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Immunocytochemical typing of ABO blood groups in vaginal swabs partly contaminated with semen*

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Summary. ABH typing by immunocytochemical method has been carried out on 163 selected vaginal swabs. 127 cases (78%) were determined correctly, 11 cases (7%) incorrectly and 25 cases (15%) could not be classified. Often, not all of the vaginal cells showed the expected positive staining, which was not counted as a false result. The incorrect results were not dependant on the secretor status, but 37% of the non-secretor cases could not be classified immunocytochemically, as compared with 12% of the secretors. In a second series, 61 vaginal swabs, dried on microscope slides, have been covered with semen from an A_1 B secretor. Absorption of heterologous antigens by the vaginal epithelia could be demonstrated only after extremely long incubation with semen and extremely long incubation with the anti-A or anti-B antibodies. From the 163 "native" swabs, 17 gave a positive reaction with the acid phosphatase test, but only one false ABH result. A possible influence of bacteria upon the results is discussed. We believe that in practice, no faults in immunocytochemical ABH typing have to be expected, due to absorption of heterologous antigens.

Key words: ABO blood groups – Immunocytochemistry – Vaginal swabs – Semen

Zusammenfassung. An 163 ausgewählten Vaginalabstrichen wurde eine AB0 Blutgruppenbestimmung immunzytochemisch durchgeführt. In 127 Fällen (78%) wurde zutreffend und in 11 Fällen (7%) falsch bestimmt, während in 25 Fällen (15%) keine Aussage möglich war. Häufig waren nicht alle Zellen eines Abstrichs antigenmarkiert, was nicht als falsch gewertet wurde. Die Fehlerquote hing nicht vom Sekretorstatus ab, allerdings konnten 37% der Nonsekretorfälle gegenüber lediglich 12% der Sekretorfälle nicht klassifiziert werden. 61 weitere Ausstriche wurden mit Sperma eines A_1B Sekretors benetzt. Die Absorption heterologer Antigene durch Vaginalepithel konnte nur nach extrem langer Inkubation mit Sperma und nur nach extrem langer Inkubation mit den anti-A bzw. anti-B Seren nachgewiesen werden. Bei den 163 „nativen" Abstrichen reagierte der Phosphatasetest 17mal positiv, aber nur in einem dieser Fälle kam es zu einer AB0 Fehlbestimmung. Die Möglichkeit bakterieller Störeinflüsse wird diskutiert. In der Praxis dürften Fehlbestimmungen infolge Absorption heterologer ABH Antigene kaum zu erwarten sein.

Schlüsselwörter: AB0 Blutgruppe – Immunzytochemie – Vaginalabstriche – Sperma

Introduction

In mixed secretions, it is often difficult or impossible to assign the ABH characteristics found to the individuals involved, dependent on the constellation of the blood groups. The proof of the ABH type of a single cell could be advantageous when compared with conventional serology of the mixture. Also, the identification of the blood group of a vaginal epithelial cell found on the penis of a suspect after rape could be important evidence. Several potentially adequate techniques have been published: Mixed cell agglutination reaction (MCAR; Coombs and Bedford 1955; Coombs et al. 1956; Swinburne et al. 1961; Swinburne 1962; Ishiyama and Okada 1975; Fregin et al. 1981), immunofluorescence technique (Coons et al. 1942; Coons and Kaplan 1950; Sulzman 1960 and 1962), immunoenzyme techniques (Mason et al. 1969; Sternberger et al. 1970; Hsu et al. 1981). In this study the PAP technique (Sternberger et al. 1970) and a modification have been used, as it is well established in forensic medicine to test ABH antigens in human tissues (Pedal 1987) and on single cells (Brinkmann et al. 1986). In the present paper, the practical meaning for the examination of sexual assaults has been investigated.

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Table 1. Results of the immunocytochemical ABH determination of 163 vaginal swabs

Vaginal swab			Positive staining			Assessment		
ABH	Se/se	*n*	A	B	H	Correct	N.c.[a]	Incorrect
A[b]	Se	39	34	2	26	33	3	3
A_1	Se	9	8	0	6	8	1	0
A_2	Se	10	10	0	7	10	1	0
A[b]	se	2	2	0	1	1	1	0
A_1	se	16	12	1	4	12	3	1
A_2	se	2	0	0	0	0	2	0
B	Se	21	1	18	8	17	3	1
B	se	1	0	0	0	0	1	0
AB[b]	Se	2	2	2	1	2	0	0
A_2B	Se	1	1	1	0	1	0	0
A_1B	se	3	2	2	0	2	1	0
A_2B	se	1	1	1	0	1	0	0
O	Se	54	4	2	46	40	8	6[c]
O	se	2	0	0	0	0	2	0
Σ		163	77	29	99	127	25	11

[a] Not classified
[b] Subtype not known
[c] Including the only incorrect result of the 17 swabs with positive acid phosphatase test

Materials and methods

Vaginal swabs from out-patients of a gynaecological clinic were prepared by applying 3 stains of 0.8 mm each in diameter on microscope slides. These were dried, stored at room temperature, and investigated within two months. ABO blood groups of the donors were partly known from blood donor passes, partly determined in blood samples and partly determined from the swabs using an absorption-inhibition test. Secretor status was determined with the same method using anti-A, anti-B and anti-H sera, and group A, B, and O red blood cells. To test the swabs for a possible contamination with seminal fluid, the acid phosphatase test was performed on every swab. Swabs which were positive with the phosphatase test and non-secretor cases were only used if the donors' blood group could be typed directly from the blood sample or was known from a donor pass. If a swab gave positive reaction in the acid phosphatase test and a blood group was known from a pass and no blood sample was present, the secretor status determined in the swab was finally attributed to the female donor; evaluation of the results under this premise showed no remarkable deviation from the overall results. Thus, from the original 200 swabs, 163 were included in the investigation. The distribution of the ABO types and the secretor status is shown in Table 1.

A total of 61 vaginal swabs with a selected distribution of ABO types and secretor status (see Table 2) were dried on microscope slides and covered with semen from an A_1B secretor donor and incubated for 1, 12, 24 and 72 h at +37°C in a humid chamber. The preparations were then rinsed and dried. Prior to the immunoenzyme determination, the dried slides were incubated in a solution of 4 parts methanol and 1 part 3% H_2O_2 for 20 min. The determination itself was performed in four steps. 1st step: Monoclonal anti-A serum (1:20; Biotest, FRG), anti-B serum (1:20; Biotest, FRG; later Dakopatts, FRG) and anti-H serum (Fresenius, FRG) was dropped onto the stains. 2nd step: rabbit anti-mouse serum (1:200; Bionetics, USA). 3rd step: swine anti-rabbit serum (Dakopatts, FRG). 4th step: PAP-complex from the rabbit (Dakopatts, FRG). Incubation times: 30 min for the 1st step, 20 min for the 2nd–4th step. Development: ACE (3-amino-9-ethylcarbazolen) substrate solution (Bourne 1983). All incubations were performed on a glass plate chilled on ice and all steps were performed in a humid chamber. Between each step the slides were rinsed with Tris-HCl buffer (1 part 50 m*M* Tris pH 7.6 and 9 parts 0.85% NaCl). All sera were diluted with 5% BSA. In a later stage of the investigation, steps 2–4 were replaced by incubation with a peroxidase-conjugated anti-mouse antibody from the rabbit (Dakopatts, FRG).

Results

Immunocytochemical tests were performed once in every case. When reading the results, the investigators knew the collective's blood group composition, but not the markings of the individual case.

Native vaginal swabs

Parameters of the immunoenzyme reaction were chosen to prevent background staining and false positive results in the controls. Staining was rated as positive if the intensity of the colour of the epithelial cells was clearly visible. The assessment "correct" was given if a reaction with the antisera corresponding to the donor's blood group was present in at least some of the epithelial cells. "Not classified" was given if the staining could not be clearly distinguished from the background, or if no staining was present. "Incorrect" was given if no colour reaction for the corresponding antigen was found, or if a false positive reaction was found. In 127 of the 163 vaginal swabs investigated, the ABO blood group was correctly determined, in 11 cases incorrectly, and in 25 cases no classification was possible.

Vaginal swabs contaminated with semen

The classification "positive" was given if the vaginal epithelial cells showed a staining with an antibody not corresponding to the female donor's ABO group. This was interpreted as absorption of heterologous ABH antigens

Table 2. Results of the immunocytochemical ABH determination of 61 vaginal swabs incubated with sperm of an A_1 B secretor for 72 h at +37°C

Vaginal swabs		Secretors		Non-secretors	
ABH	Total	Tested	Positive[a]	Tested	Positve[a]
A_1	21	10	0	11	2 (B)[b]
A_2	13	11	4 (B)[b]	2	0
B	11	10	7 (A)[b]	1	1 (B)[b]
A_1B	4	1	0	3	3 (A)[b]
A_2B	2	1	0	1	0
O	10	8	3 (AB, A)[b]	2	2 (AB)[b]
Σ	61	41	14	20	8

[a] "Positive" means absorption of heterologous ABH antigens by the vaginal epithelia in the A, B and O cases and a clearly enhanced colouring in the AB cases
[b] Letter indicates additionally present antigens

(see Discussion). After incubation with semen for periods of 1, 12, and 24 h no absorption of heterologous ABH antigens could be demonstrated. After a 72-hour incubation with semen and a prolonged incubation over further 72 h with the first (ABH) antibody, the results shown in Table 2 were obtained.

Discussion

ABO blood group typing of a single epithelial cell has been successfully performed with several methods. In this paper the usefulness of typing vaginal epithelial cells with an immunocytochemical technique was investigated for use in forensic case work. The overall result – from 163 selected vaginal swabs, immunocytochemical ABH typing was correct in 127 cases (78%), incorrect in 11 cases (7%) and 25 cases (15%) could not be classified – diverged significantly from several categories of donors and swabs, respectively.

Hormonally stimulated cells of a highly proliferated epithelium could be determined more reliably than cells with a low grade of proliferation. In fact, the most reliable results were obtained from swabs of donors in the group aged between 21 and 40 years, which included pregnant women. The most conspicuous group is the non-secretors. Only 59% of the swabs were typed correctly (16 from 27), as compared with 82% of the secretors (111 out of 136). The rate of 41% is composed of non-classifiable and false results. The low rate of false results, which was about the same in non-secretors and secretors [4% ($n = 1$) as compared with 7%] was surprising; the difference is caused by the high percentage of non-classifiable results (37% as compared with 11%). The reason for this phenomenon was detected by investigation of histological sections of the vagina: The vaginal epithelium of secretors contains ABH antigens in all layers, except the stratum basale, whereas the superficial layers of the squamous epithelium of non-secretors are free of ABH antigens (Scheithauer 1988). Consequently, in several non-secretor cases investigated, only epithelial cells free of ABH antigens may have been on the microscope slides and no blood groups could be detected. Our results do not conflict with Brinkmann et al. (1986), who determined the ABO blood group of 20 vaginal swabs correctly. Due to statistical considerations, 4 non-secretor cases have to be expected, as these occur in about 20% of our population, so only 1 non-classifiable case (37%) had to be expected in their material. In the A_2 cases, a greater percentage of epithelial cells were stained after anti-H incubation as compared with the A_1 cases. The intensity of the colour was also much higher on average. However, this statement is not valid for all cases because opposing results have also been obtained, so for case work the discrimination of A_1 and A_2 does not seem to be sufficiently trustworthy.

Eleven incorrect results were obtained: 2 A Se cases appeared as AB, 1 A Se case as O, 1 A_1 se case as B, 1 B Se case as AB, 1 blood group O Se case as AB, 4 blood group O Se cases as A and 1 blood group O Se case as B (controls were correct in every series). No clear tendency of the false results was recognized, namely, no accumulation of "acquired B" (Jenkins et al. 1972; Davies et al. 1983, 1984) was present. All together, a false B appeared in five cases and a false A in 6 cases, whereas in 2 cases the A antigen could not be demonstrated. So, results are in accordance with the experiments of Pereira (1973), who found acquired A, B and H antigens and loss of antigenic reactions in decomposing muscle tissue using two absorption-elution methods and polyclonal antibodies; monoclonal antibodies as used in our tests are not a guarantee for higher specificy (Lötterle et al. 1986; Mollicone et al. 1986). The vaginal cells determined incorrectly showed a fairly homogenous (false) immunostaining, that could easily be delimited from clumps of bacteria sometimes present on the slides. Nevertheless, bacterial enzymes diffusing from the vicinity could have altered glycoproteins of the vaginal cells producing relevant antigenic sites, as discussed by Pedal and Baedeker (1985). The tendency to a higher rate of errors in swabs with advanced cytolysis could confirm this conjecture. The clear results received in histological sections of the vagina (Scheithauer 1988) do not oppose this thesis, as the material has been stored in formaldehyde prior to immunoenzyme processing. However, neither systematic evaluation nor special investigations were performed to answer this question; finally, errors dependant on the methodology of the immunostaining procecdure are less probable.

The erroneous determination of the ABO group of male epithelial detritus contaminating the vaginal swab, leading to a misinterpretation, is possible only in one of our cases, which had the only false result (female donor: blood group O, result: B) within the collective with positive acid phosphatase test. The rate of 1 our of 17 cases, that is in a level corresponding to the overall result, was also a first indication that the absorption of heterologous antigens has no importance in forensic practice. Still, absorption of ABH antigens is conceivable, according to the literature (Rachkewich et al. 1978, Oriol et al. 1980, absorption of ABH antigens by lymphocytes; Cartron et al. 1980, Dunstan et al. 1985, by platelets; Holborow et al. 1960, Swinburne et al. 1961, by epithelia; Levine and Celano 1961, Edwards et al. 1964, Boettcher 1968, Ackerman 1969, Ragnekar and Rao 1970, Uhlenbruck and Herrmann 1972, Takeda and Hiraiwa 1985, by spermatozoa), especially from seminal fluid rich in ABH antigens (Lötterle and Scheithauer 1984). The 61 selected vaginal swabs showed numerous epithelial cells, no cytolysis and low bacterial flora; they were examined after having covered the dried material on microscope slides with fresh semen of an A_1B Se donor. Heterologous ABH antigens or a clearly enhanced antigen marking in the vaginal cells could be demonstrated only under extreme conditions of a 72-hour incubation with semen at +37°C in a humid chamber; an incubation over 24 h showed no influence. The incubation times for the immunoenzyme determination also had to be lengthened to detect "foreign antigens". No loss of antigenic activity was found, whereas A antigens as well as B antigens were acquired. Here, as well as in the native swabs, an influence of bacteria on the additional antigenic properties has to be considered: The cytological shape of the previously normal shaped

cells faded during incubation and the diagnosis "vaginal epithelium" could no longer be made. In 10 out of the 183 stains incubated with anti-A, anti-B and anti-H no eucaryotic cell material could be identified. In practice, the necessary period of contact with the seminal fluid at body temperature to cause false results will hardly be reached, as forensic investigation for vaginal epithelial cells mostly is commissioned in dried stains or penile swabs and not in vaginal swabs; unfavourable conditions are at most conceivable in swabs from the drawn back prepuce and the influence of smegma.

The proof of glycogen in the epithelia, that serves as a marker for vaginal origin of the epithelia, does not disturb the following immunoenzyme investigation. From the literature it is possible to repeat the immunoenzyme investigation on the same cell with other antibodies and other staining systems (Mason et al. 1969; Mason and Sammons 1978; Oriol and Mancilla-Jimenez 1983). Possibly, the error rate can be reduced by further improvements to the technique, although many tests were performed to optimize the parameters. What will remain for certain is an enhanced rate of non-classifiable cases if the donor is a non-secretor and the possible influence of bacteria, that can hardly be calculated under disadvantageous conditions. As compared with the potential meaningfulness of PCR technique (Saiki et al. 1985) combined with recombinant DNA techniques to individualize single cells, ABH typing is a relative poor method. However, at the moment the PCR technique is not generally recommended for case work purposes (Deutsche Gesellschaft für Rechtsmedizin 1989).

References

Ackerman DR (1969) The adsorption of anti-A and anti-B antibodies on human spermatozoa. Fertil Steril 20:324–334

Boettcher B (1968) Correlation between human ABO blood group antigens in seminal plasma and on seminal spermatozoa. J Reprod Fertil 16:49–54

Bourne JA (1983) Handbook of immunoperoxidase staining methods. Dako, Santa Barbara

Brinkmann B, Kernbach G, Rand S (1986) Cytochemical detection of ABH antigens in human body fluids. Z Rechtsmed 96: 111–117

Cartron JP, Mulet C, Bauvois B, Rahuel C, Salmon C (1980) ABH and Lewis glycosyltransferases in human red cells, lymphocytes and platelets. Rev Fr Transfus Immunohematol 23:271–282

Coombs RRA, Bedford D (1955) The A and B antigens on human platelets demonstrated by means of mixed erythrozyte platelet agglutination. Vox Sang 5:111–115

Coombs RRA, Bedford D, Rouillard LM (1956) A and B blood-group antigens on human epidermal cells. Lancet I:461–464

Coons AH, Creech HI, Jones RN, Berliner E (1942) The demonstration of pneumococcal antigen in tissues by the use of fluorescent antibody. J Immunol 45:159–170

Coons AH, Kaplan MH (1950) Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen by means of fluorescent antibody. J Exp Med 91:1–13

Davies A, Lincoln PJ, Martin PD (1983) Aberrant group B reactions detected in mixtures of semen and vaginal secretion. Referate 10. Int Kongr Ges Forens Blutgruppenkunde München 10.–15.10.1983, pp 259–263

Davies A, Lincoln PJ, Martin P (1984) Aberrent group B reactions detected in mixtures of semen and vaginal secretions possibly due to acquired B. Forensic Sci Int 25:210–208

Deutsche Gesellschaft für Rechtsmedizin (1989) DNA Resolution. Informationen der Deutschen Gesellschaft für Rechtsmedizin 37:564–565

Dunstan RA, Simpson MB, Knowles RW, Rosse WF (1985) The origin of ABH antigens on human platelets. Blood 65:615–619

Edwards RG, Ferguson LC, Coombs RRA (1964) Blood group antigens on human spermatozoa. J Reprod Fertil 7:153–161

Fregin W, Kerde C, Rommeiss S (1981) Die Bedeutung des Antigen-Antikörper-Verhältnisse bei der Untersuchung von Sperma- und Vaginalsekretspuren mittels Mischzellagglutination auf Klebeband. Kriminal Forens Wissensch 44:139–142

Holborow EJ, Brown PC, Glynn LE, Hawed MD, Gresham GA, OBrien TF, Coombs RRA (1960) The distribution of the blood group A antigen in human tissues. Br J Exp Pathol 41:430–437

Hsu SM, Raine L, Fanger H (1981) Use of Avidin-Biotin-Peroxidase Complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 29:577–580

Ishiyama I, Okada T (1975) Anwendung der modifizierten Mischzellagglutination (mixed cell agglutination reaction, MCAR nach Davidsohn) in der forensischen Serologie; MCAR auf dem Klebebandstreifen. Z Rechtsmed 77:25–40

Jenkins GC, Brown J, Lincoln PJ, Dodd BE (1972) The problem of the acquired B antigen in forensic serology. J Forensic Sci Soc 12:597–603

Levine P, Celano MJ (1961) The question of D (Rh_0) antigenic sites on human spermatozoa. Vox Sang 6:720–723

Lötterle J, Scheithauer R (1984) Sekretoreigenschaft bei frischen und fäulnisveränderten Leichen. Beitr Gerichtl Med 42:251–255

Lötterle J, Scheithauer R, Wolf I (1986) Untersuchung der Antigene des AB0-Systems an Blutspuren mit monoklonalen Antiseren. Beitr Gerichtl Med 44:87–92

Mason DY, Sammons R (1978) Alkaline phosphatase and peroxidase for double immunoenzymatic labelling of cellular constituents. J Clin Pathol 31:454–460

Mason TE, Phifer RF, Spicer SS, Swallow RA, Dreskin RB (1969) An immunoglobulin-enzyme bridge method for localizing tissue antigens. J Histochem Cytochem 17:563–569

Mollicone R, Davies DR, Evans B, Dalix AM, Oriol R (1986) Cellular expression and genetic control of ABH antigens in primary sensory neurons of marmoset, baboon and man. J Neuroimmunol 10:255–269

Oriol R, Danilovs J, Lemieux R, Terasaki P, Bernoco D (1980) Lymphocytotoxic definition of combined ABH and Lewis antigens and their transfer from sera to lymphocytes. Hum Immunol 3:195–205

Oriol R, Mancilla-Jimenez R (1983) Fluorescent staining of nuclei and amyloid substance: two useful properties of p-phenylenediamine. J Immunol Methods 62:185–192

Pedal I (1987) Blutgruppen-Immunhistochemie. AB0(H) und Lewis-Antigene menschlicher Gewebe. Thieme, Stuttgart New York

Pedal I, Baedeker C (1985) Immunenzymatische Darstellung der Isoantigene A, B und H in fäulnisverändertem Nierengewebe. Z Rechtsmed 94:9–20

Pereira M (1973) ABO grouping of decomposed human tissue. J Forensic Sci Soc 13:33–36

Rachkewich RA, Crookston MC, Tilley CA, Wherrett JR (1978) Evidence that blood group A antigen on lymphocytes is derived from the plasma. J Immunogenet 5:25–29

Rangnekar KN, Rao SS (1970) The source of blood group antigens on spermatozoa and their significance. Int Arch Allergy Appl Immunol 37:49–53

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA (1985) Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230:1350–1354

Scheithauer R (1988) Immunhistochemische Studie zum Verteilungsmuster der AB0-Blutgruppensubstanzen an weiblichen Geschlechtsorganen. Z Rechtsmed 100:149–156

Sternberger LA, Hardy PH, Cuculis JJ, Meyer HG (1970) The unlabeled antibody enzyme method of immunohistochemistry.

Preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes. J Histochem Cytochem 18:315–333

Swinburne LM (1962) The identification of skin. Med Sci Law 3: 3–12

Swinburne LM, Frank BB, Coombs RRA (1961) The A antigen on the buccal epithelial cells of man. Vox Sang 6:274–286

Szulman AE (1960) The histological distribution of blood group substances A and B in man. J Exp Med 111:785–799

Szulman AE (1962) The histological distribution of the blood group substances in man as disclosed by immunofluorescence. II. The H antigen and its relation to A and B antigens. J Exp Med 115:977–996

Takeda K, Hiraiwa K (1985) ABO blood group antigens of human spermatozoa. Tohoku J Exp Med 147:267–272

Uhlenbruck G, Herrmann WP (1972) Agglutination of normal, coated and enzyme-treated human spermatozoa with heterophile agglutinins. Vox Sang 23:444–451