MONOCLONAL ANTIBODIES TO IMMUNOGLOBULIN ALLOTYPES: SPECIFICITY AND REACTIVITY IN HAEMAGGLUTINATION AND ELISA TECHNIQUES.

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Summary

Monoclonal antibodies (McAbs) were prepared to further study the allotypes of immunoglobulins. By screening supernatants in the haemagglutination-inhibition (HAI) as well as in the ELISA techniques anti-G1m(z) (=5A1) and anti-G1m(a) (=1C2) antibodies could be obtained.
These two antibodies are specific for the corresponding allotypes in inhibition assays performed with the HAI and the ELISA, but only under special circumstances. When McAb 5A1 is used in the direct haemagglutination test (HA) there is some cross-reactivity with other IgG coated cells.
This reaction is not inhibitable. However in the HAI with G1m(z) coated cells and in the indirect ELISA with G1m(z) coated plates only G1m(z) positive samples inhibit. McAb 1C2 shows another picture, because it reacts in the direct HA as well as in the direct ELISA with IgG of all subclasses and allotypes. In inhibition tests with G1m(a) coated cells only G1m(a) positive samples inhibit; with other IgG coated cells IgG of all subclasses and allotypes inhibit. It could be shown that the epitope, detected by 5A1 is located in the CH1 domain and the epitope(s) detected by 1C2 is/are located in the CH3 domain.
In conclusion, McAbs 5A1 and 1C2 are both useful anti-Gm reagents. However McAb 1C2 seems to have a dual specificity, namely anti-Gm(a) and anti-IgG. The question is, is the G1m(a) specificity a pseudospecificity, in other words are we dealing here with an anti-IgG antibody with a prevalence for G1m(a) positive molecules.

Introduction

The immunoglobulin (Ig) allotypes belong to a polymorphic genetic system, which is very useful for paternity testing and bloodstain analysis. As the immunoglobulins are stable molecules, the allotypes can also be determined in old serum and plasma samples and bloodstains.
However it is rather difficult to obtain sufficiently strong and specific antisera. For this reason and for further study of the allotypes we undertook the production of monoclonal anti-allotype antibodies.

Material and Methods

Monoclonal antibodies

Two monoclonal antibodies (McAbs), 5A1 and 1C2, were produced by immunization of Balb/c mice with human G1m(za) positive IgG1 myeloma proteins, followed by fusion of spleen cells with cells of the non-secretor mouse myeloma cell line SP2, according to the method of Köhler and Milstein (Nature: 256, 495, 1975).Hybridomas were selected by screening of supernatants in the haemagglutination-inhibition assay (HAI) and in the ELISA. After limiting dilutions the antibody producing cells were injected into mice for the production of ascites fluid.

Human sera, myeloma proteins and fragments

Thirty Ig allotyped human sera from individuals of different races were used in the inhibition tests. Distinction between all known Gm, A2m and

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Km allotypes could be made with this panel of sera. For comparison with conventional antisera more than 1000 non-selected human sera were used. IgG, IgA and IgM myeloma proteins of the different subclasses and allotypes were used as coating antigens and as (non)inhibitors. These myeloma proteins were isolated by $(NH4)_2SO4$ precipitation followed by ionexchange chromatography (DEAE-50, Pharmacia), gelfiltration (G200, Pharmacia or AcA34, LKB) and/or affinity chromatography.
Fragments of a G1m(za) positive IgG1λ myeloma protein, were produced by digestion with papain or pepsin. Fab, Fc and pFc' fragments were isolated by gelfiltration (AcA44) and ionexchange chromatography by FPLC (Mono Q, Pharmacia).

Haemagglutination-inhibition assay

Human erythrocytes (OR2R2) were coated with incomplete Rh antibodies, mainly IgG1 and/or IgG3, or isolated IgG, IgA and IgM myeloma proteins using the chromic chloride method. The Ig coated cells were used in a 0.1% suspension in phosphate buffered saline (PBS). McAbs containing ascites was diluted in PBS with 5% foetal calf serum.

All HAI tests were performed in V-shape bottom microtitre plates by addition of 25 µl of successively (non)inhibitor, anti-allotype antibody and Ig coated cells. The plates were incubated overnight at 4°C. The results were read macroscopically over a light source after tilting the plates for about 10 minutes at an angle of about 60°.
The sera, proteins and peptides, used as (non)inhibitors were diluted in PBS.

Direct ELISA and ELISA-inhibition assay

Flat bottom polysterene plates were coated with myeloma protein (100 µl, 2 µg/ml in PBS) overnight at 4°C. After washing with PBS + 0,005% Tween 20 in the direct test 100 µl McAb containing supernatant or ascites fluid, diluted in PBS + 0.02% Tween 20 + 0.2% gelatine, were added and incubated 1 hour at 37°C.
After washing 100 µl horseradish peroxidase conjugated goat anti-mouse Ig (GM17, CLB) in a dilution of 1:5000 were added, followed by incubation at 37°C during 1 hour. As substrate 100 µl of a 0.1 mg/ml solution of tetramethyl benzidine in 0.11 M sodium acetate buffer pH 5,5 + 0.003% H2O2 was used. The reaction was stopped with 30 µl 2M $H2SO_4$ after a few minutes. The plates were read with an automated ELISA reader (Titertek, R Multiscan) at wavelength 450 nm.
In the ELISA-inhibition (or indirect ELISA) test the McAb was first incubated with the (non)inhibiting sample during one hour before the mixture was added to the Ig coated plate.

Results and discussion

Ascites fluid of two McAbs, 5A1 and 1C2, were tested in the haemagglutination (HA) test in a series of 12 ten-fold dilutions against a panel of erythrocytes coated with IgG of the different subclasses and allotypes and with IgA or IgM (Table 1). McAb 5A1 reacts strongly with the G1m(za) positive cells and weakly with some of the other IgG coated cells. McAb 1C2 reacts not only strongly with G1m(za) positive cells but also with all other IgG coated cells.
Haemagglutination-inhibition tests were performed with both McAbs with G1m(za) and with G1m(f) coated cells (Table 2). Our panels of Ig allo-

typed sera (diluted 1:20 and 1:60) and myeloma proteins (in several dilutions, starting 0.1 mg/ml) were used as inhibitors.
Only G1m(z) positive sera and proteins inhibited the agglutination by McAb 5A1 of G1m(za) coated cells. However the weak agglutination by McAb 5A1 of G1m(f) positive cells could not be inhibited. The agglutination by McAb 1C2 of G1m(za) positive cells was inhibited by G1m(a) positive samples only. This was in contrast to the agglutination of G1m (f) positive cells which was inhibited by all sera and IgG proteins. Therefore these results make the impression that McAb 1C2 has a dual specificity, depending on the circumstances that it can react as anti-G1m(a) and as anti-IgG antibody. Additional cloning experiments did not alter the specificity of this McAb. The usefulness of the McAbs 5A1 and 1C2 in the HAI test as typing reagents for G1m(z) and G1m(a) resp. could be confirmed by typing more than 1000 sera of different races. No differences were shown between the results with these McAbs and the results obtained with the conventional antisera of the same specificity.
The location of the epitopes, detectable with the McAbs 5A1 and 1C2 were investigated with Fab, Fc and pFc' fragment of a G1m(za) positive myeloma in the HAI assay (table 3). The G1m(z) specific reaction of McAb 5A1 was inhibited by the Fab fragments and not by Fc or pFc'. The G1m(a) specific reaction with McAb 1C2 was inhibited by Fc and by pFc' but not by the Fab fragment. These results are in complete agreement with the results which we have obtained in inhibition experiments with the conventional anti-G1m(z) and anti-G1m(a) antisera. This is also in agreement with the aminoacid analysis of $\gamma 1$ chains with different allotypes. It has been shown that the G1m(z) allotype depends on a difference in aminoacid 214 and the G1m(a) allotype on differences in amino acids 356-358.
The reactivity of the McAbs 5A1 and 1C2 was also tested in the direct ELISA (table 4) as well as in the ELISA inhibition test (table 5). In the direct tests McAb 5A1 reacts not only with the G1m(za) and (zax) positive myeloma proteins but also with IgG3 myeloma proteins, G3m(b) as well as G3m(g). Although the reaction with IgG3 is remarkable, it is not necessarily in contrast with the results of the HA test, because in our hands the ELISA test with IgG3 coated plates is more sensitive than with IgG of the other subclasses and thus this reactivity with IgG3 can be a reflection of the already observed weak anti-IgG reactivity of the antibody. Anyhow in the direct ELISA McAb 5A1 is not specific for G1m (z). McAb 1C2 reacts with IgG of all subclasses and allotypes in this direct test. However in the ELISA-inhibition test with G1m(za) coated plates McAbs 5A1 and 1C2 can be used as specific typing reagents for G1m(z) and G1m(a) respectively.
These results again demonstrate that the reactivity of McAbs with their epitope is strongly influenced by the presentation of the epitope on the molecule.

Table 1

McAbs 5A1 AND 1C2 IN HAEMAGGLUTINATION TEST

McAb[1]	Erythrocytes coated with: G1m(za)	G1m(zax)	G1m(f)	IgG2	IgG3	IgG4	IgA	IgM
5A1	11[2]	11	2	1	2	0	0	0
1C2	⩾12	⩾12	10	8	10	10	0	0

1) series of 12 ten-fold dilutions, starting 1:100
2) last tube in which agglutination was seen

Table 2

McAbs 5A1 AND 1C2 IN THE HAI-TEST

McAb	5A1		1C2	
dil.	10^{-6}	10^{-2}	10^{-4}	10^{-4}
	Eza	Ef	Eza	Ef
no sample	++	+	++	++
serum Gm(zaxg)	-	+	-	-
serum Gm(fnb)	++	+	++	-
serum Gm(zag)	-	+	-	-
serum Gm(afnb)	++	+	-	-
serum Gm(zfnb)	-	+	++	-
myel.prot.G1m(za)	-	+	-	-
other IgG myel.prot.	++	+	++	-
IgA	++	+	++	++
IgM	++	+	++	++
specificity	G1m(z)	aspecific	G1m(a)	IgG
strength	very strong	weak	strong	strong

++= strong agglutination
+= agglutination
-= inhibition

Table 3

LOCATION OF THE EPITOPES, DETECTABLE WITH THE McAbs 5A1 AND 1C2 BY HAI

McAb	5A1	1C2	
dilution	10^{-6}	10^{-4}	10^{-4}
	Eza	Eza	Ef
specificity	G1m(z)	G1m(a)	IgG
no sample	++	++	++
myel.prot.G1m(za)	-	-	-
Fab fragment	-	++	++
Fc fragment	++	-	-
pFc' fragment	++	-	-
	CH1	CH3	CH3

++ = strong agglutination
+ = inhibition

Table 4

REACTIVITY OF McAb 5A1 AND 1C2 IN DIRECT ELISA

McAb	5A1	1C2
dilution	10^{-5}	10^{-3}
coat		
G1m(za)	+	+
G1m(zax)	+	+
G1m(f)	-	+
IgG2	-	+
IgG3	+	+
IgG4	-	+
IgA	-	-
IgM	-	-
specificity	G1m(za)+IgG3	IgG

+ = positive
- = negative

Table 5

SPECIFICITY OF McAbs 5A1 AND 1C2 IN ELISA-INHIBITIONTEST WITH G1m(za)-COATED PLATES

McAb	5A1	1C2
dilution	10^{-5}	10^{-3}
no sample	+	+
serum G1m(z+a+)	-	-
serum G1m(z+a-)	-	+
serum G1m(z-a+)	+	-
serum G1m(z-a-)	+	+
specificity	G1m(z)	G1m(a)