TECHNICAL NOTE

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An Exploratory Study on the Characterization of A_1 and A_2 Bloodstains Using a Fluorescence Immunoassay

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ABSTRACT: A fluorescence immunoassay method is used in the differentiation of A_1 and A_2 bloodstains. Anti-A antiserum is first absorbed onto the stain. The IgM content in the eluate is then quantified by commercial Immuno-Fluor kits. The binding difference of A_1 and A_2 cells is retained in stains. This difference and the quantitative evaluation method provide a potential basis for blood typing or subtyping application.

KEY WORDS: blood, genetic typing, immunoassay

To improve the discrimination power of the ABO antigen system, forensic serologists [*1,2*] have explored the possibility of using lectins for the qualitative subgrouping of Type A bloodstains. However, it is still thought that "a completely reliable method for A_1/A_2 distinction in dried stains has yet to be developed" [*3*].

A logical approach to this problem is the use of quantitative techniques to distinguish the IgM binding site differences between A_1 and A_2 red cells [*4-7*]. In this study, commercial Immuno-Fluor (Bio-Rad Laboratories, Richmond, Calif.) kits were used for the quantification of IgM after a commercial anti-A antiserum (Dade Division, American Hospital Supply, Miami, Fla.) had been absorbed into and then eluted from test stains. It was claimed [*8*] that as little as 100 ng/mL immunoglobulin can be analyzed by the Immuno-Fluor kit.

Experimental Procedure

Bloodstain Preparation

The stain preparation method is adopted from that of Lincoln and Dodd [*9*]. Blood samples of known type, A_1, A_2, and O, were packed at 3000 rpm for 6 min and the

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supernatant plasma was removed. The cells were washed at least six times in saline and repacked between washings. Cells were then broken, and 10 μL of cells was placed on 1-cm-wide cotton cloth or cotton bandage gauze. These stains were allowed to dry for a desired duration and were then cut into equal sizes.

Absorption-Elution

Diagnostic anti-A antiserum obtained from American Hospital Supply was used for absorption. By using the fluorescence assay for human immunoglobulins described later, this serum was found to contain 765 μg/mL IgM and an insignificant amount of IgG. Two hundred microlitres of this serum of desired dilution was used for overnight absorption at 40°C. The liquid was then removed from the tube with a pipet attached to an aspirator. Stains were washed by adding 2 mL of saline at 4°C and then spinning them in a vortex for 5 s. This was repeated six times. The elution step began with the addition of 200 μL of phosphate-buffered (pH 7.3) saline to each stain. Stains were incubated, rotated at 55°C for 30 min, and then immediately removed. Aliquots (100 μL) of the eluate were removed for immunofluorescent quantification, as described below. The remaining eluate was tested against one drop of a 5% suspension of washed A_1 cells.

IgM Fluorescence Immunoassay

Immuno-Fluor kits obtained from Bio-Rad Laboratories were used for the quantification of IgM in the eluates. Each kit includes lyophilized solid phase anti-IgM antibody, lyophilized fluorescein-isothiocyanate (FITC)-labeled anti-IgM antibody, and concentrated IgM standard. For each eluate and standard, a 100 μL aliquot was incubated with 1 mL of reconstituted solid phase antibody for 1.5 h at 37°C. Then 50 μL of FITC anti-IgM solution was added, and the tubes were incubated for 1 h at 37°C. The solid phase antibody-IgM-FITC antibody complex was washed with 2 and then 3 mL of saline, and 1 mL of phosphate buffer (pH 7.3) was added to each tube. This final solution was measured for its fluorescence at 525 nm (excitation, 485 nm) with an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Silver Spring, Md.). Data obtained were calculated and graphically presented by a computer program written for this purpose. A typical standard curve is shown in Fig. 1. Concentrations of IgM in stain eluates were calculated by using the equation obtained from least-square regression.

Results and Discussion

Assuming there are 5×10^9 cells per millilitre of whole blood, that whole blood contains 50% (v/v) cells, and that there are 10^6 A sites per A_1 cell [*4–7*], a 10-μL stain prepared by the described procedure contains approximately 10^8 cells and 10^{14} antigen sites. IgM is decavalent [*10*] and, therefore, requires 10^{13} molecules to cover these antigen sites. Using a molecular weight of 850 000 for IgM, it is estimated that 14 μg of IgM will be absorbed and then eluted from each stain. With this estimation and the ratio of 5:1 antiserum/cell suggested by Boorman et al [*11*], A_1 and A_2 stains were experimentally tested with undiluted and with 50, 25, and 12.5% (v/v) anti-A antiserum. Results presented in Table 1 indicate this quantitative fluorescence method gives better resolution over the qualitative eluate agglutination score method. Since eluates from A_1 and A_2 cells were measured at different instrumental conditions without standards, direct comparison is not valid. However, it is concluded that undiluted antiserum or a 50% dilution of the commercial antiserum is suitable for absorption. Undiluted antiserum seems to give better resolution, while a 50% dilution of antiserum gives better precision.

Commercial 50% anti-B antisera obtained from American Hospital Supply were also

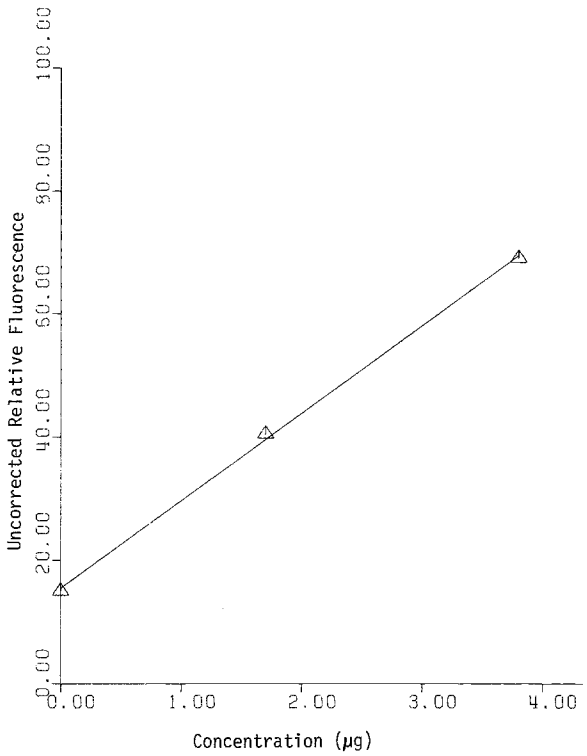

FIG. 1—*Standard curve for the evaluation of IgM content in stain eluates. The least-square fitted line is* I = *14.2*C + *15.5, where* I = *uncorrected relative fluorescence and* C = *IgM concentration. The standard deviations of the slope and the intercept are 0.41 and 0.98, respectively.*

TABLE 1—*Determination of best antigen/antibody ratio for quantitative fluorescence determination of IgM content in eluates.*

Antiserum[a] Concentration, %	Eluate Agglutination Score	Eluate[b] Relative Fluorescence
	Type A_1	
100	4+, 4+, 4+	75.5, 64.5, 85.0
50	4+, 4+, 4+	51.0, 55.0, 58.0
25	3+, 3+, 3+	41.5, 37.5, 58.0
12.5	3+, 3+, 3+	28.5, 32.0, 30.0
	Type A_2	
100	4+, 4+, 4+	33.0, 30.0, ...
50	4+, 4+, 4+	23.0, 23.5, 23.0
25	3+, 3+, 3+	20.5, 18.5, ...
12.5	3+, 3+, 3+	14.0, 12.0, 15.5

[a] 200 μL of commercial antiserum of described concentration was absorbed by stains prepared with 10 μL packed cells.

[b] 100 μL (of 200 μL total) phosphate-buffered (pH 7.3) saline eluate was measured for its fluorescence.

applied to stains prepared from various blood groups. Results in the lower part of Table 2 indicate identification of Type B blood can also be made with this procedure. Compared to the anti-A antiserum results, Type B bloodstains seem to elute less IgM. This is inconsistent with the reported lower antigen sites in B cells [*5*]. It is also possible that the IgM concentration in the anti-B antisera used for absorption was lower.

Table 3 presents results for the quantification of IgM content eluted from two sets of A_1-A_2-O stains. In either set, clear distinction of the subtypes was made. Assuming that the IgM concentration for O cell eluate represents nonspecific absorption, the IgM concentrations in the A_1 and A_2 eluates were corrected to 2.88 and 0.73 μg/mL, respectively, in the first set of stains. The A_1/A_2 ratio is almost 4, which is in excellent agreement with the ratio of antigen sites in fresh blood as established by various workers with various methods [*4–7*]. Assuming 10^8 cells per stain and an IgM valance of 10, the first set of stains yields 4.1×10^2 and 1.0×10^2 per cell for A_1 and A_2 stains, respectively. These values are considerably lower than those of fresh blood, as discussed previously. This difference is explained by the solubility of the antigenic material (lysed cells in stains), the steric arrangement of cells in the substrate, and the accessibility of antigenic sites to antibody molecules.

Results presented in the second half of Table 3 were obtained from stains aged for 27 days. The higher concentration of IgM in eluates of these aged stains is probably due to the more complete drying and subsequently greater insolubility of the antigen material in the older stains [*12*]. However, the distinction between A_1 and A_2 stains seems to decrease. Whether this points to a qualitative difference between A_1 and A_2 antigenic sites is not certain.

TABLE 2—*Fluorescence characteristics of eluates prepared from the absorption of anti-A and anti-B antisera by various blood groups.*

Blood Type (5-μL Stain)	Eluate Agglutination Score	Eluate Relative Fluorescence	Corrected Relative Fluorescence
	Anti-A Antiserum		
A_1	3+, 3+	81.0, 80.0	21.0, 20.0
A_2	3+, 3+	74.0, 76.0	14.0, 16.0
B	2+	59.0	0.0
O	−	60.0	0.0
	Anti-B Antiserum		
B	3+, 3+	55.0, 53.0	9.0, 7.0
AB	2+, 3+	52.0, 49.5	6.0, 3.5
A_1	−	44.0	0.0
A_2	−	46.0	0.0
O	−	46.0	0.0
Blank	−, −	42.0, 44.0	0.0, 0.0

TABLE 3—*IgM content in bloodstain eluate.*

Age, days	Blood Type	Eluate Relative Fluorescence	Average IgM Concentration, μg/mL	Total IgM Recovered, μg
1	A_1	69.0, 60.5, 55.0	3.54	0.708
1	A_2	31.5, 36.0, 31.0	1.39	0.278
1	O	19.5, 25.0, 24.5	0.66	0.132
27	A_1	78.5, 82.0	4.85	0.964
27	A_2	52.0, 58.0	3.10	0.620

The high IgM contents in blank and Type O stain eluates indicate the high degree of nonspecific absorption. However, these nonspecifically absorbed IgM components are not necessarily anti-A (or anti-B) antibodies. They are probably other IgM components present in the antisera. This reasoning is based on the fact that although blank and O cell eluates contain rather large quantities of IgM, they do not agglutinate with appropriate cells. Despite this apparently higher background, the quantitative nature of this method still results in better resolution than the traditional agglutination score method.

This study demonstrated that the quantitative differences between Subtypes A_1 and A_2 are retained in stains, and it explored the possibility of using quantitative fluorescence immunoassay for bloodstain typing or subtyping. However, before this technique can be used in routine work, a more complete study that considers environmental and aging effects on a large population of stains has to be conducted.

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