1973

Year Book OF DERMATOLOGY

MALKINSON
PEARSON

THE YEAR BOOK *of* DERMATOLOGY 1973

EDITED BY

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IMMUNOFLUORESCENCE IN DERMATOLOGY*†

by

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Immunofluorescence as a Technic

The time has arrived for an assessment of the impact that immunofluorescence has played on the science of dermatology. Its use has become so frequent that the complete work-up of certain groups of patients must include the fluorescent antibody technic. In fact, it has been stated, and written, that certain diagnoses rest entirely on the presence or absence of a specific antibody either circulating in the serum or fixed in vivo to the specific tissue. This review is intended to consider two facets: (1) the use of immunofluorescence as a diagnostic tool, and (2) how, or if, immunofluorescence has helped us to define the pathomechanism of every disease under consideration.

Before examining these points it seems appropriate to consider, in very general terms, some aspects of immunofluorescence from a history of its origin to methods of conjugate preparation and staining.

HISTORY

It was in 1941 that Coons, Creech and Jones published the first study utilizing the fluorescent antibody technic as a tracer to localize unlabeled antigen.[1] The principle that proteins may be labeled with chemically bound fluorescent markers without altering the functional activity of the protein is straightforward. If that protein is an immunoglobulin that is also an antibody, its use on tissue should localize antigen, if it is present. One great advantage is the exquisite sensitivity of the system, affording greater than a thousandfold increase over systems using nonfluorescent markers. Coons and co-workers attached B-anthracene to antipneumococcus type III serum to give a blue fluorescence in the presence of type III, but not type II, pneumococci. This blue fluorescence was satisfactory for bacterial smears but not for tissue sections because of the blue autofluorescence of elastic fibers in tissue. For this reason, Coons *et al.*[2] turned to the green fluorescent dye fluorescein to detect pneumococcic antigen in tissues of infected mice.

The war years interfered with further development of the technic, but in 1950 a paper by Coons and Kaplan appeared that described such refinements as preparation of frozen sections, filter and illumination

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†Many of the experiments described herein were performed while the author was associated with the Department of Dermatology, Mayo Clinic, Rochester, Minnesota.

systems for fluorescent microscopy, removal of nonspecific staining and improvements in the coupling of fluorescein isocyanate to protein.[3]

Widespread use of this clearly highly significant technic was hampered because fluorescein isocyanate was not available commercially and its preparation required use of the toxic gas phosgene. In addition, the prepared product was unstable and had to be prepared fresh each time an antibody was to be labeled. Fluorescent microscopic equipment also had to be designed by each investigator since it too was not available commercially.

Marshall, able to overcome these difficulties, described the localization of adrenocorticotropic hormone in hog pituitary and was thus the first to demonstrate that fluorescent antiserums had sufficient specificity to delineate clear differences among many native antigens in normal tissues.[4]

The so-called indirect technic was first described by Weller and Coons in 1954, when tissue culture cells containing virus were exposed to human antiserums against the virus and then to a fluorescein-tagged antiserum prepared by immunizing rabbits with human globulin.[5] This procedure would come to play an extremely important role in the widespread application of the technic since it would no longer be necessary to label each specific antibody separately. By 1957 this potential was beginning to be realized when reports appeared demonstrating presence of a serum antibody for herpes simplex[6] and Treponema pallidum[7] and the causative organism of primary atypical pneumonia.[8] About the same time, the indirect technic was employed to detect complement-binding antibodies by simply adding an additional step: serum-containing complement was added to the tissue section after serum-containing antibody and stained with a fluorescein-conjugated anticomplement.[9]

By 1958, methods were reported that provided stable fluorescein powder that could be stored for months and used at will for labeling proteins. Shortly after this, labeled antiserums as well as the labeling dyes became commercially available and the fluorescent antibody technics came into common use.

Fluorescence and Instrumentation

Fluorescence, simply described, is the absorption of light of one color and the emission of light of another color. The emitted light is always of lesser energy and, therefore, longer wavelength than that absorbed. An alkaline solution of fluorescein is golden yellow when observed with the light behind it and green when observed from the same side or at right angles to the light. When irradiated, photons of wavelengths longer than about 530 nm. (toward the red end of the spectrum) pass through the fluorescein solution without being absorbed. Blue-green light from 400-530 nm. (particularly 490 nm.) will be strongly absorbed. About 15% of the absorbed photons will have their energy dissipated in the form of heat. The other 85% will raise the absorbing molecules to a higher

energy level, which will immediately return to ground state energy level by emission of photons of yellow-green light (maximum at 517 nm.).

All of the leading manufacturers of microscopes have fluorescent systems available and most can offer knowledgeable advice regarding the light sources, the filter and the microscope for the needs of a particular laboratory or investigator. The light source for fluorescence microscopy must be rich in wavelengths in the ultraviolet and blue range. The mercury arc ("hot quartz" of the dermatologist's office) has strong mercury lines at 365, 405 and 435 nm., making it ideally suited. Probably the most widely used bulb is the HBO 200 manufactured by Osram in Germany. The bulbs are moderately expensive and have an average life of about 200 hours. Frequent starts tend to shorten bulb life and intensity gradually falls off with time. If the electrodes become pitted, the arc wanders and the lamp becomes unsuitable for use.

Proper filters are extremely important and a full discussion is beyond the scope of the present review. The reader is referred to the very excellent text on fluorescent antibody methods by Morris Goldman.[10] The object is to use a primary filter that will pass wavelengths that are: (1) produced by the mercury arc lamp, (2) absorbed by fluorescein and (3) completely absorbed by the secondary filter. Because of the physics of the various components, it is not reasonable or even possible to satisfy fully all of these criteria at maximum efficiency, but very satisfactory compromises may be made. I use a BG-12 (ultraviolet-blue) primary filter, 2-3 mm. thick. This light emits more blue than the UG-1 and gives a more intensive fluorescent image. This also increases nonspecific fluorescence, but does enable better visualization of weak specific fluorescence. Secondary filters are placed between the slide and the eyepiece and have a sharp cut-off at 500 nm.; light above this wavelength is transmitted with high efficiency. Their purpose is to absorb all of the exciting light and transmit only that light emitted by fluorescein. It is not necessary to use a deep yellow or orange filter as these greatly decrease the intensity of the fluorescent image and alter the color values so that the resulting fluorescence is yellow rather than green. For use with the BG-12 primary filter, a 2-mm. thick glass C-3384 or a gelatin W-12 proves quite satisfactory.

Either a bright- or darkfield condenser may be used, and, although there are disadvantages to both, most workers prefer the latter because specimens may be examined against a dark background without the autofluorescence of lenses which is noted when a brightfield condenser is employed. Alignment, focusing and need for matching the limiting aperture of the condenser to the numerical aperture of the objective are all critical but can be resolved by purchasing a complete system from a competent outlet.

A new illumination system known as epi-illumination or Ploem illumination (for the man who developed it) appears to offer great advantages when properly installed and operated.[11, 12] The exciting light comes from above the specimen, through the objective, and no condenser is needed. Because of the high intensity and greater efficiency,

narrower-band pass filters may be used with consequent increase in the intensity of specific fluorescence and a decrease in the nonspecific fluorescence.

Antiserum and Conjugate Preparation

Human antiserums used for dermatologic diagnosis or investigation are usually prepared in goats or rabbits by immunizing with a relatively pure immunoglobulin (monovalent) obtained from patients with monospecific myeloma. Thus, antiserums can be prepared to IgG, IgA or IgM, but the antiserum itself is an IgG antibody. Thus, if an antihuman IgM is prepared in a goat, it is designated as goat antihuman IgM. Purification of the antibody obtained from the goat is most frequently performed by precipitation of the globulin with ammonium sulfate, or cold ethanol or chromatography with diethylaminoethyl cellulose. If the immunoglobulin used to immunize the goat was pure, the resulting antibody will be pure and precipitation will yield many globulins but only one antibody. Thus, if the original human IgM is contaminated by a small amount of human IgG, the goat will make antibodies to both molecules. Purification can be achieved by precipitating the contaminating anti-IgG with pure IgG. Once purified, the antiserum can be labeled with fluorescein isothiocyanate, a preparation that is stable in the frozen or lyophilized state for many years.

Although there are a number of reliable manufacturers of both plain and fluorescein-conjugated antiserums, it is still recommended that each batch be checked for purity; that is, it should give a single precipitation line when run in immunoelectrophoresis against whole human serum. Commercial conjugates may contain excess, unbound fluorescein that will greatly increase the background staining of tissue sections, unless removed by exhaustive dialysis or gel infiltration with Sephadex.

Once prepared and purified, the concentration of antibody in the conjugate, as well as the fluorescein-protein ratio, must be determined. Fortunately, this information is now supplied by all reliable manufacturers. In addition, the optimal dilution at which the conjugate is to be used must be ascertained by performing a "chessboard" titration, which must be carried out by the individual investigator. This is necessary in order to use the conjugate at the dilution that gives minimal background or nonspecific fluorescence but still yields positive specific fluorescence.

Currently, one of the greatest needs in immunofluorescence performed in laboratories throughout the world is for adequate standardization so that results from one laboratory may be interpreted in another. A great step forward was achieved at the International Workshop of Standardization in Immunofluorescence held in Florence in 1967 and a round-table conference held in London in 1968.[13] Proper immunofluorescence cannot be carried out in a casual manner by someone with limited experience and lacking sufficient appreciation of the many pitfalls. At best, all publications should give the source of conjugate, tests for purity, antibody concentration and fluorescein-protein ratios. Details of

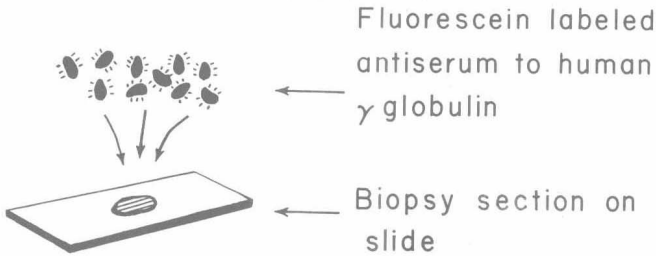
methods for these and other determinations are given in the book by Goldman[10] and that by Beutner *et al.*[14] In addition, an excellent detailed manual, *Defined Immunofluorescence in Clinical Dermatology,* edited by E. H. Beutner and R. J. Nisengard and prepared through the National Program for Dermatology, is now available for a small fee from the senior editor at the Department of Microbiology, State University of New York at Buffalo.

Tissue Preparation and Staining

Most immunofluorescence is performed on frozen sections cut at about 4 μ on a cryostat, although a method for demonstrating antinuclear antibody from specially prepared paraffin-fixed tissue has been described.[15] For direct immunofluorescence, sections of the tissue are placed on a glass slide, air-dried or fixed in acetone, and covered with the desired fluorescein-tagged anti-immunoglobulin or other conjugate (Fig. 1). Positive staining means only as much as any other histochemical technic and has no immunopathogenetic implication. That is, staining of the basement membrane zone of human skin with antihuman IgG simply means that IgG was there in vivo, just as glycogen or acid phosphatase might be present in the epidermis, and does not make any implication about how it got there or what it might mean. There has been some confusion as to what is meant by the term "direct technic"; in most publications it means labeled antibody staining a specific antigen; in dermatology it has come to mean labeled antibody staining an immunoglobulin (that may be an antibody) fixed or bound at a specific site.

Indirect immunofluorescence, also called the antiglobulin method, is used to stain a serum antibody. Cryostat sections of tissue containing an appropriate antigen (also called substrate) are applied to microscope slides, covered with serum, incubated, washed and then covered by fluorescein-tagged antiserum (Fig. 2). The source of antigen can be any appropriate tissue, but, for various reasons, certain tissues come to be used more frequently. Thus, for detection of antinuclear antibody, rabbit liver or mouse kidney is used in many laboratories. For basement membrane or intercellular immunofluorescence, guinea pig esophagus was long the most popular substrate, in large part due simply to its ready availability. However, it has recently become apparent that there are occasional patients whose antibody demonstrates species specificity and

Fig. 1.—Schematic method for direct immunofluorescent staining (test for in vivo bound γ-globulin). (Courtesy of Arch. Dermat. 102:485, 1970; copyright 1970, American Medical Association.)



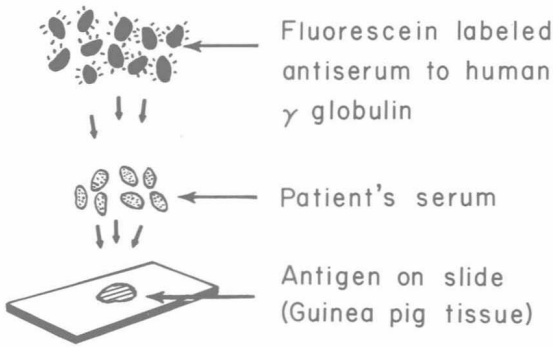


Fig. 2.—Schematic method for indirect immunofluorescent staining. Although stated here that guinea pig tissue is used as the antigen, other tissues are equally or more satisfactory (see text). (Courtesy of Arch. Dermat. 102:485, 1970; copyright 1970, American Medical Association.)

will react only with primate tissue, particularly monkey esophagus. Human skin has also been advocated[16] but lacks the advantage of an undulating basement membrane, and the epidermis is relatively thin. Katz *et al.*[16] have recommended cutting the sections horizontally to afford more intercellular spaces. It does that, but the examiner does not know the level of the cut and it is recognized that there is frequently false positive intercellular staining in the upper dermis, so that slides are read ordinarily by examining the intercellular space just above the basement membrane. A tissue source of antigen, the possibilities of which have not really been explored, is the human seborrheic keratosis.

The conjugate used is a fluorescein-tagged antibody against a protein of the particular species being studied, and the procedure used for its preparation has been briefly explained previously.

Serum is diluted serially to determine the highest dilution giving positive staining. Since the molecular combining ratio of antibody to antigen may be as high as 10 or more, but is always greater than 1, the intermediate layer of unlabeled antibody (in the serum) multiplies the number of sites to which the fluorescent conjugate can be attached. Thus, the indirect method may be several times more sensitive than the direct method.

Controls, for the indirect method, are more critical and should include: (1) saline in place of serum, (2) normal serum used at the same dilution as the unknowns and (3) a known positive antibody for each of those being examined. Some normal serums either possess antibodies in very low titer or will give false positive reactions at low titer. Thus, all laboratories must establish minimal dilutions above which any positive serum is significant. I screen all serums at a dilution of 1:20. Any antibody found at that dilution is probably significant and is titered to an endpoint.

Dermatologic Diseases and Immunofluorescence

It is unquestioned that immunofluorescent technics have played a highly significant role in certain dermatologic diseases. This role, initial-

ly, was an aid to diagnosis only, but, through the research efforts of a number of investigators, has proved to be very exciting in helping to define the pathogenesis of these diseases. Each disease will be discussed under the site of localization of the antibody.

Antinuclear Fluorescence

Diagnostic considerations.—Antinuclear immunofluorescence has come into widespread use for detecting serum antibodies to constituents of cell nuclei. Although the lupus erythematosus (LE) cell phenomenon is still widely used and the LE factor was shown by Haserick *et al.*[17] to be a 7S γ-globulin, the test has disadvantages: it is relatively insensitive, it cannot be quantitated readily, it detects antibodies to only one nuclear constituent and the physicochemical conditions of the test must be rigidly controlled. For these reasons, there are occasional false negative tests in patients with systemic LE (SLE), whereas the immunofluorescent technic, although lacking specificity, is highly sensitive and should be positive in virtually all patients with SLE. It is likely that the LE test gradually will be phased out of most clinical laboratories.

Since the immunofluorescent technic affords cytologic localization of the site of action of an antibody with a tissue antigen, it obviously follows that a serum giving immunologically specific nuclear staining must contain antinuclear antibodies. From 1950 to 1961, this immunofluorescent technic was used widely to detect antibodies in the serums of patients with connective tissue disorders. By and large it was incorrectly assumed that all serums were reacting with the same antigen, but these studies did demonstrate that the antinuclear antibodies were true autoantibodies[18] and that nucleohistone was an important nuclear antigen.[19]

In 1961, Beck[20] was the first to describe three distinct patterns. He implied that these might result from antibodies specific for distinct nuclear antigens. These patterns are (1) homogeneous: nucleus stains equally throughout without any increase or decrease at the nuclear membrane; (2) speckled: numerous minute points of fluorescence are seen throughout the nuclear substance; and (3) nucleolar: bright staining of each nucleolus, but not of the remainder of the nucleus.

These patterns were soon reported by other investigators, and Casals and co-workers[21] described a fourth or shaggy (also called peripheral) pattern in which the outlines of intact nuclei were more intensely stained than their centers. A number of other patterns have been described, including the "rim and fibrillar" consisting of bright staining of the nuclear margin with dull intranuclear fibrillar staining, and the thready pattern described by Burnham *et al.*[22] These authors recommend the use of human tumor imprints as substrate,[23] rather than the rabbit liver or mouse kidney used in most laboratories, because they can, in the authors' opinion, more clearly define a large variety of patterns, some of which have diagnostic specificity. In practice, the mixed pattern is the most common, containing a combination of two or more individual patterns.

There is presently considerable evidence that these different patterns are produced by antiserums of different specificities; the homogeneous pattern is due to antibodies to particulate DNA-histone nucleoprotein,[24] the speckled pattern is produced by antibodies to a saline-soluble nuclear protein[24] and the nucleolar pattern is due to a constituent of the nucleoli.[25] The shaggy or peripheral pattern correlates with antibodies to DNA.[26]

What is the significance of a positive antinuclear antibody and is there any diagnostic significance from any specific pattern? The immunofluorescent antinuclear antibody test, when properly performed, is highly sensitive but of low specificity. Thus it becomes positive in a number of diseases, particularly those in the connective tissue-vascular system group. In addition, it may be positive in low titer in normal individuals. For this reason, most laboratories have a dilution at which they screen serums and at which a positive test is significant, such as 1:32. Because of the high sensitivity of the procedure, a negative test virtually excludes a diagnosis of SLE. However, there are patients whose antinuclear antibodies will not react with the animal substrate ordinarily used but will react with their own leukocytes.[27]

Some immunofluorescent patterns seem characteristic of certain diseases but are not always useful in all patients. The homogeneous or mixed pattern is by far the most common and is seen in rheumatoid arthritis and in inactive as well as active SLE. The speckled pattern is characteristic of SLE, progressive systemic sclerosis[28] and mixed connective tissue disease,[29] whereas the nucleolar pattern is seen most frequently in patients with systemic sclerosis.[30] Since the shaggy or peripheral pattern is indicative of antibodies to DNA and since these specific antibodies have been demonstrated to be elevated almost exclusively only in SLE,[31, 32] this particular pattern might prove quite useful. However, in practice, one pattern might be obscured by another, and any individual pattern is not diagnostic of a specific disease, so that, as with most laboratory tests, the immunofluorescent pattern is only one of the ancillary factors in arriving at a diagnosis. A possible exception to the specificity of DNA antibody for SLE is the recent report by Mandel *et al.*[33] of elevated DNA antibody in patients with discoid LE (DLE). Most of these patients had a negative antinuclear antibody even with undiluted serum, but 2 of the 22 in the series later acquired full-blown SLE and a positive antinuclear antibody. Thus, the presence of elevated DNA antibody in patients with DLE may serve as a prognosticator and suggests that these are the patients in whom SLE is more likely to develop.

PATHOGENETIC IMPLICATIONS.—A full discussion of immune complex disease is beyond the intent of this review. Briefly, at least a portion of the development and progression of SLE is felt to result from the presence of complexes of an antigen and its corresponding antibody in the circulation.[34] When either the antigen or the antibody are in excess concentration relative to the other, the complexes remain soluble and are deposited on the glomerular basement membrane (and probably other biologic "filters" such as the basement membrane area of the

skin). When deposited, the complexes may fix complement, which would then be activated to elaborate chemotactic factors, resulting in polymorphonuclear leukocytes being deposited at the same site and releasing lysosomal hydrolases which destroy the surrounding tissue.

The reason that patients with SLE acquire antibodies to a variety of antigens is unknown, but there is likely a genetic influence since multiple members of the same family may have either overt disease or serologic abnormalities. As mentioned previously, it has recently been demonstrated that antibodies to DNA correlate better with, and seem to be more specific for, SLE than other antibodies.[31-34] The source of this antigen, or why it should be antigenic in these individuals, is unknown. However, Tan and Stoughton[35] have performed a series of interesting experiments in which they demonstrated that animals immunized with ultraviolet-irradiated DNA (UV-DNA) would make anti-UV-DNA. This antibody seemed not to cross-react with native DNA, which is only poorly antigenic by itself. A fluorescein-tagged anti-UV-DNA antibody was then used to detect UV-DNA and thymine-associated photo products in in vivo irradiated animal skin.[36] Recently, circulating antinuclear antibodies have been produced in Swiss mice after UV irradiation, particularly when kept in the dark after irradiation to prevent activation of photo-repair mechanisms.[37] A further step was taken when Natali and Tan[38] produced renal disease in rabbits immunized with UV-DNA by injecting UV-DNA intravenously. The next step, that of producing renal disease in UV-DNA-immunized animals by irradiation in vivo, has not been reported.

It seems possible, however, that UV irradiation of normal skin might release UV-DNA into the circulation, where, because of its high antigenic potential, even normal individuals might produce antibodies to it. To test this hypothesis, M. Landry and I (unpublished) have preliminary studies to show that individuals receiving large amounts of high-pressure mercury irradiation daily for treatment of psoriasis will produce detectable UV-DNA in their circulation, and, after 1-2 weeks, will also have detectable anti-UV-DNA. If a serum sample is tested immediately after irradiation, no free antibody can be detected, presumably because it is all bound to recently produced UV-DNA. A few hours after irradiation, however, anti-UV-DNA is again detectable. One presumes, therefore, that normal individuals can handle these antigens and antibodies satisfactorily, possibly by forming insoluble complexes that are readily phagocytized before they can be deposited on the renal glomeruli.

Basement Membrane Fluorescence

Lupus Erythematosus

Diagnostic considerations.—Lupus erythematosus, both discoid and systemic, is characterized by the presence of immunoglobulins and often complement attached to the basement membrane area of the skin[39] (Fig. 3). The pattern is granular or "lumpy-bumpy." The diagnostic importance of this finding is unquestioned since it is present in as high as 90% of lesions of both DLE and SLE.[40, 41] In addition, it is not