Developments in immunofluorescence: 
the need for standardization

G. D. JOHNSON, E. H. BEUTNER, and E. J. HOLBOROW

From the Medical Research Council Rheumatism Research Unit, Canadian Red Cross Memorial Hospital 
Taplow, Nr. Maidenhead, Berkshire, England

SYNOPTIS Difficulties experienced by the newcomer to the fluorescent antibody staining method largely arise from two causes: (1) the many modifications in the procedure which have been suggested over the last decade; (2) the variability of the basic reagent, fluorescent conjugate, in terms of potency and specificity. The ensuing problem of non-reproducibility is clearly an important factor for clinical laboratories now faced with increasing demands for routine tests involving immunofluorescence. In this paper a simple approach to standardization is described which, if adopted, would lead to a more efficient use of the method.

Since its description by Coons and Kaplan in 1950, the fluorescent antibody method has found wide application not only as a research tool but also in routine clinical investigation. During this time the procedure has undergone considerable modifications, largely based on developments resulting from analytical studies of various aspects of the method, but since there is little uniformity of method it is not surprising that reproducibility of findings between different laboratories is not always achieved. The increasing demand on routine clinical laboratories to carry out immunofluorescent screening tests (for autoantibodies, for example), and the commercial interest in supplying equipment and reagents to meet this demand, make it now more than ever necessary to consider the minimum requirements for standardization in order to achieve comparable and reproducible results.

The following observations, based on over 10 years’ experience of testing sera for tissue-reactive antibodies, embody a simple approach to standardization of the indirect immunofluorescent method. Similar principles should apply to most other applications.

TISSUE

A wide variety of tissues may be used as substrate in immunofluorescent staining, but suitable preparations of most of them may be obtained by one of two methods in common use. Tissue antigens that are not affected by alcohol may be successfully stained in paraffin sections of tissues fixed in cold ethanol by the method of Sainte-Marie (1962). We have found this method particularly suitable for demonstrating intracellular antibody, such as rheumatoid factor in the cells of the synovial membrane and lymph nodes. It is not, however, a suitable method for preparing sections to be used for the demonstration of, for example, antibody to smooth muscle (Johnson, Holborow, and Glynn, 1965) or thyroid cytoplasmic microsomes (Holborow, Brown, Roitt, and Doniach, 1959) where the effect of alcohol on the section is to abolish the reactivity of the antigen concerned. For antigens of this type, the alternative method of preparing cryostat sections of snap-frozen tissue must be used. A third possibility, useful in some cases, is fixation of sections, smears, or cell preparations on slides by treatment with acetone or alcohol in various concentrations.

In choosing between alternative substrates it is essential to consider their relative sensitivities in the test system. This is especially important in testing for antinuclear factors. Feltkamp (1966) has shown that the incidence of antinuclear factor varies according to the nuclear substrate employed for its detection. This may reflect differences in tissue sensitivity; for example, only sera which give a high titre on sections of thyroid are positive on sections of stomach. Among other tissues used for antinuclear factor testing, we have found smears of buccal cells unreliable and difficult to interpret; and in our hands imprints of

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2Present address: State University of New York at Buffalo, Schools of Medicine and Dentistry, Buffalo, New York, U.S.A.
rat liver (which might otherwise have offered a convenient routine substitute for tissue sections) show weak nuclear staining with many sera which we would otherwise regard as negative. Fixed tissue is usually not satisfactory for routine antinuclear factor testing. Leucocytes do not contain the saline-extractable antigen responsible for speckled nuclear staining (Beck and Paterson, 1965) and this rules out the possibility of using blood smears for screening sera for antinuclear factor, since in some sera only speckled antinuclear factor is detectable.

CONTROL SERA

The importance of using standard control sera cannot be overemphasized. The inclusion of a single 'strong positive' serum is likely to indicate only gross deviations in the test procedure and does not adequately control the sensitivity of the test. Satisfactory monitoring is better achieved by including titrations of potent standard positive sera. It is also essential to include known negative sera in each batch of tests, for the principal difficulty in immunofluorescence is not in obtaining good positives, but in recognizing negatives with confidence. Indeed, convincing negative results with negative sera are essential in evaluating unfamiliar antigen-antibody systems.

FLUORESCENT CONJUGATES

ANTISERA We are convinced that the problem of non-specific staining attributable to the conjugate is largely overcome by using very potent antisera for the preparation of conjugates so that the latter may then be considerably diluted for use in staining. Such hyperimmune antisera contain large amounts of precipitating antibody, and a simple method of assessing their potency which we have already described (Beutner, Holborow, and Johnson, 1965) is based on precipitin titration against a solution of antigen by a gel-diffusion method. If such a test is carried out under standard conditions the end-point of precipitation may be expressed in terms of 'precipitating units'. Suitably potent antisera containing 16-32 units provide conjugates that give maximum specific staining efficiency when diluted as much as 1:80.

PREPARATION OF GLOBULIN FRACTION Before proceeding to conjugation it is usual to carry out a form of fractionation procedure on the antiserum. The choice of method lies between isolation of the globulin fraction by chromatography, and simple removal of the bulk of the albumin by a crude salting-out procedure. We prefer the latter for the following reasons:

1. Traces of labelled albumin do not produce non-specific staining when conjugates are properly diluted before use. 2. The fraction obtained by salting-out may be reconstituted to a protein level suitable for conjugating without the need for concentration. (3) If the salting-out procedure is carried out entirely in the cold, the antibody activity in the globulin fraction is regularly found to be comparable to that of the original antiserum.

However, it may occasionally be desirable to prepare conjugates from more refined globulin fractions obtained by chromatography according to Goldstein, Slizys, and Chase (1961).

FLUOROCHROME We have used conjugates of fluorescein almost exclusively, and the following observations are based on experience of fluorescein isothiocyanate. A great deal of analytical work has been done by various workers to determine the requirements for satisfactory labelling of proteins, and the findings underline the need for certification and standardization of histochemical dye products generally. It has been shown by McKinney, Spillane, and Pearce (1964a) that commercial preparations of fluorescein isothiocyanate vary in purity from only 33% to 100% (crystalline); and in addition the relative amounts of isomers I and II may vary as shown by Brighton (1966). Despite its deficiencies, however, we find that satisfactory conjugates are obtained using impure fluorescein isothiocyanate provided the optimum amount for labelling has been established. For example, with a preparation only about 40% pure, 0.05 mg. of dye per 1 mg. of protein provides conjugates functionally comparable with those obtained using a proportionately smaller amount of a purer product.

The conjugating procedure itself has also been much varied. The traditional method employs gentle mixing over a period of 18 hours in the cold. However, McKinney et al. (1964b) have shown that adequate labelling occurs in one hour at 25°C. without detriment to antibody activity. A procedure that, it is claimed, results in more uniform labelling has been described by Clark and Shepard (1963); the protein solution is contained in a dialysis bag and suspended in a large volume of a dilute solution of the dye. We have found all these methods satisfactory.

IMMUNOLOGICAL SPECIFICITY Potent antisera reactive with the common light chain determinants would be useful as a screening reagent, but there are difficulties in preparing these and in interpreting their reactions. In practice, conjugates of antisera known to react with all immunoglobulin classes through their heavy chain determinants are more widely used for screening. The antigenic complexity of the immuno-
globulins necessitates the use of conjugates specific for the individual heavy chains \(\gamma, \mu\), and \(\alpha\) for detailed characterization of antibodies demonstrated by fluorescent staining. A promising method of immunizing rabbits to obtain such specific antisera has been described by Goudie, Horne, and Wilkinson (1966) who emulsified with complete adjuvant the isolated precipitin arc obtained from agar immunoelectrophoresis and injected the emulsion directly into the popliteal lymph nodes.

Van Furth, Schuit, and Hjimans (1966) have shown that the specificity of conjugates prepared from antisera to human globulin fractions may be readily tested using bone marrow preparations from patients with paraproteinaemias as substrate. In such preparations there is a high proportion of myeloma cells whose cytoplasm contains large amounts of the immunoglobulin in question.

If marrow smears from these patients are not available, an alternative procedure is to use a model system employing a characterized antibody in the first stage of the staining procedure. For example, IgG antinuclear factor may be prepared from the serum of patients with systemic lupus erythematosus by column chromatography using D.E.A.E.-cellulose (Peterson and Sober, 1960). For IgM specificity, the antibody in the serum of patients with infectious mononucleosis that stains capillary endothelium in bovine tissue (Johnson and Holborow, 1963) is invariably a macroglobulin, and provides a useful check. In the case of IgA a satisfactory substitute for myeloma bone marrow is not so far known.

**NON-SPECIFIC STAINING** A basic problem with the conjugate is to overcome its propensity to give non-specific staining. Unreacted dye is usually removed either by simple dialysis or by the equivalent procedure of molecular sieving through Sephadex (Fothergill and Nairn, 1961). The empirical practice of absorption with homogenized tissue, as originally described by Coons and Kaplan (1950) for the removal of labelled serum components responsible for non-specific staining, is still often employed. Several workers have advocated fractionation of the conjugate by ion-exchange chromatography, and have defined limits for the ratio of dye to protein. There is no doubt that fractions of conjugate with a low fluorescein-protein ratio obtained according to the method described by Goldstein et al. (1961) give no non-specific staining and are of value when that is a special problem. We have confirmed that a correlation exists between non-specific staining and fluorescein-protein ratio (Beutner, Holborow, and Johnson, 1967), but in general have found that a satisfactory solution of the problem of non-specific staining is the use of immunologically-potent conjugate diluted to the point where non-specific staining becomes negligible yet specific reactivity remains.

In summary, standardization of conjugates with the object of improving comparability between different laboratories may be accomplished by attention to three parameters: (1) precipitating antibody content; (2) dilution employed for staining; and (3) fluorescein : protein ratio, which may be conveniently expressed as a ratio of the respective optical densities at 490 \(m_u\) (fluorescein) and 280 \(m_u\) (tyrosine) under defined conditions.

**STORAGE** The most satisfactory method of storing conjugate is by lyophilization. We have found that an economical scheme is to prepare a dilution suitable for use and lyophilize appropriate small aliquots which when reconstituted provide 1 ml. (say) of working solution.

**COMMERCIAL PRODUCTS** Commercial conjugates vary in their efficiency and should be used with caution. The tendency of manufacturers to dispense them in diluted form precludes definitive analysis by the user. In this connexion we welcome the move made by the Blood Group Reference Laboratory\(^1\) to undertake the preparation and issue of standardized antiglobulin conjugates.

**STAINING PROCEDURE**

**DILUTION OF PATIENTS' SERA** The indirect method is widely used for the demonstration of antibodies to tissue constituents. Although in some systems the reactions are not easily demonstrable when diluted serum is used, it is preferable whenever possible to screen sera at a dilution which minimizes the so-called 'non-specific staining' that is attributable to specific staining of non-specifically attached serum protein. For example, carrying out the test for antinuclear factor on sections of calf thyroid at a dilution of 1 : 10 sharply demarcates the sera of patients with active systemic lupus erythematosus from those of matched normal population controls, the incidence of positive tests in the patients being near 100\%, compared with less than 1\% in the controls.

**WASHING PROCEDURE** Some form of mechanical aid to increase the efficiency of the washing procedure in removing non-specifically attached serum or conjugate is desirable. With a magnetic stirring device, the time required for washing tissue sections after treatment with serum is only 10 minutes; after staining with conjugate, however, at least one hour should be allowed for adequate washing.

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\(^1\)Blood Group Reference Laboratory, Medical Research Council, Gatlib Road, off Ebury Bridge Road, London, S.W.1.
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MICROSCOPY

The light source conventionally used with fluorescein is the high-pressure mercury vapour lamp, which has a characteristic 'spiky' emission spectrum. Exciting filters that give satisfactory results include those which transmit mainly in the ultraviolet region, e.g., Schott and General UG1, and those whose transmission extends from ultraviolet to blue, e.g., Schott and General BG12. Many workers favour the latter which gives maximum intensity of specific fluorescence emission against a blue background of unstained tissue. Dark field condensers are the most useful, and the recently available Japanese Tomild condenser, which is suitable for use with both high- and low-power objectives, is a promising innovation.

An alternative light source to the mercury burner is the relatively cheap iodine-quartz lamp. This does not require an expensive starter unit, and has the advantage that maximum illumination is available immediately on switching on. The emission spectrum of these lamps is linear over the visible range, but they have not yet been employed extensively for immunofluorescence work because of lack of a satisfactory filter system. This difficulty has been resolved by Tomlinson (1967) who has found that the combination of two Wratten primary filters (32 and 38A) used with a yellow secondary filter gives good results. We have confirmed that with this filter combination specific fluorescence is bright green against a brownish-red background of unstained tissue that gives good contrast for photography.

CONCLUSIONS

In conclusion, it may be said that although many elaborate modifications of the original immunofluorescent method of Coons have been proposed, a simple routine procedure such as we describe here provides reliable and reproducible results, and is practicable for use in laboratories wishing to use immunofluorescence for screening tests. It is not suggested, however, that the adoption of a single technique is desirable: diversity of methods is most likely to yield new observations. The function of standardization is to provide a basis for comparison by reference to specific activity of significant parameters. In immunofluorescence these are: (1) the nature and state of substrate; (2) dilution of serum employed (in indirect staining); (3) specificity and potency of conjugate; and (4) behaviour of standard sera in the system.

REFERENCES