FLUORESCENT ANTIBODY TECHNIQUES AND THEIR APPLICATIONS

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PREFACE

Almost two decades have passed since Dr. Albert H. Coons and his co-workers first established the principles of the fluorescent antibody technique. This revolutionary technique, based on more than ten years of investigation, made it possible, by using the specific reactivity of labeled antibody, to locate precisely the corresponding antigen in tissue. Subsequently a number of improvements and refinements were devised, giving this simple and reproducible technique a degree of specificity and sensitivity which has led to its increasing application in a variety of fields, including immunology, microbiology, pathology, histology and clinical diagnostics.

A number of monographs and articles dealing with the theoretical and practical aspects of this technique have been published to date, but most of these have dealt with specific applications rather than the general methodology of the technique itself. Our fluorescent antibody (FA) study group at the Institute of Medical Science began work on synthesizing a fluorescent dye, fluorescein isocyanate, more than ten years ago. When Riggs and others succeeded in synthesizing the dye and it had become readily available to us, we directed our efforts to eliminating nonspecific fluorescence—the most troublesome problem encountered in using the fluorescent antibody technique. The problem, both in terms of antigen and antibody, could be approached only on the basis of the extensive knowledge accumulated over the years by the members of the Institute and through their multi-disciplinary cooperation. A number of important improvements in the optical system were also achieved by the physicists of our staff. The total number of contributors to these studies was well over 180. The improved technique which resulted from these investigations can now be used to achieve fluorescent antibody staining with a minimum of nonspecific fluorescence and a maximum of clarity and excellence.

The details of our method were presented at a meeting of the Training Course of the FA Technique, organized by WHO in Copenhagen in 1965, and have subsequently been discussed with many investigators here and abroad. We have at present determined to publish them in the hope that they will be of value to other workers and that they will stimulate further research on methodology.

The present book is divided into two sections: the first includes theories and the general method, the second describes specific applications. The first section was written by the core of our study group, namely the editor, Assoc. Prof. Kawamura and Prof. Matsuhashi, Drs. Kawashima (immunology) and Nakamura (immunochemistry), Prof. Kusano, Assoc. Prof. Aoyama and Dr. Hayashi (pathology), and Mr. Wada (optics). The second section was also written by members of our group, all leading authorities in their fields in Japan, and is

based on actual experiments and methods which we devised. We have used as many color photographs as possible to illustrate particular points in these chapters.

Some sections of the book were originally written in Japanese and were translated by Assoc. Prof. Watanabe with the cooperation of Dr. Ebisawa. The entire manuscript has been revised by Mrs. Annik L. Chamberlain, the second section by Prof. Ishikawa, and part of the first by Dr. Colin. Their dedicated labor is greatly appreciated. Much valuable advice and a number of useful suggestions were obtained on the general plan and various aspects of this book from Profs. Yamamoto and Tsunematsu; Mr. Noda kindly provided assistance with the photographic techniques. I would also like to express my cordial thanks to Mr. Shigeo Minowa for his help in furthering the publication of this book.

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Editor

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PART I

CHAPTER I

INTRODUCTION

For a long time, it was a far reaching dream for many workers engaging in biomedical research to visualize the distribution and localization of antigen in tissue and cells directly under the microscope. Although the presence of antigenic material in tissue can be deduced, with more or less certainty, by histochemical or physical methods depending upon the nature of the antigen, it was indispensable to develop a new method which could trace antigen by the specific combination of the corresponding labeled antibody, in order to identify the specific antigen and to learn about its distribution *in situ*.

The dream came true with the development of techniques for using several kinds of labeled antibodies, such as fluorescent antibody and antibody labeled with ^{131}I or with ferritin. Although the latter two methods have certain advantages, autoradiography is necessary with ^{131}I-antibody and the ferritin-labeled antibody along with electron microscopy. For general use, therefore, the fluorescent antibody technique has the advantage of being a relatively simple and rapid procedure.

Within a very short period after its discovery by Coons and his co-workers, the fluorescent antibody (FA) technique was being applied to a variety of biomedical problems. The technique is based on the successful conjugation of antibody with a dye of high quantum yield without denaturation. In the original method a new dye, fluorescein, with an intense greenish yellow fluorescence, was used instead of azo-dyes and conjugated with antibody through carbamide bonding in place of diazo-bonding in order to minimize the denaturation of antibody. Fluorescein isocyanate, however, is so labile that its synthesis is difficult. Hence, attention was focused on the synthesis of some other dye which would be less problematical.

Meanwhile, Riggs and others succeeded in devising a method for synthesizing fluorescein isothiocyanate which could largely fulfill the various demands of workers; subsequently its crystallization was achieved in the Baltimore Biological Laboratory. Popular use of the FA technique started only after the dye became available commercially. A simple and efficient method of conjugating the dye without denaturation and of purifying the conjugate was established by investigators following advances in immunochemistry. Improved methods of pretreating or fixing tissue sections also brought about further refinements in the technique.

These manifold improvements resulted in the successful elimination of nonspecific fluorescence, the most serious flaw in the technique. Together with

improvements in the optical system, these developments have largely nullified the requirement for special skills in using the FA technique and have made it suitable for general use in the laboratory.

The first part of this book gives the theory and the general characteristics and critical points of the FA technique. The preparation of materials, staining methods, and microscopic observation of the stained preparations are all described in detail. All of these methods are the product of our own investigations, and we have tried to organize them so as to provide a clear and useful guide for the actual laboratory use of this technique. In the second part, some of the results which we obtained by the application of our methods to specific tissues are illustrated with photographs, many of them in color. We hope that these will provide additional information and also evidence that the FA technique can be carried out successfully by anyone with a minimum of training in cytology.

CHAPTER II

SYNOPSIS OF THE FLUORESCENT ANTIBODY TECHNIQUE

1. Historical Review

Classical histochemistry aims at visualizing and differentiating nucleic acids, enzymes, fats and microorganisms. Immunohistochemistry is an advanced technique of histochemistry which uses antigen-antibody systems with high specificity. Antibodies used as specific staining agents for antigen in tissue or cells are not, however, visible unless tagged with a tracer such as dye or isotope. This technique was attempted by Reiner (1930)[25] and Heidelberger et al. (1933)[15] more than 35 years ago with azo-dyes, but did not achieve general use because of its low sensitivity.

Hopkins et al. (1933),[17] Fieser et al. (1939)[11] and Creech et al. (1941)[10] tried using fluorescent substances as tracers, but they were too deleterious to the antibody itself to be acceptable. In 1941–42, Coons et al.[6,7] succeeded for the first time in tracing pneumococcus soluble polysaccharide antigen in tissue sections of mice infected with pneumococcus, using a fluorescein labeled antibody. In 1950 they developed a new fluorescein isocyanate to label the antibody globulin, and thus paved the way for the modern development of the fluorescent antibody technique.[8] Later refinements were contributed by Riggs et al. (1958)[26] in the synthesis of fluorescein isothiocyanate (FITC), which can easily be conjugated to globulin (Marshall et al., 1958),[19] and by Goldstein et al. (1960)[13] in the use of gel filtration and column chromatography for the purification of labeled antibody.

Other methods used in immunohistochemistry, such as autoradiography and the ferritin antibody technique,[29,30] are also now available. The former is more sensitive than the fluorescent antibody technique, but the specimen must be reacted with sensitive emulsion for a few days in order to observe distribution of radioactive grains and the location of antigen within the tissue or cells is difficult. The latter also has numerous technical disadvantages, especially non-specific binding, but it is useful for ultrastructure studies and localization of antigen. In many cases in medical and biological research, combined use of these methods can be most rewarding.

2. Principle

The fluorescent antibody technique combines histochemical and immu-

nological methods to pinpoint specific antigen-antibody complexes present in tissue sections or cellular smears with the aid of fluorochrome substances conjugated to the antibody. Fluorochromes and ultraviolet light sources are the mainstay of the techniques. A fluorochrome is a substance which emits light of longer wavelength than the exciting radiation. This is called fluorescence when the substance emits light only while it is being excited, and phosphorescence when the light continues to be emitted even after the energy source has been cut off. Fluorescent substances can be tagged or conjugated to the antibody globulin without interfering with its immunological specificity and its ability to combine with antigen.

When tissues or cells containing a particular antigen, e.g. influenza A2 virus, are stained with its specific fluorescein-conjugated antibody and examined under a fluorescence microscope, only cells containing or infected with influenza A2 virus fluoresce. (This procedure is the direct method; see Fig. 1.) Cells infected with influenza B virus will not fluoresce at all if the fluorescent antibody has been carefully checked for specificity and is appropriately diluted. The fluorescent antibody technique is applicable to any antigenic substance, either within the cells or outside them——protozoa, bacteria, rickettsia, viruses, tissue antigens, hormones and enzymes.

Cells containing a particular antibody can also be stained with its specific fluorescein-labeled antigen; this procedure is called the fluorescent antigen technique. In practice, however, the structure and physicochemical properties of antigens are so varied that the labeling conditions must be examined in every case. If serum protein is the antigen, the same method of conjugation as that used in the fluorescent antibody technique (antibody staining method) can be applied, although technically this is known as the fluorescent antigen technique.

Another method used in the fluorescent antibody technique is the indirect method (Fig. 1). First, an unknown antibody is reacted with a known antigen (serological diagnosis) or an unlabeled antibody is reacted with a known or unknown antigen; these antibodies are called primary antibodies. In the second step, the serum globulin of the antigen-antibody complex formed is stained with specific antiglobulin conjugate (secondary antibody). If specific fluorescence is detected, we have indirect evidence of the presence of the specific antigen-antibody complex formed in the first step (application of the Coombs test).

There is still another method, the complement method, in which the antigen-antibody-complement complex is stained with specific anti-complement conjugate.

3. Characteristics of the Fluorescent Antibody Technique

A. Specificity

The antibody globulin with which the fluorochrome substance is conjugated is a protein which has a highly specific reactivity for antigen. Fluorescent antibody staining with this globulin is, therefore, highly specific and affects only the

antigen (bacterial, viral, tissue or cellular) to which the antibody has been prepared. Hence it is possible to localize virus antigen within the cells, or to confirm the relation between the inclusion body and virus particles.

B. Rapidity

The staining procedure and microscopic examination can be completed in one to two hours. Japanese encephalitis virus antigen from human brains, rabies virus antigen from dog brains, or influenza virus antigen in nasal smears can be identified in one to two hours after the specimen is sent to the laboratory. This is much more rapid than the isolation of viruses or serological tests, which often take more than one or two weeks.

C. Sensitivity

The sensitivity of the direct fluorescent antibody technique is very similar to that of the complement fixation test, but differs from the neutralization or hemagglutination tests. The precipitin reaction B method also corresponds to that of the antibody dilution technique but not to that of the antigen dilution technique. It is impossible to determine the exact sensitivity of the method because the strength of the specific fluorescence cannot be measured quantitatively. With this technique the labeled antibody is used in excess and only two units of primary antibody can detect the antigen in sufficient amounts. Hence, the sensitivity of the technique depends on the amount of antigen. Coons (1956)[9] stated that the concentration of pneumococcal capsular polysaccharide must be at least 0.8×10^{-4} $\mu g/mm^2$ to be detected by the fluorescent antibody technique. Pressman et al. (1958)[24] suggested a similar figure of 1.4×10^{-4} $\mu g/mm^2$ for the antigen concentration in a tissue segment 5 microns thick.

The sensitivities of the indirect and complement method of the fluorescent antibody technique are about 5–10 times higher than that of the direct method.

4. Prerequisites

A. Antigen

The material must be prepared so that the antigen loses little or none of its reactivity and is made accessible to the antibody. The pretreatment or fixation of the material is intended to keep the antigenic material fixed firmly on the slide as well as to remove fats and other substances which interfere with the antigen-antibody reaction. Sometimes, however, fixation may enhance auto- and nonspecific fluorescence, or untreated material may be better for observation. Preparation and observation of appropriate control materials are indispensable in these cases.

The concentration of antigen in the material must be high enough to be detected by the technique, or else the most carefully prepared material will still be unsatisfactory for observation.

B. Antibody

A highly potent and specific fluorescent antibody with very weak negative charge is needed. The starting antiserum must have a high titer and should contain little or no antibody for the tissues which are to be examined. If the antiserum contains antibody for normal tissues, this must be absorbed with a preparation of the particular tissue or acetone-dried powder of other organs (see p. 50). Adequate dilution of the antibody will obviate this procedure when the antibody titer of the serum is very high. The immunizing antigen should also contain little or no normal tissue substances, and the animal should not be infected with the particular agent or any related agent.

Antibody globulin prepared from this antiserum must keep its antibody titer and be free from degenerate albumin and α-globulin, and the negative charge of the final fluorescent antibody must be as weak as possible. The negative charge of the protein molecule increases as more fluorochrome substance is conjugated with it, resulting in nonspecific adsorption to the normal tissue or increased nonspecific fluorescence. A proper ratio of fluorochrome substance to protein molecule (F/P molar ratio) is approximately 1 to 2.

Labeled and unlabeled globulins of the same antibody titer differ in that the former are more weakly charged. This is readily shown in electrophoresis by the greater mobility towards the anode of the labeled globulin and is utilized in DEAE cellulose chromatographic separation of two substances. Unlabeled globulin, on the other hand, reacts with antigen faster than labeled globulin and this characteristic is used in the one-step inhibition test.

C. Fluorochrome

Fluorochrome substances used in this technique should conjugate easily and combine firmly with antibody globulin, should not interfere with antibody reactivity on conjugation, should be stable, have a good quantum yield (fluorescence efficiency), and have as much difference as possible between the maximal absorption and maximal emission wavelengths.

D. Conditions of Reaction

Dilution of the fluorescent antibody, time and temperature of staining, pH of buffered saline and washing must be checked carefully. The staining titer must be determined beforehand with known tissue preparations containing a sufficient amount of antigen, and the working dilution should contain 2 to 4 staining untis.

E. Control Specimens

The following controls are required to ensure specificity of the staining reactions. Antigens: untreated or uninfected tissue, fixed unstained tissue, and tissue containing heterologous antigen. None of these controls should show fluorescence on microscopy. Antibody: one-step or two-step inhibition tests, staining with heterologous antibody or normal globulins labeled with fluoro-