Research Report

Antibody-Based Approach to High-Volume Genotyping for MIC-1 Polymorphism

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Abstract

Macrophage inhibitory cytokine-1 (MIC-1) is a divergent member of the TGF-β superfamily. There are at least two known alleles of MIC-1 that are due to a G→C point substitution at position 6 of the mature protein, which alters a histidine to an aspartic acid (MIC-1 H *and MIC-1* D*). We have determined the phenotype of MIC-1 circulating in serum by exploiting the differences in the affinity of the two monoclonal antibodies to the* H *and* D *alleles of MIC-1. A PCR-RFLP-based method for genotyping MIC-1 is also described. We validate these two assays using DNA sequencing of 19 subjects as the standard. We then used the validated assay to determine the frequency of the two MIC-1 alleles in a population of 261 adult blood donors. Inter-assay and sequencing concordance was 100%. The frequency of the three common MIC-1 genotypes was homozygous (*H H*), 54%; heterozygous (*H D*), 39%; and homozygous (*D D*), 7%. This novel antibody-based assay confidently determines the genotype of MIC-1. It offers the advantages of an ELISA—ease of automation, high-volume throughput of samples, and ease of use in a routine, clinical laboratory.*

INTRODUCTION

Macrophage inhibitory cytokine-1 (MIC-1) is a recently described divergent transforming growth factor (TGF-β) superfamily cytokine, first cloned by us on the basis of increased mRNA expression associated with macrophage activation (1) and subsequently by others (8). The production of antibodies to MIC-1 has allowed us to develop a sensitive sandwich ELISA that is capable of detecting this cytokine in human serum samples. We have previously reported that serum MIC-1 levels are markedly elevated in pregnant women (7), and early data have also suggested that elevated serum MIC-1 levels occur in colorectal, breast, and prostate carcinoma and may be related to tumor cell mass. Immunohistochemistry has indicated that there is increased MIC-1 staining in these neoplasms compared with surrounding normal tissue (2). Increased MIC-1 mRNA production caused by colorectal and prostate tumors has also been detected by both SAGE and microarray analysis (3,9). This suggests that local production by these tumors is responsible for the elevation of serum MIC-1 levels. These studies all suggest a possible clinical utility for the measurement of cytokine serum levels.

In our early work on the cloning of MIC-1, it was apparent that it was present in at least two allelic forms because of a C→G point substitution (4). This altered a histidine (*H*) to an aspartic acid (*D*) at position 6 of the amino acid sequence of the mature protein (MIC-1 *H* to MIC-1 *D*). This represents a nonconservative substitution of a weakly basic, aromatic to a strongly acidic, acyclic amino acid. It is possible that this polymorphism has functional consequences for MIC-1 biology, and genotyping may thus have clinical utility. A simple way to assess this appeared when epitope mapping studies indicated that one of our monoclonal antibodies (MAbs) (13C4H4) had a markedly reduced affinity for the MIC-1 *D* protein (5).

Using the above antibodies, we have developed an immunoassay that allows accurate inference of an individual's MIC-1 genotype from serum phenotype. We describe the antibody-based genotyping assay and a complementary PCR-RFLP-based method for genotyping DNA. Using the validated ELISA-based assay, we have determined the MIC-1 genotype in a population of 261 normal blood donors.

MATERIALS AND METHODS

Serum and DNA Samples

Serum and DNA samples were obtained from 19 ambulatory laboratory workers. DNA was isolated into samples using a QIAamp® DNA blood mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol. These samples were used for assay validation.

Normal serum samples (288) were obtained from the Red Cross Blood Bank (Adelaide, Australia). The age distribution was from 17 to 71 years ($\bar{x}$ = 48; SD = 13), with 139 males and 122 females. All donors met the selection criteria for blood donation, according to standards from the Australian Red Cross

Blood Services South Australia. All samples were collected with informed consent, as approved by St. Vincent's Hospital Ethics Committee.

Anti-MIC-1 Antibodies

Mouse ascites were obtained from previously described mouse MAb-producing hybridomas, designated MAb 13C4H4 and MAb 26G6H6 (5–7). Polyclonal antiserum 233-P was obtained from sheep immunized with recombinant hMIC-1 as previously described (5). Polyclonal anti-MIC-1 IgG antibody was purified from serum using caprylic acid, followed by ammonium sulfate precipitation (6).

Immunoprecipitation

MAb 13C4H4 was adsorbed to protein-A Sepharose® beads (Amersham Biosciences, Uppsala, Sweden). Conditioned culture medium (1 mL) from prostate carcinoma cell line PC3 was immunoprecipitated by incubating the sample with the antibody-bound beads overnight at 4°C, and the beads were then washed five times with PBS containing 1% (v/v) Triton® X-100. Bound proteins were eluted using nonreducing SDS sample buffer and analyzed by SDS-PAGE, followed by immunoblot analysis with the MAb 26G6H6 (7). MAb 26G6H6 was diluted 1:5000 in PBS containing 1% BSA and 0.05% Tween® 20 (all from Sigma, St. Louis, MO, USA). Specificity was further confirmed by blocking the MAb 26G6H6 with 10 µg purified recombinant MIC-1. Anti-mouse IgG-biotin (Jackson Immunoresearch, West Grove, PA, USA) was used at a dilution of 1:2000 as the secondary antibody.

Direct ELISA

Maxisorp™ 96-well ELISA plates (Nalge Nunc International, Roskilde, Denmark) were coated at 4°C for 24 h with 100 µL/well of either 20 ng/mL rhTGF-β1 (R&D Systems, Minneapolis, MN, USA) or 18 ng/mL MIC-1 in coating buffer (0.1 M carbonate-bicarbonate in distilled water, pH 9.6). Plates were washed three times with 300 µL/well wash buffer [PBS containing 0.05% (v/v) Tween 20]. Nonspecific binding was blocked with the addition of 250 µL/well of 1% (w/v) BSA in PBS for 2 h at 37°C. Antibodies 13C4H4 and 26G6H6, polyclonal sheep antibody 233-P, and IgG-enriched normal sheep and mouse IgG1 (R&D Systems) used as controls were then added to the plates (100 µL/well) and incubated for 1 h at 37°C as previously described (7). The plates were washed three times, followed by the addition of 100 µL/well biotinylated donkey anti-sheep IgG or biotinylated goat anti-mouse IgG (both from Jackson Immunoresearch) at a 1:10 000 dilution and incubated for 1 h at 37°C. The plates were then washed again three times, and 100 µL/well HRP-conjugated streptavidin (Genzyme, Cambridge, MA, USA) at a 1:2000 dilution were added to the plates and incubated for 30 min at 37°C. Plates were then washed four times, and 100 µL/well 1 mg/mL o-phenylenediamine dihydrochloride (Sigma) were added, using the manufacturer's recommended buffer. Color development was allowed to proceed for 5–15 min, and the development was terminated with the addition of 100 µL 2 M sulfuric acid. The absorbance was measured at 490 nm in a Sanofi® LP400 microplate reader (Pasteur Diagnostics, Saltzberg, Austria).

Total MIC-1 Concentration Determination

The MIC-1 sandwich ELISA used for the quantification of total MIC-1 in serum was performed essentially as previously described (7). In brief, the mouse anti-hMIC-1 MAb 26G6H6 was used for antigen capture, and sheep anti-MIC-1 antibody 233-P was used for detection. The hMIC-1 serum concentration was determined by reference to a standard curve constructed using recombinant hMIC-1 protein as the standard. The optimum concentration for both antibodies was determined by checkerboard titration and then used for all subsequent assays. Three human serum samples that had undergone multiple serum MIC-1 determinations in this assay format were used as controls. Sample diluent was used as a background control. All samples were assayed in triplicate, and the CV for sample triplicates was generally less

than 10%. The three serum controls, representing the three genotypes, were used in each assay as described earlier. The CV for more than 40 repeated readings of these samples was 10%–12%.

MIC-1 Genotyping Assay

The genotyping assay is an immunoassay-based method to detect phenotype and, from the phenotype, deduce the genotype of the circulating serum protein. In the case of MIC-1, this is determined based on an estimation of the serum MIC-1 levels with an antibody (13C4H4) with low affinity for MIC-1 *D*, compared with an antibody (26G6H6) capable of binding both MIC-1 *H* and MIC-1 *D* as described above. A MIC-1 sandwich ELISA was established utilizing the mouse anti-hMIC-1 MAb 13C4H4 for antigen capture and the sheep PAb 233-P for detection as described above. The optimum concentration of both antibodies was determined by checkerboard titration and then used for all subsequent studies. Maxisorp 96-well ELISA plates were coated with MAb 13C4H4 supernatant diluted 1:500 in coating buffer at 4°C for 24 h. ELISA plates were then washed and blocked as for a direct ELISA. Recombinant human MIC-1 standards and controls, representing the three allelic combinations (*HH*, *HD*, and *DD*), and serum samples in antibody diluent (1% BSA and 0.05% Tween 20 in PBS) were then added to the plates (100 µL/well) and incubated for 1 h at 37°C. The plates were washed three times, followed by the addition of 100 µL/well sheep PAb 233-P diluted 1:25 000 in antibody diluent and incubated for 16 h at 4°C. Washings and the addition of secondary antibody and reagents for detection were performed as for a direct ELISA. Color development was allowed to proceed until a clear difference was seen between the lowest rhMIC-1 standard and the zero standards. The reaction was allowed to continue until the zero standard, when assessed with the naked eye, had a perceptibly higher absorbance than the *DD* serum control. When these criteria were satisfied and the 1000 pg/mL standard had an absorbance of greater than one, the ELISA development was terminated as described above. All samples were assayed in triplicate.

To determine the MIC-1 phenotype, the observed MIC-1 concentration obtained from the 13C4H4 assay was divided by the total MIC-1 concentration determined in the 26G6H6 assay. Preliminary studies had previously indicated that these ratios fell into three distinct groups when rounded to one decimal point: below 0, between 0.0 and 0.5, and greater than 0.7, representing homozygous D (*DD*), heterozygous (*HD*), and homozygous H (*HH*) genotypes, respectively. These ratios were then chosen for further evaluation.

Ratiometric PCR-RFLP Assay for MIC-1 Genotyping

A standard master mixture for *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA) was made according to the manufacturer's recommendations with 1 µL each 10 pM 5p and 3p (5p, 5′-GCCGCCGCCGTCGCAGTCGGA-3′; 3p, 5′-CAGGCGGTGCAGGCTCGTCTTGAT-3′) primers to a final volume of 20 µL/reaction. Genomic DNA (100 ng) was used as a template. Denaturation was performed at 94°C for 1 min, annealing at 65°C for 1 min, and extension at 72°C for 2 min. Forty cycles were then performed in a PTC-200™ Peltier thermal cycler (MJ Research, Waltham, MA, USA). This yielded a specific product of 318 bp that was subsequently digested with the restriction enzyme *Ava*II (New England Biolabs, Beverly, MA, USA).

Three microliters of PCR product were directly digested at 37°C overnight with *Ava*II, according to the manufacturer's instructions, in a total volume of 20 µL. The digest was run on a 3% agarose gel containing 0.02% (w/v) ethidium bromide at 80 V until separate bands were observable. Genotype was determined by comparing the band pattern and intensity.

DNA Sequencing

Products from the above reaction were purified from the agarose gel using the QIAEX® II Gel Extraction Kit (Qiagen), according to the manufacturer's instructions. The fragments were then sequenced using the ABI PRISM® BigDye™ terminator RR mixture (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's recommended protocol, and analyzed using an ABI PRISM DNA 377 sequencer. Each fragment was sequenced in both directions, using the above PCR primers.

RESULTS

Sensitivity and Specificity of Antibodies

The epitope binding sites of these antibodies have been extensively characterized (5). The specificity of the MAb 13C4H4 and the PAb 233-P has also been described (7). The specificity of the 26G6H6 MAb was further deter-

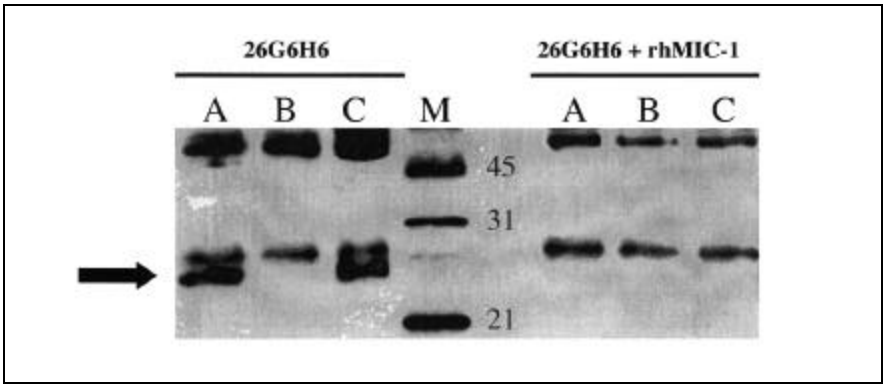

Figure 1. MAb 26G6H6 is specific for MIC-1. Western blots were stained with MAb 26G6H6 and MAb 26G6H6, with excess rhMIC-1 to block antibody binding. The arrow represents MIC-1. Additional lanes at the top and just above MIC-1 represent the heavy and light chains of the precipitating MAb 13C4H4. M, Bio-Rad® Broad molecular markers (kDa) (Hercules, CA, USA). (A) PC3-conditioned medium, (B) unconditioned medium, and (C) rhMIC-1.

mined by the immunoprecipitation of the purified recombinant MIC-1 with MAb 13C4H4, followed by immunoblot analysis with MAb 26G6H6. MAb 26G6H6 specifically recognized the 25-kDa dimeric MIC-1. Additionally, preincubating the antibody with purified recombinant MIC-1 before Western blot analysis greatly reduced the staining of the MIC-1-specific 25-kDa band (Figure 1).

One of the unusual but consistent features of the MIC-1 ELISA, with MAb 13C4H4 used for antigen capture, was that subjects with *DD* protein exhibited an absorbance lower than zero. We hypothesized that the cause of an absorbance reading below the zero standard for these samples was due to the reduced affinity of the 13C4H4 capture antibody for the *DD* protein (5). In this ELISA, there is prolonged incubation of the MIC-1 *DD*, bound to the 13C4H4 capture antibody, with the higher affinity 233-P antibody. The low affinity of this MAb for the *DD* protein, coupled with the higher affinity of the PAb, results in a transfer of the protein from the solid phase-bound monoclonal to the liquid phase. In the liquid phase, the *DD* protein remains strongly bound by the PAb. This leads to a substantial increase in the amount of PAb-bound MIC-1 in solution. The antigen-bound polyclonal is then unavailable to bind nonspecifically to the plate, thereby decreasing the background. This situation leads to a reduced absorbance level below the zero standard. To provide evidence to support this view, we used doubling dilutions of serum from a known homozygous *D* subject in the 13C4H4 sandwich ELISA. As expected, with reducing serum concentrations, the absorbance returned to the zero value, which supports this hypothesis (Figure 2).

Comparison between MIC-1 Genotype Determined by ELISA with that of DNA Sequencing

Figure 3 shows a typical DNA sequencing trace for the *HH*, *HD*, and *DD* genotypes. The genotype of the 19 ambulatory laboratory workers was determined in the genotyping assay (Table 1). There was 100% agreement between these two methods.

Table 1. Comparison of MIC-1 Genotype Determined Using the Antibody-Based Method, PCR-RFLP, and DNA Sequencing

ID	R26	R13	13/26	Antibody, PCR-RFLP, and DNA Sequencing
A	45.3	<0	<0	*DD*
B	49.9	12.0	0.2	*HD*
C	41.1	8.8	0.2	*HD*
D	40.3	13.7	0.3	*HD*
E	82.9	21.0	0.3	*HD*
F	35.8	6.3	0.2	*HD*
G	45.6	6.5	0.1	*HD*
H	838.1	215.8	0.3	*HD*
I	54.0	14.5	0.3	*HD*
J	37.0	6.6	0.2	*HD*
K	124.0	117.6	0.9	*HH*
L	32.5	38.4	1.2	*HH*
M	38.3	45.2	1.2	*HH*
N	503.2	520.9	1.0	*HH*
O	73.7	70.7	1.0	*HH*
P	36.5	38.4	1.1	*HH*
Q	182.3	122.8	0.7	*HH*
R	65.6	74.4	1.1	*HH*
S	58.2	66.3	1.1	*HH*

R26 represents the MIC-1 level derived using 26G6H6 as the capture antibody, R13 represents the MIC-1 level derived using 13C4H4 as the capture antibody, and 13/26 is the ratio obtained from dividing R13 by R26.

Comparison between MIC-1 Genotype Determined by PCR-RFLP with that of DNA Sequencing

We developed an additional DNA-based method for MIC-1 genotyping based on the PCR amplification of a region of the MIC-1, followed by PCR-RFLP analysis. RFLP assays were a mainstay of mutational analysis for many years; however, they have been superseded by less labor-intensive PCR assays. In the case of MIC-1 DNA, the area around the point mutation respon-

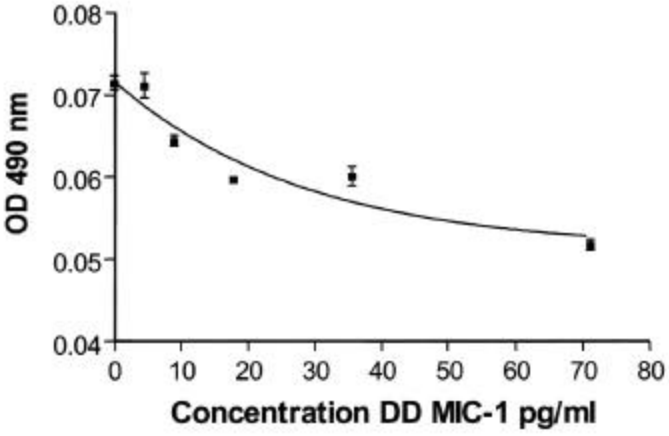

Figure 2. Monoclonal capture of MIC-1 from *DD* homozygotes. ELISA results showing MIC-1 capture using 13C4H4 MAb. Results shown from triplicate experiments.

sible for the allelic determinant has a very high GC content (approximately 90%). This makes it very difficult to use strategies such as competitive PCR assays to determine allelic differences. However, we were able to develop an assay combining both PCR and RFLP to determine the MIC-1 genotype.

The generation of this assay involved the exploitation of several characteristics of agarose gel electrophoresis. The high-percentage gel used (3%) allowed maximal separation of the relatively small (147 and 102 bp) bands produced by cleavage. The small size of the bands relative to the change in base pair numbers by cleavage (45 bp) led to large differences in the amount of ethidium bromide intercalated to magnify the band intensity differences between the 147- and 102-bp fragments. The differences in band separation would have been impossible to resolve if the change in band size was, for example, from 1002 to 1047 bp. The magnified difference in band intensity (147 vs. 102) made it easy to determine the different genotypes based on the band pattern and brightness. Homozygous *H H* subjects had about equal band intensity in the 147- and 102-bp fragments. In heterozygotes *H D*, the 147-bp band was significantly brighter than the 102-bp fragment. In the case of the homozygous *D D* samples, only the 147-bp band is present. An additional factor magnifying the band intensity difference was that in the homozygous *H* DNA, there were equimolar amounts of the 102- and 147-bp bands. This produced a pattern of two bands, the upper (147 bp) with a slightly greater intensity than the lower (102 bp). In the case of the heterozygote, there was a 3:1 ratio of the 147- to the 102-bp band, producing a very bright upper band (147) compared to the lower band (102) (Figure 4).

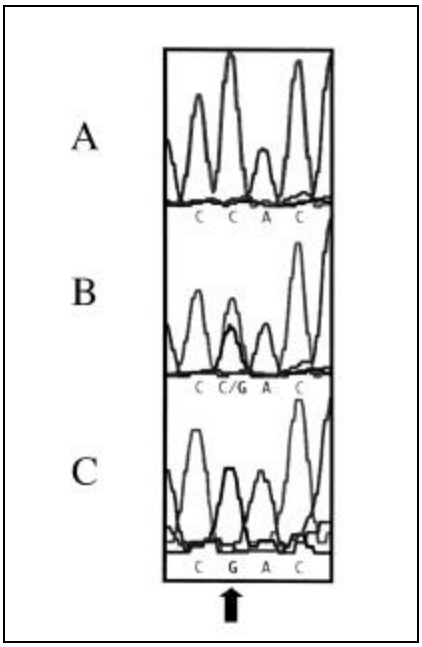

Figure 3. A representative trace of sequencing of PCR-amplified region of the MIC-1 gene. The arrow represents the nucleotide changed between the genotypes. (A) Homozygous *H* genotype. (B) Heterozygous *H D*. (C) Homozygous *D*.

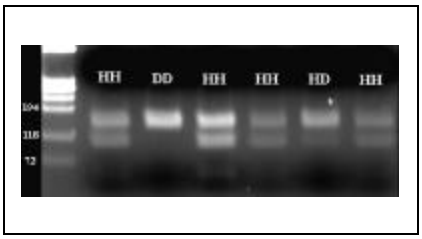

Figure 4. *Ava*II restriction digest of a PCR-amplified region of MIC-1 from the genomic DNA of six patients. Each lane is labeled with the phenotypes confirmed by DNA sequencing. Note that the predicted 45-bp product may be seen in the homozygote *H*.

When comparing the results of ratiometric PCR-RFLP analysis to the concordant genotyping assay and sequencing data, there was 100% agreement (Table 1).

Genotype of MIC-1 in a Normal Population

We also mapped the frequency distribution of the ratios obtained in the genotyping assay for each subject. The frequency distribution histogram indicates that there were clear differences between the genotypes with no overlap (Figure 5). The ratios obtained were comparable to the data derived from the original validation study, which provided additional validation for the genotyping assay. The *D D* absorbances obtained from the genotyping assay were all less than zero. This means that it is impossible to derive a meaningful genotype ratio from the standard curve, except to say that it is less than zero. Because there is a clear separation from zero for heterozygous *H D* genoytype ratios, it follows that these ratios are clearly separated from the homozygous *D D* ratios.

Using the genotyping assay, the gene frequencies of MIC-1 *D* and *H* were de-

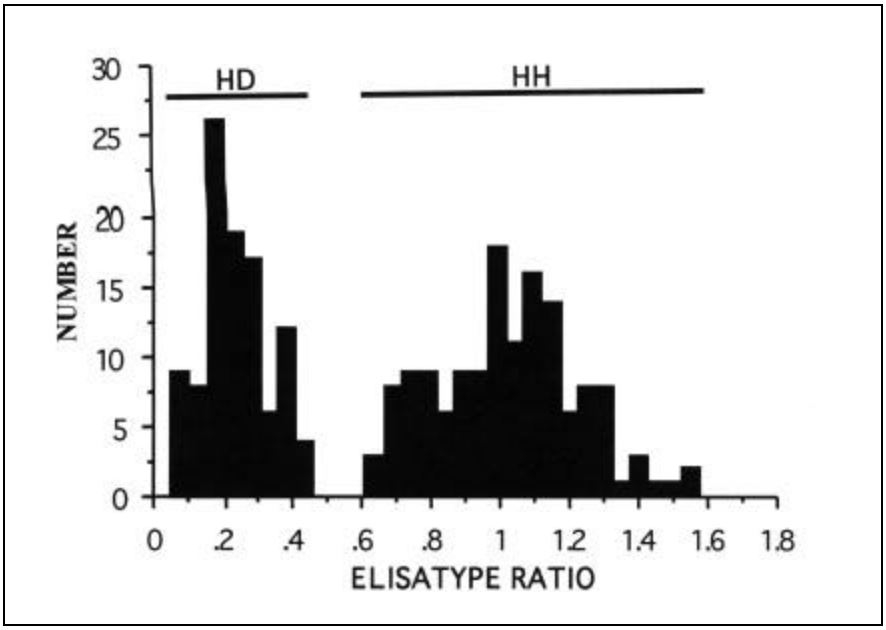

Figure 5. Frequency distribution histogram of 261 normal individual raw (not rounded to one decimal point) genotyping ratios. There is clear separation between zero and the heterozygous *H D* subjects and between the heterozygous *H D* and homozygous *H H* The homozygous *D D* results are not plotted because they cannot be derived from the standard curve. Because all the homozygous *D D* genotyping ratios are below zero, it follows that there is clear separation between the heterozygous *H D* and homozygous *D D* genotyping ratios.

termined in a normal population. The genotype frequency was 54% for *H H*, 39% for *H D*, and 7% for *D D*. This conforms to the ratios predicted by the Hardy-Weinberg equilibrium. The presence of these two relatively common alleles raises the possibility of there being a survival advantage for one or both allelic products in different circumstances. This assay may be used as a tool to determine MIC-1 allele frequencies in various disease populations.

DISCUSSION

With the widespread availability of genomic databases, the number of point mutations and their relation to functional biology are increasing exponentially. Genotyping to predict risk or the likely course of disease is becoming increasingly important and moving from the research laboratory to clinical practice. For this to become commonplace, it is important that assays be developed that are suitable for use in clinical pathology laboratories. The assays must be cost-effective, simple to perform, and ideally, utilize existing technology.

Here we describe a MAb-based genotyping assay to determine the major allelic forms of MIC-1, a TGF-β superfamily cytokine. This method of genotyping has several advantages. The equipment and reagents required for this method are inexpensive, easy to maintain, and available in most reasonably equipped laboratories. The format of the assay allows a large number of samples to be analyzed simultaneously with relatively inexpensive reagents and can be easily automated. The principle of this assay and the circumstances that lead to its development may indicate a strategy to develop other protein genotyping assays.

Several broad principles seem to have emerged for the development of these types of assays. It is likely that point mutations associated with changes in the function of a secreted protein will have alterations in antigenic characteristics. This is because this type of alteration is more likely to occur on the exposed protein surface and be associated with a nonconservative substitution. Therefore, it will be possible to generate MAbs or even anti-peptide antibodies directed at these determinants. It is quite likely that even now MAbs capable of discriminating single amino acid substitutions in proteins occur frequently. However, they probably go unrecognized because they are not screened in a manner appropriate for detecting this difference. In the case of MIC-1, one of four MAbs discriminated between MIC-1 alleles. This affinity difference was exploited to produce this assay.

In addition to antibody-based genotyping, we have developed a strategy to overcome the problem faced in RFLP analysis when no unique restriction cleavage sites are available. This method, although more labor intensive than the antibody-based method, can be easily performed with the most basic DNA amplification equipment. This analysis requires no specialized training or equipment beyond the ability to perform SAGE, which makes this an ideal additional confirmatory assay to accompany the above method in the routine diagnostic laboratory.

CONCLUSION

Here we describe a novel MAb-based assay capable of detecting different allelic forms MIC-1. This ratiometric ELISA-based assay is capable of determining the MIC-1 phenotype from protein circulating in the blood of all individuals. From this phenotype, it is then possible to determine patient genotype. The approach we have adopted could be the forerunner of a range of immunoassays capable of undertaking high-throughput genotyping, a procedure that is going to be important, based on the genetic diversity data arising from the human genome project.

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