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Optimal Fluorescein-to-Protein Ratios of Bacterial Direct Fluorescent-Antibody Reagents

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A number of bacterial systems were studied with specific direct fluorescent-antibody reagents prepared from rabbit antiserum fractions and having a wide range of fluorescein-to-protein ratios. These systems included *Bacteroides*, *Bordetella*, *Clostridium*, *Escherichia*, *Legionella*, *Listeria*, *Salmonella*, *Shigella*, and *Streptococcus*. For all systems studied, a fluorescein-to-protein ratio of 30 was optimal for conjugates prepared from ammonium sulfate fractions (>75% gamma globulin) and pure immunoglobulin G desorbed from the Sepharose-bound protein A of *Staphylococcus aureus*. A pepsin digestion procedure is described that yielded the $F(ab')_2$ piece of pure immunoglobulin G; this was labeled and studied at two fluorescein-to-protein ratios.

Our laboratory has been involved in fluorescent-antibody methodology research, training, and consultation for many years. Throughout all of these activities the most frequently asked question was, and still is, "What fluorescein/protein (F/P) ratio should I have?" The answer has always been "It depends on the intended application, but for all bacterial systems we prefer a high F/P ratio."

An optimal F/P ratio is that which provides the maximum specific staining titer with a minimum of nonspecific (non-immunological) staining. This paper deals with the direct relationship between specific staining and F/P ratios for a number of bacterial immunofluorescence systems. The data presented have been accumulated over a number of years. Some new techniques have been introduced from time to time, but the results are remarkably similar.

MATERIALS AND METHODS

Bacterial systems representing 9 genera and 13 species were examined during this study. These were *Bacteroides fragilis*, *Bordetella pertussis*, *Clostridium septicum*, *Escherichia coli*, *Legionella pneumophila* (2), *Legionella bozemanii* sp. nov. (1), *Legionella micdadei* sp. nov. (12), *Legionella dumoffii* sp. nov. (1), *Listeria monocytogenes*, *Salmonella*, *Shigella dysenteriae*, *Streptococcus mutans*, and *Streptococcus pyogenes*.

All of the data presented were obtained from rabbit antisera. Most of the antisera were fractionated by three successive precipitations in 35% saturated ammonium sulfate, as described previously (9). Immunoglobulin G (IgG) was isolated from some of the antisera by affinity chromatography on a column of protein A (*Staphylococcus aureus*) covalently bound to Sepharose CL-4B (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.), as previously described (7). Some of the IgG was further fractionated to $F(ab')_2$ by pepsin digestion of the Fc fragment; the method described by Nisonoff (16) was modified, and the procedure followed is described below.

(i) The pH of the IgG solution (22 mg of IgG per ml in borate saline) was lowered to 4.5 by adding 2 parts of 0.1 M acetic acid, in 0.15 M sodium chloride, to 3 parts of protein.

(ii) A pepsin solution (0.5 mg/ml) was prepared by rehydrating crystallized pepsin in 0.2 M sodium acetate-1.0 M acetic acid-0.15 M sodium chloride reagent, pH 3.8.

(iii) The pepsin solution was added to antibody for a final ratio of 1 mg of pepsin for each 50 mg of IgG desorbed from protein A; the pH was checked to be sure it was between 3 and 4.

(iv) The mixture was placed in an incubator at 37°C overnight (16 to 18 h).

(v) After incubation, the mixture was centrifuged to remove the acid-insoluble glycoproteins.

(vi) The clear supernatant antibody mixture was carefully decanted, and its pH was raised to 10 with concentrated sodium hydroxide to inactivate the pepsin.

(vii) The total volume of the antibody mixture was measured, and an equal volume of saturated ammonium sulfate was added to precipitate and recover the $F(ab')_2$.

(viii) After centrifugation to pack the precipitate, the supernatant was discarded; the precipitate was dissolved in distilled water and then reprecipitated with ammonium sulfate to wash the protein.

(ix) After centrifugation to recover the second sulfate precipitate, the protein was redissolved in distilled water and then dialyzed in 0.05 M borate-0.15 M sodium chloride (pH 7.4) to remove the sulfate before column chromatography.

(x) The sulfate-free antibody was applied to a column of protein A-Sepharose CL-4B and eluted with the borate-saline solution. The final $F(ab')_2$ product did not bind to protein A and contained approximately one-half the total protein of the original IgG sample.

No other protein was obtained during acid desorption of the column.

The total protein concentrations of the globulin fractions were measured by the biuret method (8). The protein concentrations of the IgG and $F(ab')_2$ samples were estimated by absorption at 280 nm, assuming $E_{1\,cm}^{1\,mg/ml} = 1.4$. All antibody fractions were then labeled with fluorescein isothiocyanate (FITC) by the direct method, in which various dye weights were used to achieve the desired F/P ratios (11). Unreacted FITC was removed by dialysis in pH 9.0 to 9.5 phosphate-buffered saline.

All of the conjugates for a given antigen were prepared from the same lot of antisera and were adjusted to approximately equal protein concentrations. All conjugates were titrated with their homologous antigens. The test antigens were bacterial cell suspensions in 0.85% sodium chloride containing 1% Formalin. Smears were prepared and then stained with serial twofold dilutions of the conjugates according to a standard direct fluorescent-antibody procedure (14). The stained smears were examined by fluorescence microscopy, and the conjugate titers were expressed as reciprocals of the highest dilutions, relative to the initial adjusted protein concentration, that retained a 3+ or greater staining of the bacterial cells. All of the conjugates were also examined for physicochemical characteristics. Protein concentrations were measured by the biuret method with optical density readings at 560 nm, FITC concentrations were determined by absorbance in 0.1 N sodium hydroxide at λ_{max} (493 nm), F/P ratios were expressed as micrograms of FITC per milligram of protein, and protein compositions were examined by cellulose acetate strip electrophoresis (11).

RESULTS

All of the globulin preparations obtained by fractionation with ammonium sulfate were at least 75% gamma globulin and contained no more than 1% albumin. The characteristics of the conjugates prepared from these fractions are shown in Table 1. With each of these eight bacterial systems, the highest specific staining titer was obtained at the highest F/P ratio prepared and tested. Most of these maximum F/P ratios were between 27 and 31 μg of FITC per mg of protein. Another relationship calculated was the F/T ratio, or micrograms of FITC at the specific titer dilution. In all systems studied, the lowest F/T ratio was associated with the maximum specific staining titer and the highest F/P ratio.

The first seven conjugates in Table 2 and all of the conjugates in Table 3 were prepared from the same pool of *L. pneumophila* antisera obtained by combining the sera of the last three bleedings from a rabbit immunized with the Philadelphia 2 strain (4) of serogroup 1. The characteristics of conjugates prepared with IgG fractions desorbed from protein A are shown in Table 2. Again, the specific staining titer increased and the F/T ratio decreased as the F/P ratio increased from below 20 up to approximately 30. Above an F/P ratio of approximately 32, the specific staining titer decreased and F/T ratio increased as the F/P ratio increased.

The $F(ab')_2$, IgG, and globulin conjugates prepared from *L. pneumophila* antisera were all examined at a protein concentration of approximately 5 mg/ml (Table 3). The labeling efficiency of the $F(ab')_2$ preparations was extremely low compared to the IgG and globulin (88% IgG) fractions. The same high specific staining titer was obtained with all three preparations, but the globulin, IgG, and $F(ab')_2$ conjugates with this 1:320 titer had quite different F/P ratios of 30, 28, and 8, respectively.

DISCUSSION

The optimal F/P ratio for globulin and IgG preparations with all of the systems studied was approximately 30; although this is higher than most reports recommend, it is necessary with bacterial systems in order to obtain high titers and bright staining. The nonspecific staining expected with high F/P ratios was not a problem with any of these systems because the resulting high titers allowed greater dilution without sacrificing specific staining intensity, and dilution is very effective in reducing nonspecific staining (10). This relationship is expressed in the F/T ratio, which is the micrograms of protein-bound FITC in 1 ml of conjugate at the specific titer dilution. Nonspecific staining is directly related to the FITC concentration (10), so the F/T ratio is a measure of the nonspecific staining potential of a reagent. In this study, the lowest F/T ratios were always associated with the highest specific staining titers and the high F/P ratios near 30, but further increases in F/P ratio caused lower specific staining titers and higher F/T ratios.

Under identical labeling conditions at 5 mg of protein per ml with 40 μg of FITC per mg of protein, the globulin, IgG, and $F(ab')_2$ preparations had labeling efficiencies (microgram ratio of bound FITC to original reaction mixture weight) of 75, 70, and 20% for final F/P ratios of 30, 28, and 8, respectively (Table 3). Despite these differences, all three conjugates had the same high specific staining titer of 1:320. Since IgG is only 50% $F(ab')_2$ by weight, the remainder being Fc piece, the IgG conjugate and the globulin conjugate of this antiserum appear to be about twice as effective as the $F(ab')_2$ conjugate on a molar basis for direct fluorescent-antibody staining. This can be readily accounted for on the basis of the higher degree of labeling obtained with intact IgG relative to that obtained

TABLE 1. *Characteristics of conjugates prepared from ammonium sulfate-fractionated rabbit antisera*

Labeled antibody	F/P ratio (μg/mg)	Specific staining titer[a]	Protein concn (mg/ml)[b]	FITC concn (μg/ml)	F/T ratio (μg/ml)[c]
Escherichia coli O18ac:B21:H7	3	32	9	27	0.84
	10	128	9	90	0.70
	17	256	9	153	0.60
	29	512	9	261	0.51
Bordetella pertussis	5	20	6	30	1.50
	18	80	6	108	1.35
	23	160	6	138	0.86
	27	320	6	162	0.51
Streptococcus pyogenes (group A)	3	<1	1.6	4.8	<5
	6	10	1.6	9.6	0.96
	9	50	1.6	14.4	0.29
	16	100	1.6	25.6	0.26
	28	500	1.6	44.8	0.09
Listeria monocytogenes (type 4b)	8	16	5	40	2.5
	14	32	5	70	2.2
	27	256	5	135	0.5
Salmonella (polyvalent)	11	10	14	154	15.4
	17	20	13	221	11.1
	37	160	9	333	2.1
Shigella dysenteriae (A1)	14	16	11	154	9.6
	31	64	6	186	2.9
Clostridium septicum	11	64	7	77	1.2
	31	256	7	217	0.8
Bacteroides fragilis	10	20	3	30	1.5
	16	32	3	48	1.5
	21	40	3	63	1.6
	28	100	3	84	0.8

[a] Specific staining titer = reciprocal of the highest dilution that retained a 3+ or greater staining of the homologous cells.
[b] Measured by the biuret method.
[c] F/T ratio = FITC concentration at the specific staining titer.

with the $F(ab')_2$ preparation. If we assume the same 20% labeling efficiency with the $F(ab')_2$ portion of the IgG in the IgG conjugate as was observed in labeling $F(ab')_2$ that was freed of the Fc piece, then approximately 86% of the FITC was bound to the Fc piece in the IgG conjugate. It appears, therefore, that the Fc piece serves as an area for attaching a heavy load of fluorescein without interfering with the antigen-antibody reaction.

The use of $F(ab')_2$ conjugates eliminates the well-known reaction of intact IgG molecules with *S. aureus* strains that contain cell-bound *Staphylococcus* protein A. This undesired activity can be blocked, however, by using rhodamine-labeled preimmunization IgG (5) as a diluent for the FITC-IgG conjugate. In this laboratory we prefer to use rhodamine-labeled preimmunization whole serum as the diluent. The added benefit of the rhodamine counterstain has been well documented (6, 17) and is particularly useful when examining deparaffinized tissue sections and sputum specimens (3). Although Danielsson and Forsum (6) reported no significant difference between IgG and $F(ab')_2$ conjugates at comparable F/P ratios, several differences in methods of preparation, labeling, and analysis make it difficult to directly compare our results. Further studies are, therefore, desirable, using IgG and $F(ab')_2$ conjugates to stain microbial antigens in diseased tissue sections where background and nonspecific fluorescence are diagnostic problems.

Conjugation procedures have been developed to produce reagents with any degree of labeling (F/P ratio) desired. The chosen F/P ratio is best

TABLE 2. *Characteristics of conjugates prepared from rabbit antibody (IgG) desorbed from protein A-Sepharose CL-4B*

Labeled antibody	F/P ratio (μg/mg)	Specific staining titer[a]	Protein concn (mg/ml)[b]	FITC concn (μg/ml)	F/T ratio (μg/ml)[c]
Legionella pneumophila Philadelphia 2	7.6	20	5.0	38	1.90
	14.7	80	5.1	75	0.94
	21.2	160	5.0	106	0.66
	27.9	320	5.1	143	0.45
	31.3	320	5.3	166	0.52
	37.7	160	5.3	200	1.25
	44.3	80	5.1	226	2.83
L. pneumophila Togus 1	18.0	320	3.8	68	0.21
	26.0	640	4.1	107	0.17
L. bozemanii (WIGA)	30.1	1,024	10	301	0.29
L. micdadei (TATLOCK)	28.4	1,024	10	284	0.28
L. micdadei (HEBA)	31.0	1,024	10	310	0.30
L. dumoffii (Tex-KL)	29.0	1,024	10	290	0.28
Streptococcus mutans (type d)	29.8	2,048	12.6	375	0.18
	31.7	2,048	12.6	400	0.20

[a] Specific staining titer = reciprocal of the highest dilution that retained a 3+ or greater staining of the homologous cells.
[b] Measured by the biuret method.
[c] F/T ratio = FITC concentration at the specific staining titer.

TABLE 3. *Characteristics of F(ab')$_2$, IgG, and globulin conjugates prepared from the same pool of L. pneumophila antisera*

Labeled antibody	Reaction mixture ratio[a]	Labeling efficiency (%)[b]	F/P ratio (μg/mg)	Specific staining titer[c]	Protein concn (mg/ml)[d]	FITC concn (μg/ml)	F/T ratio (μg/ml)[e]
F(ab')$_2$	20	11	2.1	5	4.9	10	2.0
IgG	20	74	14.7	80	5.1	75	0.94
F(ab')$_2$	40	20	7.9	320	4.9	39	0.12
IgG	40	70	27.9	320	5.1	143	0.45
Globulin (88% IgG)	40	75	30.0	320	5.1	153	0.48

[a] Reaction mixture ratio = micrograms of FITC per milligram of protein during labeling at 25°C, pH 9.5.
[b] Labeling efficiency = [bound FITC (micrograms per milligram of protein)/reaction mixture FITC (micrograms per milligram of protein)] × 100.
[c] Specific staining titer = reciprocal of the highest dilution that retained a 3+ or greater staining of the homologous cells.
[d] Measured by the biuret method.
[e] F/T ratio = FITC concentration at the specific staining titer.

achieved by controlling the labeling reaction conditions and using FITC of known high purity. The Tabeling reaction is most efficient chemically at 25°C with a pH of 9.5 in 0.05 M Na_2HPO_4. The pH is critical to achieve the higher F/P ratios: for example, a volume of globulin was given 40 μg of FITC per mg of protein; one-half of the mixture was removed and its pH was adjusted to 7.0; the remaining one-half was adjusted to pH 9.5, and then both preparations were set aside to react. The final F/P ratio of the pH 7 sample was only 11, but the pH 9.5 sample had the desired F/P ratio of 31. The pH is also critical during dialysis and storage of conjugates with high F/P ratios. These reagents must be kept at pH 9.0 to 9.5 to avoid precipitation. If some precipitate develops during dialysis or storage, the pH should be checked and adjusted if indicated.

Studies of viral and rickettsial reagents recommend medium F/P ratios of 10 to 15 μg/mg because conjugates with higher ratios exhibit a prohibitive degree of background fluorescence (13, 15, 18). The data in this report show why

we can recommend a high F/P ratio of 30 μg/mg for bacterial conjugates; the high ratio gives us the optimal conditions of maximum specific staining titer with a minimum of nonspecific staining, and that allows full utilization of the potential of a given lot of antibody as a diagnostic reagent for fluorescent-antibody identification of bacteria.

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