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Quantitative Studies of Immunofluorescent Staining

I. ANALYSES OF MIXED IMMUNOFLUORESCENCE

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Summary. Mixed anti-globulin immunofluorescence (or mixed IF) was analysed using the following model system: calf thyroid sections, human anti-nuclear factor (ANF), rabbit antisera to human IgG and fluorescein-labelled human IgG. This system was characterized on the basis of: (1) the immunoelectrophoretic demonstration of antibodies to human IgG in anti-globulins; (2) titration of the anti-globulins and labelled globulins; and (3) determination of molar fluorescein to protein (F: P) ratios. Titration of anti-globulins (rabbit anti-human IgG) by gel precipitation afforded an assay of 'units' of antibody activity. Similarly, 'units' of labelled globulin antigen were determined by a gel precipitation titration. Block titrations of these components of the indicator system against ANF serum yielded constant titres or 'plateaux' of nuclear staining over a range of units of anti-globulin and of labelled immunoglobulin. Thus, the following predictions can be made. Optimal mixed IF staining may be attained with 4 units or more of an anti-globulin (anti-human IgG) and with 1 unit or more of a fluorescein-labelled human immunoglobulin if the latter has an F: P ratio in the range of about 1: 1 to 3: 1. The titre of the ANF appears to be proportional to the F: P ratio over this range.

INTRODUCTION

As reported previously (Beutner, Holborow and Johnson, 1965), immunofluorescent staining can be achieved with antibodies to move human tissue by the method of mixed antiglobulin immunofluorescence (IF). This method is based on the same principle as the mixed anti-globulin agglutination test of Coombs, Mark and Bedford (1956). An anti-human immunoglobulin is first allowed to combine with the human tissue antibody that has become attached to the tissue section. The free antibody combining sites of the anti-globulin are then allowed to react with fluorescein-labelled immunoglobulin (antigen) to render the reaction visible under the fluorescence microscope. It was found that anti-nuclear factors, thyroid microsomal antibodies, skeletal muscle antibodies (associated with myasthenia gravis) and antibodies to different components of skin could be detected by mixed IF staining. The staining patterns as well as the titres observed were essentially the same as those obtained by the indirect immunofluorescent or so-called 'sandwich' technique of Weller and Coons (1954). Since the anti-globulin and labelled antigen

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used in mixed IF can be varied independently, the method lends itself to quantitative analysis.

The object of the studies summarized in this report was to relate precipitation titres of anti-globulins, and molar fluorescein to protein (F: P) ratios of labelled immunoglobulins, to their mixed IF staining reactivities. The reason for undertaking these studies was that simple, reliable and objective criteria for quantitative evaluation of anti-globulins would be an asset in defining IF staining systems and rendering them more reproducible from one laboratory to another. In view of the similarities between indirect and mixed IF staining in terms of their underlying principles, in the staining patterns observed and in the titres obtained, the quantitative relationships which obtain for mixed IF staining assume some relevance to indirect IF staining.

METHODS AND MATERIALS

Immunization

Rabbits were variously immunized with: (1) commercial Cohn fraction II (Nutritional Biochemical Corp., Cleveland, Ohio) of human plasma; (2) purified IgG fraction of human serum obtained by DEAE-cellulose chromatography; (3) crude β_{1A} fraction of human serum prepared according to the method of Müller-Eberhart, Nelsson and Aronson (1961); and (4) rabbit red blood cells sensitized with human antibodies as described by Milgrom, Dubiski and Wozniczko (1956). The first three preparations of antigen were mixed with Freund's complete adjuvant.

Conjugation and fractionation

Fluorescein isothiocyanate (FITC) conjugation was carried out by the standard method of Riggs, Loh and Eveland (1961). Various amounts of FITC were used, ranging from 0·01 to 0·1 mg FITC/mg protein. Most conjugates were prepared from commercial fraction II from human plasma (Koch Light Ltd, Colnbrook, Bucks). Bicarbonate buffer was added to the reaction mixtures to bring the pH to 9·2. DEAE-cellulose fractionation of conjugates was performed by a modification of the methods of Goldstein, Slizys and Chase (1961), equilibrating the column with 0·01 M, pH 7·0 phosphate buffer and eluting the active conjugate with 0·26 M NaCl in the starting buffer.

Characterization of anti-globulins and labelled globulins

(a) *Immunoelectrophoresis* of anti-globulins was performed on microscope slides in borate buffer by standard methods (Crowle, 1961).

(b) *Assay of anti-globulins and immunoglobulins* was performed by Ouchterlony gel diffusion tests to determine 'units' of precipitating activity: (1) A 'micro' method used about 1 mm thickness of 1 per cent agar (Ionagar No. 2: 'Oxoid Division of Oxo Ltd, London) (in pH 7·0, 0·158 M phosphate buffered saline) on a microscope slide. Two sets of six wells 8 mm (centre to centre) from a central well, each 2·5 mm in diameter were cut on a slide with a die; (2) A 'macro' method employed a Petri plate with about 20 ml of 1 per cent agar (as above). With a standard die (Shandon Scientific Co., London), six peripheral wells 4 mm in diameter, were cut 14 mm (centre to centre) from a central well which was 12 mm in diameter. These two types of precipitin tests yielded comparable results. In each, a 1 mg/ml solution (by Biuret determination) of γ-globulin (fraction II) was placed in the central well and serial dilutions in buffered saline of an anti-globulin in the form of whole serum, globulin or conjugate in peripheral wells. The highest dilution giving a visible line

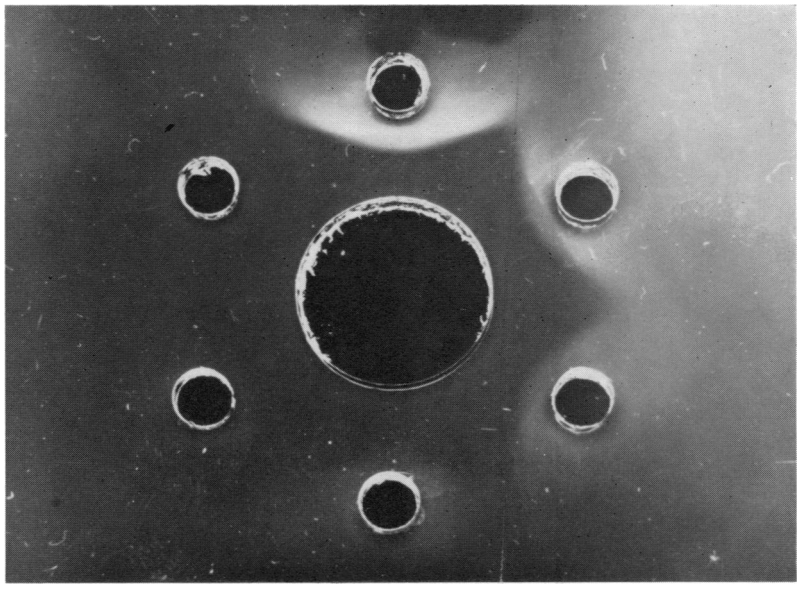

FIG. 1. Centre well 1 mg fraction II per ml. Top well: 1: 4 dilution of rabbit antiserum to human IgG. Clockwise: the peripheral wells contain serial two-fold dilutions of the same material.

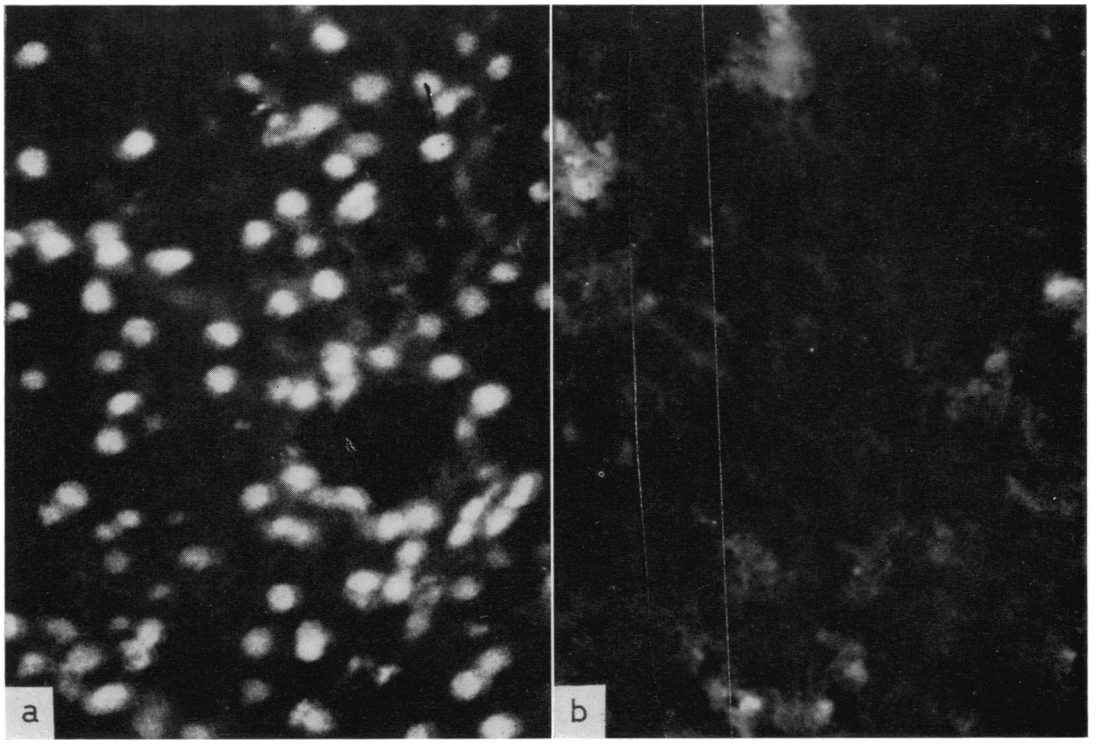

FIG. 2. Mixed immunofluorescence (MIF) staining of sections of monkey liver. The sections were treated with human sera, 2 units of rabbit anti-human IgG and 2 units of labelled IgG. × 200. (a) MIF staining of liver section with human anti-nuclear factor, 1:25 dilution. Nuclear staining is evident. (b) MIF staining of liver section with normal human serum, 1:25 dilution. The white spots were areas of blue-white autofluorescence.

(*Facing p.* 328)

of precipitation after 48 hours was attributed 1 'unit' of anti-globulin per ml. Fig. 1 illustrates such a test.

The lower right-hand well in Fig. 1 contained a 1:16 dilution of anti-globulin which gave the last visible line of precipitation. Thus, this serum contained 16 units of anti-globulin per ml. Labelled antigen titrations were performed in the same way. One 'unit' of labelled immunoglobulin is defined as the amount per ml in the highest dilution of this antigen which yields a visible line of precipitation with 2 units of anti-globulin in the central wall.

(c) *Fluorescein to protein (F: P) ratios* were calculated on a molar basis. Protein assays were performed by the Biuret method with readings at 560 mμ as described by Goldwasser and Shepard (1958). For fluorescein assays a fresh solution of commercial, chromatographically pure FITC (FITC—Baltimore Biological Laboratories, Baltimore, Maryland) diluted in 0·1 N NaOH was employed as a standard. It yielded the following extinction coefficient:

$$E\,{}^{1\ \mu g/ml}_{1\ cm}\ 492\ m\mu = 0{\cdot}20\ (\pm 0{\cdot}02) \text{ or } E\,{}^{molar}_{1\ cm}\ 492\ m\mu = 7{\cdot}8\ (\pm 0{\cdot}8) \times 10^4.$$

This accords with the extinction coefficient 0·219 for FITC reported by McKinney, Spillane and Pearce (1964). According to these authors the commercial FITC standard employed in our studies yields essentially the same extinction coefficient as their purified preparation. Estimates of fluorescein concentration were frequently found to be affected by irrelevant absorption by protein at 492 mμ. It was found that a correction for this irrelevant absorption could be achieved by subtracting half of the value for the absorption at 320 mμ from the absorption at 492 mμ. Experimental evidence for this will be reported at a later date. Molar fluorescein to protein (F: P) ratios were calculated from the fluorescein and protein concentrations and the molecular weight ratios (taking the molecular weights of FITC and γ-globulin as 390 and 160,000 respectively).

Mixed IF staining

The staining procedure was performed as described previously (Beutner *et al.*, 1965). The source of human anti-nuclear factor (ANF) used for most of these studies was ascitic fluid from a patient with systemic lupus erythematosus. Most of the microscopy was performed with a Reichert Fluorescence microscope. Some of the later studies were performed with a Zeiss unit. For readings of IF staining reactions, preparations were randomised. Readings were made by two or more investigators.

RESULTS

COMPARISON OF MIXED AND INDIRECT IF STAINING

The appearance of mixed anti-globulin immunofluorescence (IF) staining reactions of anti-nuclear factors (ANF) was indistinguishable from that observed by the conventional indirect IF method. The appearance of mixed IF reaction of an ANF is illustrated in Fig. 2(a). The corresponding negative control is shown in Fig. 2(b).

The titres obtained by mixed and indirect IF staining were compared by employing the same anti-globulin, the same anti-nuclear factor (homogeneous type) and sections of the same tissue in the two methods. The anti-globulin (No. 2708) when tested by immunoelectrophoresis yielded a single line characteristic of IgG and two lines in the beta region.

For indirect IF staining an IgG fraction af this anti-globulin was prepared by DEAE-cellulose fractionation and conjugated with FITC. It had an F:P ratio of 1·5:1 and was employed for IF staining at a dilution containing 1/5 of a unit per ml. For mixed IF staining the same anti-globulin was used as whole serum diluted to contain either 1 or 2 units per ml, and a fluorescein-labelled preparation of human fraction II with an F:P ratio of 3·8:1 was employed as top layer antigen. The characteristics of the reagents and results obtained with the two systems are summarized in Table 1.

TABLE 1

COMPARISONS OF INDIRECT AND MIXED IMMUNOFLUORESCENCE TITRATIONS OF HUMAN ANTI-NUCLEAR FACTORS ON SECTIONS OF CALF THYROID

Case (diagnosis)	Sera dilution	ANF reactions		
		Test 1	Test 2	Test 3
Be (normal)	1:10	–	–	–
Au (SLE)	1:50	++	+	++
	1:100	W	W	++
	1:200	–	W	W
	1:400	–	–	–
Titre		1:100	1:200	1:200
Characteristics of test systems				
Method		IIF	MIF	MIF
Anti-IgG		2708	2708	2708
(a) Form used		γ_2 labelled	Whole serum	Whole serum
(b) Units used		1/5	1	2
Labelled antigens			FII	FII
F:P ratio		1·2	3·8	3·8

IIF, Indirect immunofluorescence; MIF, mixed (anti-globulin) immunofluorescence; FII, Cohn fraction II of human plasma; ANF, anti-nuclear factor; SLE, systemic lupus erythematosus; W, weak positive.

Fluorescein to protein ratios (F:P) are expressed as molar ratios of FITC to IgG protein.

It may be seen that the ANF serum employed yielded clearly visible staining to a dilution of 1:100 by the indirect IF staining method while the mixed IF method gave a titre of 1:200 under each of the two conditions used. The fact that mixed IF staining yielded a higher titre than the indirect IF method is probably associated with the fact that the F:P ratio of the conjugated anti-globulin was lower than that of the labelled fraction II used in mixed staining (see data in Table 6). Also, the amount of anti-globulin (as expressed in units) employed in the mixed IF procedure was greater. As shown in Table 1, 1/5 of a unit, or 1/5 the concentration of conjugated anti-globulin necessary to give a visible precipitation reaction, yielded a positive indirect IF staining reaction. Thus, it is apparent that indirect IF staining may be attained in the absence of readily demonstrable precipitating antibody. The mixed IF titrations using 1 and 2 units of anti-globulin both yielded the same ANF titres, but the intensity of staining attained with 2 units was greater than with 1 unit. Thus, the titre and intensity of IF staining appear to be independent variables, a relationship typically found in block titrations performed at higher dilutions of anti-globulins.

ANF TITRATIONS BY MIXED IF WITH DIFFERENT ANTI-GLOBULIN SERA

A group of eleven rabbit sera containing antibodies to IgG was selected for studies on mixed immunofluorescent staining, and their characteristics are summarized in Table 2. Antisera obtained by a variety of immunization procedures were selected for study because a spectrum of anti-globulins was desired. The immunizing antigens included β_{1A} preparations containing IgG as a minor contaminant, rabbit red cells coated with human antibody, a purified IgG, and a partially purified preparation (human fraction II). These antisera were examined by immunoelectrophoresis for reactivity with whole human serum,

TABLE 2

CHARACTERISTICS OF RABBIT ANTISERA TO HUMAN IgG SELECTED FOR STUDIES OF MIXED IF STAINING

Rabbit No.	Immunizing antigen	Immunoelectrophoretic analyses			Precipitin* titres with 1 mg IgG/ml
		Antigens electrophoresed: Normal human serum	IgG	IgG-Fl†	
2708	FII‡	IgG+2 lines near beta	+		1:16
118	FII	IgG line only	+		1:32
82-16	Sensitized RBC§	IgG line only	+	+	1:1
82-II	Sensitized RBC	IgG line only	+		negative
Pd	FII	IgG+IgM+line near beta	+	+	1:4
52/I	IgG	IgG line only	+	+	1:16
52/II	IgG	IgG line only	+	+	1:16
82-15	Sensitized RBC	IgG line only	+	+	1:4
121-3	Crude β_{1A}	IgG+IgM+5 other lines¶	+	+	1:4
121-2/I	Crude β_{1A}	IgG+IgM4 other lines¶	+	+	1:8
121-2/II	Crude β_{1A}	IgG(?)+3 other lines¶	−	+	negative

* Precipitin endpoints were determined by testing doubling dilutions of the antiglobulins in a standard gel diffusion test (see 'Methods').

† IgG-FI, Fluorescein labelled FII with an estimated molar fluorescein to protein ratio 3·6.

‡ FII, Human serum fraction II Koch Light Laboratories Ltd, Colnbrook, Bucks, England.

§ Sensitized RBC or antibody coated rabbit red blood cells were prepared according to Milgrom, Dubiski and Wozniczko (1956).

¶ Serum 121-3 yielded three lines in the beta region and two lines in the alpha region; serum 121-2/I gave four lines in the beta and alpha regions while a later bleeding of the same rabbit 121/2-II gave only three lines in the same regions.

with chromatographically purified IgG and, in most cases, with a fluorescein-labelled IgG preparation employed in the mixed IF staining. In addition, the titre of antibody to IgG was determined by serial dilution of the anti-globulin in standard gel diffusion precipitation tests (see 'Methods'). The activity of the sera was expressed as 'units' of precipitating anti-globulin, e.g. serum 2708 in Table 2 contained 16 units/ml.

The relationship between anti-globulin activity and reactivity in mixed immunofluorescence staining was studied by performing block titrations. Thus, serial dilutions of the standard anti-nuclear factor were tested with dilutions of anti-globulins, and a single labelled human IgG preparation (F:P ration, 1·5:1) was used throughout as the third layer antigen. The antigenic activity of this labelled globulin was determined by a gel precipitation test as described above and it was used at a concentration of 1–2 units/ml. (The use of different concentrations from 1 to 16 units of this labelled human IgG preparation yielded identical results as will be shown subsequently.) The results in block titrations were expressed as titres of the anti-nuclear factor. Anti-globulin dilutions were

expressed as units of precipitating antibody. Some typical results of such block titration are shown in Table 3. It may be seen from the ANF titres shown that the end-points varied by a factor of ±1 doubling dilution. One unit of the anti-globulin 52-II was employed in ten replicate titrations of the standard ANF using the same labelled immunoglobulin. The end points obtained ranged from 1:100 to 1:400, with titres of 1:200

TABLE 3

TITRATIONS OF AN ANF SERUM BY MIXED IMMUNOFLUORESCENCE STAINING: MAXIMUM TITRE 'PLATEAUX' OBTAINED WITH THREE ANTI-GLOBULINS SERIALLY DILUTED TO CONTAIN THE SAME RANGE OF PRECIPITIN UNITS

Rabbit antisera to IgG*	Experiment No.	ANF titres observed						
		Units of anti-IgG per ml						
		8	4	2	1	1/2	1/4	1/8
52-I	1	100			25			neg
	2	100		100	50			
	3		100			neg	neg	
52-II	1	200			200†			neg
	2		200			25	neg	
	3		200	100	100			
121-2I	1	100			25			neg
	2		100		25		neg	
	3		100	25	25			

The following standard mixed IF test system was employed: A standard ANF (antinuclear factor) was examined at serial two-fold dilutions starting at 1: 25. A fluorescein labelled preparation of fraction II with a molar F:P ratio of 1·5:1 was used as the top or third layer antigen throughout. Mixed IF staining tests were performed under standard conditions as described in 'Methods'.

* All rabbit antisera to human immunoglobulins contained antibodies to IgG as revealed by immunoelectrophoresis. For details see Table 2.

† Ten replicate titrations with one unit of the antiglobulin 52-II yielded the following ANF titres: 100 (3×), 200 (6×) and 400 (1×). Only two of these ten titrations are listed in Table 3.

in six of the ten experiments. Within single experiments consistent titres were observed. For example, the three titrations with serum 52-II that yielded end-points of 1:100 were all carried out in a single experiment. The results in Table 3 indicate that a constant titre was maintained over a range of anti-globulin dilutions (though the intensity of staining decreased with decreasing unitage, as indicated before). This range of anti-globulin dilutions yielding constant ANF titres is referred to as the 'plateau'. As may be seen in Table 3, this plateau appeared only with 4 and 8 units of anti-globulin 121-2I, while with serum 52-II it extended from 1 to 8 units. Table 4 shows the end-points obtained, with the eleven anti-globulin sera tested.

It may be seen that all anti-globulins tested at dilutions containing 4 units/ml or more yielded usable reactions. Indeed, all of the anti-globulins gave ANF titres in the range of 1:100 to 1:400. Dilutions of anti-globulins containing ¼–1 unit ml yielded variable results. At 1 unit/ml some anti-globulins yielded maximal staining titres while others did not, and at ¼ unit/ml some were positive and others negative. These results show that while the best fluorescent staining is obtained with *potent* precipitating anti-globulin sera, antisera of equal precipitating potency do not necessarily give the same end-point in titrations of a given specific antibody by immunofluorescence. All of the anti-globulins included in these experiments gave negative reactions when tested at ⅛ unit/ml. In

TABLE 4

TITRATIONS OF AN ANF SERUM BY MIXED IMMUNOFLUORESCENCE STAINING: DIFFERENCES BETWEEN ELEVEN SERIALLY DILUTED ANTI-GLOBULIN SERA IN RANGE OF PRECIPITIN UNITS GIVING USEFUL STAINING

Anti-IgG		Units* of anti-IgG				
Rabbit No.	Unit/ml	8	4	1	1/4	1/8
2708	16	400	400	400	100	neg
118	32	400	200	200	100	neg
82-16	1			200	100	neg
82-II†	1				100	neg
Pd	4		200	200	neg	neg
52-I	16	100	200	25	neg	neg
52-II	16	200	100	200	neg	neg
82-15	4		100	50		neg
121-3	4		200	50	neg	neg
121-2I	8	100	100	25		
121-II	1				neg	
		Usable range		Variable range		Negative range

Mixed IF staining with a standard ANF was performed as for Table 3. MIF staining endpoints in titrations of this standard ANF varied by ± 1 doubling dilution.

* Precipitin end-points (see Table 2 and 'Methods') provided the basis for adjusting dilutions of anti-globulins to the desired unitage, e.g. serum 2708 which had a precipitin end-point of 1:16 was diluted 1:4 to obtain 4 units/ml; 1:16 to obtain 1 unit/ml, etc.

† Mixed IF titres of the standard ANF with undiluted anti-globulins having negative precipitin reactions are listed under 1/4 units.

subsequent studies one hyperimmune anti-globulin obtained after 6 months immunization of a rabbit with chromatographically purified human IgG yielded a weak doubtful ANF reaction when tested at $\frac{1}{8}$ unit/ml.

INFLUENCE OF F:P RATIO ON NON-SPECIFIC STAINING

Sixteen conjugates of human γ-globulin mostly in the form of fraction II with various F:P ratios were prepared and tested. The non-specific staining activity of these conjugates was recorded in a number of mixed IF staining experiments on sections of calf thyroid or monkey thyroid or both. The relation between the F:P ratios and the level of non-specific staining is shown in Table 5.

TABLE 5

SUMMARY OF RELATION BETWEEN F:P RATIOS AND NON-SPECIFIC STAINING BY SIXTEEN CONJUGATES OF HUMAN γ-GLOBULIN IN MIXED IF STAINING

Range of F:P ratios tested	No. of conjugates	Nonspecific staining
0·6:1 to 2·7:1	6	Negative to negligible
3·0:1 to 5·2:1	6	Variable 2 negative 4 positive
5·6:1 to 18·1	4	Positive*

* Positivity renders the conjugate unusable for titration of specific antibody.

The conjugates prepared and examined fell into three categories. Those with F:P ratios of 2·7:1 or less were essentially negative for non-specific staining (one of these with an F:P ratio of 2·2:1 yielded a trace of non-specific staining which was deemed negligible). Six conjugates with F:P ratios in the range of 3·0:1 to 5·2:1 yielded variable results. That is, two with F:P ratios of 4·8:1 and 5·2:1 were essentially negative while four with ratios of 3·0:1, 3·4:1 and 4·3:1 yielded sufficient non-specific staining to interfere with ANF titration. Conjugates with F:P ratios of 5·6:1 or more were regularly unusable because of their strong non-specific staining reactions.

INFLUENCE OF F:P RATIO ON ANF TITRE

Further mixed IF block titrations were performed with constant amounts of anti-globulin and with serial dilutions of selected labelled human immunoglobulins as the top layer reagent. Tests were performed on calf thyroid sections. The first step (human ANF) was the same as in previous experiments. For the second step, a single anti-globulin (52-II) was used throughout at a concentration of 2 units/ml. Four labelled human IgG preparations used as third layer antigens had F:P ratios ranging from 0·6:1 to 5·2:1. Three of these conjugates were made from commercial fraction II while the fourth was prepared from a DEAE-cellulose purified preparation of IgG. Concentrations were adjusted to 16 units/ml. Serial four-fold dilutions down to 1/64 unit/ml were tested against serial dilutions of the standard ANF. The results of these block titrations are summarized in Table 6.

TABLE 6

TITRATION OF AN ANF SERUM BY MIXED IMMUNOFLUORESCENCE STAINING: RELATION BETWEEN F:P RATIO OF LABELLED IMMUNOGLOBULIN, AND ANF TITRE

Labelled antigens	F:P ratios	Units of labelled human IgG					
		16	4	1	1/4	1/16	1/64
FII	0·6:1	50*	50	50	25	25	neg
FII	2·2:1	200	200	200	100	25	neg
IgG	4·8:1	400	400	400	400	V(50)	neg
FII	5·2:1	400	400	400	400	V(200)	neg

A single anti-globulin diluted to 2 units/ml was used throughout. V, Variable titre. The titres given in brackets were those of typical but not reproducible end-points.

* Observed titre of ANF.

Each of the four conjugates employed in the block titrations shown in Table 6 yielded well defined plateaux of ANF staining titres, the first two in the range of 16–1 units of antigen per ml and the latter two from 16 to ¼ units/ml. At higher dilutions of conjugate the ANF titres declined, all tests being negative at dilutions of 1/64 units of labelled antigen per ml. That is, the first two conjugates had plateau end-points at 1 unit/ml while the latter two had end-points at ¼ unit/ml. Of particular interest in these block titrations is the relation between the molar F:P ratios and the plateau ANF titres. The ANF titres appeared to be approximately proportional to the F:P ratios. In order to substantiate this relationship five labelled human γ-globulins with graded F:P ratios were prepared and tested at 2 units/ml on monkey liver sections with another human ANF-containing serum and

another anti-globulin at 2 units/ml. Again, a comparable proportionality between the ANF titres and the F:P ratios of the conjugate was observed.

To answer the question whether high concentrations of both anti-globulin and labelled immunoglobulin would increase the apparent ANF titres the following titrations were performed. Serial dilutions of the standard ANF were titrated with dilutions of a rabbit anti-human IgG antiserum and labelled fraction II dilutions selected on the basis of data shown in Table 6. These tests were again performed on sections of calf thyroid. The results of the three way block titration are shown in Fig. 3.

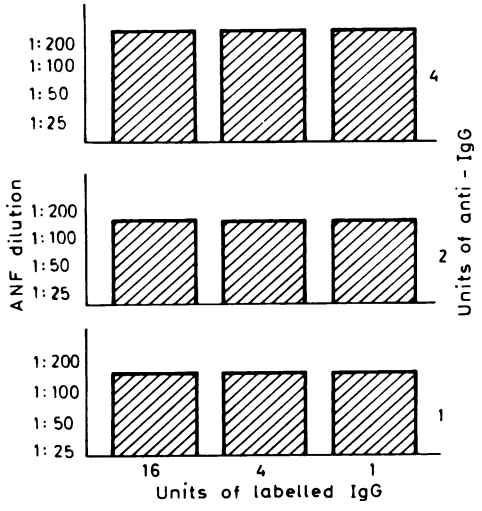

FIG. 3. Titrations of an ANF serum by mixed IF staining, showing failure of high concentrations of labelled IgG to enhance titre.

It is evident that the combined use of 4 units/ml of rabbit anti-human γ-globulin and 16 units/ml of the labelled human γ-globulin did not give rise to an enhanced ANF titre. Indeed, it may be seen that plateaux of ANF titres were maintained over the range of labelled human γ-globulin dilutions at each of the dilutions of rabbit anti-human γ-globulin tested. On the other hand, dilution of the anti-globulin was accompanied by some decline in the ANF titres.

DISCUSSION

The most valuable piece of information that came out of these studies was the finding that in block titrations, the primary or specific antibody (ANF in this work) appeared to have a constant titre over a range of dilutions of anti-globulin—that is to say, a 'plateau' effect was demonstrated. Such plateaux may be ascribed at least in part to a saturation of reactive sites by the indicator system. The plateau effect was seen in three titration systems. When anti-globulins were titrated against the specific antibody with a constant amount of the top layer labelled antigen a well defined plateau of apparent titres of specific antibody over a broad range of anti-globulin dilutions was usually obtained. This suggested a saturation of the antigenic sites of the specific antibody over the whole plateau range. When constant amounts of anti-globulin and serial dilutions of the labelled immunoglobulin were used a sharply defined plateau of specific antibody titres was observed in all

experiments. This points to a saturation of the free antibody combining sites of the anti-globulin over the whole plateau range of dilutions. Similarly, in studies of block titrations in indirect IF staining (not described here) well defined plateaux of specific antibody titres were found over a broad range of dilutions of labelled anti-globulin. This again, points to saturation of the antigenic sites on the specific antibody when higher concentrations of labelled anti-globulin are used.

One important, practical corollary of the demonstration of plateaux of antibody titres in block titration in mixed IF staining is that the sensitivity of the method is not enhanced by using reagents at concentrations higher than those that obtain at the plateau end-point. Unpublished studies indicate that in indirect IF staining also the use of corresponding dilution of labelled anti-globulin not only affords the advantage of more economical use of conjugates, but also diminishes or abolishes non-specific staining.

Finally, the mechanisms underlying mixed and indirect immunofluorescence may be considered. The plateau end-point of antiglobulin activity in one rabbit antiserum (2708) was found to be 1 unit/ml by mixed IF and $\frac{1}{16}$ unit/ml by indirect IF. A possible explanation of this discrepancy is that bivalent antibody molecules in combining with antigen may occlude both their combining sites, or only one. It is clear from Fig. 4 that in mixed

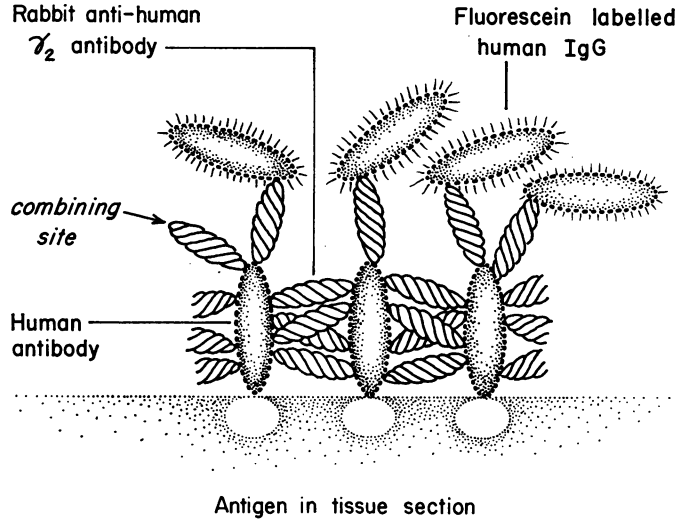

FIG. 4. Diagram of mixed IF staining method.

IF, fluorescent immunoglobulin is taken up only by free combining sites on anti-globulin molecules. In indirect IF, in contrast, each anti-globulin molecule is labelled and contributes to the total fluorescence.

Observations on the relationship between non-specific staining and the F:P ratios of the labelled human globulin preparations are consistent with those reported by others (Goldstein *et al.*, 1961; Frommhagen, 1965). It is noteworthy that some variability in the degree of non-specific staining was encountered in the range of 3:1 to 5:1. This may be a result of the procedure employed for conjugation. The dialysis-labelling method of Clarke and Shepard (1963) which provides a more uniformly-conjugated reagent might be an advantage.

Comparisons of mixed and indirect IF staining procedures reveal several advantages and disadvantages of each. The *advantages* of mixed IF staining may be summarized as follows:

(1) The fact that anti-globulins and the labelled immunoglobulin antigen can be varied independently in mixed IF staining facilitates quantitative analysis of IF staining with anti-globulins.

(2) Mixed IF staining with anti-globulins containing antibodies to other serum proteins and labelled immunoglobulin preparations free of these other serum proteins yields staining reactions specific for the immunoglobulins. This permits the use of polyvalent antiserum (for example) for the purpose of identifying reactions involving the various immunoglobulin classes. Conversely, monospecific anti-globulin sera could be used with a mixture of labelled serum proteins.

(3) A further possible use for a mixed IF procedure is in identification of antigens in tissue sections or other material. This would involve the use of antiserum containing specific antibody, and labelled antigen, and would be the converse of the method used by White (1954) to demonstrate specific antibody in plasma cells.

The *disadvantages* of mixed IF staining as compared with the indirect staining method may be summarized as follows:

(1) The mixed method entails an extra step in processing and therefore requires more time, though the sensitivity of the two methods is approximately the same.

(2) The concentration of anti-globulin required to obtain plateau titres is approximately 16 times greater for mixed IF staining than it is for indirect staining.

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