A RAPID SCREENING ASSAY FOR BLOOD GROUP SPECIFICITY OF MONOCLONAL ANTIBODIES USING THE MICROTITER PLATE SYSTEM:

Alois Poschmann, Volker Müller, Hans H. Hoppe and Konrad Fischer

Abteilung für Klinische Immunpathologie der Universitäts-Kinderklinik and Zentralinstitut für Transfusionsmedizin, Hamburg, Germany.

When producing monoclonal antibodies of blood group specificity it is important to screen as early as possible and rapidly hundreds of hybridoma supernatants on antibody activity and specificity. The standard techniques for erythrocyte antibody screening and identification are time consuming and expensive. Therefore, we tested the microtiter plate system which was adapted to blood grouping firstly by Wegmann and Smithies in 1966 (10), using V-bottom wells. In 1970 Crawford et al. (2) used U-bottom wells and modified the microtiter plate system for antibody screening and identification, titration and cell typing. Since then, this microtiter hemagglutination method has achieved increasing importance(1,7,9). The method is simple, rapid, economic and makes it possible to read, interprete and record automatically the agglutination reactions. The advantages of the microtiter hemagglutination method are demonstrated by rapid detection and isolation of antigen-positive hybridomas after fusion and final cloning. Our experience with this method is based upon more than 40.000 reactions.

MATERIALS AND METHODS:

Immunization and somatic cell hybridization (4,5,8):

8-week-old female BALB/c mice were immunized according to the schedule of Stähli et al. (8) with washed erythrocytes of a healthy donor of the following type: B MN ss P Le(a-b+) Rh pos CcD.ee K neg Fy(a+b+) Jk(a+b+). Spleen cells were obtained 1 day after the final boost and were fused in the ratio 5:1 x 10^7 with BALB/c, P3-X63-Ag8-U1 (11) in the presence of 0,5 ml of 40 % polyethylene glycol 4000 (Merck) and 15 % dimethylsulfoxide.

Selection and growth of hybridomas (4,5):

RPMI 1640 culture medium containing 20 % fetal calf serum and supplemented with glutamine, penicillin and streptomycin plus hypoxanthine, aminopterin and thymidine (complete HAT medium) was used for the selection and primary culture of growing colonies(4). The fusion mixture was suspended in 50 ml of complete HAT medium and distributed into 48 wells of 2 Greiner fusion plates each containing normal spleen cells (1-2 x 10^5/well). They were used as feeder cells prepared 1 day prior to fusion. 10 days after fusion cellular growth was observed macroscopically in all primary culture wells. Culture supernatant from each well was tested for hemagglutinating activity. With the small wells at the bottom of a big well the Greiner fusion plate provides higher resolving power than any other multi-well tissue culture plate (early cloning). Several cell clones in a big well are not mixed with each other. All cell clusters of the antigen-positive wells are transferred using 50 or 100 µl Eppendorf pipettes into microtiter plates. After several days of proliferation the supernatant of each well is tested again for antigen reactivity: each positive

Advances in Forensic Haemogenetics 1
(c) Springer-Verlag Berlin Heidelberg 1986

Advances in Forensic Haemogenetics 1
Edited by B. Brinkmann and K. Henningsen
© Springer-Verlag Berlin Heidelberg 1986

well indicates an antigen-positive clone or at least highly enriched clone. The antigen-positive hybridomas are transferred into cluster trays and expanded in Petri dishes until enough cells are available for storage in liquid nitrogen and for final cloning. Thus a particular antigen-positive clone can be isolated under optimal conditons after 2-3 tests. Antigen-positive hybridomas are isolated by final cloning by limiting dilution in microtiter plates (4) and evaluated according to Fazekas de St. Groth (3). Since hybridomas may stop synthesizing antibodies, the supernatants are tested against red cells from time to time, and only positive clones are expanded and aliquots of the cells are frozen and stored in liquid nitrogen.

Microtiter hemagglutination method:

Rigid plastic microtiter plates containing 96 U bottom wells (Greiner) are used for all tests. 25 µl of hybridoma supernatant are distributed by a repetitive dispenser (Multipette Eppendorf) and mixed with 25 µl of a 2 % red cell suspension in PBS (red cells used for immunization, and appropriately selected cell sets for antibody screening and identification). Plates are covered to prevent evaporation and are incubated 10 - 30 min at room temperature. After centrifugation 30 - 60 sec at 100 x g, using a centrifuge adapted for plates (Hettich), the plates are agitated by a shaker (Titertek, Flow) for 10 - 30 sec, to resuspend the cells completely. The agglutination reactions are read from the bottom of the plate by a reading device with an illuminated and magnifying mirror (Biotest).

INDIRECT ANTIGLOBULIN TECHNIQUE (AGT):

Applying monoclonal antibodies the AGT can be performed WITHOUT WASHING because the hybridoma supernatant contains only specific antibodies, and the total amount of immunoglobulin is too small to inhibit the antiglobulin serum. Microplates are centrifuged 30 - 60 sec at 700 x g and the supernatant fluid is removed by flicking the plate over a sink. 50 µl of appropriately diluted anti-mouse-immunoglobulin (IgG + IgM, Jackson) are added by a multi-microdiluter (8 oder 12-channel graduated pipette Multistepper, Titertek, Flow). Plates are centrifuged again for 30 - 60 sec at 100 x g, agitated and read. Coombs control cells may be added to each negative AGT to confirm the antiserum's reactivity.

INTERPRETATION:

Positive reactions are to be interpreted based on the normal appearance of agglutination reactions. A negative reaction will appear as a smooth cell suspension in the bottom of the well. Verification of weak reactions may be accomplished by interpreting the settled red blood cell pattern after 20 - 30 min. The settled pattern ist then read by observing the underside of the well through the magnifying mirror. Negative reactions appear as smooth round cell buttons, whereas strong positive reactions will appear as jagged clumps of cells. Weak positive reactions will appear as a cell button with a "halo" surrounding it.

Advances in Forensic Haemogenetics 1
(c) Springer-Verlag Berlin Heidelberg 1986

RESULTS AND DISCUSSION:

In contrast to polyclonal antisera containing many unrelated immunoglobulins beside the specific antibody, in hybridoma supernatants the total amount of immunoglobulin is a specific antibody and too small to inhibite the antiglobulin serum. Therefore, during the screening and identification of blood group specific monoclonal antibodies, the AGT can be performed WITHOUT WASHING. Thus, the microtiter method including AGT is extremely rapid without decreased hemagglutination sensitivity.

	Microplates	Cluster trays	Petri dishes
supernatant (Vol.)	50-100 µl	1-2 ml	10 ml
growing hybridoma	721	624/721 (87%)	152/721 (21%)
antigen positive	500/721 (69%)	363/721 (50%)	118/721 (16%)
		363/500 (73%)	118/500 (24%)
		363/624 (58%)	118/152 (78%)
			118/363 (32%)

Table 1: cell growth and agglutinating activity of hybridomas during expanding after cell hybridization (B-28-1-85).

Supernatants of growing cells in Greiner fusion plates, microplates, cluster trays and Petri dishes are tested systematically for hemagglutinating activity using the microtiter hemagglutination method. As shown in talbe 1, 500 out of 721 cells growing in microplates were reactive with the red cells used for immunization, whilst in cluster trays the results were 363. In Petri dishes only 118 supernatants were angigen positive. There is a remarkable loss of antibody production during the first days after hybridization: 31 % after 10 days (microplates), 42 % after 15 days (cluster trays), whilst 4 weeks later (Petri dishes) the clones are more stable (only 22 % loss of antibody production). 118 out of 500 (24 %) primary antigen-positive clones could be preserved and stored in liquid nitrogen.

64 (54%) with defined blood group specificity:

- 34 Anti-B
- 13 Anti-AB
- 14 Anti-N
- 3 Anti-N/Anti-B

54 (46%) with undefined specificity:

- 27 Panagglutinins (saline): 10 VCN -/- PAP; 12 VCN +/- PAP; 5 VCN +/+ PAP
- 27 IgG-Panagglutinins (AGT): 6 VCN -/- PAP; 5 VCN +/- PAP; 16 VCN +/+ PAP

Advances in Forensic Haemogenetics 1
(c) Springer-Verlag Berlin Heidelberg 1986

Table 2: Specificity of 118 antigen-positive hybridoma cells (VCN=Vibrio cholerae neuraminidase, PAP=Papain)

Out of 118 antigen-positive hybridomas 64 (54 %) showed defined blood group specificity, whilst 54 (46 %) reacted with all native testcells. However, if red cells were treated with neuraminidase or papain, different agglutination reaction patterns could be observed.

Subclone	cell growth	reactivity	yield	reactivity
I J 12-10	61	0		
-25	85	0		
-50	86	0		
I J 13- 5	17	15 (88%)	15	15/15 (100%)
-25	51	47 (92%)		
-50	72	70 (97%)		
I K 4-10	23	0		
-25	54	2 (4%)	1	1/2
-50	79	3 (4%)	2	1/3
I T 12- 5	18	5 (28%)	3	3/5 (60%)
-25	76	15 (20%)	14	13/15(87%)
-50	92	90 (98%)		

Table 3: cell growth and hemagglutinating activity of some selected subclones with Anti-B specificity.

24 selected hybridoma cells were finally cloned by limiting dilution (9 anti-B, 4 anti-AB, 2 anti-N and 9 panagglutinins). Hybridomas may stop synthesizing antibodies despite good cell growth (see table 3). Therefore the supernatants have to be tested frequently. In subclone I J 12 the antibody producing cell line is lost. In subclone I K 4 only 4 % of growing cells show hemagglutinating activity. The many "non-producers" may overgrow the "producers". To avoid the loss of these few antibody producing cells, the hybridomas have to be recloned. Other subclones like I J 13 and I T 12 are good antibody-producers and stable cell lines.

Subclone		Erythrocytes								
II V 10	ONN	VCN	TRY	CHY	PAP	BMN	VCN	TRY	CHY	PAP
D 1	+++	+	0	+++	0	++	0	0	0	0
D 2	+++	+	0	+++	0	++	0	0	0	0
D 5	+++	++	++	+++	0	+++	0	++	+	0
E 10	+++	++	++	+++	0	+++	0	++	+	0
E 11	+++	+++	+++	+++	0	+++	0	+++	+++	0
H 10	+++	+++	+++	+++	0	+++	0	+++	+++	0

Table 4: effect of enzyme treatment of tested erythrocytes on the reactivity of different subclones with anti-N specificity (VCN = Vibrio cholerae neuraminidase, TRY = Trypsin, CHY = Chymotrypsin, PAP = Papain).

Advances in Forensic Haemogenetics 1
(c) Springer-Verlag Berlin Heidelberg 1986

When different subclones of hybridoma II V 10 with anti-N specificity are tested against red cells treated with various enzymes, their reactivities are reduced differently, suggesting that each recognized a distinct and different epitope (table 4). Hemagglutinating reactivity for all subclones are readily destroyed by papain, whilst the reactive sites for subclones E 11 and H 10 are resistant to chymotrypsin and trypsin. The reactive sites of MN (heterozygous) red cells demonstrated greater sensitivity to neuraminidase treatment than those of NN (homozygous) red cells, possibly due to dosage effect. There may be distinct epitopes on the N antigen: The sensitivity of subclones D 1 and D 2 epitopes to neuraminidase and trypsin treatment is in contrast to the resistance of sublones E 11 and H 10. Similar results regarding the M antigen were demonstrated by Nichols et al. (6), who postulated two blood group M epitopes applying anti-M monoclonal antibodies.

In conclusion, the microtiter hemagglutination method provides considerable advantages. In our experience, based upon more than 40.000 reactions, it es a simple, rapid and economic method. If large numbers of samples are examined (as in the case of production of monoclonal antibodies with blood group specificity)economy of equipment, reagents, and especially time, is achieved in comparison to standard techniques carried out in tubes. Furthermore, the microtiter hemagglutination method gives important information about comparative strength of reactions. For reading reactions, agitation of the plates gives more uniform cell dispersal as by shaking individual tubes. However, the most important advantage is the obvious rapidity. One technologist can perform as many as 600 reactions per hour including reading and manual recording. Last, but not least, the method allows the automatic evalution of agglutination reaction patterns by a photometer combined with a computer, programmed to produce print outs.

REFERENCES:

1) American red cross blood services, special services section, Bethesda, Maryland, USA: blood services directive 6.44, September 1981
2) Crawford,M.N., F.E.Gottman and C.A.Gottman: Transfusion 10: 258 (1970)
3) Fazekas de St.Groth,S.: J. Immunol.Methods 35:11 (1980)
4) Goding,J.W.: monoclonal antibodies: principles and practice. Academic press, London (1983)
5) Köhler, G. and C.Milstein: Nature, London 256:495 (1975)
6) Nichols, M.E., R.E.Rosenfield and P.Rubinstein: Vox Sang. 49:138 (1985)
7) Parker, J.L., D.A.Marcoux,E.B.Hafleigh and F.C.Grumet: Transfusion 18:417 (1978)
8) Stähli,Chr.,T. Staehelin,V.Miggiano,J.Schmidt and P.Häring: J. Immunol. Methods 32:297 (1980)
9) Uthemann,H., I.Alberding and M.Großer: Ärztl.Lab. 30:259 (1948)
10) Wegmann,T.G. and O.Smithies: Transfusion 6:67 (1967)
11) Yelton,D., B.A.Diamond, S.-P.Kwahn and M.D.Scharff: Curr.Top.Microbiol.and Immunol. 81:1 (1978)

Advances in Forensic Haemogenetics 1
(c) Springer-Verlag Berlin Heidelberg 1986