Immunogenetics (2009) 61:91–100
DOI 10.1007/s00251-008-0344-9

ORIGINAL PAPER

Polymorphisms of MICA recognized by human alloantibodies

Yizhou Zou · Zhiqiang Qin · Allison Silveus · Yisun Fan · Peter Stastny

Received: 8 October 2008 / Accepted: 12 November 2008 / Published online: 10 December 2008

Abstract MICA antigens are polymorphic glycoproteins expressed on the surface of human endothelial cells and other cells. Antibodies against MICA have been found in transplant recipients and were found to be associated with decreased survival of kidney allografts. In the present work, we investigated the polymorphisms that are recognized by antibodies against MICA. Soluble MICA recombinant proteins representing 11 common alleles, two hybrid alleles, and two single amino acid mutated alleles were produced. Patterns of reactivity were determined with MICA bound to Luminex beads. In some studies, sera containing antibodies against MICA were absorbed by cell lines transfected with MICA*001, MICA*002, MICA*008, and MICA*009 or with untransfected cells, followed by testing of antibody reactivity against MICA proteins bound to beads. The monoclonal antibodies and sera used in this study were found to recognize up to 14 distinct MICA epitopes as demonstrated by their differential absorption/reactivity patterns. Among these, nine epitopes correlated with a single unique amino acid: one shared two signature amino acids, one shared three signature amino acids in close proximity, and three epitopes involved multiple amino acids in a nonlinear sequence. Two groups of public epitopes (MICA-G1 and MICA-G2) were characterized. MICA shared epitopes were determined by reactivity loss in single MICA antigen bead assays by absorption with MICA transfectants. Since these epitopes may be targets for antibody binding and possibly antibody-mediated allograft rejection, epitope identification may help understand the development of MICA antibodies and to identify suitable donors for sensitized transplant recipients.

Keywords MHC class I related gene A (MICA) · Epitope · Alloantibodies

Y. Zou · Z. Qin · A. Silveus · Y. Fan · P. Stastny (✉)
Department of Internal Medicine,
Division of Transplant Immunology,
The University of Texas Southwestern Medical Center,
5323 Harry Hines Blvd,
Dallas, TX 75390-8886, USA
e-mail: ps@sta08.swmed.edu

Introduction

The MICA antigens are products of a locus situated close to HLA-B in the human MHC (Bahram et al. 1994). MICA genes are polymorphic, with 54 distinct proteins having been described, according to the most recent published report (Robinson et al. 2003). When recombinant MICA proteins were produced in our laboratory, we found that they were able to react with antibodies in the sera from some organ transplant recipients (Zwirner et al. 2000). Moreover, the presence of antibodies against MICA in patient serum before transplantation was found to be associated with shortened graft survival of kidney transplants (Zou et al. 2007). However, because DNA samples were not available in that study, it could not be determined whether MICA antibodies that correlated with reduced survival of grafts were specific for the MICA antigens of the donors.

The determinants of MICA molecules that can be recognized by antibodies in human sera are not known. It is important to identify and characterize the antigenic epitopes in order to analyze the specificity of MICA antibodies associated with rejection. Such knowledge would go a long way toward the development of a virtual crossmatch, which might be useful for the selection of donors for sensitized recipients if antibodies against donor MICA antigens are shown to be harmful to the graft.

The method established in our laboratory for screening sera for antibodies against MICA antigens is based on the use of recombinant MICA alleles coupled to polystyrene microspheres (Zou et al. 2006b). In the analysis of the results obtained, certain patterns of reactivity were found repeatedly, and the amino acids that determine the epitopes could often be deduced from analysis of the distribution of the polymorphic residues and their correlation with the observed reactions of the sera.

When the antibody reactions were analyzed, it was observed that in addition to sera that recognized only one allele or two to three MICA antigens, two broad groups of sera reacting with many alleles could be identified. These complex patterns were analyzed by producing hybrid MICA molecules consisting of part of an allele belonging to one group and part of another allele belonging to the other group. In addition, site-directed mutagenesis was used to modify key amino acids and determine their role in the binding of antibodies that produced these complex patterns.

The analysis has led to the definition of 14 distinct antibody-binding epitopes that explain most of the serological patterns that we have observed in the course of testing several thousand sera. While it is possible that by using this approach we could have missed some epitopes that are rarely seen, the method used has the advantage that it is based on experimental data and therefore likely to be highly reproducible when additional work is performed using similar methods.

Materials and methods

Stable transfected cells and monoclonal antibodies

The cloned cDNA containing full-length MICA was transfected into HMy2.C1R cells, which do not express HLA-A, B, or MICA, but have small amounts of HLA-Cw4 (ATCC, Rockville MD, USA). Genes for transfection were composed of leader peptide, α1, α2, α3, TM, and cytoplasmic domains (Fig. 1c). MICA was expressed with a fused EGFP sequence, and successfully transfected cells could be monitored by fluorescence microscopy and sorted by flow cytometry. MICA-expressing HMy2.C1R cells were cultured in RPMI-1640 complete medium plus 500 μg/ml of G418. Stable transfectants expressing high levels of the alleles MICA*001,*002, *008, and *009 were produced. Expression was demonstrated by flow cytometry with the monoclonal antibodies 6B3 and 3.2H3 (Fig. 1d). Human

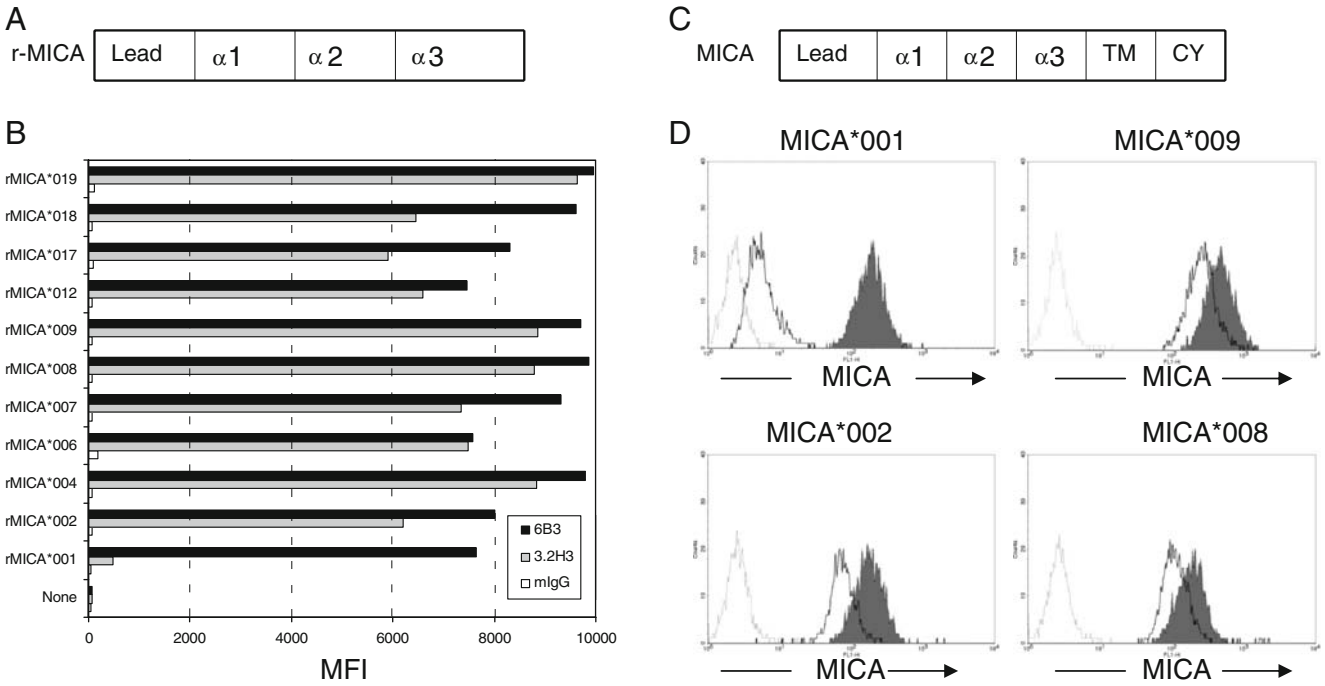

Fig. 1 Reaction of single antigen beads and MICA-expressing transfected cells with MICA-specific mAbs. **a** Soluble MICA recombinants were produced containing the extracellular leader peptide, α1, α2, and α3 domains. **b** The MFI was detected with mAbs 6B3 (*black bar*) or 3.2H3 (*gray bar*) and normal mouse IgG (mIgG, *open bar*) from 11 single antigen beads named as rMICA*001, *002, *004, *006, *007, *008, *009, *012, *017, *018, and *019. The negative beads (*None*) were used for serum background control. **c** The full-length MICA molecules expressed on the surface of transfected cells. **d** Surface MICA expression was confirmed by mAb 6B3 (*black profile*), 3.2H3 (*light profile*), and mIgG (*open profile*) in HMy2.C1R cells transfected with MICA*001, *002, *008, and*009

fibroblasts stably transfected with MICA*002 and MICA*008 by retroviral transduction were obtained as previously described (Zou et al. 2005). These cells were cultured in DMEM complete medium plus 500 μg/ml of G418. Surface expression of MICA on transfectants was also determined by flow cytometry.

Monoclonal antibodies (mAb) specific for MICA, including the monomorphic antibody 6B3, recognizing native MICA protein, the polymorphic mAb 3.2H3, which does not react with MICA*001 but recognizes all other MICA alleles tested, produced by us (Zou et al. 2002) were cultured in DMEM with 10% IgG-free FCS. mAb were isolated and purified from supernatants of cultures with an ImmunoPure IgG purification kit (Pierce, Rockford, IL, USA). Isotype-matched, purified normal mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at the appropriate concentrations as negative controls in some experiments.

Absorption and elution

Sera with antibodies against MICA antigens selected for absorption studies were obtained from ten kidney allograft recipients and six heart recipients. Also, a panel of 18 sera from healthy volunteers was used. For absorption of antibodies, 1.0×10^7 transfected cells were harvested and washed with PBS at pH 7.4 three times. Fifty microliters of selected serum was added to washed MICA-expressing cells and incubated at room temperature for 1 h. When the purpose was to remove specific antibodies from serum, absorptions were performed two to four times until no detectable antibodies remained. When the purpose was to obtain specific antibodies bound to transfected cells after serum absorption, cells were washed with PBS three times, and the bound antibodies were eluted by adding 45 μl of elution buffer (0.13 M citric acid, 60 mM Na_2HPO_4, pH 3.0). The eluates were immediately neutralized with 5.0 μl of 1 M NaH_2PO_4 (pH 9.0).

Antibody detection by single antigen MICA Luminex flow cytometry

Tests for IgG antibodies against MICA antigens were performed with the use of soluble MICA antigens coupled to polystyrene microbeads (Luminex). These antigens were based on our previous complementary DNA constructs of MICA*001, MICA*002, MICA*004, MICA*008, and MICA*009 (Zou et al. 2005). In this study, we produced seven more MICA alleles, including MICA*006, MICA*007, MICA*012, MICA*017, MICA*018, and MICA*019. Briefly, fragments encoding the signal peptide and the extracellular domains of these proteins, including a recognition sequence for a six-histidine coding sequence with a stop codon, were cloned into pFastBac-1 vector (Invitrogen) and selected after sequencing. Two hybrid MICA cDNAs were produced by combining pFastBac-MICA*002 and pFastBac-MICA*008 vectors using multiple-enzyme digestions and ligations. Two mutants were obtained using site-directed mutagenesis also from the mentioned vectors. A Bac-to-Bac Baculovirus Expression system was used to produce soluble proteins in High Five insect cells (Invitrogen). These recombinant proteins were affinity-purified with nickel-affinity agarose and coupled to polystyrene microspheres (Luminex, Austin, TX, USA). For the assay, 11 purified recombinant MICA alleles, and four modified alleles were individually coupled to 15 beads. In addition, negative and positive control beads were prepared by coating with and without normal human IgG according to the manufacturer's protocol (Luminex).

For the antibody measurements, 15 μl of test serum or control samples was incubated with beads in the dark for 30 min at room temperature (RT) and then washed with wash buffer (PBS, 0.05% Tween-20). Sixty microliters of goat anti-human IgG or goat anti-mouse IgG secondary antibody conjugated with R-phycoerythrin (Jackson, West Grove, PA, USA) was added to the beads and incubated for 30 min in the dark at RT. After washing, beads were re-suspended with 60 μl of PBS. Mean fluorescence intensity (MFI) was read in a Luminex 100 flow cytometer (Luminex). For every assay, we ran the same negative and positive control sera or a normal mouse IgG control (Jackson). A threshold was determined for each bead from the mean value for the relative amount of binding plus 3 SD, determined from serum samples from 18 healthy persons.

Data analysis and epitope determination

MFI were obtained from the output files (*.csv) generated with trimmed mean fluorescence values for each bead from the Luminex-100 flow machine. Reactions to a MICA allele were considered positive if MFI was greater than the threshold of the corresponding beads. Amino acid sequences of the MICA antigens were downloaded from the IMGT/HLA Database (release 2.21.0, April 08, 2008). For epitope searching, MICA alleles were arranged according to shared amino acids correlating with serologic reactions as shown in Table 2. Amino acids in nonpolymorphic positions in the tested alleles are not shown in the table. Antibody-reaction patterns were compared to the amino acid alignments and were considered to be generated by recognition of one or more amino acids shared by the reactive alleles. Antibody absorption and elution experiments were utilized to further refine the definition of the epitopes as described below in the "Results".

Results

Recognition of MICA antigens on beads and on the surface of transfected cells

Luminex beads were coated with 11 preparations of single MICA antigens, representing 11 MICA alleles. All of these beads gave strong signals when they reacted with the monomorphic MICA-specific mAb 6B3, which is a monoclonal antibody recognizing native MICA proteins. All were positive also with 3.2H3, except for MICA*001, which is known not to be recognized by this antibody (Zou et al. 2002). The mean fluorescence intensities varied between 6,000 and 10,000. Normal mouse IgG and beads without antigen showed very low background (Fig. 1b). Four full-length MICA molecules (MICA*001, *002, *008, and *009; Fig. 1c) expressed on the surface of human cell lines were recognized by mAb 6B3, and three of them (MICA*002, *008, and *009) were also stained by mAb 3.2H3 (Fig. 1d). Live MICA-expressing transfected cells were used to remove and/or elute MICA antibodies from human sera.

Determination of the key amino acids in epitopes using patient sera that reacted with a single MICA antigen

An initial analysis was based on antibodies that reacted with only one of the 11 MICA alleles being tested. The sera were P5-49, G-495, P7-47, and RJ, each reacting only with rMICA*001, *004, *006, and *012, respectively. Four distinct amino acids, one for each allele-specific epitope, could be identified in the linear alignment of variable amino acids as shown in Table 2. For example, since antibodies in serum P5-49 reacted with rMICA*001 but not with any other alleles, it appeared that the lysine (K) in position 125 was involved in the epitope that distinguishes MICA*001 from other alleles. Three other epitopes that characterized MICA*004, MICA*006, and MICA*012 were mapped in the same way and are highlighted in Table 2. Thus, serum G-495, which recognized MICA*004, appears to react with an epitope based on arginine (R) in position 181; serum p7–47, specific for MICA*006 binds an epitope based on isoleucine (I) in position 176; and serum RJ reacted with MICA*012, which involves leucine (L) in position 155. Other distinct amino acids in MICA antigens, for example, arginine (R) at position 91 in the case of MICA*017, were not recognized with the sera we have tested.

Identification of shared epitopes by antibody absorption

In the course of screening human sera for antibodies against MICA, it was observed that most alloantisera with positive reactions appeared to recognize more than one allele. It was not clear whether such sera contained one antibody against an epitope shared by the positive alleles or whether there were multiple antibodies in the same serum that were responsible for the complex patterns. Four typical reaction patterns of MICA antibodies found in patients are illustrated in Fig. 2.

In the case of serum Abs224E13, it recognized MICA*001, *012, and *018 but not others (Fig. 2a). This pattern appears to correlate with the presence of threonine (T) at position 24 since all of the other alleles have alanine (A) in this position (Table 2). After this, serum was absorbed with transfectant cells expressing MICA*001; the test with all the beads was found to be negative (Fig. 2a).

In another case, serum p5–58 (Fig. 2b) was positive with MICA*002 and MICA*017. After absorption with MICA*002 transfected cells, reactions to MICA*002 and to MICA*017 antigen beads were removed. The results were best explained by the recognition of glycine (G) in position 014 in the α1 domain of the MICA amino acid sequence in both of these alleles (Table 2).

Another reaction pattern was observed with serum p10–64, which had antibodies against three antigens. In this case, the beads that carry MICA*004, *006, and *009 gave positive signals. After this serum was absorbed with MICA*009-transfected cells, the positive signals with all three beads became negative. This was best explained by a shared epitope and could be mapped to valine (V) in position 122 (Table 2). However, serum p5-31 (Fig. 2c) not only reacted with beads having MICA*004, *006, and *009 but also gave positive signals with bead MICA*019. Removing antibodies from this serum by absorption with MICA*009 transfectant cells led to the loss of all of the positive signals. These results indicated that there was a shared epitope present in the four reactive alleles. This pattern of reactions may correspond to S in residue 175 (Table 2).

Serum p5–62, shown in Fig. 2d, reacted uniquely with MICA*008 and MICA*019, with all the other alleles being negative. This pattern appears to be associated with an epitope in the α3 domain formed by isoleucine (I) in position 213 and arginine (R) in position 251 (Table 2). Absorption with cells having MICA*008 removed reactivity with beads conjugated with the same allele, as well as with MICA*019, which shares this epitope. These absorption results confirmed the sharing of certain epitopes that were postulated on the basis of correlations between serum reactions and distribution of polymorphic amino acids.

Analysis of sera with complex reaction patterns

In addition to the patterns already described, there were many MICA positive sera that reacted with two reciprocal broad groups of antigens. Serum 224E13 reacted with beads coated with MICA*001, *002, *007, *012, *017, and *018 (Fig. 3a, before absorption). Serum 226E03 recognized the remaining alleles, namely MICA*004, *006,

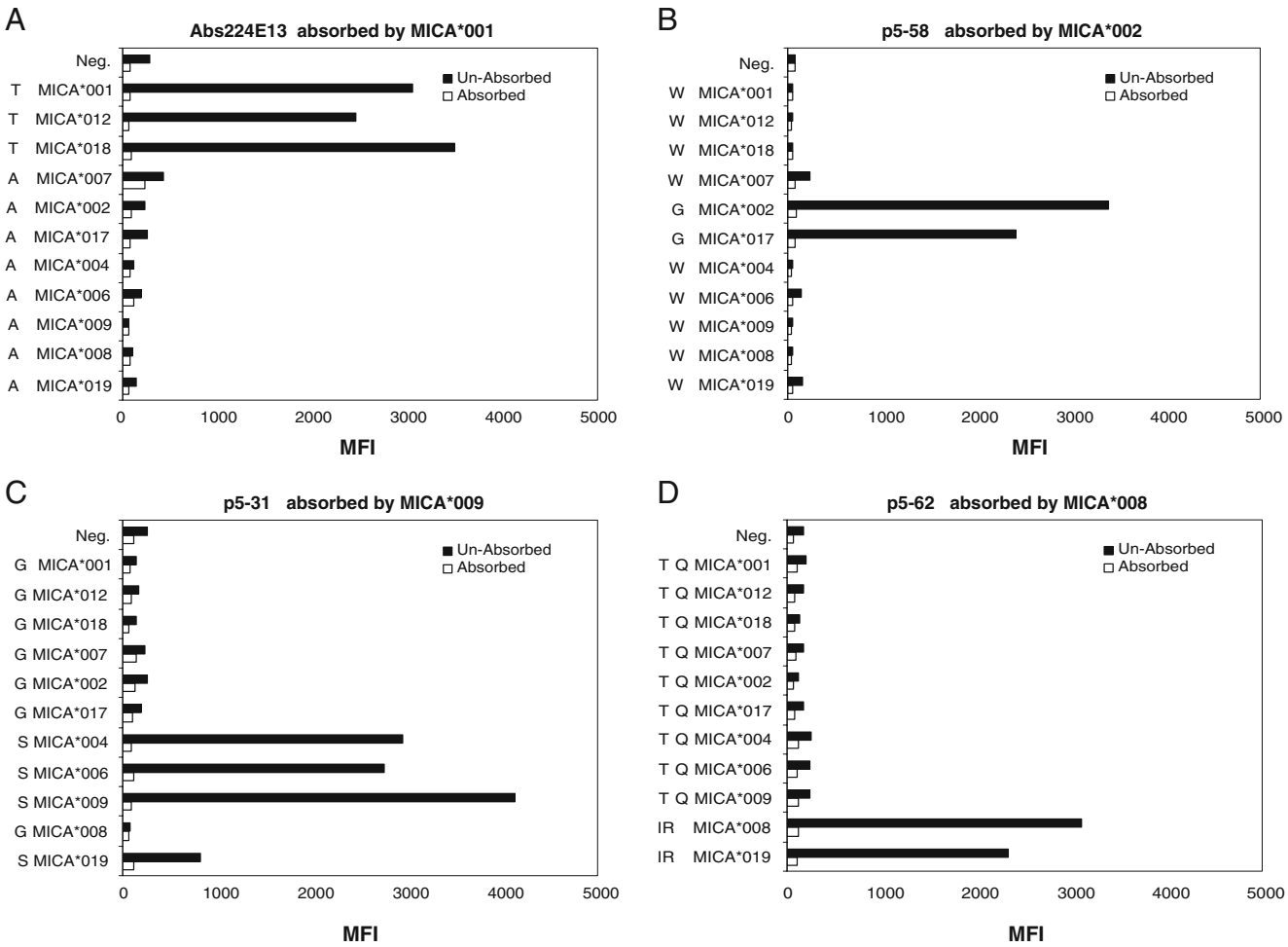

Fig. 2 Determination of MICA epitopes recognized by antibodies in four selected human sera using MICA single antigen beads. The data labels in *Y* axes of the graphs identify the single antigen beads with different MICA alleles. The *letters to the left of the labels* represent the amino acid(s) located in variable positions 24 (**a**), 14 (**b**), 175 (**c**), and 213 and 215 (**d**) of the MICA alleles. Values of MFI were obtained from sera with (*open bar*) and without (*black bar*) absorption by transfected cells, which are listed in the *top of graphs*

*008, *009 ,and *019 (Fig. 3b, before absorption). In order to analyze these complex patterns, antibody absorption was performed. For example, serum 224E13 corresponding to the first pattern, named MICA-G1, was absorbed sequentially with cells transfected with MICA*001 and MICA*002. This removed the reactivity with beads having the alleles included in group 1 (MICA-G1, absorbed, Fig. 3a). Absorption of serum 226E03 with MICA*008 and MICA*009 transfected cells removed the reactivity against MICA alleles of group 2 (MICA-G2) in this serum (absorbed, Fig. 3b).

Mapping epitopes for group-specific antibodies MICA-G1 and MICA-G2 using antibody absorption and elution

From the variable amino acid alignment, six amino acids appear to divide the MICA alleles into two groups: 36C, 129M, 173K, 206G, 210W, and 215S are associated with MICA-G1; 36Y, 129V, 173E, 206S, 210R, and 215T are associated with MICA-G2 (Table 2). In order to define the key epitopes involved in the reaction of these long groups, we produced hybrid recombinant proteins having part of MICA*002 and part of MICA*008, the two most common alleles in many populations. These hybrid molecules were made by exchanging the sequences at the middle. In addition, using site-directed mutagenesis, position 173 of the α2 domain in MICA*002 was changed from K to E (rMICA-02E, Table 1). Purified antibodies were obtained by absorption and elution from cells transfected with MICA*001 or MICA*009. Antibodies for MICA-G1 were obtained by absorption with MICA*001 transfectants and were measured on rMICA*002 beads, thus avoiding the recognition by the allele-specific alloantibody against MICA*001 in eluates. Similarly, antibodies for MICA-G2 were obtained by absorption with MICA*009 transfectants and were measured on rMICA*008 coupled beads. Table 1 shows the results of the reactions of the antibodies from these absorption–elution experiments.

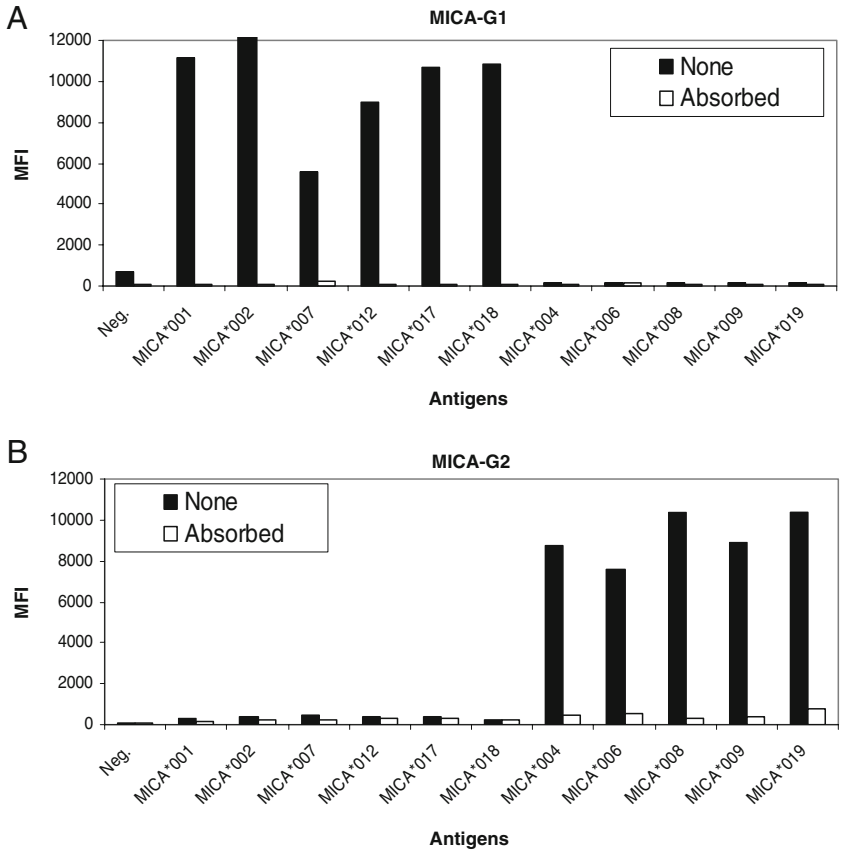

Fig. 3 Determination of MICA epitopes from serum samples containing MICA antibodies with group-specific patterns. Group-specific antibodies MICA-G1 (**a**) or MICA-G2 (**b**) in serum samples were detected by single antigen beads before (*black bar*) and after absorption (*open bar*) by transfected cells. Serum 224E13 (**a**) was absorbed with MICA*001 transfected cells followed by MICA*002 transfected cells, and serum 226E03 (**b**) was absorbed by MICA*009 transfectants following by MICA*008 transfectants. Single MICA antigen beads listed in *X* axes were ranked for MICA antigen groups

Table 1 Identification of shared epitopes by Luminex single antigen beads using antibodies eluted from transfectant cells

Antigen	Epitopes[a]	MICA-G1[b]	MICA-G2[c]
rMICA*002	C M K G W S	+ +	—
rMICA*008	Y V E S R T	—	+ +
rMICA-288[d]	C M E S R T	+	+ +
rMICA-822[d]	Y V K G W S	+	—
rMICA-02E[e]	C M E G W S	+ +	—
rMICA-08M[e]	Y M E S R T	—	+ +

[a] Epitopes listed are based on amino acids thought to be recognized by group-specific antibodies. They are 36C/Y in α1, 129M/V and 173K/E in α2, and 206G/S, 210W/R, and 215S/T in the α3 domains
[b] Antibodies absorbed and eluted from MICA*001 transfected cells
[c] Antibodies absorbed and eluted from MICA*009 transfected cells
[d] Hybrid recombinants of MICA*002 and MICA*008
[e] Mutated recombinant by site-directed mutagenesis

Table 2 Mapping MICA epitopes based on detection with antibodies against MICA found in patient sera

	[a]α1			α2									α3				
	0[b]	0	0	0	1	1	1	1	1	1	1	1	2	2	2	2	2
	1[c]	2	3	9	2	2	2	5	7	7	7	8	0	1	1	1	5
Allele	4[d]	4	6	1	2	5	9	5	3	5	6	1	6	0	3	5	1
MICA*001	W	T	C	Q	L	K	M	H	K	G	V	T	G	W	T	S	Q
MICA*01201	–	–	–	–	–	E	–	L	–	–	–	–	–	–	–	–	–
MICA*01801	–	–	–	–	–	E	–	–	–	–	–	–	–	–	–	–	–
MICA*00701	–	A	–	–	–	E	–	–	–	–	–	–	–	–	–	–	–
MICA*00201	G	A	–	–	–	E	–	–	–	–	–	–	–	–	–	–	–
MICA*017	G	A	–	R	–	E	–	–	–	–	–	–	–	–	–	–	–
MICA*004	–	A	Y	–	V	E	V	–	E	S	–	R	S	R	–	T	–
MICA*006	–	A	Y	–	V	E	V	–	E	S	I	–	S	R	–	T	–
MICA*00901	–	A	Y	–	V	E	V	–	E	S	–	–	S	R	–	T	–
MICA*00801	–	A	Y	–	–	E	V	–	E	–	–	–	S	R	I	T	R
MICA*019	–	A	Y	–	–	E	V	–	E	S	–	–	S	R	I	T	R

a: α-MICA extracellular domain; b, c, d represent location numbers of polymorphic amino acids.

Antibodies against MICA-G1 antigens appeared to react with epitopes formed by residues in α1 and/or α3 domains because the two hybrid recombinants rMICA-288 (formed by combining the first part of MICA*002 and the second part of MICA*008, Table 1) and rMICA-822 (formed by combining the second part of MICA*002 and the first part of MICA*008, Table 1) remained reactive. M in position 129 of the six amino acids could be excluded as a target for recognition by these antibodies because the mutated recombinants (rMICA-08M, Table 1) in which 129V was changed to M in position 129 did not react with these antibodies. Since eluted antibodies to MICA-G1 reacted strongly with rMICA-02E (Table 1) with K in position 173, this position did not appear to be needed; however, since the sample might contain multiple antibodies, this position could still be involved in the reactions of this group. The antibodies recovered from sera with the MICA-G2 reaction pattern appeared to recognize epitopes defined by three key amino acids, which were always found together (206S, 210R, and 215T) in the α3 domain (Table 2). Because the hybrid recombinant rMICA-822, which carried the α1 and half of α2 of rMICA*008 did not react, the amino acids 36Y and 129V could be excluded in the reactivity of these antibodies. The molecule formed by switching amino acid K to E from rMICA*002 (rMICA-02E) was not recognized by these antibodies; therefore, the three amino acids 206S, 210R, and 215T in α3 domain of MICA*008 and other antigens belonging to MICA-G2 group were identified as the key amino acids for this reactivity. Table 2 shows the variable amino acid alignments of the 11 MICA alleles tested. In all, there were 17 positions where polymorphism could be detected in at least one allele. Highlighted amino acids define the groups we have identified. About 25–30% of sera, which contained antibodies against MICA, were found to have specificity for either group MICA-G1 or group MICA-G2.

Determination of key amino acids involved in other group-specific antibodies

Besides the two types of reciprocal group-specific antibodies (MICA-G1 and MICA-G2, Table 3), three serum samples with long group-specific reactivity were observed in MICA antibody screening using 11 MICA antigens. The first one reacted to all alleles tested except rMICA*001. Since this sample lost its reactivity by absorption with any one of the transfected cells with MICA*002, MICA*008, or MICA*009, the reaction is best explained as due to a unique amino acid 125E, which is shared by all the reactive antigens (MICA-G3, Table 3).

Serum CP1-64 reacted to all alleles tested except MICA*008 and MICA*019 (MICA-G4, Table 3). The pattern could best be explained by presence of two antibodies, against MICA-G1 and against an epitope defined by 122V (pattern 7, Table 3), because the reactivity of MICA-G1 was removed by transfected cells of MICA*001, and the remaining reactivity to rMICA*004, rMICA*006, and rMICA*009 could be removed after absorption by transfected cells with MICA*009 (data not shown). In the same way, serum p07-16 (pattern 14, Table 3) might contain two antibodies, one reacting to

Table 3 Determination of MICA epitopes recognized by human patient sera

Pattern ID	Antigen Name	Allele(s) included	Position/amino acid(s)
1	MICA-1	MICA*001	125K
2	MICA-4	MICA*004	181R
3	MICA-6	MICA*006	176I
4	MICA-12	MICA*012	155L
5	MICA-1-12-18	MICA*001, MICA*012, MICA*018	24T
6	MICA-2-17	MICA*002, MICA*017	14G
7	MICA-4-6-9	MICA*004, MICA*006, MICA*009	122V
8	MICA-8-19	MICA*008, MICA*019	213I, 251R
9	MICA-4-6-9-19	MICA*004, *006, *009, *019	175S
10	MICA-G1	MICA*001, *002, *007, *012, *017, *018	36G, 173K, 206G, 210W. 215S
11	MICA-G2	MICA*004, *006, *008, *009, *019	206S, 210R, 215T
12	MICA-G3	Not MICA*001	125E
13	MICA-G4	Not MICA*008, MICA*019	36G, 173K, 206G, 210W, 215S, 122V
14	MICA-G5	Not MICA*001, MICA*0012, MICA*018	206S, 210R. 215T, 14G

MICA-G2 group antigen and another recognizing epitope 14G of MICA like Pattern 6 (Table 3), because all antigens, which have these epitopes, gave positive reactions. Furthermore, the fact that these reactions were removed after absorption by transfected cells either with MICA*009 or with MICA*001 confirmed the presence of two antibodies in this serum (data not shown).

Discussion

The antibody response against MICA is in many ways similar to the antibody response against HLA antigens. However, MICA antigens are expressed more selectively; the amounts of MICA protein on the cell surface where it is expressed are smaller, and the polymorphism is not as great as either HLA class I or HLA class II.

Because of these factors and the relatively lower frequency of sensitization against MICA, the method of immunization against MICA in persons who have not been transplanted is not well understood (Zou et al. 2007). It is clear that most healthy persons do not have antibodies against MICA. However, sera from patients with endstage renal disease awaiting kidney transplantation have been found to have such antibodies. When we examined the role of transfusions, which have been shown previously to induce the production of antibodies against HLA (Zou et al. 2007), the evidence is not that clear. Patients who had received six or more transfusions were found to have a definite increase in the frequency of antibodies against HLA, but antibodies against MICA were not affected (Zou et al. 2007). It is possible that MICA is expressed only on a very small population of endothelial cell precursors and monocytes (Vermehren and Sumitran-Holgersson 2002), and therefore, immunization with blood cells is relatively weaker. Perhaps many more transfusions are required and perhaps other factors prevalent in patients with endstage renal disease being prepared for transplant also play a role.

Some nonspecific binding to MICA-coated beads has been observed in our experiments. Such reactions are easily recognized because all the beads give positive signals, while the negative control bead is usually clearly negative. The hallmark of these nonspecific reactions is that the sera producing them do not bind to MICA on live cells when performing flow cytometry. These nonspecific reactions may be due to antibodies binding to sugars on the heavily glycosylated recombinant MICA antigens or to other contaminating materials, including perhaps hidden determinants found in denatured recombinant antigen preparations. Denatured MICA was demonstrated using mouse monoclonal antibodies known to recognize only denatured linear peptides of MICA. Separately, it has recently been suggested that recombinant glycoproteins made in High Five insect cells may contain carbohydrate that can be recognized by crossreactive antibodies sometimes found in human subjects (Hancock et al. 2008).

Specific antibodies against MICA appear to arise against mismatched epitopes, which can be defined by traditional methods. Analysis of the sera included in this study has led to the definition of antigenic epitopes based on the distribution of polymorphic amino acids and antibody-reactive patterns to 11 common MICA alleles tested. It was easy to define the polymorphic amino acids that were associated with recognition of only one of the 11 MICA antigens tested even though other alleles were not tested in this panel. Examples are MICA*001 defined by 125K, MICA*004 characterized by 181R, MICA*006 correlated

with 176I, or MICA*012 associated with 155L (Table 2). When a serum sample reacted with two or three MICA alleles of the 11 antigens tested, sharing of unique amino acids was postulated, and antibody absorption confirmed the sharing of epitopes. Analyzing the antibody reaction patterns, a unique amino acid may be shared by two (14G in MICA*002 and MICA*017) or three (24T in MICA*001, MICA*012, and MICA*018; 122V in MICA*004, MICA*006, and MICA*009) or four (175S in MICA*004, MICA*006, MICA*009, and MICA*019) MICA alleles. In another instance, a pair of amino acids (213I, 251R) are shared by two alleles (MICA*008, MICA*019). All of these patterns were based on observed examples of such sera shown in the Luminex antibody detection assay combined with antibody absorption with transfectant cells expressing single MICA alleles at high levels.

In addition, we have found two reciprocal broad patterns of reactivity with MICA, which appear to be due to complex epitopes involving six key amino acids. These patterns were quite frequent and involved group 1, which included MICA*001, MICA*002, MICA*007, MICA*012, MICA*017, and MICA*018, and group 2, consisting of MICA*004, MICA*006, MICA*008, MICA*009, and MICA*019. Some of the complexity was resolved by performing absorptions with transfectant cells expressing single MICA alleles at high levels. Further characterization of the key amino acids involved was achieved by using hybrid molecules and antibodies against the antigen groups purified by absorption and elution.

The hybrid molecules contained part of MICA*002 (a member of group 1) and part of MICA*008 (from group 2, Table 1). Mutated molecules consisted of MICA*002, in which position 173 was changed from K to E and MICA*008 with a switch in position 129 from V to M. From the results of these experiments, we concluded that neither of the central positions (129, 173) was essential for the reaction of the group-specific antibodies. Group 1 antibodies appeared to require variable amino acids in the alpha-1 and/or alpha-3 domains. The antibodies eluted from group 2 alleles required three key amino acids in the alfa-3 domain (206S, 210R, and 215T).

Thus, as in HLA serology, we can define “short” and “long” patterns in the serologic reactions of antibodies against MICA. The presence of multiple antibodies in human sera makes the determination of epitopes more difficult, but confirmation was possible in most cases by performing absorptions and elutions using stable MICA transfectant cells. The picture that develops from these experiments gives credence to the antibody studies and confirms their specificity. With this information, we can gain a better understanding of the immunogenicity of the mismatches of MICA alleles in recipients and donors of organ transplants. The role of specific antibodies can now be correlated with the outcome of transplants as it becomes possible to define antibodies against specific donor-mismatched MICA epitopes. Finally, this information should prove invaluable for the development of a virtual crossmatch based on antibody testing and high-resolution typing for MICA and may lead to the recognition of mismatches that might be of high risk to the transplanted organ and therefore considered unacceptable in the same way as high-risk donor HLA antigens are to be avoided. This work is therefore a necessary preparation for the studies in which we plan to correlate specific antibodies against donor MICA antigens and to correlate the presence or the new development of such antibodies with the outcome in organ transplants. To determine whether antibodies against donor MICA antigens are needed for the correlation with kidney transplant outcome we previously reported (Zou et al. 2007), in an ongoing project, we have identified recipients in the Collaborative Transplant Study database for whom serum and donor DNA samples are available. Sera will be tested for antibodies against MICA, and donor DNA will be analyzed by sequence-based typing for high-resolution MICA allele determination (Zou et al. 2006a). Results will be analyzed using the new information about MICA epitopes described in the present report.

References

Bahram S, Bresnahan M, Geraghty DE, Spies T (1994) A second lineage of mammalian major histocompatibility complex class I genes. Proc Natl Acad Sci USA 91:6259–6263 doi:10.1073/pnas.91.14.6259

Hancock K, Narang S, Pattabhi S, Yushak ML, Khan A, Lin SC et al (2008) False positive reactivity of recombinant, diagnostic, glycoproteins produced in High Five insect cells: effect of glycosylation. J Immunol Methods 330:130–136 doi:10.1016/j.jim.2007.08.002

Robinson J, Waller MJ, Parham P, de Groot N, Bontrop R, Kennedy LJ et al (2003) IMGT/HLA and IMGT/MHC: sequence databases for the study of the major histocompatibility complex. Nucleic Acids Res 31:311–314 doi:10.1093/nar/gkg070

Vermehren D, Sumitran-Holgersson S (2002) Isolation of precursor endothelial cells from peripheral blood for donor-specific cross-matching before organ transplantation. Transplantation 74:1479–1486 doi:10.1097/00007890-200212150-00001

Zou Y, Mirbaha F, Lazaro A, Zhang Y, Lavingia B, Stastny P (2002) MICA is a target for complement-dependent cytotoxicity with mouse monoclonal antibodies and human alloantibodies. Hum Immunol 63:30–39 doi:10.1016/S0198-8859(01)00349-4

Zou Y, Bresnahan W, Taylor RT, Stastny P (2005) Effect of human cytomegalovirus on expression of MHC class I-related chains A. J Immunol 174:3098–3104

Zou Y, Han M, Wang Z, Stastny P (2006a) MICA allele-level typing by sequence-based typing with computerized assignment of polymorphic sites and short tandem repeats within the trans-

membrane region. Hum Immunol 67:145–151 doi:10.1016/j.humimm.2006.02.016

Zou Y, Heinemann FM, Grosse-Wilde H, Sireci G, Wang Z, Lavingia B et al (2006b) Detection of anti-MICA antibodies in patients awaiting kidney transplantation, during the post-transplant course, and in eluates from rejected kidney allografts by Luminex flow cytometry. Hum Immunol 67:230–237 doi:10.1016/j.humimm.2006.02.017

Zou Y, Stastny P, Susal C, Dohler B, Opelz G (2007) Antibodies against MICA antigens and kidney-transplant rejection. N Engl J Med 357:1293–1300 doi:10.1056/NEJMoa067160

Zwirner NW, Marcos CY, Mirbaha F, Zou Y, Stastny P (2000) Identification of MICA as a new polymorphic alloantigen recognized by antibodies in sera of organ transplant recipients. Hum Immunol 61:917–924 doi:10.1016/S0198-8859(00)00162-2