IgM-type GM-CSF autoantibody is etiologically a bystander but associated with IgG-type autoantibody production in autoimmune pulmonary alveolar proteinosis

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In 1999, we discovered a high concentration of granulocyte-macrophage colony-stimulating factor (GM-CSF) autoantibody (GMAb) in sera of patients with idiopathic pulmonary alveolar proteinosis (aPAP). It consists primarily of the IgG isotype. At present, information on other isotypes of the autoantibody is limited. We detected serum the IgM isotype of GMAb (IgM-GMAb) in more than 80% of patients with aPAP and 22% of healthy subjects, suggesting that a continuous antigen pressure may be present in most patients. Levels of the IgM isotype were weakly correlated with that of IgG-GMAb. The mean binding avidity shows high avidity and strong neutralizing capacity against GM-CSF, it is rational to consider that the antibody blocks GM-CSF bioactivity in the lung, resulting in maturation arrest of alveolar macrophages and the development of PAP (4). This hypothesis was recently proven by establishing a PAP model in primates by injecting patient-derived GMAb (10, 11).

In our earlier studies, we reported that GMAb in aPAP only consisted of the IgG isotype (6) predominantly featuring IgG1 and IgG2. GMAb in pharmaceutical IgG preparations was shown by Svenson et al. (15), suggesting the presence of GMAb in the normal human IgG fraction, which was later confirmed to be ubiquitous in the sera of normal subjects (18). In contrast, GMAbs in cord blood are exclusively IgM, and those from maternal peripheral blood are both IgM and IgG (8). Recently, Sergeeva et al. (12) reported that patients with myeloid leukemia or myelodysplastic syndrome had not only IgG (52% of patients) but also IgA (33%) and IgM-GMAb (20%). Thus the isotypic distribution of GMAb is heterogeneous among both healthy and diseased states.

In the present study, we reevaluated the occurrence of immunoglobulin heavy-chain isotypes of GMAb other than IgG in aPAP. We found that many patients had low concentrations of serum IgM- and IgA-GMAb, but only the concentration of IgM-GMAb was specifically elevated in the patients. Characterization of IgM-GMAb was performed for binding avidity and neutralizing capacity. We assessed the role of IgM-GMAb in the pathogenesis of aPAP.

MATERIALS AND METHODS

Study subjects. Serum samples from 71 patients with aPAP and 23 healthy subjects as controls were collected from nine hospitals in Japan participating in a study of aPAP and stored in Niigata University Medical and Dental Hospital at −80°C. We also collected and stored BALF supernatants at the diagnosis of PAP similarly from nine of the above patients. Written informed consent was obtained under protocols approved by the institutional review boards of the hospitals. A diagnosis of aPAP was confirmed by cytological analysis of BALF, pulmonary histopathological findings, or both (17).
**Analysis of peripheral mononuclear cell population by flow cytometry.** Peripheral blood mononuclear cells (PBMCs) were separated from whole blood by density gradient centrifugation using Ficoll (GE Healthcare, Uppsala, Sweden). PBMCs were washed twice with PBS supplemented with 2% BSA and 0.2% sodium azide and taken in different flow cytometry tubes and stained with mAbs for 60 min at 4°C in the dark. After incubation, the cells were washed and analyzed by flow cytometry (Epics XL; Beckman Coulter, Brea, CA). The following mAbs were used in this study: FITC-conjugated anti-CD3 was purchased from BD Bioscience (San Diego, CA); phycoerythrin (PE)-conjugated anti-CD4 were purchased from Dako (Carpinteria, CA); PE-cyanine 5 (PE-Cy5)-conjugated anti-CD8 and CD56 and PE-Texas Red-conjugated CD19 were purchased from Beckman Coulter; PE-conjugated anti-CD14 was purchased from Nichirei Bioscience (Tokyo, Japan).

**Reagents.** Recombinant human GM-CSF (rhGM-CSF; Leukine, Immunix, WA) was dialyzed against PBS (pH 7.4) and biotinylated using the NHS-PSEO-biotin kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer’s instructions. The purity of biotinylated GM-CSF (bGM-CSF) was almost 100%. IgG was purified from a pooled serum or plasma sample of aPAP using protein A/G (mixed 7.5; GE Healthcare, affinicy chromatography) according to the manufacturer’s instructions. The bound IgG was eluted with glycine-HCl (10 mM, pH 2.8; bound fraction). For isolating IgG-GMAb, the eluate containing IgG or the flow through (containing both IgM and IgA) were independently loaded onto GM-CSF-coupled NHS HiTrap columns and eluted with glycine-HCl (10 mM, pH 2.8). The IgM/AgMAB fraction was further purified by loading onto HiTrap IgM Purification HP (GE Healthcare) columns, according to the manufacturer’s instructions. The purities of isolated GMAbs were close to 100% for both IgG and IgM, as determined by ELISA assay (Bethyl Laboratories, Montgomery, TX). Pharmaceutically prepared immunoglobulin was kindly provided by Benesis, Osaka, Japan (Venoglobulin IH) and Kaketsuzan (The Chemo-sero Therapeutic Research Institute, Kumamoto, Japan) (Kenketsu Venilon-I). Monoclonal IgG-GMAb was kindly provided from Dr. Kenzo Takada (Evec, Sapporo, Japan) and was used as the standard in ELISA. Monoclonal IgM-GMAb was obtained from immortalized Epstein-Barr virus-transformed peripheral mononuclear cells from patients with aPAP as described previously (8), and the stable cell culture supernatant with the highest IgM-GMAb production was used as the standard antibody for IgM-GMAb-ELISA as described below.

**GMAB-ELISA.** The GMAB concentration in the serum, culture medium, and BALF was measured using direct ELISA as described previously (18). Each isotype-GMAb was detected using peroxidase-labeled anti-human Fcγ, Fcα, or Fcε antibody (Dako), and Fcε antibody (GeneTex, Irvine, CA). The sera were diluted to 1 in 3,000 to measure the concentration of IgG-GMAb. For measurement of IgM- or IgA-GMAb, serum samples were diluted 1 in 300. The lower detection limit of IgG- and IgM-GMAb, which was defined as the concentration corresponding to mean blank optical density (OD) + 2 SD, were 0.26 and 0.12 μg/ml, respectively. For IgA-GMAb, the OD value at lower detection limit was 0.062. For measurement of GMAB in BALF, BALF was diluted consistently to 1 in 10 with the lower detection limit of 0.009 and 0.004 μg/ml for IgG- and IgM-GMAb, respectively.

**Characterization of GMAB.** The GM-CSF binding avidity of purified GMAB was determined using bGM-CSF binding assay. After being coated with anti-Fcγ or anti-Fcα antibody (Dako) overnight, 96-well microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were washed five times with PBS/0.1% Tween 20 (PBST) and blocked with Stabilcoat (SurModics, Eden Prairie, MN). The purified IgG- or IgM-GMAb was applied to each well, incubated for 1 h at room temperature, washed three times with PBST, and reacted with bGM-CSF (75 μg/ml) for 1 h at room temperature. After being washed five times with PBST, bGM-CSF-bound GMAB was reacted with alkaline phosphatase-streptavidin (Innovojet, Carlsbad, CA). The activity of alkaline phosphatase was detected by CDP-Star (Applied Biosystems, Carlsbad, CA) using a chemiluminescence plate reader (Berthold Technology, Bad Wildbad, Germany). On the basis of the Lineweaver-Burk plot, the dissociation constant (Kd) was determined from the concentration of bGM-CSF at 50% of maximal binding.

**GM-CSF neutralizing capacity.** The GM-CSF neutralizing capacity of GMAB was estimated by a conventional bioassay using a GM-CSF-dependent cell line, TF-1 cells, as described previously (5, 19). Briefly, TF-1 cells (20,000 cells/well) were cultured (37°C, 5% CO2) in microtiter plates for 3 days in macrophage serum-free medium (Invitrogen) containing rhGM-CSF in concentrations of 0.5 ng/ml with GMAB ranging 0–12 μg/ml. TF-1 cell survival was evaluated using the MTT assay as described previously (4). The percentage of growth was calculated using the equation, % growth inhibition = 100 – 100 × (A – B)/(C – B), where A is the absorbance of TF-1 cells grown in the presence of rhGM-CSF and GMAB, B is the absorbance of TF-1 cells grown in medium only, and C is the absorbance of TF-1 cells grown without GMAB but containing GM-CSF (20 ng/ml).

**Immunoblotting of GMABs.** The highly purified IgG-GMAb, non-IgG-GMAb, and GMAB including whole isotypes described above was analyzed using SDS-PAGE with 2–15% gradient gels under reducing conditions using 2-mercaptoethanol (30 mA, 50 min). Fractionated proteins were transferred onto PVDF membranes by electroblotting (12 V, 75 min). Membranes were incubated with blocking solution [PBS containing 1% (wt/vol) BSA and 0.1% (vol/vol) Tween 20] at 4°C overnight, washed, incubated with horseradish-peroxidase-conjugated anti-human IgG or IgM antibody (room temperature, 60 min), and visualized by ECL Plus (GE Healthcare).

**Statistical analysis.** Statistical analyses were performed on a microcomputer using JMP software (SAS Institute, Cary, NC). Numerical data were evaluated for a normal distribution using the Shapiro-Wilk test and for equal variance using the Levine median test. Statistical comparisons of nonparametric data were made with the Mann-Whitney U-test for two-group comparisons. Paired samples were evaluated with the Wilcoxon signed-rank test. Spearman’s correlation coefficients were calculated to assess a correlation between two parameters. P values of <0.05 were considered significant.

**RESULTS**

**Profile of PBMCN population in aPAP.** To characterize the immunological modulation by aPAP, we compared the PBMCN population between 24 subjects with aPAP and 23 healthy subjects. As shown in Table 1, there was no difference in cell counts of CD3+, CD4+CD3+, CD19+, and CD14+ cells, corresponding to total T cells, CD4+ T cells, total B cells, and monocytes, respectively. Only CD8+CD3+ cells significantly decreased in the patients compared with controls (P < 0.01). Thus aPAP seemed to alter no remarkable changes in major populations of PBMCNs.

**Occurrence of Ig isotypes of GMAB.** In previous studies, we demonstrated that GMABs in patients with aPAP were mainly of the IgG isotype but did not rule out the occurrence of low concentrations of other isotypes. To elucidate this, free GMAB (able to bind with GM-CSF) was isolated from pooled patient sera by GM-CSF-coupled affinity chromatography, concentrated, and applied to immunoblotting. As shown in Fig. 1A, bands specific for IgG-, IgM-, and IgA-GMAb were detected at the predicted molecular weights, but neither IgD- nor IgE-GMAb were detected. The binding of IgG-, IgM-, or IgA-GMAb to biotinylated GM-CSF was completely blocked by an excess amount of nonlabeled GM-CSF, indicating the specificity of the binding (Fig. 1B).

**Concentrations of IgG-, IgM-, and IgA-GMAb.** All sera from patients (n = 71, 51 males, 20 females, median 44.0 yr, range...
Using monoclonal IgM-GMAb, the concentrations of those IgM-GMAb were estimated to be 1.1 μg/ml (0.2–10.7) and 0.4 μg/ml (0.1–1.0) for patients and controls, respectively (median, P < 0.001). When the cut-off value was set at 0.13 μg/ml, the sensitivity and specificity were 78.3% and 80.3%, respectively (Fig. 2A, middle). Because an IgA-GMAb standard was not available, the concentration was not determined, but the frequency of detection and the OD values were comparable between aPAP and controls (Fig. 2A, right). The lower detection limit was 0.12 μg/ml for IgM-GMAb and was 0.062 for IgA-GMAb (as absorption at 450 nm). We could detect both IgM- and IgA-type GMAb in 57 (81%) and 45 (63%) of

<table>
<thead>
<tr>
<th>Populations In Peripheral Blood</th>
<th>aPAP (n = 24, counts/μl)</th>
<th>Healthy Subjects (n = 23, counts/μl)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total lymphocytes</td>
<td>1183.7 ± 701.6 (212.0–2908.6)</td>
<td>1407.3 ± 383.6 (706.5–2383.9)</td>
<td>NS</td>
</tr>
<tr>
<td>T cells</td>
<td>736.5 ± 583.4 (77.4–2188.3)</td>
<td>906.6 ± 259.3 (467.2–1463.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Total CD3+ cells</td>
<td>397.8 ± 340.8 (27.9–1164.2)</td>
<td>419.4 ± 155.5 (180.1–917.1)</td>
<td>NS</td>
</tr>
<tr>
<td>Total CD4+ cells</td>
<td>266.4 ± 182.2 (21.4–667.4)</td>
<td>388.6 ± 155.5 (67.7–701.6)</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>B cell</td>
<td>235.4 ± 195.1 (28.5–815.1)</td>
<td>215.3 ± 93.1 (62.1–406.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Monocyte</td>
<td>226.3 ± 160.4 (60.9–623.8)</td>
<td>199.2 ± 140.6 (66.2–710.2)</td>
<td>NS</td>
</tr>
</tbody>
</table>

All data are expressed as means ± SD (range). *Statistical difference between patients with autoimmune pulmonary alveolar proteinosis (aPAP) and healthy subjects. Data are analyzed by Mann-Whitney U-test.
71 patients, respectively. Five (22%) of 23 control subjects were positive for IgM-GMAB, and 13 (57%) were positive for IgA-GMAB. Although IgG, IgM, and IgA GMAB were detected in the sera of both patients and healthy controls, our data indicated that only IgG and IgM GMAB specifically increased in aPAP.

When the concentration of IgG, IgM, and IgA-GMAB were compared, a weak correlation was recognized between the concentrations of IgG- and IgM-GMAB (Fig. 2B, R² = 0.35, P < 0.01). Other clinical parameters including age, sex, smoking status, occupational dust inhalation, arterial oxygen pressure, and serum KL-6 and SP-D were not correlated with either IgG- or IgM-GMAB.

Characterization of IgM-GMAB properties. To assess the pathophysiological roles of IgM-GMAB, we investigated the properties of IgM-GMAB compared with those of IgG-GMAB. The mean binding avidity of IgM-GMAB to GM-CSF was 10.9 ± 9.0 nM (n = 7), which was 136-fold lower than that of IgG-GMAB (n = 5, 80 ± 60 pM, Fig. 3A and Table 2). Previous studies have demonstrated that IgM-GMAB in neonatal or maternal subjects is nonneutralizing. The neutralizing capacity of purified IgM-GMAB from pooled patient sera was
more than 1 µM, whereas that of IgG-GMAb was 50 pM, indicating that IgM-GMAb was only a very weak neutralizer of GM-CSF (Fig. 3B).

**Concentration of GMAb in BALF.** To investigate the isotypic distribution of GMAb in BALF, which derived from the major involved lesion in aPAP, we measured IgG-, IgM-, and IgA-GMAb (Table 3). In nine patients tested, IgG-GMAb was consistently detected, whereas IgM- or IgA-GMAb was not detected in eight patients. Thus the result confirmed that IgG-GMAb is the causative isotype of aPAP.

**DISCUSSION**

In this study, we first evaluated the profile of PBMNCs population in aPAP comparing with healthy subjects. Then we reevaluated the isotypic distribution of GMAbs in aPAP and detected low concentrations of IgM- and IgA-GMAb for the first time. Our previous studies had paid little attention to the contribution of IgM-GMAbs to the pathogenesis of aPAP because their role is likely to be minor considering their low concentrations in patient sera. Using ELISA, the concentrations of IgM-GMAbs were found to be 1–10% of IgG-GMAb in patients. Noticeably, IgM-GMAb was present in more than 80% of patients but only 20% of healthy subjects. Moreover, there was a weak correlation in the patients, but not in the controls, between IgM-GMAb and IgG-GMAb concentrations. These results suggest that the occurrence of IgM-GMAb might be associated with the process of IgG-GMAb development. In contrast, the occurrence of IgA-GMAb was similar between patients and controls.

In general, immunoglobulins in BALF were lower than 0.1% of the serum level (16). The concentrations of IgG-GMAb in BALF with less than 1% of the serum level shown in the study were reasonable. In contrast to IgG-GMAb levels in BALF, both IgM- and IgA-GMAb were negligible in most patients. Taken together with high avidity and neutralizing capacities of IgG-GMAb, our present results reconfirm the pathogenic importance of IgG-GMAb.

Although the mechanism for production of GMAb is still unknown, the high retention of IgM-GMAb in patients compared with controls suggests the possibility of recurrent or persistent stimulus of B-lymphocytes by GM-CSF and expansion of the B cell repertoire reacting to self-GM-CSF in lymphatic organs in patients. Thus B cells expressing IgM-GMAb may have many chances for class-switching, differentiating to IgG-GMAb-producing B cells. In addition, as the avidity of IgG-GMAb is extremely high, affinity maturation by somatic hypermutation and clonal expansion may repeatedly proceed in germinal centers of lymphatic organs in the patients.

Previous reports demonstrated that nonneutralizing IgM-GMAb mainly developed in adult cancer patients during the early phase of exogenous rhGM-CSF administration (7, 9), followed by a predominance of IgG-GMAb after repeated injections. However, neutralizing IgG-GMAb occurs in only 40% of patients, whereas IgG-GMAbs in aPAP are consistently neutralizing (6, 19). Therefore, isotypic changes from IgM to IgG and phenotypic changes from nonneutralizing to neutralizing forms are critical events in the pathogenesis of aPAP. Taken together, we suppose that B cells self-reactive to GM-CSF that have escaped apoptosis by B cell receptor editing in bone marrow may undergo somatic hypermutation and class-switching in response to repeated or continuous stimulation by GM-CSF in secondary lymphatic tissues in patients with aPAP.

The present study has clarified the isotypic distribution and characterized IgM-GMAb in patients with aPAP. Although IgM-GMAb is etiologically a bystander, it is frequently present and increased in the patients, suggesting the occurrence of a continuous antigen pressure in vivo.

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**GRANTS**

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


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**Table 2. Binding avidity of IgM- and IgG-GMAb**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Kav, nM</th>
</tr>
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<tbody>
<tr>
<td>IgM-GMAb</td>
<td>20.7</td>
</tr>
<tr>
<td>Purified IgM-GMAb</td>
<td>6.54</td>
</tr>
<tr>
<td>Case 1</td>
<td>3.57</td>
</tr>
<tr>
<td>Case 2</td>
<td>2.34</td>
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<tr>
<td>Case 3</td>
<td>24.6</td>
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<tr>
<td>Case 4</td>
<td>43.4</td>
</tr>
<tr>
<td>Case 5</td>
<td>4.34</td>
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<tr>
<td>Case 6</td>
<td>14.1</td>
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<tr>
<td>IgG-GMAb</td>
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<tr>
<td>Purified IgG-GMAb</td>
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<tr>
<td>Case 1</td>
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<tr>
<td>Case 2</td>
<td>0.07</td>
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<tr>
<td>Case 3</td>
<td>0.07</td>
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</tbody>
</table>

*Each sample was purified from pooled sera derived from 40 of the present 71 patients with aPAP (described in MATERIALS AND METHODS). GMAb, granulocyte-macrophage colony-stimulating factor autoantibody.

REFERENCES


