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Whole Antiserum versus the Gamma-Globulin Fraction of Antiserum in the Indirect Fluorescent Antibody Technique

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Summary. Two antisera were compared with their γ-globulin fractions for their effectiveness as the middle or unlabelled layer in the indirect fluorescent antibody technique. The γ-globulin fraction did not produce better results in either case than the equivalent concentration of whole serum. Control sections were prepared with normal rabbit serum substituted for the whole antiserum and with normal rabbit γ-globulin substituted for the γ-globulin fraction of the antiserum. The control sections with γ-globulin showed considerably more non-specific fluorescence than those with whole serum. In one case the non-specific fluorescence in the γ-globulin control interfered with interpretation of the experimental section.

INTRODUCTION

The indirect or 'sandwich' fluorescent antibody technique of Weller and Coons (1954) is more sensitive than the direct technique (Nairn, 1962, p. 124). It has the added advantage that many antigens can be studied with a single conjugated antiserum. While much attention has been paid to non-specific fluorescence caused by unconjugated fluorochromes and conjugated non-antibody proteins, non-specific reactions caused by the non-antibody proteins in the unconjugated middle layer have apparently not been a problem and no mention of such reactions is found in several standard references to the technique (Mellors, 1959; Nairn, 1962; Smith, Metzger and Hoggan, 1962).

While studying the antigens of rat liver, we attempted to increase the fluorescent staining of a low titre rabbit antiserum to rat liver by using the concentrated γ-globulin fraction in the indirect technique. We were unable to interpret the experiment as a control section with concentrated normal rabbit γ-globulin substituted for the immune γ-globulin also gave bright fluorescence. When we repeated the experiment with concentrated whole antiserum, the control section treated with concentrated normal rabbit serum showed only minimal fluorescence. Consequently, we studied this system and another which did not require concentrated antiserum in order to determine whether whole antiserum or the γ-globulin fraction is preferable as the middle layer in the indirect technique.

The antibody used in the second system, anti-normal mouse serum, has been shown to localize on the basement membranes and reticulin of mouse tissues (Myers, Frei and Rose,

1964) probably due to antibodies which cross-react with the β-globulin fraction of mouse serum (Tan and Kaplan, 1963).

MATERIALS AND METHODS

Antisera

Fluorescein-labelled goat anti-rabbit γ-globulin was obtained from Hyland Laboratories, Los Angeles, California, and absorbed with guinea-pig liver homogenate prior to use.

Rabbit anti-rat liver antisera (anti-rL) were produced as follows. Rabbits were immunized with 20 mg of lyophilized whole homogenates of rat liver suspended in 0·5 ml of 0·9 per cent NaCl and an equal volume of complete Freund's adjuvant. The injections were given in multiple subcutaneous sites every 10 days for a total of four such immunizations. The rabbits were bled 10 days after the last injection. After absorbing with rat serum, and homogenates of rat heart, kidney and lung, the antisera reacted specifically with rat liver when tested by the tanned red blood cell method of Boyden (1951). The antisera were stored separately at −20° until used. One antiserum with a low titre by tanned cell agglutination was selected for these experiments.

Rabbit anti-normal mouse serum (anti-NMS) was produced as follows. Five mg of lyophilized mouse serum in 0·5 ml of complete Freund's adjuvant was injected intramuscularly into two rabbits followed in 3 days by 5 mg of mouse serum intravenously. Two weeks after the initial injection, the rabbits received a second intramuscular injection of mouse serum in adjuvant and were bled 5 days later. The antisera were pooled and stored at −20°.

Normal rabbit γ-globulin (RGG) and immune rabbit γ-globulin (anti-rL-GG and anti-NMS-GG)

Normal rabbit serum (NRS) and the rabbit antisera were precipitated with 40 per cent saturated ammonium sulphate at room temperature. After centrifugation the precipitates were suspended in a minimal volume of phosphate buffered saline, pH 7·4 (PBS) and dialysed overnight against PBS at 4°. The anti-NMS-GG and an aliquot of the RGG were restored to their original volume with PBS. The anti-rL-GG and an aliquot of the RGG were taken to one-fifth their original volume with PBS to make a concentrated immune γ-globulin and a concentrated control γ-globulin.

Concentration of NRS and anti-rL-serum

NRS and anti-rL-serum were concentrated five times by dialysis against polyethylene glycol (average molecular weight 20,000).

Tissues

Normal rat liver and normal mouse kidney were fixed in cold 95 per cent ethanol, dehydrated in the cold and embedded in paraffin by the method of Sainte-Marie (1962).

Fluorescent staining

The stains and controls for each antigen were performed at the same time to minimize the differences due to technique. The sections were cleared with cold xylene and washed in cold PBS for 10 minutes. The appropriate antiserum, antiserum γ-globulin, NRS and RGG were applied for 30 minutes and the slides were then washed in PBS for 10 minutes.

The fluorescent antiserum was applied for 30 minutes. After a final wash in PBS, coverslips were mounted with 1 : 9 PBS : glycerol. Control sections with the middle layer omitted were included to determine the degree of fluorescence due to non-specific reactions between the labelled antiserum γ-globulin and the tissues.

Photomicrography

The slides were examined under a Reichert Zetopan microscope fitted with an Osram HBO 200 light source. For photographing on high-speed Ektachrome daylight film, a fresh field was selected and the film exposed for 5 minutes. The photographs were copied on Agfa IFF film through a Kodak 15 G gelatin filter. A constant exposure time was selected for each tissue determined by the light meter reading of the dimmest colour slide (the NRS control in each case). Enlarged prints on Kodabromide F5 paper were made with constant exposure for each group.

TABLE 1

Tissue	Unlabelled antiserum or γ-globulin fraction	Labelled anti-γ-globulin (γ-globulin fraction)	Results
Rat liver	Anti-rL-serum-concentrated	Goat anti-rabbit	Uniform parenchymal cytoplasmic staining
Rat liver	NRS-concentrated	Goat anti-rabbit	Very faint background stain
Rat liver	Anti-rL-GG-concentrated	Goat anti-rabbit	Uniform parenchymal cytoplasmic staining
Rat liver	RGG-concentrated	Goat anti-rabbit	Uniform parenchymal cytoplasmic staining
Rat liver	PBS (labelled antiserum control)	Goat anti-rabbit	Very faint background stain
Mouse kidney	Anti-NMS-serum	Goat anti-rabbit	Specific fluorescence of basement membranes
Mouse kidney	NRS	Goat anti-rabbit	Faint uniform background stain
Mouse kidney	Anti-NMS-GG	Goat anti-rabbit	Specific fluorescence of basement membranes
Mouse kidney	RGG	Goat anti-rabbit	Non-specific fluorescence of tubular cytoplasm
Mouse kidney	PBS (labelled antiserum control)	Goat anti-rabbit	Faint uniform background stain

RESULTS

Concentrated anti-rL-serum and concentrated anti-rL-GG stained the cytoplasm of liver parenchymal cells with approximately equal intensity (Figs. 1 and 2). However, the concentrated RGG used as the control for the anti-rL-GG also gave good staining of the liver parenchymal cytoplasm (Fig. 3), while the concentrated NRS used as the control for the anti-rL-serum gave only minimal non-specific staining (Fig. 4) which did not differ in intensity or distribution from the staining present on the section from which the middle layer was omitted.

Rabbit anti-NMS localized along the basement membranes of tubules, glomeruli and blood vessels. Little difference was seen in the staining when anti-NMS-serum was compared with anti-NMS-GG, although there was a suggestion of slightly more intense background fluorescence in the case of the γ-globulin (Figs. 5 and 6). The RGG control

slides showed faint non-specific fluorescence of renal tubular cytoplasm and no fluorescence in the glomeruli; this pattern did not cause any difficulties in interpretation of the experimental slide. The NRS control and the control with the middle layer omitted showed almost no background fluorescence and no differences were observed between tubules and glomeruli. The results are summarized in Table 1.

DISCUSSION

While non-specific fluorescence in the indirect fluorescent antibody technique usually results from unconjugated fluorochromes or from adherence of non-specific labelled proteins, it appears that some non-specific staining may result from non-specific reactions with proteins in the unconjugated antibody. No other explanation can account for the fluorescence of the γ-globulin control sections which was not observed in the other controls. Since these reactions are of low intensity, they should not ordinarily interfere with the observation of specific bright fluorescence or with differentiating control slides from experimental slides. Smith *et al.* (1962) have shown that albumin labelled with rhodamine makes an effective counterstain for antibody labelled with fluorescein. They concluded that the labelled albumin reacts non-specifically with tissue proteins. Although such reactions are thought to result from a tendency of conjugated proteins to react with tissue proteins (Smith *et al.*, 1962; Mellors, 1959, p. 15; Nairn, 1962, p. 116), our results indicate that unlabelled proteins also react with tissues. Whether the lack of staining in control sections utilizing whole serum results from a predilection of non-γ-globulin serum proteins to react with tissues or from simple dilution of γ-globulin in a mixture of proteins cannot be determined from the present experiments; in either case there would be little γ-globulin on the tissue available for binding the specific labelled antibody. On the other hand, γ-globulin in the absence of other serum proteins appears to be bound to the tissues.

Two factors apparently contributed to the inability to distinguish between the experimental section of rat liver stained with anti-rL-GG and the control section. One of these was the use of concentrated γ-globulin which increased the intensity of the non-specific staining; the other was the general cytoplasmic distribution of the antigen—the same areas that showed non-specific staining in control sections.

Figs. 1–6 are enlarged from magnifications of 200 diameters. The figures are excessively grainy because of the over-development needed to show the fluorescence of the NRS control sections.

FIG. 1. Rat liver stained by the indirect technique with concentrated anti-rL-GG and fluorescein-labelled anti-rabbit-GG.

FIG. 2. Rat liver stained by the indirect technique with concentrated anti-rL-serum and fluorescein-labelled anti-rabbit-GG.

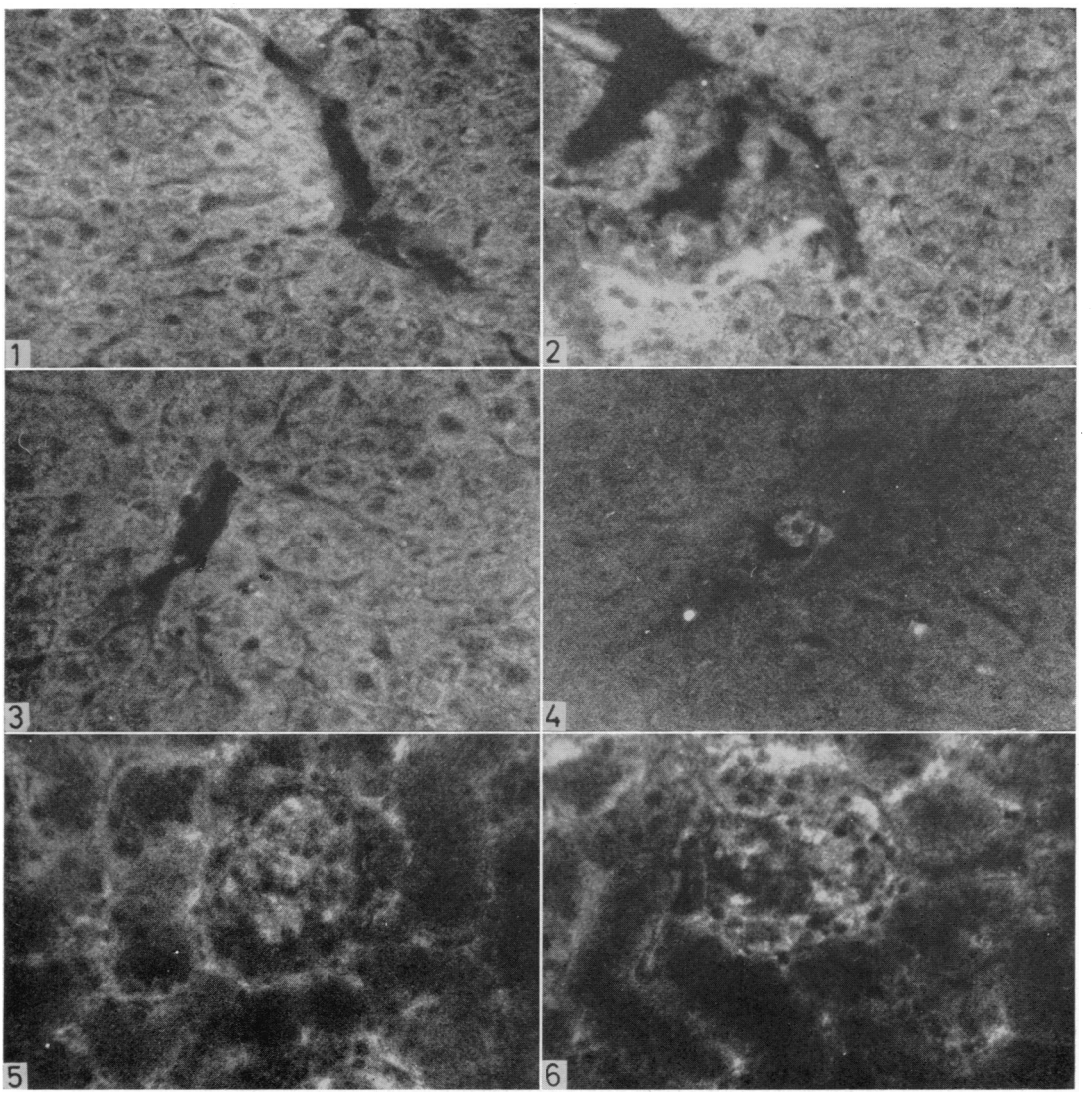
FIG. 3. Rat liver stained by the indirect technique with concentrated RGG and fluorescein-labelled anti-rabbit-GG. This section was intended to serve as a control section for Fig. 1. The diffuse staining on this section indicated that any specific staining in Fig. 1 was masked by the non-specific staining.

FIG. 4. Rat liver stained by the indirect technique with concentrated NRS and fluorescein-labelled anti-rabbit-GG. This was the control section for Fig. 2. The minimal background fluorescence is easily distinguished from the specific fluorescence of the experimental slide. The bright dot is an artifact.

FIG. 5. Mouse kidney stained by the indirect technique with anti-NMS-GG and fluorescein-labelled anti-rabbit-GG. Specific fluorescence is seen along basement membranes in tubules and glomeruli. Faint non-specific fluorescence is seen delineating the cytoplasm of the tubules.

FIG. 6. Mouse kidney stained by the indirect technique with anti-NMS-serum and fluorescein-labelled anti-rabbit-GG. Specific fluorescence is as good as in Fig. 5 with less non-specific fluorescence, although the cytoplasm of a tubule is seen adjacent to the glomerulus.

Indirect Fluorescent Antibody Technique



(*Facing p.* 104)

In the mouse kidney slides, the γ-globulin control showed a similar localization on tubular cytoplasm which was easily differentiated from the more intense specific staining in a characteristic distribution.

Our results indicate that the γ-globulin fraction of an antiserum gives no better specific staining than the equivalent concentration of the whole antiserum when used as the middle layer in the indirect fluorescent antibody technique. It has the disadvantage of causing more non-specific staining which may produce false-positive fluorescence in control sections or conceivably mask a pattern of specific fluorescence. Since it is simpler to use the whole antiserum, using the γ-globulin fraction appears unwarranted.

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