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The Detection of Soluble ABH Blood Group Substances in Semen and Saliva Using Monoclonal Blood Grouping Reagents

REFERENCE: Mudd, J. L., **"The Detection of Soluble ABH Blood Group Substances in Semen and Saliva Using Monoclonal Blood Grouping Reagents,"** *Journal of Forensic Sciences*, JFSCA, Vol. 34, No. 5, Sept. 1989, pp. 1082-1089.

ABSTRACT: Using an enzyme-linked immunosorbent assay (ELISA), this study investigated the use of monoclonal antibodies for detecting secreted ABH blood group substances in semen and saliva. The results demonstrated that the behavior of some monoclonals were unpredictable and often failed to detect the corresponding antigen in a number of the specimens tested. The suitability of the monoclonal reagents for detecting soluble blood group antigens could not be predicted by their behavior with red cell antigens. Consequently, care must be taken in the selection of monoclonal reagents for use in the detection of secreted blood group antigens.

KEYWORDS: forensic science, genetic typing, semen, saliva, blood groups, ABO blood group system, monoclonal antibodies, ELISA, immunoassay

Monoclonal antibodies are becoming increasingly important tools in the field of blood group serology. Monoclonals have been used to detect the antigens of the ABO, Lewis, Gm, and Rh blood group systems [*1-6*]. This technology offers a stable and reproducible antibody of a desired specificity [*7*]. Although monoclonal antibodies offer certain advantages over conventional human polyclonal blood grouping reagents [*7,8*], some monoclonals reagents may, under certain conditions, produce unexpected results [*9-12*].[2] This study reports on some unexpected grouping results, using monoclonal blood grouping reagents, that were obtained during an evaluation of an enzyme-linked immunosorbent assay (ELISA) for detection of secreted ABH substances in semen and saliva.

Materials and Methods

Semen samples were obtained from a Washington, DC fertility clinic and were maintained at −30°C until assayed. Using standard procedures [*13*], saliva samples were obtained from volunteer donors in the Scientific Analysis Section, FBI Laboratory, Washington, DC, and the FBI Academy, Quantico, Virginia. Mouse monoclonal antibodies against human blood group antigens A, B, and H were obtained from Dako Corporation, Santa Barbara, Califor-

This is Publication 88-05 of the Laboratory Division of the Federal Bureau of Investigation. Names of commercial manufacturers are provided for identification only and inclusion does not imply endorsement by the Federal Bureau of Investigation. Received for publication 23 July 1988; accepted for publication 16 Nov. 1988.

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nia, Allotype Genetic Testing (AGT), Atlanta, Georgia, and Ortho Diagnostics (anti-A and anti-B only), Raritan, New Jersey. Additional mouse monoclonal antibodies against human blood group antigen B (Bioscot, Celtech, and Chembiomed) were supplied by Dr. Steven Fletcher, Home Office Forensic Science Service, Central Research Establishment, Aldermaston, U.K. Human polyclonal anti-A and anti-B, as well as Affirmagen Group A and B and Selectogen Group O red blood cells (RBC), were obtained from Ortho Diagnostics. Affinity purified lectin from *Ulex europeaus* (UEA I), crystallized/lyophilized bovine serum albumin (BSA), 30% BSA solution, goat anti-mouse IgM (u-chain specific) alkaline phosphatase conjugate, and Sigma 104 phosphatase substrate (*p*-nitrophenyl phosphate [PNPP]) were purchased from Sigma Chemical Company, St. Louis, Missouri. EIA purity Tween-20 (polyoxyethylene sorbitan monolaurate) was obtained from Bio-Rad, Richmond, California.

The 96-well V-bottom polystyrene and flat bottom Immulon II microplates were obtained from Dynatech Laboratories, Chantilly, Virginia.

Buffered saline (HBS), used as a RBC and body fluid diluent, consisted of 0.144 *M* sodium chloride and 0.01 *M* HEPES (N-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (Sigma Chemical Co.) at pH 7.2. Buffered saline containing 1% (w/v) BSA was used as a diluent for blood grouping reagents used in the adsorption-inhibition hemagglutination assay. Buffered saline containing 1 (v/v) and 3% (v/v) fish skin gelatin (Hipure liquid gelatin, Norland Products, New Brunswick, New Jersey) was used in the ELISA as an antibody diluent and blocking solution, respectively. The enzyme substrate consisted of PNPP (6 mg/mL) in 0.1 *M* glycine buffer, pH 10.4, containing 0.001 *M* magnesium chloride ($MgCl_2$) and 0.001 *M* zinc chloride ($ZnCl_2$). The ELISA wash (T-20) was prepared as 1 : 20 dilution of a 3*M* sodium chloride (NaCl) stock solution containing 10% (v/v) Tween-20.

The adsorption-inhibition (AI) assay was performed as described by Baechtel [*14*] with the following modification. Before use in the assay, those microplate wells receiving the UEA I lectin were precoated for 30 min at room temperature with 50 μL of 30% BSA, washed three times with HBS, and blotted dry.

The ELISA was configured as a direct binding assay in which 10 μL of the serially diluted antigen (semen or saliva) was deposited in Wells 4 to 12 of Rows A-B, D-E, and G-H of the microplate. The wells in Column 1 received only PNPP and were used as a substrate blank. Wells in Columns 2 and 3 received HBS (10 μL per well) in place of the antigen and were used as no antigen controls. Following the addition of antigen or HBS, the microplate was placed, uncovered, on the surface of slide warmer (Fisher Scientific, Columbia, Maryland) set at the maximum temperature and the liquid evaporated to dryness (approximately 30 to 40 min). The wells were then blocked for 1 h at room temperature using 3% fish gel (200 μL per well). Immobilized blood group substances were subsequently detected following the sequential addition (50 μL per well) of the appropriately diluted monoclonal blood grouping reagent (anti-A to Rows A and B, anti-B to Rows D and E, and anti-H to Rows G and H) and enzyme conjugate. All wells were washed manually five times between each addition of the monoclonal, conjugate, and substrate. After addition of the monoclonal and conjugate, the plate was covered and incubated at room temperature for 1 h. Following addition of the PNPP substrate, the plate was covered and incubated at 37°C for 1 h. The absorbance was read at 410 nm using an automated microplate reader (Titertek Multiscan, Flow Laboratories, McLean, Virginia).

Blood group antigen titers from semen and saliva were determined using serial doubling dilutions. Adsorption-inhibition titers were recorded as the reciprocal of the last dilution producing 2+ agglutination [*15*]. ELISA titers were determined separately for each antigen tested. Titers were recorded as the reciprocal of the last dilution for which both replicate wells produced an optical density (OD) that exceeded 0.050, or the mean OD plus three standard deviations of the corresponding no antigen controls, whichever was greater. When titers were compared, a difference of two doubling dilutions was necessary before the titers

were considered significantly different [*16*]. The mean titer values were calculated using the $\log_2$ of the individual titers.

Results

Semen and saliva samples were obtained from 111 and 66 donors, respectively. Since the blood type of the semen donors was unavailable, the assignment of blood group phenotype was based on the grouping results obtained by adsorption-inhibition and adsorption-elution (data not shown). Of the individuals tested, 88 (79%) of the semen donors and 57 (85%) of the saliva donors were classified as secretors. The distribution of the ABO phenotypes determined for these samples is given in Table 1 and is similar to frequency data previously reported [*14*].

The BGS titers, as determined by adsorption-inhibition hemagglutination and ELISA, for the 88 semen specimens are given in Table 2. As shown, the A, B, and H titers for the different phenotypes varied over a wide range with both assays. Of the Group A semen tested ($N = 30$), the levels of A exceeded that of the H in 26 of the samples when tested by inhibition and in 27 when tested by ELISA. Analysis of 12 Group B semen using adsorption-inhibition revealed 10 samples for which the level of the B BGS exceeded that of H. However, when these samples were assayed using ELISA, the level of the B BGS exceeded that of H in only 1 sample. Although the sensitivity obtained by ELISA for detection of A and H BGS greatly exceeded the sensitivity obtained by adsorption-inhibition, no difference in sensitivity was

TABLE 1—*Distribution of ABO blood group phenotypes for semen and saliva specimens used in study.*

Blood Group	Semen ($n = 88$), %	Saliva ($n = 57$), %
O	46.6	49.1
A	34.1	33.3
B	13.6	14.0
AB	5.7	3.5

TABLE 2—*Titers of soluble blood group substances in 88 human semen specimens as determined by adsorption-elution and enzyme-linked immunosorbent assay.*

Blood Group	Number of Specimens	Substance Present	Titer[a]			
			Inhibition		ELISA	
			Mean	Range	Mean	Range
O	41	H	1200	200-51K	185K	26K-819K
A	30	A	7700	400-26K	620K	26K-6500K
		H	880	50-26K	118K	26K-819K
B	12	B	5700	800-102K	12K	ND[b]
		H	1500	100-51K	205K	51K-819K
AB	5	A	10K	50-102K	800K	13K-6550K
		B	19K	2K-205K	670	ND-26K
		H	600	400-800	235K	102K-1600K

[a]Reciprocal of last tube dilution producing a positive signal.
[b]Antigen not detected at lowest dilution tested.

observed between the 2 assays when testing for the B BGS. Of the 12 group B semen tested, the ELISA failed to detect the presence of the B BGS in 2 specimens at the lowest dilution tested (1/200).

A summary of BGS titers determined for the 57 saliva samples identified as having originated from secretors is presented in Table 3. For most specimens, the ELISA proved more sensitive than AI for detecting soluble BGS. While both AI and ELISA revealed a wide variation in BGS titers, 3 of the samples (1 O and 2 A) showed no detectable H BGS by AI at the lowest dilution (1/12) tested. Of these three samples, only 1 showed no detectable H antigen by ELISA at the lowest dilution (1/400) tested. Further, 6 specimens, which had produced H titers between 12 and 100 by AI, showed no detectable H BGS by ELISA.

The inability of the Dako anti-B monoclonal reagent to detect the B antigen in two of twelve semen and in one of eight saliva, as well as the poor response of the monoclonal reagent with the remaining Group B semen and saliva samples, prompted the evaluation of six additional monoclonal anti-B blood grouping reagents for use in ELISA. These reagents were tested in both the AI and ELISA procedures.

These six monoclonal reagents, as well as the Dako monoclonal and the Ortho polyclonal anti-B reagents, were used to determine the inhibition titers for the twelve group B semen identified in the initial study. These results are presented in Table 4. Using the polyclonal anti-B reagent, the B antigen was successfully detected in the twelve Group B semen with an average titer of 5K. However, only two of the seven monoclonal reagents tested by AI (Bioscot and AGT-2) were capable of detecting the B antigen in all twelve samples tested. The mean B titers determined for the Bioscot (13K) and AGT-2 (2K) reagents were significantly higher than the titers obtained using the other monoclonal reagents (160–650).

When these MAB reagents were used in ELISA to titer the same group B semen (Table 5), only Bioscot, Bioclone, and Celtech reacted with all twelve Group B specimens. The MAB AGT-2, which previously tested positive by inhibition with the twelve semen, failed to react with these same specimens when tested by ELISA. Of the monoclonals tested, only Bioscot had reacted in both assays with all specimens tested. In addition, the Bioscot reagent was the most reactive in the ELISA, producing titers that were six to thirteen times greater than the titers obtained using the other monoclonal reagents.

The seven MABs were further tested against Group B saliva. As before, the MABs were tested in both the AI and ELISA procedures using the six Group B saliva identified in the initial study. The results of these studies are presented in Tables 6 (AI) and 7 (ELISA).

TABLE 3—*Titers of soluble blood group substances in 57 human saliva specimens as determined by adsorption-elution and enzyme-linked immunosorbent assay.*

Blood Group	Number of Specimens	Substance Present	Titer[a]			
			Inhibition		ELISA	
			Mean	Range	Mean	Range
O	28	H	130	12-400	2700	ND[b]-51K
A	19	A	2300	200-13K	82K	26K-410K
		H	57	ND-400	1100	ND-13K
B	8	B	1K	ND-3200	1200	ND-13K
		H	30	ND-50K	70	ND-1600
AB	2	A	2300	800-6400	102K	51K-205K
		B	570	200-1600	57	ND-3200
		H	25	12-50	40	ND-1600

[a]Reciprocal of last tube dilution producing a positive signal.
[b]Antigen not detected at lowest dilution tested.

TABLE 4—*Adsorption-inhibition titers from semen as determined using different monoclonal anti-B blood grouping reagents.*

Monoclonal Anti-B Reagents	B-Antigen Detection Ratio[a]	Titer	
		Mean	Range
Bioscot	12/12	13K	4K-16K
Bioclone	5/12	400	50-2K
Celtech	6/12	160	25-1K
Dako	10/12	200	25-16K
AGT(1)	7/12	650	25-8K
Chembiomed	6/12	160	25-128K
AGT(2)	12/12	2K	500-32K
Polyclonal	12/12	5K	1K-32K

[a]Number positive/number tested.

TABLE 5—*ELISA titers from semen as determined using different monoclonal anti-B blood grouping reagents.*

Monoclonal Anti-B Reagents	B-Antigen Detection Ratio[a]	Titer	
		Mean	Range
Bioscot	12/12	215K	32K-1024K
Bioclone	12/12	36K	2K-1024K
Celtech	12/12	40K	4K-1024K
Dako	9/12	32K	ND[b]-1024K
AGT(1)	9/12	47K	ND-1024K
Chembiomed	8/12	16K	ND-128K
AGT(2)	0/12	ND	ND

[a]Number positive/number tested.
[b]Antigen not detected at lowest dilution tested.

TABLE 6—*Adsorption-inhibition titers from saliva as determined using different monoclonal anti-B blood grouping reagents.*

Monoclonal Anti-B Reagents	B-Antigen Detection Ratio[a]	Titer	
		Mean	Range
Bioscot	6/6	1400	800-6400
Bioclone	2/6	100	100
Celtech	2/6	140	100-200
Dako	3/6	500	200-1600
AGT(1)	4/6	400	100-1600
Chembiomed	3/6	250	100-800
AGT(2)	5/6	600	200-1600
Polyclonal	6/6	2300	800-13K

[a]Number positive/number tested.

TABLE 7—*ELISA titers from saliva as determined using different monoclonal anti-B blood grouping reagents.*

Monoclonal Anti-B Reagents	B-Antigen Detection Ratio[a]	Titer	
		Mean	Range
Bioscot	5/6	21K	4K-128K
Bioclone	4/6	10K	4K-16K
Celtech	6/6	20K	4K-128K
Dako	6/6	7K	2K-32K
AGT(1)	5/6	12K	2K-32K
Chembiomed	6/6	4K	1K-8K
AGT(2)	0/6	ND[b]	ND

[a]Number positive/number tested.
[b]Antigen not detected at lowest dilution tested.

As expected, the polyclonal anti-B generated positive AI grouping results with the six saliva specimens. However, as was observed when testing semen, the various anti-B monoclonal reagents failed to behave as expected with the Group B saliva (Table 6). Of the reagents tested, only Bioscot produced positive AI grouping results with the six saliva tested. The Bioclone and Celtech reagents were found to be the least reactive by AI, producing positive grouping results for only two of the six saliva. The AGT-2 reagent, which previously tested positive by inhibition with the twelve Group B semen, yielded positive AI results for five of the six saliva.

ELISA grouping results for the Group B saliva samples are shown in Table 7. The monoclonal reagents from Celtech, Dako, and Chembiomed, which failed to respond with approximately half the saliva tested by AI, produced positive results with all of the saliva when tested by ELISA.

Additionally, the AGT-2 MAB, which produced positive inhibition results for five of six saliva, failed to react with any of the saliva when tested by ELISA.

Discussion

Because of their serological properties, monoclonal anti-A and anti-B blood grouping reagents are gaining widespread use in blood banking. In some instances, these reagents are now beginning to replace the human polyclonal anti-A and anti-B blood grouping sera (Ortho Diagnostics, Product Notice 1032R, January 1988).

The monoclonal blood grouping sera that are available have been thoroughly tested against large panels of red cells using hemagglutination assays. However, the use of hemagglutination to screen monoclonal blood grouping reagents appears to favor the selection of those monoclonal reagents that recognize and react strongly with the major structural antigen on the red cell.[2]

In this study, 111 semen specimens and 66 saliva specimens were screened for ABH blood group substances by adsorption-inhibition hemagglutination and ELISA. As expected the sensitivity of the ELISA for the A and H BGS, expressed as a function of the semen BGS titers in Table 2, greatly exceeded the titers obtained with the inhibition assay. A comparable increase in sensitivity was not obtained for the B antigen using the Dako anti-B reagent. For at least 2 of these specimens, the Dako monoclonal failed to react with the corresponding BGS. As shown in Table 3, the mean salivary ABH titers obtained by ELISA were lower than expected when compared with the corresponding inhibition titers. In particular, the Dako anti-H reagent, which performed well in the ELISA with semen, did not react as strongly against the H antigen in saliva.

[2]S. Fletcher, personal communication, Central Research Establishment, Home Office Forensic Science Service, Aldermaston, U.K.

Using adsorption-inhibition (Table 4) and ELISA (Table 5), seven different monoclonal anti-B reagents were tested against the semen from twelve Group B donors. Of the seven MABs tested by AI, only the Bioscot and AGT-2 reagents reacted with the twelve semen specimens. However, when tested using ELISA, the AGT-2 reagent failed to react with any of the Group B semen. Additionally, the Bioclone and Celtech reagents, which responded poorly in the inhibition assay by failing to react with approximately half of the semen tested, performed well in ELISA and reacted with all the samples tested. Of the MABs tested, the Bioscot reagent provided the greatest sensitivity in both assays for detecting the B BGS in semen.

These same monoclonals were also tested against six Group B saliva samples. Of the MABs tested in the inhibition assay, only the Bioscot reagent reacted with the six specimens tested. When tested in the ELISA, the Bioscot reagent failed to react with one specimen. Only the Celtech, Dako, and Chembiomed reagents were successful in detecting the B BGS in the six saliva specimens.

The results of this study clearly demonstrate that some ABO monoclonal blood grouping sera, particularly the anti-B reagents, do not always behave as expected with the BGS from semen and saliva regardless of the assay used. The strong reaction observed between the monoclonal reagents and the corresponding red cell antigens could not be used to predict the behavior of MABs with soluble blood group antigens. This may result from the heterogeneous structure of water-soluble blood group antigens as well as subtle differences in the specificities of the monoclonal reagents [*12*]. Further, the behavior of a particular MAB reagent with one body fluid could not be used to predict its behavior with the same antigen from another body fluid. Before one can take advantage of the increased sensitivity offered by ELISA for the analysis of body fluid stains, the MABs must be thoroughly screened against each body fluid in question to provide the strongest reactivity and ensure against the occurrence of false negative results.

References

[*1*] Voak, D., Sacks, S., Alderson, T., Takei, F., Lennox, E., et al., "Monoclonal Anti-A from a Hybrid-Myeloma: Evaluation as a Blood Grouping Reagent," *Vox Sanguinis*, Vol. 39, 1980, pp. 134-140.

[*2*] Sacks, S. H. and Lennox, E. S., "Monoclonal Anti-B as a New Blood Grouping Reagent," *Vox Sanguinis*, Vol. 40, 1981, pp. 99-104.

[*3*] Takatori, T., Tustsubuchi, Y., and Terazawa, K., "Lewis Typing of Human Bloodstains by Enzyme-linked Immunosorbent Assay (ELISA) Using Monoclonal Anti-Le^a and Anti-Le^b," *Journal of Forensic Sciences*, Vol. 32, No. 4, July 1987, pp. 900-905.

[*4*] Cowles, J. W., Cox, M. T., McMican, A., and Blumberg, N., "Comparison of Monoclonal Antisera with Conventional Antisera for Lewis Blood Group Antigen Determination," *Vox Sanguinis*, Vol. 52, 1987, pp. 83-84.

[*5*] Francois-Gerard, Ch. and Hoste, B., "A Double-Sandwich ELISA Test for Glm(3) Phenotyping of Bloodstains with a Mouse Monoclonal Antibody," *Journal of the Forensic Science Society*, Vol. 27, 1987, pp. 31-38.

[*6*] Bourel, D., Lecointre, M., Genetet, N., Gueguen-Duchesen, M., and Genetet, B., "Murine Monoclonal Antibody Suitable for use as an Rh Reagent, Anti-e," *Vox Sanguinis*, Vol. 52, 1987, pp. 85-88.

[*7*] Voak, D. and Lennox, E., "Principles of Monoclonal Antibodies in Blood Transfusion Work," *Biotest Bulletin*, Vol. 4, 1983, pp. 281-290.

[*8*] Martin, P. D. and Parkin, B. H., "The Use of Monoclonal Antibodies in Forensic Science," in *Advances in Forensic Haemogenetics 2, 12th Congress of the Society for Forensic Haemogenetics*. W. R. Mayr, Ed., Springer-Verlag, New York, 1988, pp. 284-288.

[*9*] "A Scientific Forum on Blood Grouping Serum Anti-A (Murine Monoclonal Blend) Bioclone," May 1987, available from Ortho Diagnostic Systems Inc., Raritan, NJ.

[*10*] Molinaro, G. A., Eby, W., and Reimer, C., "A Monoclonal Antibody May Show Cross-Reactivities in Ouchterlony Assays but not in Other Assays," *Journal of Immunological Methods*, Vol. 96, 1987, pp. 219-224.

[*11*] Gane, P., Vellayoudom, J., Mollicone, R., Breimer, M. E., Samuelsson, B. E., "Heterogeneity of Anti-A and Anti-B Monoclonal Reagents: Agglutination of Some Weak ABH Erythrocyte Variants

and Recognition of Synthetic Oligosaccharide and Tissue Antigens," *Vox Sanguinis*, Vol. 53, 1987, pp. 117-125.

[*12*] Chen, H. T. and Kabat, E. A., "Immunochemical Studies on Blood Groups: The Combining Site Specificities of Mouse Monoclonal Hybridoma Anti-A and Anti-B," *Journal of Biological Chemistry*, Vol. 260, No. 24, 1985, pp. 13208-13217.

[*13*] "Saliva Testing for ABH and Lewis," in *Technical Manual of the American Association of Blood Banks*, 8th ed., F. K. Widmann, Ed., American Association of Blood Banks, Arlington, VA, 1981, pp. 122-123.

[*14*] Baechtel, F. S., "Secreted Blood Group Substances: Distributions in Semen and Stabilities in Dried Semen Stains," *Journal of Forensic Sciences*, Vol. 30, No. 4, Oct. 1985, pp. 1119-1129.

[*15*] Mudd, J. L., "A Mictoplate Method for Reverse ABO Typing of Bloodstains," *Journal of Forensic Sciences*, Vol. 31, No. 2, April 1986, pp. 418-425.

[*16*] "ABO System," in *Technical Manual of the American Association of Blood Banks*, 8th ed., F. K. Widmann, Ed., American Association of Blood Banks, Arlington, VA, 1981, pp. 105-120.

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