Simultaneous Analysis of Serum Immunoglobulins in Patients With M Protein Using Cellulose Acetate Membrane Isoelectric Focusing

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We developed a method for the simultaneous analysis of microheterogeneity of human serum IgG, IgA, IgM, IgD, and IgE, and serum protein pattern using cellulose acetate membrane isoelectric focusing, and analyzed in 11 healthy subjects and 67 patients with M protein (17 cases of multiple myeloma [MM] and 50 cases of monoclonal gammopathy of undetermined significance [MGUS]). Using this method, bands indicating the microheterogeneity of each immunoglobulin could clearly be detected. Among healthy subjects, the detected IgG, IgA, and IgM bands did not vary, but the detected IgE and IgD bands did vary. Therefore, IgA, IgM, and IgG were selected for comparison of serum immunoglobulins in MM and in MGUS. In the IgA-type M protein group, normal IgM and IgG bands were decreased in MM patients compared to MGUS patients, while the M band and other bands were increased in

MM patients compared to MGUS patients, but the differences between the two groups were not significant. In the IgG-type M protein group, normal IgM, IgA, and IgG were significantly decreased in MM patients compared to MGUS patients. We examined the changes in electrophoretic pattern in six MM patients and eight MGUS patients with IgA-type M protein after neuraminidase treatment. The width of the M band in MM patients with IgA-type M protein decreased with neuraminidase treatment. On the other hand, the width of the M band in MGUS patients with IgA-type M protein increased with neuraminidase treatment. We concluded that the decrease of the normal immunoglobulins in MM patients with IgG type M protein could be detected by this method, and IgA type of M protein binding sugar chain were different between MM and MGUS patients. *J. Clin. Lab. Anal.* 13:145–150, 1999. © 1999 Wiley-Liss, Inc.

Key words: human serum immunoglobulin; multiple myeloma; monoclonal gammopathy of undetermined significance

INTRODUCTION

When serum protein fraction is analyzed using agarose gel electrophoresis, the abnormal band that appears in the immunoglobulin region is called M protein or M band. The M protein is often found in the serum of patients with multiple myeloma (MM) and monoclonal gammopathy of undetermined significance (MGUS). MM is an immunodeficiency disease characterized by the proliferation of malignant plasma cells, abnormal production of monoclonal immunoglobulin, and a low level of normal immunoglobulins. MGUS is a monoclonal γ -globulinaemia, as is MM, but the normal immunoglobulin level does not decrease in MGUS. The incidence of monoclonal gammopathies increases with aging (1). The presently used diagnostic criteria of MM were proposed by Kyle (2). They are based on the following findings: the

existence of the M protein, bone punched-out lesion figures, the proliferation of malignant bone marrow puncture cells, and the reduction in normal levels of immunoglobulin. MM can be diagnosed by these criteria at present, but it is difficult to discriminate between MM and MGUS. Although there are many cases that satisfy some of these criteria, they could not be diagnosed as MM. Therefore, long-term observation is needed for accurate diagnosis (3). Immunoglobulin microheterogeneity occurs due to variations in the types and num-

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bers of sugar chains, and in the amino-acid sequence. Sialic acid, which is negatively charged, is often combined to the terminal of sugar chains, and increased activity of sialyltransferase which combines sialic acid to the sugar chain terminus has been reported in MM (4). Isoelectric focusing (IEF) has been shown to be a useful technique for the analysis of these microheterogeneous molecules. There have been some reports of the analysis of the M protein using IEF (5–7), but there are no reports yet on the simultaneous detection of different immunoglobulins. We developed a method for IEF using cellulose acetate membrane (CA membrane), and previously reported that it could detect microheterogeneity of five different immunoglobulins (IgM, IgA, IgG, κ -chain, and γ -chain) and a protein fraction simultaneously (8–10). In this study, we attempted to examine differences in the isoelectric pattern of each immunoglobulin, in the pI of M protein, and in the M protein binding sugar chain between MM and MGUS patients using this IEF method.

MATERIALS AND METHODS

Chemicals

Separax EF (CA membrane) and 20% Sepaline 3.5–10 (carrier-ampholyte) were from Fuji Photo Co. (Tokyo, Japan). 40% SERVLYT 4–6, 40% SERVLYT 9–11 (carrier-ampholyte) were from Boehringer Ingelheim (Heidelberg, Germany). Coomassie brilliant blue G-250 was from Sigma Chemical Co. (St. Louis, MO). Neuraminidase III was from TOYOCO Co. (Osaka, Japan). Peroxidase-conjugated rabbit antihuman IgE immunoglobulin (lot no. 027), peroxidase-conjugated rabbit antihuman IgD immunoglobulin (lot no. 012), peroxidase-conjugated rabbit antihuman IgM immunoglobulin (lot no. 050), peroxidase-conjugated rabbit antihuman IgA immunoglobulin (lot no. 055), and peroxidase-conjugated rabbit antihuman IgG immunoglobulin (lot no. 115) were from Dako Chemicals (Glostrup, Denmark). Blockace (blocking solution) was from Dainihon Pharmaceutical Co. (Osaka, Japan). PBS buffer was from MBL Co. (Nagoya, Japan). Other chemicals of reagent grade were purchased from Wako Pure Chemicals (Osaka, Japan).

Samples

Serum samples collected from 67 (MM 17, MGUS 50) inpatients or outpatients with M protein who underwent clinical examinations at Gunma University Hospital were used in this experiment. The presence of the M protein in these sera was confirmed by CA membrane electrophoresis, and the type of M protein was identified using immunofixation. Control sera were collected from 11 normal volunteers.

Isoelectric Focusing on Multilayer Membrane

We used a thermo-module cooling IEF apparatus (HIPEP ISOPHOR MK-1, COSMO BIO, Tokyo, Japan). The condi-

tions for electrophoresis were those we previously reported for this method, with some modifications (10). The carrier-ampholyte solution was composed of 9.4% Sepaline 3.5–10, 0.2% SERVLYT 4–6, 1% SERVLYT 9–11 and 10% saccharose. The 30 min preliminary electrophoresis at 500 V was followed by sample application (3 μ l) and stepwise electrophoresis as follows: at 300 V for 10 min, 500 V for 20 min, 1,000 V for 25 min, 1,500 V for 30 min, and 2,000 V for 5 min.

Protein Staining and Western Blotting

After electrophoresis, the six sheets of CA membrane were peeled off one by one. The top CA membrane was stained with Coomassie brilliant blue G-250 according to the method of Toda et al. (8).

The proteins of the five CA membranes, except for the top membrane, were transferred onto polyvinylidene difluoride (PVDF, Immobilon, Millipore Co., Bedford, MA) membranes using a transfer module (AE-6656, ATTO Co., Osaka, Japan). Western blotting was performed according to the reported method (11), except that the transfer was at a run constant current of 1.5 mA/cm² for 5 min, and the methanol concentration in the transfer buffer was 5%. After blotting, the PVDF membranes were blocked for 1 hr with a blocking solution. These PVDF membranes were washed three times in T-PBS (containing 0.05% Tween 20 in phosphate buffered saline) for 1 min each time. Peroxidase-conjugated rabbit antihuman IgD, IgE, IgM, and IgA antibodies were diluted 1,000-fold in T-PBS, and peroxidase-conjugated rabbit antihuman IgG antibody was diluted 500-fold in T-PBS. The PVDF membrane to which the second CA membrane had been transferred was immersed in diluted anti-IgD solution, the PVDF membrane to which the third CA membrane had been transferred was immersed in diluted anti-IgE solution, the PVDF membrane to which the fourth CA membrane had been transferred was immersed in diluted anti-IgM solution, the PVDF membrane to which the fifth CA membrane had been transferred was immersed in diluted anti-IgA solution, and the PVDF membrane to which the bottom CA membrane had been transferred was immersed in diluted anti-IgG solution. Each PVDF membrane was incubated overnight at room temperature. After incubation, these PVDF membranes were washed three times in T-PBS for 5 min each time. Staining was performed according to our previously described method (10).

Desialylation

Desialylation was carried out in a microtest tube where we added 10 μ l serum and 10 μ l of 1 U/mL neuraminidase III diluted in 0.1 M phosphate buffer, 5 mM EDTA, pH 6.0 at 37°C overnight.

Analysis of Electrophoretic Patterns and Statistical Analysis

Electrophoretic patterns were analyzed on a Macintosh 6100/60AV computer using the public domain NIH Image

program. Statistical comparisons were made using the Student's *t*-test.

RESULTS

Simultaneous Detection of IgD, IgE, IgM, IgA, and IgG Microheterogeneity in Healthy Subjects

The present method provides six different types of information simultaneously. Thus, we attempted the simultaneous detection of serum IgD, IgE, IgM, IgA, and IgG microheterogeneity, and of protein fraction using six CA membranes. The electrophoretic patterns of IgD, IgE, IgM, IgA, and IgG microheterogeneity, and the serum protein isoelectric patterns from four healthy subjects are shown in Figure 1. All of the membranes showed clear electrophoretic patterns. Among healthy subjects, the IgG, IgA, and IgM bands detected did not differ in number or intensity, but the IgE and IgD bands differed in intensity and pI range. The isoelectric point ranges of the immunoglobulin bands were pI 4.7–8.3 for IgD, pI 4.9–9.0 for IgE, pI 4.9–7.3 for IgM, pI 3.9–6.1 for IgA, and pI 4.9–9.0 for IgG.

Comparison of the Electrophoretic Patterns of IgG, IgM, and IgA in MM Patients and in MGUS Patients

We compared the electrophoretic patterns of IgG, IgM, and IgA in MM and MGUS patients. At first, we looked for differences between the pI of the M protein band of MM patients and MGUS patients, but we could not observe any

clear differences. Then, we examined the possibility that the decrease of production of normal immunoglobulin in MM patients could be detected by this method. The electrophoretic patterns of 2 MM patients, of an MGUS patient, and of a healthy subject are shown in Figure 2. Lanes 1 and 4 show the electrophoretic patterns for the IgG-type M protein in an MM patient and in a healthy subject, respectively. In the MM patients, the M protein was clearly seen, but the normal IgG bands detected in healthy subjects were hardly seen in that patient. We also examined the electrophoretic patterns for IgA and IgM in this MM patient also; the IgA and IgM bands were located in the same region as normal IgA and IgM, but the intensities of these bands were decreased compared to those of healthy subjects. On the other hand, lane 2 shows the electrophoretic pattern for an MGUS patient with IgG-type M protein. The levels of the IgM, IgA and IgG bands were the same as those of healthy subjects, except for the M band. No decrease in the production of normal immunoglobulin was detected. Lane 3 shows the electrophoretic pattern of an MM patient with IgA-type M protein. Increases of M protein and other bands were seen in the IgA electrophoretic pattern, whereas the IgG and IgM bands detected in the same region as healthy subject were decreased. The ratios between the level of each immunoglobulin, in MM and MGUS patients and in healthy subjects, were calculated (except the M protein portion) and the results are shown in Table 1. In the IgA-type M protein group, the IgM and IgG ratios in MM patients were lower than those in MGUS patients, and the IgA ratio in MM patients was greater than that in MGUS patients, but there were no significant differences between the two groups.

Fig. 1. Isoelectrophoretic patterns of immunoglobulin microheterogeneity in healthy subjects on 6 layers of CA membrane. 1, For the top membrane, serum protein detection was used; 2, for the second membrane, IgD detec-

tion; 3, for the third membrane, IgE detection; 4, for the fourth membrane, IgM detection; 5, for the fifth membrane, IgA detection; 6, for the bottom membrane, IgG detection.

Fig. 2. Isoelectrophoretic patterns of immunoglobulins in MM patient, in MGUS patient and in healthy subjects. 1, Electrophoretic pattern obtained by IgM detection; 2, electrophoretic pattern obtained by IgA detection; 3, electrophoretic pattern obtained with IgG detection. The electrophoretic immunoglobulin patterns in lane 1 are from an MM patient with IgG-type M protein; those in lane 2 are from an MGUS patient with IgG-type M protein; those in lane 3 are from an MM patient with IgA-type M protein; those in lane 4 are from a healthy subject.

In IgG-type M protein group, the IgM, IgA, and IgG ratios in MM patients were significantly lower than those in MGUS patients. This result indicated that the decrease in the production of normal immunoglobulin in MM patients with IgG-type M protein could be detected by this method.

Change of Serum Electrophoretic Pattern in MM Patients and in MGUS Patients With IgA M Protein After Neuraminidase Treatment

The electrophoretic patterns for IgA were stained strongly in almost all MM patients with IgA-type M protein as shown

in lane 3 of Figure 2. The sera of six MM patients and eight MGUS patients with IgA-type M protein were treated with neuraminidase, and the electrophoretic patterns of IgA with and without neuraminidase treatment were compared. The results are shown in Figure 3. Lane 1 shows the electrophoretic pattern in the MGUS patient without neuraminidase treatment and lane 1' shows the electrophoretic pattern in the MGUS patient with neuraminidase treatment. The width of the M band in MGUS patients increased after neuraminidase treatment, and IgA with a wide range of pIs was stained strongly. On the other hand, in MM patients, the IgA-type M protein band and wide pI range of IgA were strongly stained without neuraminidase treatment (lane 2), but the pI range of IgA was reduced by neuraminidase treatment, and only IgA in a part of the pI range was stained strongly (lane 2'). We performed the densitometric analysis of the patterns in these patients with and without neuraminidase treatment, and we compared the half peak of the width of the M protein bound in the densitometric pattern (Table 2). The width of the M protein band in MGUS patients was increased 1.78 ± 1.10 mm (mean $\pm$ SD) by neuraminidase treatment, but the width of the M protein band in MM patients was decreased 1.65 ± 0.95 mm by neuraminidase treatment.

DISCUSSION

The most important feature of the method used in this study is that IEF can be performed using layers of CA membranes. With this method, it is possible to peel off the CA membranes one by one after electrophoresis and use a different detection method for each CA membrane. In this study, we attempted the simultaneous detection of serum IgD, IgE, IgM, IgA, and IgG microheterogeneity, and protein pattern using six CA membranes. Clear electrophoretic patterns were obtained in all membranes using this method.

We compared isoelectric pattern of each immunoglobulin of MM patients with that of MGUS patients, and pI of M protein of two groups of patients were compared using IEF also. The IgG, IgA, and IgM bands detected in healthy subjects did not vary much in number or concentration, but the

TABLE 1. Normal Immunoglobulin Level Ratio Between MM/MGUS Patients and Healthy Subjects

Type of M protein	n	Optical density ratio (patients ^a /healthy subjects ^b)		
		IgM	IgA	IgG
IgA type M protein				
MM	6	0.560 ± 0.371	2.757 ± 1.962	0.701 ± 0.531
MGUS	8	1.468 ± 1.314	2.164 ± 1.046	1.587 ± 1.106
IgG type M protein				
M	11	0.595 ± 0.589**	0.724 ± 0.489**	0.699 ± 0.457*
MGUS	42	1.352 ± 0.861	1.483 ± 0.883	1.085 ± 0.535

^aOptical density of IgM, IgA, and IgG, except the M protein portion, in MM and MGUS patients.

^bOptical density of IgM, IgA, and IgG in healthy subjects, except the M protein portion detected in patients. Each value represents the mean $\pm$ S.D.

* $P < 0.05$.

** $P < 0.01$, vs. MGUS patients with IgG-type M protein. The significance of differences of values was determined by the Student's *t*-test.

Fig. 3. Isoelectrophoretic patterns of immunoglobulin for an MM patient with IgA M protein and an MGUS patient with IgA M protein with or without neuraminidase treatment. Lanes 1 and 1' show the electrophoretic pattern in an MGUS patient without and with neuraminidase treatment, respectively. Lanes 2 and 2' show the isoelectric pattern in an MM patient without and with neuraminidase treatment, respectively.

IgE and IgD bands did. We therefore selected IgA, IgM, and IgG for comparison of serum immunoglobulins in MM and in MGUS. Many researchers have attempted to analysis the M protein using IEF (5–7), but they have not been able to find clear differences between the pI of the M protein in MM and in MGUS, nor to discriminate between MM and MGUS using IEF. We looked for differences between the pI of the M proteins between the two groups, but we also failed to detect any. It has been reported that the frequency of actuarial probability of malignant transformation was from about 15 to 19% for 10 years based on long-term follow up data in MGUS (1,12,13). We think that one possible reason explaining the lack of difference between the pI of the M protein in MM and

in MGUS may be as follows. If transformation from MGUS to MM is often observed over the long term, then the amino acid sequence of abnormal immunoglobulin produced by plasma cell clones would not vary.

It is well known that the production of normal immunoglobulin is blocked in MM patients. Separate quantitative analysis of normal immunoglobulin and M protein is difficult using the ELISA or the immunoturbidity methods, which are generally used for quantitative analyses. When a great reduction in the production of normal immunoglobulin occurs, as in the reduction of the γ -globulin fraction, except for the M protein in the serum protein fraction, it can be easily detected. However, a small reduction of normal immunoglobulin production is not easy to detect. We therefore evaluated the possibility for detecting the decrease of production of normal immunoglobulins in MM patients, because our ultimate purpose was the easy determination of disease condition of a patient based on the findings of this method. In the IgG-type M protein group, the levels of normal IgM, normal IgA and normal IgG in MM patients were significantly decreased in MM patients as compared to the levels in MGUS patients. In the IgA-type M protein group, there were slight decreases in the levels of normal IgM and normal IgG in MM patients as compared to MGUS patients, but there were no significant differences between the two groups. Thus, the decrease in the production of normal immunoglobulins in MM patients with IgG-type M protein could be detected by this method. Moreover, qualitative changes in the levels of immunoglobulins were detectable. These results could not be obtained by quantitative analysis methods such as the ELISA or immunoturbidity methods.

On the other hand, in IgA-type M protein group, the IgA ratio in MM patients was slightly higher than that in MGUS

TABLE 2. Width of M Protein Band in IgA Patients With and Without Neuraminidase Treatment

Patient	No.	Width of M protein without neuraminidase treatment (A) ^a	Width of M Protein with neuraminidase treatment (B) ^a	A–B ^b
MM	1	1.95	1.15	0.80
	2	2.80	1.40	1.40
	3	5.10	3.45	1.65
	4	3.40	2.35	1.05
	5	5.75	2.05	3.70
	6	4.90	3.60	1.30
	Total	3.98 $\pm$ 1.36	2.33 $\pm$ 0.93	1.65 $\pm$ 0.95
MGUS	1	2.00	3.30	–1.30
	2	2.00	1.90	0.10
	3	3.90	7.20	–3.30
	4	1.50	4.90	–3.40
	5	4.90	6.70	–1.80
	6	1.00	3.20	–2.20
	7	4.65	5.80	–1.15
	8	2.00	3.15	–1.15
	Total	2.74 $\pm$ 1.41	4.52 $\pm$ 1.79*	–1.78 $\pm$ 1.10*

^aEach value represents the mean (mm) $\pm$ S.D.

^bThe value was obtained by subtracting B from A.

* $P < 0.01$, vs. MGUS in patients with IgA-type M protein. The significance of differences between values was evaluated by the Student's *t*-test.

patients, but there were no significant differences between the two groups. The electrophoretic pattern of IgA revealed staining over a wide range of pI. IgA is dimeric, and it has nine bound sugar chains. However, it has been reported that the number of binding sugar chains increases in MM, and that the structure of the sugar chains bound to IgA displays a high degree of heterogeneity not only in the number of carbohydrate chains, but also in their composition (14). The peripheral blood mononuclear cell and serum sialyltransferase activities in MM were higher than in MGUS (4). Crook et al. suggested that the sialylation status of the immunoglobulin paraproteins may be different for IgG, IgA, and IgM in MM patients groups (15). We speculate that this was due to changes of sugar chain structure. To test this idea, we treated sera from MM patients and from MGUS patients with neuraminidase, and compared the width of the M bands with and without neuraminidase treatment. The width of the M band in MM patients decreased with neuraminidase treatment. On the other hand, the width of the M band in MGUS patients increased with neuraminidase treatment. Although Schipper et al. reported that the IgG-type M protein in MM patients had more subfractions extending over a wider pI range than the M protein in patients with benign monoclonal gammopathy, and that the M protein in MM is less stable and more likely to release charged amino groups (5), we speculate that the reason that the width of the M band in MM patients decreased with neuraminidase treatment is as follows. Since there are various types and numbers of sugar chains bound to the IgA M protein in MM patients, increased width of the M band is detected using IEF, which can detect even very small differences of charge. When the effect of the sugar chain charge is excluded, the M protein charge depends only on the protein charge, and therefore a uniform M protein band can be detected after neuraminidase treatment. It remains unknown why the width of the M protein in MGUS patients increased with neuraminidase treatment. One of the reasons may be that two types of IgA M protein existed, as suggested by the sialic acid release upon neuraminidase treatment (16).

Kyle reported that frequency of malignant transformation was about 15% in MGUS patients for 10 years (1). Development of methods to distinguish cases of MGUS that will progress to myeloma from those that will not is desired. But long-term follow up data in MGUS is needed for development of the method. Therefore, we tried to compare M protein of MM to that of MGUS patients using IEF at first for our ultimate purpose: diagnosis of difficult cases to differen-

tiate MM from MGUS, and determination of the state of transformation from MGUS to MM. In the present study, although only a few cases were analyzed, the difference was detected in IgA-type M protein. For the purpose described above, evaluation of other types of M proteins and determination of many cases will be needed using this method in the future.

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