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Keiko Shiiya, Mariko Iizuka, Yuko Ito, Seino Utako, Hashimoto Atsushi, et al.. Estimation of the True Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) Autoantibody Concentration in the Sera of Normal Subjects. *Journal of Bioanalysis & Biomedicine*, 2017, 09, <10.4172/1948-593x.1000192>. <hal-04033679>

HAL Id: hal-04033679

<https://hal.science/hal-04033679v1>

Submitted on 17 Mar 2023

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Estimation of the True Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) Autoantibody Concentration in the Sera of Normal Subjects

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Abstract

According to our previous studies, Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) autoantibodies (GMABs) are normally present in healthy people. To date, antigen-antibody capture assay has measured the concentrations. However, nonspecific IgG other than GMABs and immune complex formation with intrinsic GM-CSF prevents us from measuring the accurate concentrations. To solve these problems, we developed a liquid phase high-sensitivity assay using biotinylated GM-CSF (bGM-CSF) and streptavidin coated plate, which could measure the concentration of GMABs over 0.02 ng/ml. Using this method, the true GMABs concentration in 30 normal subjects was estimated to be 52.6 ng/ml for the median value, ranging 10.6 to 799.5 ng/ml. Thus, we propose a novel method that enables us to estimate the true concentration of GMABs in the sera of healthy subjects.

Keywords: Pulmonary alveolar proteinosis; Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF); Autoantibody; Liquid phase reaction

Introduction

In 1999, we discovered high concentrations of GMABs in both sera and bronchoalveolar lavage fluids from patients with autoimmune pulmonary alveolar proteinosis (aPAP) that was called “idiopathic pulmonary alveolar proteinosis” at that time [1,2]. These autoantibodies showed high avidity, specificity, and neutralizing capacity to GM-CSF and had multiple epitopes on the molecule [3]. As gene-targeting mice against GM-CSF or its receptor develop PAP phenotype [4,5]. These autoantibodies had been considered as the causative agent for aPAP. Ten years later, Trapnell and his colleagues proved this hypothesis by reproducing PAP in nonhuman primates that were injected with purified GMABs from patients with aPAP [6].

On the other hand, Svenson et al. first reported that GMABs occurred consistently in pharmaceutically prepared intravenous immunoglobulin (IVIG) [7]. They also demonstrated that these autoantibodies showed high avidity with GM-CSF. As IVIG is produced through purification of IgG from pooled plasma obtained from normal subjects, the consistent presence of GMABs suggested the occurrence in the sera of normal subjects. In this regard, Svenson et al. reported that GMABs was rarely present in 0.3% plasma of normal subjects [7]. Alternatively, Bazin et al. demonstrated that artificial activation of cryptic IgG auto reactivity to GM-CSF for explanation that GMABs are consistently present at low levels in IVIG [8].

On the other hand, a ubiquitous occurrence of autoantibodies to various cytokines in the sera of normal subjects has been reported [9]. We demonstrated that GMABs occurred ubiquitously at low levels in the sera of healthy subjects [10]. Currently available sandwich ELISA method to quantify GMABs is accurate and precise for the diagnosis of autoimmune PAP discriminating from the values in healthy individuals, of which the detailed method was described and the results were intensively evaluated [11]. However, these reports did not determine the accurate concentrations of GMABs in the sera of healthy subjects because at very low levels, non-specific IgG binding to coated GM-CSF cannot be ignored by the ELISA system. Thus, assay development

to detect low levels of GMABs in high specificity should be necessary to quantify the accurate concentration of GMABs in the sera healthy individuals.

When we measure the concentrations of GMABs in the sera of normal subjects accurately, we are also confronted with difficulties as GMABs form immune complexes with intrinsic GM-CSF, and thus the complexes are not necessarily trapped by recombinant GM-CSF coated on the plate. To solve the problem, we developed a liquid phase high-sensitivity assay using chemiluminescence reaction that was able to detect GMABs even in the form of immune complex. Subsequently, we estimated the true concentration of GMABs by subtracting non-specific GM-CSF binding IgG from total GM-CSF binding IgG.

The concentrations of GMABs in the sera of normal subjects measured by this novel method were lower than those estimated by the conventional ELISA in the previous reports [10,11].

Materials and Methods

Subjects

The institutional review board of the Niigata University Medical & Dental Hospital approved this study. All participants gave written informed consent in accordance with the Declaration of Helsinki. Both thirty normal subjects and patients with autoimmune PAP patients were enrolled in the study. Healthy subject group included 21 male and 9 female with median age was 44.5 (22-64).

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Received October 30, 2017; **Accepted** November 14, 2017; **Published** November 21, 2017

Citation: Shiyya K, Iizuka M, Ito Y, Utako S, Atsushi H, et al. (2017) Estimation of the True Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) Autoantibody Concentration in the Sera of Normal Subjects. J Bioanal Biomed 9: 272-276. doi:10.4172/1948-593X.1000192

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Preparation of standard GMABs

GMABs were purified from pooled sera from 30 patients with aPAP as previously described [3]. The purity of isolated GMABs was estimated to be 82.8% using ELISA (Bethyl Laboratories Inc., TX). Serum IgG fractions were purified by protein A/G Sepharose (GE Healthcare, UK) according to the manufacturer's instructions.

bGM-CSF

The rhGM-CSF (salgramostim) was purchased from Genzyme-Sanofi Corporation (Cambridge, MA) and biotinylated using the NHS-PEO-biotin kit and monomeric avidin kit (Pierce Biotech, Rockford, IL, USA) according to the manufacturer's instructions. The purity of bGM-CSF was almost 100%.

Artificially formed immune complex

Purified GMABs (100 ng/ml) were incubated with 2.5 ng/ml of rhGM-CSF overnight at 4°C to form immune complex.

Measurement of GMABs concentration: Conventional method

Serum GMABs levels were measured using direct ELISA as previously reported. In brief, micro-ELISA plates (Maxisorp™ flat-bottom, clear, 96-well plates; Nunc, Roskilde, Denmark) were coated with rhGM-CSF at 1 µg/ml with phosphate-buffered saline (PBS) at 4°C overnight. After washing with PBS containing 0.1% Tween™-Twenty (0.1% PBS-T) (MP Biomedicals, Solon, OH, USA), plates were stabilized with blocking solution (Stabilcoat™, SurModics, Eden Prairie, MN, USA) for 1 h at room temperature. Human monoclonal GMAB that was kindly provided by Dr. Kenzo Takada (Evec Co. Ltd., Sapporo, Japan) was diluted in PBS containing 1% bovine serum albumin (1% BSA/ PBS) and was used as a standard for antibody measurement. Serum samples were diluted into 1% BSA/PBS at a 100-fold dilution, and a volume of 50 µl of prepared samples and standard was transferred to plates. After keeping plates at room temperature for 1 h and washing with 0.1% PBS-T, GMABs captured by rhGM-CSF were detected by peroxidase-labeled anti-human Fcγ (Dako Corporation, Carpinteria, CA, USA). After washing, color was developed using tetramethylbenzidine (TMB) solution, and the absorbance was measured at 450 nm. The mean optical density and the standard deviation at 450 nm were shown in the Table 1 for various concentrations of standard human monoclonal antibody (Table 1).

Measurement of GM-CSF binding IgG concentration by a novel method

To detect low concentrations of GMABs in the sera of normal

Conc ng/mL	Mean OD	Mean OD-mean blank	SD	CV%
1.5625	972	627	12	1.87
0.78125	647	302	8	2.71
0.390625	513	168	5	3.07
0.1953125	428	83	8	9.03
0.09765625	395	50	5	10.95
0.048828125	367	22	8	37.68
0.0244140625	357	12	8	69.99
0	345	0	5	-

Table 1: Concentration response of the calibration curve and accuracy of measuring the standard monoclonal GM-CSF autoantibody in the conventional ELISA system. Conc: Concentration of standard monoclonal GM-CSF autoantibody, J152-4C. OD: Optical Density, SD: Standard deviation, CV: Coefficient of Variation.

subjects, we developed a novel method as follows (Figure 1). Briefly, each 50 µl of sera from healthy subjects was diluted with 1%BSA/PBS-T at 100-fold and incubated with excess amount of bGM-CSF (50 ng/ml) overnight at 4°C and transferred into 96 well streptavidin coated plate (Immobilizer streptavidin F96 White, Nunc, Roskilde, Denmark) previously hydrated with PBS. The plate trapped GMABs bound with bGM-CSF for 1 h at room temperature. After washing the plate with PBS-T five times, each well was blocked with 100 µl of Stabilcoat for 1 h, and further washed with PBS-T five times.

GMABs captured by bGM-CSF were detected by alkaline phosphatase-labeled anti-human Fcγ (Dako Corporation, Carpinteria, CA, USA). After washing, the activity of alkaline phosphatase was detected by CDP-Star® (Applied Biosystems, CA) using a chemiluminescence plate reader (Berthold. Tec. GmbH & Co., Germany). For standard GMABs, purified polyclonal GMABs described above was used for this assay.

Statistical analysis

Test for normality of continuous variables and homogeneity of variance were performed using Shapiro-Wilk test and Leuven test, respectively. Normally distributed continuous variables were reported as mean ± standard error and data that were not normally distributed were reported as medians and interquartile ranges (median 25%, 75%). The paired t test was used for comparisons between normally distributed data and the treatment periods. Comparisons of nonparametric data were made using the Wilcoxon signed-rank test. For group comparisons, unpaired t tests and Wilcoxon rank-sum tests were used. Correlation coefficient between two continuous variables was obtained using Spearman's correlation method. P values less than 0.05 were considered statistically significant.

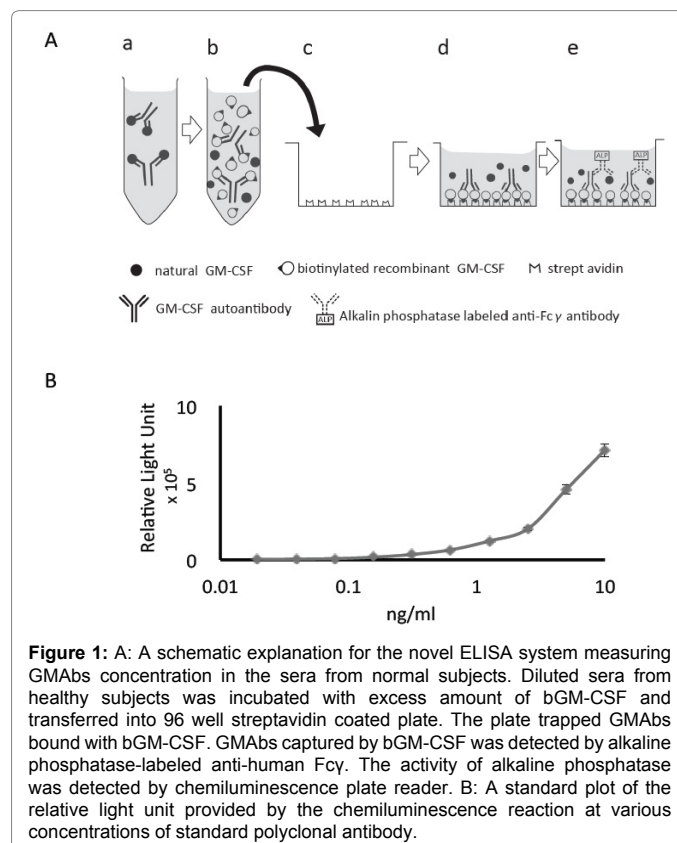


Figure 1: A: A schematic explanation for the novel ELISA system measuring GMABs concentration in the sera from normal subjects. Diluted sera from healthy subjects was incubated with excess amount of bGM-CSF and transferred into 96 well streptavidin coated plate. The plate trapped GMABs bound with bGM-CSF. GMABs captured by bGM-CSF was detected by alkaline phosphatase-labeled anti-human Fcγ. The activity of alkaline phosphatase was detected by chemiluminescence plate reader. B: A standard plot of the relative light unit provided by the chemiluminescence reaction at various concentrations of standard polyclonal antibody.

Results

A novel method for measuring GM-CSF binding IgG

To estimate the low concentration of GMABs in the healthy subjects which forms immune complex with intrinsic GM-CSF [10], we developed a novel method in which low concentration of GMABs are trapped with excess concentration of bGM-CSF followed by capture on streptavidin-coated plate and then binding with alkaline phosphatase labeled anti-IgG Fc antibody followed by chemiluminescence reaction to detect alkaline phosphatase activity (Figure 1A). The standard curve of purified polyclonal GMABs appeared a downward convex shape (Figure 1B). When we determine the detection limit as the end concentration with CV less than 10%, the lower detection limit for polyclonal standard was 0.02 ng/ml (Table 2). In contrast, when the lower detection limit was determined using the conventional method as described in “Material and Methods”, it was 0.195 ng/ml, indicating the sensitivity of the novel method was 10 fold higher than that of the conventional method (Table 1).

Feasibility of the novel method to measure the concentration of GMABs in the immune complex

Artificially formed immune complex described in the Materials and Methods (containing 2.5 ng/ml standard purified GMABs) was incubated with various concentrations of bGM-CSF (0-25 ng/ml) and the concentration of bound GMABs was measured by the novel method. The concentration was dose-dependently increased as the concentration of added bGM-CSF reaching 2.5 ng/ml of GMABs at 12.5 ng/ml of bGM-CSF (Figure 2A). On the other hand, when variable concentrations of GMABs in artificially formed immune complex were measured by the novel method, the values were almost compatible with the values for GMABs alone (0-5 ng/ml) and slightly lower with 6% reduction at 10 ng/ml of GMABs (Figure 2B). These results suggest that the novel method is able to estimate the concentration of GMABs even in the form of immune complex.

Estimation of the true GMABs concentration

To estimate the true concentration of GMABs in the sera of normal subjects, we need to know the concentration of IgG that bind with GM-

CSF nonspecifically. When the 100-fold diluted serum was incubated with various concentrations of non-labeled GM-CSF (0-10 mg/ml) and bGM-CSF (50 ng/ml) and then the concentration of IgG bound with bGM-CSF was measured by trapping with streptavidin coated plate, the total IgG bound bGM-CSF reduced as the added non-labeled GM-CSF increased, reaching a bottom that is presumed to be the concentration of IgG bound nonspecifically (Figure 3). The inhibition rate of the bound IgG by the added non-labeled GM-CSF appeared a saturation-binding curve (Figure 3A). The Michaelis-Menten plot provided a V_{max} value that was considered to be the ratio of true GMABs concentration per total IgG bound bGM-CSF (Figure 3B).

Distribution of true GMABs concentrations in 30 normal subjects

Based on each V_{max} value and each total IgG concentration bound bGM-CSF (Figure 4A), each true GMABs concentration was calculated. In 30 normal subjects, the value was estimated to be 10.6 to 799.5 ng/ml with median value at 52.6 ng/ml (Figure 4B). As the lower detection

Conc ng/ml	Mean RLU	Mean RLU-mean blank	SD	CV%
10	711697	711033	42461	5.97
5	457907	457243	30663	6.71
2.5	201140	200477	11532	5.75
1.25	122350	121687	9216	7.57
0.625	64927	64263	1849	2.88
0.3125	35243	34580	1449	4.19
0.15625	17280	16617	1366	8.22
0.078125	8440	7777	366	4.70
0.0390625	5087	4423	125	2.83
0.01953125	3000	2337	121	5.17
0.009765625	1850	1187	161	13.54
0	663	0	57	-

Table 2: Concentration response of the calibration curve and accuracy of measuring the standard polyclonal GMABs in the novel ELISA system. Conc: Concentration of standard polyclonal GMABs that was prepared from pooled sera of patients with autoimmune PAP as described in Materials and Methods. RLU: Relative Light Unit, SD: Standard deviation, CV: Coefficient of variation.

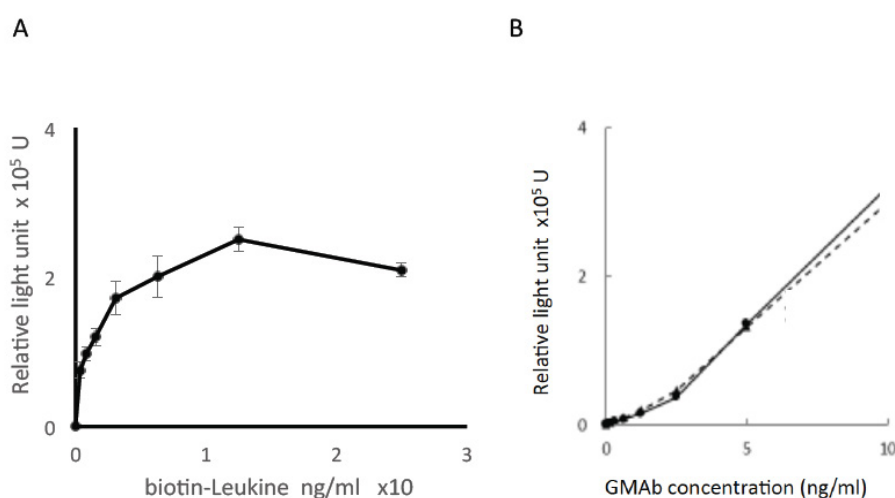
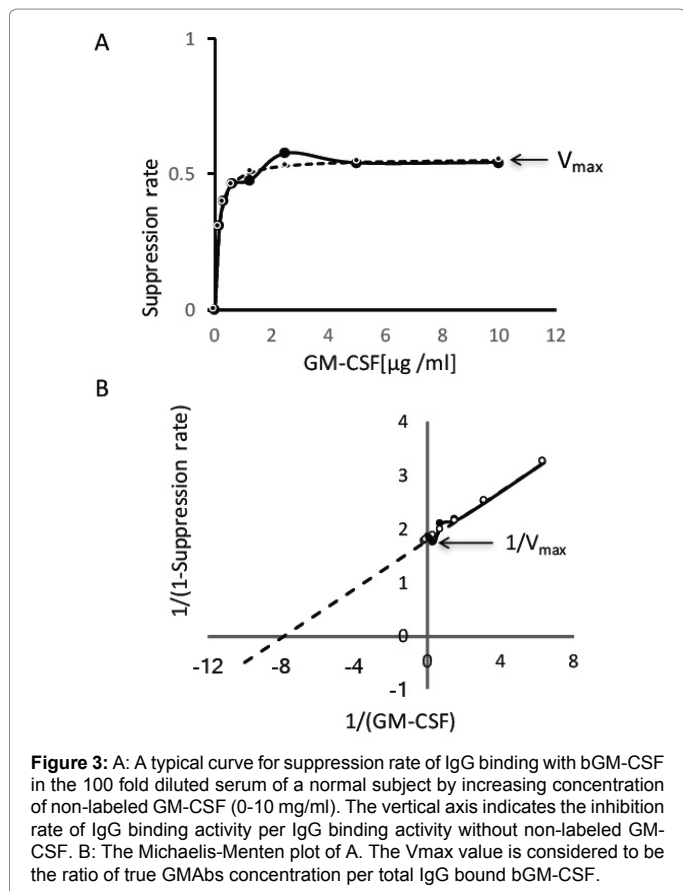


Figure 2: Binding of artificial GM-CSF/GMABs complex with bGM-CSF. Binding activity is expressed as the relative light units in chemiluminescence reaction. A: Binding of artificial GM-CSF/GMABs complex containing 2.5 ng/ml of the antibody described in Material and Methods with various concentrations of bGM-CSF (0-2.5 ng/ml). B: Binding of various concentrations (0-10 ng/ml) of GMABs (solid line) or artificial GM-CSF/GMABs complex (dotted line) with bGM-CSF (50 ng/ml).



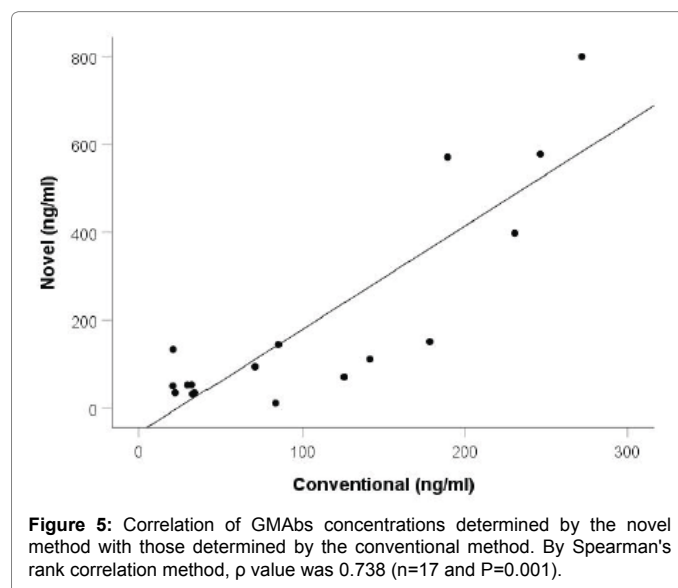
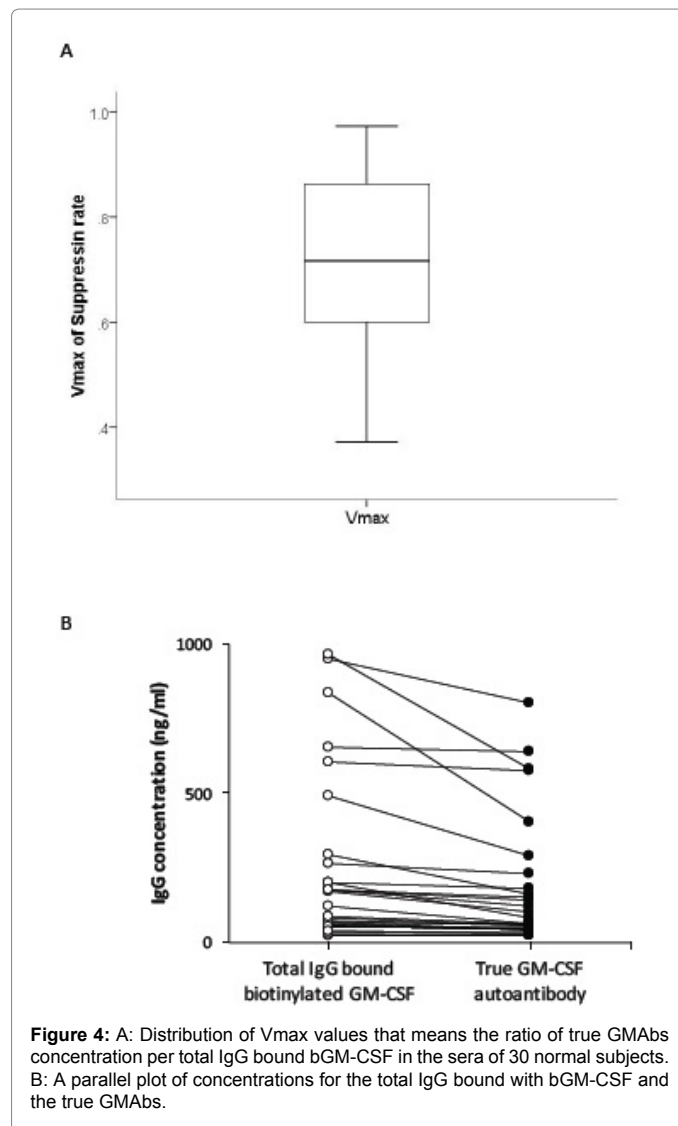
limit for the polyclonal standard was 0.02 ng/ml and the serum samples were diluted at 100-fold, the lower detection limit was 2.0 ng/ml. Thus, all of 30 samples were positive for GMABs with mean value of 164.0 ng/ml. These data suggested that the novel method revealed that the true GMABs concentrations were lower than the value reported previously ((0.28 [0.20–0.51] µg/ml; n=38) [11].

Validation of the data obtained by the novel ELISA method

To evaluate the accuracy of the novel ELISA system, we compared the GMABs concentrations in the same 30 normal subjects between the novel and conventional ELISA system. Of all, 13 samples were under the detection limit in the conventional method (19.5 ng/ml, Table 1), whereas all samples were over the detection limit in the novel method (2 ng/ml). Thus, only 17 data were available in the conventional method, there was still a strong correlation between the novel and conventional methods (Figure 5, $\rho=0.738$, $P=0.001$), suggesting the reliability of the novel method.

Discussion

In this study, we developed a novel quantitation system for measuring the low concentration of GMABs in the sera of normal subjects. The system demonstrated that low concentrations of GMABs were detected in 30 of 30 normal subjects, whereas 17 of 30 subjects by the conventional ELISA system. The accuracy and reliability of the data obtained by the novel quantitation system was confirmed by comparing the data with those obtained by the conventional method. The presence of GMABs in the blood of normal subjects was first demonstrated by Svenson et al. in 1998 [7], who reported that IgG type of GMABs were consistently detected in every batch of pharmaceutically prepared



immunoglobulin preparation. Although the occurrence of GMAbs in the normal sera was thought to be infrequent, but IVIG is produced from pooled plasma of several thousands of healthy donors, and thus, Svenson et al. demonstrated that the consistent detection of GMAbs in every IVIG might reflect the presence of relatively high concentration of GMAbs in a few donors [7]. On the other hand, we previously thought that low concentration of GMAbs was consistently present in the sera of all normal subjects using conventional ELISA method and immunoblotting for some part of subjects. In that study, we did not mention about nonspecific binding of IgG other than GMAbs with GM-CSF coated on the ELISA plate. In this regard, Meager et al. submitted a criticism to our report that nonspecific binding of IgG other than GMAbs might affect the results of measuring GMAbs concentrations in the sera of normal subjects [12]. According to their criticism, we evaluated the nonspecific binding of IgG other than GMAbs. We realized that the nonspecific binding was not negligible in normal subjects in whom the true concentration of GMAbs was expected to be very low, and thus, we applied a high sensitivity detection system to measure even low concentrations of GMAbs in nano gram order.

Because pretreatment of the serum is likely to cause artificial activation of cryptic IgG that may bind GM-CSF [8], we did not perform any pretreatment on the sera with acid or detergent to dissociate GM-CSF/GMAbs immune complex. Our data showed that liquid-phase preincubation with excess amount of extrinsic bGM-CSF efficiently substituted the intrinsic GM-CSF in the complex.

In the previous report, bound GM-CSF in the immune complex of normal sera was estimated to be approximately 3 ng/ml in average [10], whereas the mean concentration of GMAbs was 164.0 ng/ml in the present study. When the molecular weight of GM-CSF and IgG were presumed to be 23 and 150 kDa, respectively, the molar ratio of these molecules involved in the sera of normal subjects was approximately 1: 8.4. Anyway, the immune complex in the sera of normal subjects is consisted of little GM-CSF bound with excess GMAbs. The binding sites of GMAbs in the complex are likely not occupied completely, and thus, the free sites may easily bind to the extrinsic GM-CSF.

In the conventional method, the detection of GMAbs bound with GM-CSF coated on the plate was performed using peroxidase labeled anti-Fc gamma second antibody followed by color reaction with TMB solution. By this system, we can detect GMAbs concentration higher than 19.5 ng/ml as shown in this study. To increase the sensitivity of detection, we applied alkaline phosphatase labeled anti-Fc gamma second antibody followed by chemiluminescence reaction. This system detected as low as 2.0 ng/ml of GMAbs. Moreover, improvement in the detection sensitivity did not increase the detection rate of GMAbs in the sera of normal subjects, because the novel system estimates the binding of nonspecific IgG with GM-CSF in each serum independently.

GMAbs in healthy subjects were found to reduce GM-CSF stimulation of myeloid cells as reported previously [10]. Serum levels of GM-CSF and GMAbs may be exquisitely balanced in healthy individuals [10]. Namely, most serum GM-CSF is present in the form of an immune complex, and at levels much higher than the free GM-CSF levels previously reported [10]. Thus, GMAbs in healthy subjects may play an indispensable physiological role to keep the homeostasis of cytokine environment. It may attenuate the bioactivity of excess GM-CSF and eradicate the inflammation.

In conclusion, we confirmed the occurrence of serum GMAbs in healthy subjects, however, the concentration were lower than demonstrated previously. The overestimation in the previous study is likely due to the underestimation of nonspecific IgG binding in the

healthy sera. We believe that the present study will lead physicians and researchers to proper understanding on the significance of GMAbs in healthy subjects.

Acknowledgements

We thank Kanji Uchida and Masato Watanabe for valuable suggestions and discussions.

Funding

The Japan Society for the Promotion of Science supported this work in part with a grant from Category 15H04829 (K.N.), 15K15321(K.N.). Japan Agency for Medical Research and Development also provided a grant, 15ek0109079h0001 (KN) and 15Aek0109063h0002 (YI) to support this research.

Conflict of Interest

None of the authors has a financial relationship with a commercial entity with an interest in the subject of this manuscript.

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