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Evaluation of the Immunological Specificity of Fluorescein-Labelled Anti-Human IgM Conjugates

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Summary. The immunological specificity of two anti-IgM fluorescein-labelled antisera was evaluated in gel diffusion, by the direct immunofluorescent technique on characterized bone marrow preparations taken from patients with myeloma and by an indirect staining method employing virus-infected cells and selected post-infection sera. The results show that neither gel diffusion nor direct immunofluorescent methods provide a reliable index of specificity for conjugates to be used in indirect procedures.

INTRODUCTION

Fluorescein-labelled antisera are frequently used in indirect immunofluorescent procedures to determine the immunoglobulin class of specific antibody following infection with bacteria (Julian, Logan and Norins, 1969), viruses (Baublis and Brown, 1968; Brown, Baublis and O'Leary, 1970; Haire and Hadden, 1970; Connolly, Haire and Hadden, 1971) and parasitic organisms (Kane, Matossian and Batty, 1971). Although methods of preparing fluorescein-labelled antisera to the heavy chains of immunoglobulin molecules have been described, little information has been presented concerning the specificity of these reagents in fluorescence tests (Takahashi, Yagi and Pressman, 1968; Reisberg, Rossen and Butler, 1970). A method of evaluating specificity by a direct immunofluorescent test using bone marrow preparations obtained from patients suffering from IgG, IgA or Bence-Jones myeloma of either type or from Waldenström's macroglobulinaemia has been described (Hijmans, Schuit and Klein, 1969). Direct immunofluorescent procedures are, however, generally less sensitive than indirect techniques; this is related to the increased number of sites available for combination with labelled antibody in the latter procedure (Nairn, 1969). In view of this we thought it of value to establish whether specificity as defined by the pattern of staining obtained with characterized bone marrow preparations provided a reliable index of specificity for conjugates to be used in indirect techniques. The specificity of two anti-human IgM conjugates was evaluated in direct tests performed on bone marrow preparations and in indirect systems employing selected post-infection sera and virus-infected tissue culture cells.

EXPERIMENTAL METHODS

Preparation of anti-human IgM conjugates

(a) *Preparation and selection of antisera.* Antisera were raised in sheep by multiple injections of purified IgM obtained from Waldenström sera. At least three different IgM preparations

were used for immunization. Hyperimmune sera were selected for conjugation on the basis of titre by a standard gel diffusion technique. Dilutions of antisera were titrated against a standard solution of purified human IgM (1 mg/ml). Sera giving a clear precipitation line at a dilution of 1:16 or more were used for subsequent labelling (Beutner, Sepulveda and Barnet, 1968).

(b) *Fractionation of antisera.* The globulin fraction was separated from whole serum by treatment with Rivanol (2-ethoxy-6,9-diaminoacridine lactate) followed by precipitation with 50 per cent saturated ammonium sulphate.

(c) *Absorption with insoluble antigen polymers.* The globulin fraction obtained from whole serum was absorbed prior to labelling with insoluble cord serum polymers prepared by the method described by Avrameas and Ternynck (1969). Cord sera were screened for the presence of IgG, IgM and IgA in gel diffusion; IgM deficient samples were used as antigen. Following absorption the specificity of the solution was assessed in gel diffusion against normal human serum and purified preparations of IgG, IgM and IgA and Fab_2. Labelled globulin fractions (conjugates) were absorbed with cord serum polymers and insolubilized IgG and IgA immunoglobulin rich fractions obtained by ion-exchange chromatography.

(d) *Conjugation.* Globulin fractions shown to be specific for IgM in gel diffusion were labelled with fluorescein isothiocyanate (isomer 1). Unreacted dye was separated from the labelled fraction by gel filtration on Sephadex 50 and by dialysis against phosphate buffered saline.

Titration of labelled anti-human IgM conjugates

The specific titres of labelled anti-human IgM conjugated were assessed in the following.

(i) A direct immunofluorescent test on polyclonal bone marrow preparations as described by Hijmans *et al.* (1969).

(ii) Indirect immunofluorescent tests: (1) cryostat sections of calf thyroid were treated with dilutions of infectious mononucleosis serum prior to incubation with dilutions of anti-IgM conjugate. A positive result was indicated by staining of the vascular endothelium (Johnson and Holborow, 1963; Holborow and Johnson, 1970); (2) coverslip tissue cultures infected with measles or rubella virus were treated with dilutions of sera selected from patients with measles or rubella diagnosed by conventional serological tests and shown by previous titration with conjugates of known specificity to have antibodies of IgM class only. Following incubation and washing, the cultures were treated with dilutions of conjugate (Haire and Hadden, 1970; Connolly, Haire and Hadden, 1971).

The specificity of the conjugates was assessed by the direct staining technique on characterized monoclonal bone marrow preparations and by the indirect technique on virus-infected cells. Two groups of antiviral sera were selected for these specificity tests; early post-infection sera which contained specific antiviral IgM antibodies in the absence of demonstrable IgG class antibodies, and late post-infection sera containing specific antibodies of IgG type only. These sera were selected with the aid of standard anti-human IgG and anti-human IgM conjugates (Table 1).

RESULTS

Two anti-human IgM conjugates were prepared and evaluated by these procedures. The working dilutions (i.e. those giving intense specific fluorescence with minimal background staining) of these conjugates in both direct and indirect tests are shown in Table 2.

TABLE 1

ANTI-VIRAL ANTIBODY LEVELS IN SELECTED POST-INFECTION SERA DEMONSTRATED BY THE INDIRECT IMMUNOFLUORESCENT TEST USING STANDARD ANTI-HUMAN IgG AND IgM CONJUGATES

Serum	Post-infection interval		Reciprocal of highest dilution of post-infection serum giving fluorescence with:	
			Anti-IgG FITC	Anti-IgM FITC
*M2A	3 days	acute	—	10
M6A	4 days		—	5
M17A	3 days		—	5
M19A	2 days		—	5
†R122A	3 days		—	5
R135G	14 weeks		20	—
R131G	15 weeks		80	—
R122H	19 weeks		40	—
R98B	26 weeks		40	—
R57C	35 weeks		20	—
R37C	36 weeks		40	—
M2C	4 years		20	—
M6C	4 years		20	—
M19C	4 years		20	—

* Sera from patients with measles infection.
† Sera from patients with rubella infection.

TABLE 2

THE WORKING DILUTIONS OF TWO ANTI-HUMAN IgM CONJUGATES IN DIRECT AND INDIRECT IMMUNOFLUORESCENT TESTS

Conjugate	Direct IF bone marrow	Indirect IF	
		Infectious mononucleosis	Viral
1	1:2–1:8	1:4–1:64	1:4–1:64
2	1:2–1:8	1:4–1:64	1:4–1:64

TABLE 3

THE PATTERN OF FLUORESCENT STAINING OBTAINED WITH TWO ANTI-HUMAN IgM CONJUGATES ON A SERIES OF CHARACTERIZED MONOCLONAL BONE MARROW PREPARATIONS

Conjugate	Dilution of conjugate	Staining observed with bone marrow preparations											
		IgM*		IgG*					IgA*			Light chains	
		1	2	1	2	3	4	5	1	3	4	κ	λ
1	1:4	+	+	+	+	+	tr	N	+	+	N	+	—
2	1:4	N	+	N	N	tr	N	—	N	+	+	N	—

* Numbers identify marrow samples from different patients.
tr = Trace fluorescence.
N = Not tested.

The staining reactions obtained on a series of characterized bone marrow preparations are shown in Table 3. Rhodamine labelled bovine serum albumin was used as a counter-stain in these experiments in order to eliminate reactions which might be attributable to non-specific fluorescence. Weak but definite green fluorescence was observed with most samples of IgG, IgA and κ light chain myloma bone marrows tested despite the fact that

TABLE 4

THE PATTERN OF FLUORESCENT STAINING OBTAINED WITH REABSORBED ANTI-HUMAN IgM CONJUGATES ON A SERIES OF CHARACTERIZED MONOCLONAL BONE MARROW PREPARATIONS

Conjugate	Dilution of conjugate	Staining observed with bone marrow preparations									
		IgM*	IgG*					IgA*		Light chains	
		1	1	2	3	4	5	6	2	3	κ	λ
1a	1:4	+	—	—	+	—	N	N	—	tr	—	—
2a	1:4	+	N	N	—	tr	—	—	—	tr	—	—

* Numbers identify marrow samples from different patients (code as for Table 3).
tr = Trace fluorescence.
N = Not tested.

TABLE 5

LEVELS OF VIRAL-SPECIFIC ANTIBODIES IN SELECTED POST-INFECTION SERA OBTAINED WITH STANDARD AND TEST ANTI-HUMAN IgM CONJUGATES BY AN INDIRECT IMMUNOFLUORESCENT PROCEDURE

Post-infection sera		Reciprocal of highest antiserum dilution of serum giving fluorescence with conjugates			
		Standard		Tested	
Early	Late	Anti-IgG	Anti-IgM	1a* Diluted 1:32	2a† Diluted 1:32
M2A		—	10	40	20
M6A		—	5	20	20
M17A		—	5	20	N
M19A		—	5	20	20
R122A		—	5	5	5
R135A		—	5	5	N
	R37C	40	—	N	5 weak
	R131G	80	—	N	5 weak
	R57C	20	—	N	5 weak
	R135G	20	—	20	5 weak
	R98G	40	—	10	N
	R122H	40	—	10	N
	M2C	20	—	40	—
	M6C	20	—	10	—
	M19C	20	—	20	—

* Conjugate 1 reabsorbed with cord polymer.
† Conjugate 2 reabsorded with cord polymer.

gel diffusion tests performed with the concentrated globulin fraction used for conjugation had shown complete specificity for IgM.

As a result of these findings conjugates 1 and 2 were reabsorbed with cord serum polymers (1a and 2a) and retested on bone marrow preparations (Table 4) and by the indirect immunofluorescent technique on virus-infected tissue cultures (Table 5). It may be seen from Table 4 that trace reactions were obtained with one out of four IgG marrows and one of the two IgA marrows tested. It is of interest to note that the fluorescence obtained with

the two conjugates was against different IgG marrow samples. However, when the conjugates (1a and 2a) were tested in the indirect immunofluorescent technique the presence of undesirable cross reactivity was plainly apparent (Table 5). Samples of late post-infection sera which had been shown not to contain antiviral IgM antibodies by tests with standard conjugates showed positive fluorescence with conjugate 1a. The results obtained with conjugate 2a were less striking but none the less weak positive reactions were observed with some late post-infection sera.

After further absorptions, on this occasion with an insoluble preparation of IgG and IgA, the conjugates (1b and 2b) were retested and found to be specific in both direct and indirect procedures. Table 6 shows the results in the indirect test before and after the final absorption.

TABLE 6

LEVELS OF VIRAL SPECIFIC ANTIBODIES IN SELECTED POST-INFECTION SERA OBTAINED WITH TWO ANTI-HUMAN IgM CONJUGATES BEFORE AND AFTER FINAL ABSORPTION WITH IMMUNOGLOBULIN POLYMER

Post-infection sera		Reciprocal of highest dilution of serum giving fluorescence with conjugates:			
Early	Late	1a* diluted 1:32	1b† diluted 1:16	2a* diluted 1:32	2b† diluted 1:32
M2A		40	10	20	20
M6A		20	10	20	20
M17A		20	10	N	N
M19A		20	10	20	20
R122A		5	—	5	5
	R37C	N	—	5 weak	—
	R131G	N	—	5 weak	—
	R57C	N	—	5 weak	—
	R135G	20	—	5 weak	—
	R98G	10	—	N	N
	R122H	10	—	N	N
	M2C	40	—	—	—
	M6C	10	—	—	—
	M19C	20	—	—	—

* Conjugate before absorption with immunoglobulin polymer.
† Conjugate after absorption with immunoglobulin polymer.
N=Not tested.

DISCUSSION

These results show that specificity in gel diffusion cannot be taken as a criterion of specificity for fluorescein-labelled antisera.

Characterized bone marrow preparations provide excellent substrates for defining specificity in an immunofluorescent system because it is possible to pinpoint cross reactions between the three major immunoglobulin classes and against light chains. However, although the number of antigenic sites available for combination with labelled antibody in plasma cells synthesizing large amounts of immunoglobulins might be expected to be similar to that available for the second stage of an indirect technique, the results presented in this paper do not support this view. Reactions due to contaminating anti-IgG antibodies in the conjugates were more readily detected by indirect test procedures than by direct tests on bone marrow cells. Evaluation of conjugates by the former procedure does therefore provide a more accurate measure of specificity.

Conjugates 1a and 2a gave weak reactions against different IgG positive marrow preparations (Table 4). This is likely to be a reflection of configurational differences between heavy chains of the same immunoglobulin class (Cohen and Milstein, 1967; Rowe, 1968) and indicates that the failure to get a reaction with a single bone marrow sample does not preclude reactions against preparations obtained from other patients suffering from the same class of immunoglobulin paraproteinaemia. This problem would not be encountered in indirect tests employing whole serum which contains a heterogeneous population of immunoglobulins.

It is evident that indirect techniques should be used in preference to direct tests for defining the immunological specificity of anti-human immunoglobulin conjugates as the former are more sensitive and less likely to give false negative results. However, although the presence of unwanted anti-IgG or IgM reactivity may be detected in this way by appropriate selection or fractionation of intermediary antisera, indirect systems for assessing anti-IgA reactivity are not readily available. Until they become so, the presence of anti-IgA reactivity must still be assessed by the direct technique using IgA myeloma bone marrow preparations.

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