner
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Boris Albini

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DEFINED IMMUNOFLUORESCENCE AND RELATED CYTOCHEMICAL METHODS[a]

Editors

ERNST H. BEUTNER, RUSSELL J. NISENGARD, AND BORIS ALBINI

CONTENTS

[a]This volume is the result of a conference entitled Seventh International Conference Defined Immunofluorescence, Immunoenzyme Studies and Related Labeling Techniques, organized by the Department of Microbiology of the School of Medicine and the Department of Periodontology of the School of Dentistry of the State University of New York at Buffalo and the International Service for Immunodermatology Laboratories (ISIL) with advice from the IUIS-WHO Subcommittee on Standardization in Immunofluorescence, held on June 8–11, 1982 in Niagara Falls, NY.

III. Immunoenzyme Methods and Their Application

IV. Substrate Processing for Immunofluorescence

V. Fluorescent Tracing in Tumor Immunology

VI. Autoimmunity and Immunopathology of Skin and Kidney

VII. Immunochemical Procedures in Virology, Bacteriology, and Parasitology

Recognition of Antibodies as Labeled Globulins

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As early as the end of the nineteenth century it was widely suspected that antibodies were proteins, since they normally were found in the globulin fractions of serum or plasma. Proof of their nature was, however, impossible because of the merely qualitative, comparative, and often misleading dilution titers by which antibodies were presumed to be measured.[4] As a result, the belief persisted that antibodies might be substances of unknown nature merely adsorbed to globulins. Even as late as 1922 or 1923 a "protein-free antibody" could be had for the treatment of pneumococcal pneumonia: O.T. Avery and I found that "antibody-free protein" was more descriptive.

Announcement of the precipitin reaction between antigen and antibody by R. Kraus in 1897 stimulated speculation as to whether antigens rendered antibodies insoluble by denaturing them, and whether the precipitates formed contained antigen. This led to the first recorded use, in 1902, of labeled antigens. Von Dungern raised antibodies in rabbits with crab blood containing the copper-protein hemocyanin.[1] The antigen-antibody precipitate turned blue when shaken with air, proving the presence of hemocyanin. P.T. Müller used the phosphoprotein casein as antigen and reported a much higher content of phosphorus in the immunoprecipitate than in a volume of serum equal to that taken for precipitation.[19] Labeled antibodies entered the picture much later, as we shall see, although antibodies in their native state are already labeled globulins separable from accompanying proteins by their combining groups for homologous antigen.

As a chemist suddenly exposed to immunology in the early 1920s, I marveled at the progress that had been made with the primitive, purely relative methods of titration then available to the immunologist, progress that often depended upon a brilliant idea or a shrewd guess rather than sound scientific evidence. Yet such fundamental questions as whether antibodies were proteins, whether precipitins, agglutinins, and protective antibodies were the same or different, and whether complement was merely a colloidal state of fresh serum (Bordet) or a substance (Ehrlich) could not be approached with methods as subjective and grossly inaccurate as those in use. After fascinatedly reading Bordet's *Traité d'Immunité*, papers by Ehrlich, and Arrhenius and Madsen's *Immunochemistry*, I resolved to try to end the polemics that were plaguing immunology by applying the rigid criteria of microanalytical chemistry. This had been attempted previously by Wu *et al.*[21] who used hemoglobin and iodinated egg albumin as labeled antigens, but the results were equivocal.

The time was ripe for such an effort, for new tools had become available. Avery and I, later with W.F. Goebel, had established that the capsular slimes of types II and III pneumococci were chemically different, nitrogen-free polysaccharides,[5,6] so that any nitrogen found in their precipitates with homologous antisera would necessarily come from the sera. Even partially purified antibodies were available, for Felton had found that when potent antipneumococcal horse sera were poured into water most of the

antibodies precipitated with the so-called euglobulins, leaving most of the other proteins in solution.[2] With these materials, Forrest E. Kendall and I were able to measure accurately in units of weight, mg per ml, the actual content of precipitating antibodies in antipneumococcal sera[9] and to develop a quantitative theory of the precipitin reaction that explained much, and from which useful predictions could be made.[10] When a single polysaccharide antigen was involved, precipitins, protective antibodies,[14] and agglutinins[7] were found to be identical and the intractable dispute as to complement was decided in favor of Ehrlich's view[3]: complement was found to have weight.

But most antigens are proteins, and so it became necessary to distinguish between antigen nitrogen and antibody nitrogen. Yellow to orange azoproteins were known, but as quantitative colorimetric estimations were to be made, it seemed desirable to strive for deeper colors. Accordingly, benzidine was tetrazotized and combined on one side with R-salt, the salt of 2-naphthol-3,6-disulphonic acid, known to yield red dyes, and on the other side with crystalline egg albumin (Ea).[18] The resulting dark purplish-red protein was rather drastically purified until it reacted only faintly with anti-Ea, as we did not want it said that any antibodies due to DEa were actually only anti-Ea.

DEa was antigenic in rabbits and yielded precipitates with the dye that were pink to red depending upon the proportions in which dye and antiserum were mixed.[11,15] For analysis, the washed precipitates were dissolved in alkali, quantitatively rinsed into a colorimeter cup, and compared with an alkaline solution of DEa of known content of nitrogen. This had to be done visually, for photoelectric colorimeters had not yet been invented. The solutions of the precipitates were then quantitatively rinsed into micro-Kjeldahl flasks and analyzed for total N from which the colorimetrically determined antigen N was subtracted to give antibody N. Study of the characteristics of this precipitin reaction made it possible to extend our quantitative method to colorless native proteins.[12] Although Ea precipitated anti-DEa, the quantitative reaction curve was like that expected for a cross-precipitation and totally different from that of a homologous reaction.[12,13]

With the polysaccharide-antibody system we showed, with Torsten Teorell, that strong salt solutions shifted the equilibrium toward precipitates containing less antibody.[16] This release of antibody theoretically promised a means of isolating pure antibody and the conditions for accomplishing this were found after some difficulty. Analytically pure antibody, 100% precipitable or agglutinable, proved to be a typical globulin,[8] settling the old dispute once and for all and putting immunology on a firm scientific basis. Once the chemical nature of antibodies was proven, immunologists could begin to ask where and how they were elaborated, starting a tremendous, continuing expansion.

Kendall, Kabat, and I also began tentative attempts to label antibodies with R-salt or with malachite green, but the products tended to become insoluble and were not intensely enough colored to use in a projected study of phagocytosis. It remained for Albert Coons to think of fluorescence.

As labeled proteins, antibodies served well in a collaborative study with Rudolph Schoenheimer, Sarah Ratner, and David Rittenberg[17,20] who were using the uptake and release of ^{15}N in their physiological studies of protein behavior. Because of the rapid shuttling in and out of ^{15}N, Schoenheimer proposed that amino acid linkages were labile, opening and closing readily. It was Henry P. Treffers' idea to test this hypothesis with two different immune labels. We immunized a rabbit with killed type III pneumococci, gave it a diet containing ^{15}N, and also injected antiserum to type I pneumococci from another rabbit. Repeated quantitative type I and type III precipitin analyses showed that only the type III antibody, which the rabbit was synthesizing, had taken up ^{15}N—the type I precipitates contained virtually none. Schoenheimer greeted

the data with "O Weh!," for the antibodies with their specific markers had required an alteration of his hypothesis.

It is apparent, then, that the specificity of the combining groups of antibodies has served as an adequate label for many purposes. Antibodies even possess a second marker, idiotypy, which has given rise to a whole literature and may even be awaiting further exploitation. However, the conjugation of antibodies with enzymes or radioactive elements or substances has enormously increased the sensitivity of their detection and the advantages of their use, as the preceding conferences of this series and the present one have demonstrated.

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ALBERT H. COONS

Albert H. Coons

In memoriam

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As we are gathered here it is my privilege to take you back some years in remembrance of Albert Coons. Since I am his oldest friend in this society, I have been asked to bring homage to his memory. It is exactly 30 years and 30 days since I met him the first time at Harvard.

As we all know his scientific achievements starting in 1942 with the finding of pneumococci in tissue by fluorescent antibody technique, I am not going to discuss his scientific contributions. I will instead give some personal glimpses. Albert Coons was a Harvard boy, or let us rather call him a Harvard gentleman. He graduated from Harvard in 1937. He was a classmate of another famous research worker, Lewis Thomas. In his book *The Medusa and The Snail,* Thomas has a chapter, "Note on Medical Economics." Albert Coons, like the majority of his class that later went into research and teaching, was quite sure when he graduated he would be a practitioner. Harvard graduates were better paid because they were better physicians than graduates from other schools; that was what they told themselves. Coons also wanted to practice. He chose internal medicine. He wanted to do something socially important and not just earn money like many of the other graduates. We are grateful that he did not set up this practice.

Indeed, Coons did do something essential in research—and perhaps he did not earn so much money, at least not to start with. One example: In June 1952, I visited Albert Coons who demonstrated the brand new sandwich technique on cells forming antibodies to diphtheria toxoid. In 1953, during the Sixth International Congress of Microbiology in Rome, I was asked to arrange a symposium on antibodies. I was glad to succeed in the end to have Albert Coons present, to explain his immunofluorescent technique to a larger international audience. "In the end"—that is where the money comes in again—Albert Coons got no governmental money for travelling. His father's economic support made the participation in the Rome congress possible. Things were more difficult in those days and Albert Coons was a very modest, self-critical man, not very clever in selling himself. But not even his modesty could stop the fantastic success of his technique. He initiated a new field of study in pathology, cell biology, microbiology, and, of course, in immunology.

In 1961, Albert Coons gave a presidential address to the American Association of Immunologists. To read this address is to learn what qualifications make a great pioneer research worker. He finished his address by giving what might be called a scientific will. It concerned the education of young people in laboratory research work and reflects his own life experience. Firstly, the problem you attack, Coons said, should be a principle one; secondly, it is no doubt better and more stimulating to work on a problem of your own than to carry out a set task. Albert Coons found his own research problem and he never gave up the idea he got in 1939 while sitting alone in a room in Berlin: to label antibodies for the study of Aschoff's nodules. He returned in 1940 to Harvard Medical School with this idea hammering away in his mind. At Harvard he

had a most stimulating environment. People listened, as for example John Enders, who told him that he did not believe that Coons should solve the rheumatoid disease problem by hooking a fluorescent dye on antibodies but that the idea was very good for many other purposes. Enders also introduced him to chemists working with isocyanate for other purposes. Coons thus was fortunate to carry on his pioneer work in an outstanding environment, but his success depended on his character. He had scientific imagination; he could create enthusiasm in workers in other research fields and he could cooperate. He was keen on giving credit to his co-workers and was absolutely loyal to Harvard until his death. Last, but not least, he never gave up and he was himself a hard worker. He was in the lab most weekends and holidays. One of his maxims was the Greek saying "Strive for grace under pressure." But in spite of being so scientifically involved he had time for his large family, he was interested in music, especially the chamber music of Beethoven, and in poetry. He had many good friends who appreciated his sense of humor and listened to his laughter around a good dinner table. We are grateful to have known him.