Immunology, 1970, **18**, 319.

BRIEF COMMUNICATION

Impurities in Conjugated Globulins for Immunofluorescence Techniques

W. D. BRIGHTON AND JANET E. LAMPARD

Division of Immunological Products Control, Medical Research Council, Hampstead, London, N.W.3

(*Received 27th June* 1969)

Summary. Conjugated albumin and free uncombined fluorochrome are two constituents of preparations of immunoglobulins conjugated to fluorochromes which produce non-specific staining. The maximum permissible levels of these two components have been determined using as substrate coverslip preparations of monkey kidney cells.

INTRODUCTION

Preparations of immunoglobulins conjugated to fluorescent dyes are now widely used for the detection of many antigen–antibody systems. The most usual technique employed in making these preparations is to fractionate an antiserum containing a high concentration of antibodies to the antigen which it is designed to detect by means of a procedure which precipitates the immunoglobulin fraction of the serum, leaving the albumin and many other serum proteins in solution. The immunoglobulin fraction is then redissolved and coupled by means of a reaction involving free NH_2 groups of the protein with the desired dye by means of either a carbamide, thiocarbamide or sulphonamide linkage. The excess of free dye is removed by dialysis or gel filtration. Detailed explanation of these procedures has been given by Nairn (1968).

Not all conjugates are equally efficient in use, and often artefacts which lead to misinterpretation of results are produced. Recent attempts (Brighton, 1966; Brighton, Taylor, Tomlinson and Wilkinson, 1967; Holborow, Brighton, Taylor and Sander, 1970) to define the desired characteristics of conjugates have shown that no precise data have been published which give the maximum permissible levels of two of the most common contaminants of these preparations. In the present work an assessment has been made of the non-specific staining ability of conjugated albumin and of free, uncombined fluorescein isothiocyanate (FITC) which are often present in conjugates (Brighton, 1966). The levels here reported have been determined using only one form of substrate, that of monkey kidney cell cultures on coverslips, because in these preparations the cellular organization of DNA and of RNA are typical of the many different substrates used for the demonstration by immunofluorescence of many viruses and also for the organ-section substrates used for the demonstration of antibodies in the autoimmune diseases. The maximum permissible levels may be higher for work which involves pure cultures of bacteria and other parasites, though where bacteria are present together with much extraneous material as in the detection of *Shigella sonnei* in faeces by the method of Taylor, Heimer and Lea (1965) this in unlikely to be so.

MATERIALS AND METHODS

Substrate

Primary monkey kidney cultures on coverslips were incubated for 4 days at 35° (after trypsinizing) and were then fixed in acetone at −60° by the method of Sander (1969).

Conjugated albumin

A solution of bovine serum albumin (BSA) Fraction V (Armour Pharmaceutical Co. Ltd, Eastbourne, England) at a concentration of 11 mg/ml was conjugated by the method of Brighton (1966) so that the ratio of optical densities (495/280 nm = 1). Absence of free FITC in this preparation was shown by thin-layer chromatography on Sephadex G-200 (Brighton, 1966).

Free FITC

As the method of conjugation employed a 10 per cent dispersion of FITC on celite the solution containing free FITC was also made from the same starting material. Ten milligrams of 10 per cent FITC/celite dispersion were shaken in 0·05 M phosphate–0·15 M saline, pH 7·2 (PBS) until the FITC had dissolved. This solution contained 0·1 mg FITC/ml.

Stained coverslips

Doubling serial dilutions were made in PBS from both solutions. Two series of conjugated BSA dilutions were made, and to one of these was added a solution of conjugated swine antihuman immunoglobulins from a commercial source (Nordic Diagnostics, Tilburg, The Netherlands) which was known not to give non-specific staining in the 1+9 dilution used.

A drop of the appropriate solution was placed in the centre of a fixed coverslip culture preparation and permitted to react for 30 minutes. The solution was then removed and the preparation washed with tap water, air dried and mounted on a microscope slide in DPX prepared with tolyl phosphate instead of the more usual dibutyl phthalate.

Microscopic examination

Each of the three series was examined by epi-illumination in a Zeiss Photomicroscope II using the light from an HBO 200/W mercury lamp filtered through a 1·5 mm UG1 glass and the dichroic mirror of the instrument, with eyepiece filter GC41. Comparison was made of the stained area with the unstained area surrounding it and the fluorescence of each slide was graded on an arbitrary scale of +++ (very bright) ++ (bright) + (definite) and 0 (negative, including 'trace' and '±').

RESULTS

The results of FITC, conjugated BSA and conjugated BSA with 1 : 10 dilution of conjugated immunoglobulin are given in Tables 1–3.

TABLE 1
FITC

Dilution	1	1 : 500	1 : 5000	1 : 10,000	1 : 20,000	1 : 40,000
FITC (mg/ml)	1×10^{-1}	2×10^{-4}	2×10^{-5}	1×10^{-5}	5×10^{-6}	$2{\cdot}5 \times 10^{-6}$
Brightness	+++	+++	++	0	0	0

TABLE 2

CONJUGATED BSA

Dilution	1	1 : 5	1 : 10	1 : 40	1 : 80	1 : 160	1 : 320	1 : 640	1 : 1280
Albumin (mg/ml)	11	2·2	1·1	$2{\cdot}75 \times 10^{-1}$	$1{\cdot}37 \times 10^{-1}$	$6{\cdot}87 \times 10^{-2}$	$3{\cdot}43 \times 10^{-2}$	$1{\cdot}73 \times 10^{-2}$	$8{\cdot}59 \times 10^{-3}$
Brightness	+++	+++	++	++	++	+	0	0	0

TABLE 3

CONJUGATED BSA WITH 1 : 10 DILUTION OF CONJUGATED IMMUNOGLOBULIN

Dilution	1	1 : 5	1 : 10	1 : 40	1 : 80	1 : 160	1 : 320	1 : 640	1 : 1280
Albumin (mg/ml)	11	2·2	1 · 1	$2{\cdot}75 \times 10^{-1}$	$1{\cdot}75 \times 10^{-1}$	$6{\cdot}25 \times 10^{-2}$	$3{\cdot}12 \times 10^{-2}$	$1{\cdot}56 \times 10^{-2}$	$6{\cdot}26 \times 10^{-3}$
Brightness	+++	+++	++	++	+	+	0	0	0

DISCUSSION

Non-specific staining, i.e. all cells in the preparation stained evenly, was seen with the higher concentrations of all three series. With free FITC the staining was more concentrated on the nuclei, nucleoli, nuclear membrane and cytoplasmic RNA than over the remainder of the cell, but with conjugated albumin in addition to these brightly stained discrete areas there was an overall lower level coloration which suggested that not only was the dye adhering to nucleic acid, but also the protein moiety was adhering to the cell membrane.

In the definition of specifications for immunofluorescence conjugates it is suggested that where the conjugate is to be used in tests involving the kind of substrate used in this work the maximum permissible level of conjugated albumin should be $3{\cdot}0 \times 10^{-2}$ mg/ml and that of free FITC 1×10^{-5} mg/ml in the dilution of the conjugate applied to the test substrate.

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