ippines). Enrolled patients with secondary infections were randomly assigned to the IVIG group and non-IVIG treatment groups by means of sealed envelopes.

Treatment with IVIG. Human immunoglobulin (2.5 g/vial, Gammamune; Bayer Health Care, Brea, CA) at a dose of 0.4 g/kg/day was given intravenously to each patient in the IVIG group intravenously at a constant rate of 0.1g/kg/hour on day 2, and was continued each day until the fourth day after admission (day 4) for three days. The dose and frequency of IVIG in this study was chosen on the basis of previous investigations of IVIG to treat ITP.[16,17] Standard treatment including intravenous fluids was given to all patients, regardless of group assignment, according to WHO guidelines.[11]

Evaluation. To determine the effects of a high dose of IVIG, patients in both groups were requested to remain hospitalized until the seventh day after admission (day 7). The primary measure of efficacy was an increase in platelets. Platelet counts in patients of both groups were monitored daily during hospitalization (days 1–7) and on day three after discharge (day 10) at an outpatient clinic. The target sample size could not be estimated for this study because no previous studies had determined the effects of IVIG on the thrombocytopenia during acute dengue virus infection. A interim target sample size of 30 was chosen to ensure that there would be at least a 70% chance for detecting a difference of 40% (100% versus 60%), with a one-sided alpha level of 0.05, in the frequency of platelet counts higher than 80,000/μL on the fourth day after admission in patients with dengue illness and a low platelet count between 20,000 and 80,000/μL.[8,9]

Statistical analysis. All the data are expressed as the mean ± SD. Differences in the demographic and clinical data between the IVIG group and non-IVIG groups were tested using either a chi-square test or a Fisher's exact test for nominal variables. Differences in laboratory data between the IVIG and non-IVIG groups were analyzed using a Student's *t*-test for continuous variables. A *P* value less than 0.05 was considered significant.

RESULTS

Demographic data. Thirty-one patients with secondary infection were randomized to either the IVIG group (n = 15) or the non-IVIG group (n = 16) (Table 1). Ten DF cases and 5 DHF cases (1 DHF I and 4 DHF II) were assigned to the IVIG group, and 9 DF cases and 7 DHF cases (6 DHF II and 1 DHF III) were assigned to the non-IVIG group. The increase in the hematocrit (mean ± SD) was significantly greater in DHF patients than in DF patients for both treatment groups (30.0 ± 6.3% versus 13.8 ± 5.3% in the IVIG group and 29.9 ± 12.9% versus 8.9 ± 4.4% in the non-IVIG group; $P < 0.001$). No significant differences were found between the two groups with respect to demographic and laboratory data including peripheral platelet count and levels of aspartate aminotransferase and alanine aminotransferase at day 1.

TABLE 1

Comparison of clinical features of patients with secondary dengue virus infection between the IVIG group and the non-IVIG group*

Parameter	Group		*P*
	IVIG (n = 15)	Non-IVIG (n = 16)	
Mean age, years (SD)	16.3 (3.6)	14.3 (3.4)	0.108
Male %	53.3	43.8	0.594
Mean body weight, kg (SD)	37.8 (10.6)	37.3 (8.7)	0.891
Days after onset (SD)	3.9 (0.6)	3.7 (1.1)	0.462
Laboratory data on day 1			
Mean platelet count, $\times 10^3/\mu L$ (SD)	54.9 (12.7)	48.0 (13.1)	0.147
Mean AST, U/L (SD)	129.1 (130.5)	124.0 (69.5)	0.893
Mean ALT, U/L (SD)	100.6 (105.2)	59.5 (31.5)	0.146
Days of severe thrombocytopenia (SD)	3.1 (1.0)	2.5 (0.8)	0.11

* IVIG = intravenous immunoglobulin; AST = aspartate aminotransferase; ALT = alanine aminotransferase. Period of severe thrombocytopenia indicates the days of platelet count between 20,000 μL and 80,000/μL.

Treatment with IVIG. Because the lowest platelet counts were found on day 2, the recovery phase, but not the phase of development for severe thrombocytopenia, was evaluated in these patients (Figure 1). Although patients who received IVIG were carefully monitored for adverse events, none were observed in patients in this group either during or after treatment. Despite treatment with a high dose of IVIG, no significant change in the platelet counts between day 2 (day of initiation of IVIG treatment) and day 7 was observed in either treatment group. Likewise, no difference was found in the duration of severe thrombocytopenia between the IVIG and non-IVIG groups (Table 1). No significant difference was found in platelet counts in the DF and DHF subgroups between the IVIG and the non-IVIG groups during the same period.

Levels of PAIgG also were examined in patients in both groups on days 2, 5, and 10. The PAIgG levels (mean ± SD ng/10^7 platelets) increased from baseline on day 2 in both the IVIG and non-IVIG groups (24.5 ± 15 versus 33.8 ± 28.6), decreased on day 5 (18.5 ± 8.4 versus 13.7 ± 5.2), and returned to normal levels (11.0 ± 7.8 versus 8.7 ± 3.7) on day 10, which is consistent with our previous findings.[9] No significant difference was found in levels of PAIgG in patients in the IVIG group and the non-IVIG group.

DISCUSSION

Although the patients were enrolled in an early phase of the illness (less than 4 days after onset), the effect of IVIG on

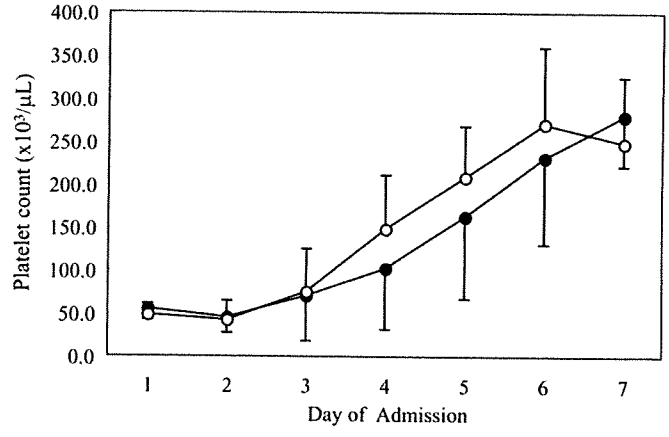

FIGURE 1. Comparison of platelet counts in patients with secondary dengue virus infections receiving a high dose of intravenous immunoglobulin (IVIG) and intravenous fluids (closed circle) and patients receiving intravenous fluids alone (open circle). The IVIG at a dose of 0.4 g/kg/day was given to each patient in the IVIG group on the second, third, and fourth day after hospital admission.

recovery of platelet count could be evaluated, but the inhibitory effect on the development of thrombocytopenia could not evaluated. No adverse effect of IVIG was noted, but there was no shortening of the time for platelet counts to return to normal levels in patients with secondary dengue infections. Because a rapid recovery of platelet counts is typically found in most patients with severe thrombocytopenia, no additional management of hemostatic abnormalities, including a high dose of IVIG, is required for such patients.

Our group recently developed an *in vitro* assay of phagocytosis of human platelets using flowcytometry. With this assay, it was shown that phagocytosis of platelets from patients with secondary infections by macrophages is significantly increased compared with that of platelets from healthy control subjects (Oishi K. and others, unpublished data). These data suggest that platelet clearance by macrophages plays a role in thrombocytopenia in this disease. Collectively, the lack of efficacy of IVIG in treating severe thrombocytopenia in secondary dengue virus infection shown in this study suggests that platelet clearance by macrophages through Fc γ receptors is not a primary mechanism of thrombocytopenia in secondary dengue virus infection. Other immune mechanisms in this disease may involve platelet clearance by macrophages through complement receptor 3 (CR3) and complement-mediated platelet lysis because complement activation mediated by circulating viral antigen is involved in the pathogenesis of this disease.[6,18]

Alternatively, de Castro and others recently conducted a pilot study to determine whether anti-D (Rh_0 D) immunoglobulin treatment, which resulted in a platelet increase of more than 70% in Rh+ non-splenectomised patients with ITP, was effective in increasing platelet counts among pediatric and adult patients with dengue illness.[19] Anti-D immunoglobulin also facilitates immune-mediated clearance of antibody-coated erythrocytes and spares sensitized platelets because of preferential destruction of erythrocytes by the mononuclear phagocytic system in ITP.[7,20] Although de Castro and others demonstrated a trend toward higher platelet counts after treatment with anti-D immunoglobulin among such patients, there was no significant difference in the kinetics of platelet counts between pediatric and adult patients with dengue virus infection who received anti-D immunoglobulin and those who received placebo.[19]

In conclusion, the present study demonstrated a lack of efficacy for a high dose of IVIG in hastening the recovery of platelet counts in patients with secondary dengue virus infection. These data suggest that platelet clearance by macrophages through Fc γ receptors is not a primary mechanism of thrombocytopenia in secondary dengue virus infection. Further studies are required to identify the immune mechanisms of thrombocytopenia in secondary dengue virus infection.

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Mechanisms underlying glycosylation-mediated loss of ecotropic receptor function in murine MDTF cells and implications for receptor evolution

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A *Mus dunni* tail fibroblast (MDTF) cell line is highly resistant to infection by ecotropic Moloney murine leukemia virus (Mo-MLV). The cationic amino acid transporter type 1 (CAT1) paralogues of murine NIH 3T3 and MDTF cells (mCAT1 and dCAT1, respectively) contain two conserved *N*-linked glycosylation sites in the third extracellular loop (ECL3, the putative Mo-MLV binding site). Glycosylation of dCAT1 inhibits Mo-MLV infection, but that of mCAT1 does not. Compared with mCAT1, dCAT1 possesses an Ile-to-Val substitution at position 214 and a Gly insertion at position 236 in the ECL3. To determine the residues responsible for the loss of dCAT1 receptor function, mutants of mCAT1 were constructed. The mCAT1/insG receptor (with a Gly residue inserted at mCAT1 position 236) had greatly reduced Mo-MLV receptor function compared with mCAT1. Treatment of mCAT1/insG-expressing cells with tunicamycin, an *N*-linked glycosylation inhibitor, increased the transduction titre. In addition, the reduced susceptibility to Mo-MLV observed with mCAT1/insG-expressing cells correlated with impaired binding of Mo-MLV. These results show that a single amino acid insertion confers mCAT1 receptor properties on dCAT1 and provide an important insight into the co-evolution of virus–host interactions.

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INTRODUCTION

The entry of ecotropic murine leukemia virus (Eco-MLV) into host cells is initiated by the interaction between the viral envelope glycoprotein (Env) and its receptor, cationic amino acid transporter type 1 (CAT1) (Albritton *et al.*, 1989; Kim *et al.*, 1991; Wang *et al.*, 1991). The CAT1 protein is a 14-transmembrane multi-spanning molecule comprised of seven extracellular and six cytoplasmic loops with two conserved *N*-linked glycosylation sites (Asn 223 and Asn 229). The third extracellular loop (ECL3) appears to be critical for ecotropic retrovirus receptor function (Albritton *et al.*, 1989; Overbaugh *et al.*, 2001; Sommerfelt, 1999; Tailor *et al.*, 2003). Previous studies have shown that the integrity of the ^{235}YGE^{237} motif in ECL3 is essential for MLV receptor function (Albritton *et al.*, 1993; Yoshimoto *et al.*, 1993).

Susceptibility to Eco-MLV is restricted to murine and certain rat cells. Glycosylated murine CAT1 paralogue (mCAT1) receptors retain full Mo-MLV receptor function (Kubo *et al.*, 2002; Wang *et al.*, 1996). Hamster CAT1 (hCAT1) orthologues fail to function as the Eco-MLV receptor but can be rendered functional after treatment with tunicamycin, an inhibitor of *N*-linked glycosylation (Miller & Miller, 1992; Wilson & Eiden, 1991). In addition, the rat CAT1 orthologue (rCAT1) and the *Mus dunni* CAT1 paralogue (dCAT1) function poorly as Mo-MLV receptors due to glycosylation-dependent inhibition (Eiden *et al.*, 1993, 1994; Kubo *et al.*, 2002; Tavoloni & Rudenholz, 1997). The ECL3 of rCAT1 is 2 aa longer than the ECL3 of mCAT1 (due to deletion of the Lys residue at position 222 and insertions of Ser, Pro and Leu at positions 226–228 compared with mCAT1). We previously reported that the extra amino acids in rCAT1 (compared with mCAT1) are associated with the inhibition of Eco-MLV infection by rCAT1 glycosylation (Kubo *et al.*, 2004).

M. dunni tail fibroblast (MDTF) cells are permissive for all four classes of MLV: ecotropic, xenotropic, polytropic and amphotropic, with the notable exception of the ecotropic Moloney murine leukemia virus (Mo-MLV) (Chesebro & Wehrly, 1985; Lander & Chattopadhyay, 1984). It has been shown previously that pretreatment of MDTF cells with tunicamycin renders MDTF cells susceptible to Mo-MLV (Eiden *et al.*, 1994). Compared with the mCAT1 protein, the dCAT1 protein possesses a substitution of Ile for Val at position 214 and a Gly insertion at position 236 in the ECL3, in addition to a substitution of Asn for Asp at position 373 and a substitution of Thr for Ala at position 590 (Eiden *et al.*, 1993) (Fig. 1a). To determine which amino acid changes are responsible for the low susceptibility of dCAT1 to Mo-MLV infection, we constructed mutants of the mCAT1 receptor and elucidated the mechanism by which Mo-MLV infection is blocked in *M. dunni* cells. The results observed in this study showed that the Gly insertion impairs the mCAT1 receptor function, and provide important insights into CAT1 gene evolution as the ecotropic virus receptor.

METHODS

Expression plasmids. The cDNA clones of mCAT1 and dCAT1 were kindly provided by Dr J. M. Cunningham (Harvard Medical School, USA; Albritton *et al.*, 1989) and Dr M. Eiden (NIAID, USA; Eiden *et al.*, 1993). A plasmid encoding the mCAT1/M3 mutant was kindly provided by Dr D. Kabat (Oregon Health Science University, USA; Wang *et al.*, 1996). PCR was performed using these cDNA clones as templates with the following primers for C-terminal haemagglutinin (HA) tagging: 5′-TTACTGCAGACAGATTTGCTC-AGCGCGATG-3′ and 5′-TCATGCGTAATCCGGAACATCGTACG-GGTATTTGCACTGGTCCAAGTTGCTGTCAGGAGTCTT-3′. The latter antisense primer contains the HA epitope sequence as reported previously (Kubo *et al.*, 2002). The PCR product was cloned into the pTargeT vector (Promega) by TA cloning. The *Eco*RI–*Not*I fragment containing the HA-tagged CAT1 sequence was subcloned into the pMXpuro retrovirus vector (Onishi *et al.*, 1996). A vesicular stomatitis virus G protein (VSV-G) expression plasmid (pHEF-VSVG) was obtained from Dr L. Chang through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, USA (Chang *et al.*, 1999).

Mutagenesis. PCR-mediated mutagenesis was performed on the HA-tagged mCAT1- and mCAT1/M3-expressing plasmids using 5′-phosphorylated BamX (5′-CTATAGGGCGAATTCCGATCCTTGCT-AGCC-3′) and mutation primers. The sequences of the mutation primers used to construct mCAT1/insG and mCAT1/I214V were 5′-AAACCCTCCCTCACCCCCGTATTTCACGTTTGT-3′ and 5′-GAG-CTGCCAGTTTTTAACGGAGCCTTTCACGAA-3′, respectively. The BamX primer has a nucleotide substitution that disrupts the unique *Bam*HI site of the pTargeT vector. These primers were synthesized by Nissinbo Inc. The PCR amplified fragments of approximately 900 bp, which in turn provided PCR primers for amplification of the

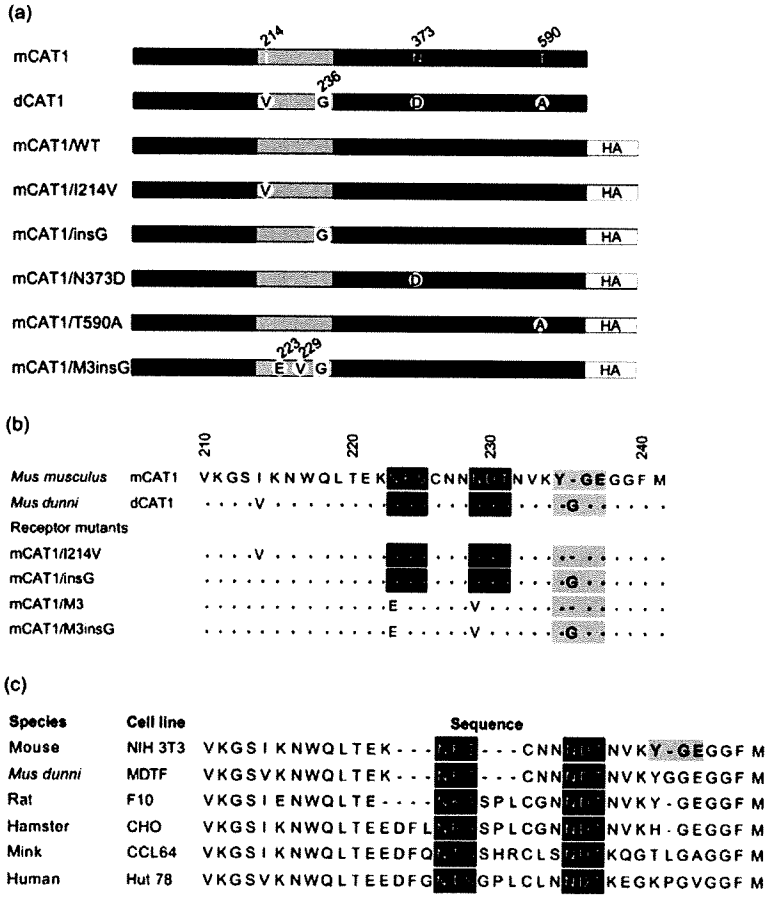

Fig. 1. Comparison of the amino acid sequences of mCAT1, dCAT1 and constructed mCAT1 mutants. (a) Structure of HA-tagged mCAT1 mutant proteins. Four amino acid differences distinguish mCAT1 from dCAT1. The wild-type mCAT1 (mCAT1/WT) and mCAT1 mutants were constructed to contain a C-terminal HA epitope tag. ○, Substitution mutation, □, insertion mutation. ECL3 is indicated by grey shading. (b) Comparison of the ECL3 amino acid sequences of mCAT1, dCAT1 and mCAT1 mutants. Filled boxes, *N*-linked glycosylation sites; shaded box, Mo-MLV Env-binding domain. (c) Comparison of the ECL3 amino acid sequences of CAT1 proteins from various animals. Filled boxes, *N*-linked glycosylation sites; shaded box, Mo-MLV Env-binding domain in mCAT1.

complete plasmid containing the desired mutations. The PCR product was treated with *Bam*HI to digest the template plasmid and transformed into *Escherichia coli* DH5α competent cells (Takara). The *Eco*RI–*Not*I fragment containing the HA-tagged CAT1 mutant sequence was subcloned into the pMXpuro retroviral vector. The nucleotide sequence of the mutant was confirmed by sequencing (GeneNet and Applied Biosystems).

Cells. Human glioma NP2 cells (Soda *et al.*, 1999), human rhabdomyosarcoma TELCeB6 cells expressing both Mo-MLV gag–pol and the *lacZ* vector (Cosset *et al.*, 1995), human embryo kidney 293T cells and MDTF cells (Lander & Chattopadhyay, 1984) were cultured in Dulbecco's modified Eagle's medium (Sigma) at 37 °C in 5% CO_2. MDTF cells were provided from the ATCC. The culture media were supplemented with 8% fetal bovine serum (Biofluids). CAT1-expressing NP2 cells were constructed as follows: human embryo kidney 293T cells were transfected with 3 μg Mo-MLV gag–pol, 3 μg CAT1-expressing retroviral vector and 3 μg VSV-G expression plasmid (Chang *et al.*, 1999) using 30 μl TransIT LT1 reagent (Mirus). Cells were washed 24 h after transfection and cultured for 24 h in fresh medium. The culture supernatant of the transfected cells was inoculated into NP2 cells, which were subsequently selected using puromycin (10 μg ml^{-1}). The puromycin-resistant cell pool was utilized in this study.

Semi-quantitative RT-PCR. Total RNA samples were isolated from NP2 cells expressing the HA-tagged wild-type mCAT1 and untagged dCAT1. First-strand cDNA was synthesized using reverse transcriptase (Takara) from total RNA samples (1 μg). Semi-quantitative PCR was performed to detect CAT1 and GAPDH mRNAs. The sequences of the primers for the CAT1 RT-PCR were 5′-TCAAGCGTGGCAAGAGCCTGGAG-3′ and 5′-TGCCCCTGACAGGACACCAGAGAA-3′, and for the GAPDH RT-PCR were 5′-AGGTCGGAGTCAACGGATTTGGT-3′ and 5′-GTGGGCCATGAGGTCCACCAC-3′, and were synthesized by GeneNet.

Transduction assay. The ecotropic retroviral vector encoding the *lacZ* gene was generated by stable transfection of TELCeB6 cells, which express the Mo-MLV gag–pol genes and the *lacZ*-containing retroviral vector genome (Cosset *et al.*, 1995), using the wild-type Mo-MLV Env expression plasmid (Kubo & Amanuma, 2003). Target cells (2×10^5) were plated onto a 60 mm culture dish and cultured for 24 h in the presence or absence of tunicamycin (400 or 800 ng ml^{-1}). Cells were washed with medium to remove tunicamycin and inoculated with the retroviral vector in the presence of polybrene (4 μg ml^{-1}). After an additional 24 h culture in fresh medium, cells were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Wako); cells that stained blue were counted for an estimation of transduction titre.

Immunofluorescence microscopy. HA-tagged CAT1-expressing NP2 cells were cultured on four-well culture slides (Miles) in the presence or absence of tunicamycin (800 ng ml^{-1}) for 24 h. Cells were fixed with cold methanol and incubated with a mouse anti-HA antibody (Covance), followed by indocarbocyanine (Cy3)-conjugated anti-mouse IgG antibody (Sigma). Cells were observed using a confocal fluorescence microscope (Leica).

Western immunoblotting. HA-tagged CAT1-expressing cells (2×10^5) were seeded and cultured for 48 h. Cells were then cultured for an additional 24 h in the presence or absence of tunicamycin (400 or 800 ng ml^{-1}). Cell lysates were subjected to 7.5% SDS-PAGE followed by Western blotting using mouse anti-HA antibody and horseradish peroxidase-conjugated anti-mouse IgG antibody (Bio-Rad).

Virus binding. A virus-binding assay was performed as reported previously (Lavillette *et al.*, 2000). Cells (5×10^5) were incubated with viral stock for 30 min at 4 °C, after which unbound virus was removed by two washes with 0.2% BSA in ice-cold PBS; virus–cell complexes were incubated sequentially at 4 °C with goat anti-gp70 antiserum (diluted 1:500) and phycoerythrin (PE)-conjugated donkey anti-goat antiserum (diluted 1:100; Jackson Laboratories). Cells were then fixed in 1% formalin (Sigma). Fluorescence intensity was analysed by flow cytometry (Coulter or Becton Dickinson). Parental NP2 cells and NP2/mCAT1 mutants were incubated in virus-free medium and served as negative controls.

Statistical analysis. Differences between groups of data were determined by Student's *t*-test. Statistical significance was set at $P<0.05$ for all tests.

RESULTS

Characterization of dCAT1 in human NP2 cells

MDTF cells are resistant to ecotropic Mo-MLV infection, but this resistance can be counteracted by treatment with tunicamycin, an *N*-linked glycosylation inhibitor. To determine whether the ecotropic receptor of MDTF cells or another cellular factor is responsible for this event, dCAT1-expressing human NP2 cells were constructed. To detect dCAT1 protein expression, dCAT1 was C-terminally tagged with the HA epitope. Unfortunately, the HA-tagged dCAT1 protein was not detected by Western blotting using an anti-HA antibody, and NP2 cells transduced by the HA-tagged dCAT1-encoding retrovirus vector were not susceptible to the Mo-MLV vector infection, indicating that the HA-tagged dCAT1 was not expressed. Therefore, untagged dCAT1-expressing NP2 cells were constructed.

The transduction titre of the Mo-MLV vector in the untagged dCAT1-expressing NP2 cells was less than 10% of that in untagged and tagged mCAT1-expressing cells (Fig. 2a and c), although mRNA levels of dCAT1 and mCAT1 were similar (Fig. 2b). Serial dilution of the template first-strand cDNA induced lower levels of PCR product, indicating that these PCR conditions were semi-quantitative. In addition, tunicamycin treatment (400 ng ml^{-1}) of the dCAT1-expressing cells enhanced the transduction titre approximately tenfold (Fig. 2c), as in MDTF cells (Eiden *et al.* 1994). These results indicated that dCAT1 is a determinant for the lower susceptibility of MDTF cells to Mo-MLV infection and for the glycosylation-mediated loss of receptor function.

Functional comparison of various mCAT mutant proteins

The dCAT1 protein has an Ile-to-Val substitution at position 214 and a Gly insertion at position 236 in the ECL3, compared with the mCAT1 sequence (Fig. 1a) (Eiden *et al.*, 1994). The C-terminally HA-tagged dCAT1 protein was not detected by Western blotting using anti-HA antibody, similar to hCAT1 (Kubo *et al.*, 2004). Therefore, we constructed mutants of HA-tagged mCAT1 (Kubo *et al.*, 2002). The C-terminal HA tagging of mCAT1 did not affect its receptor function (Fig. 2a).

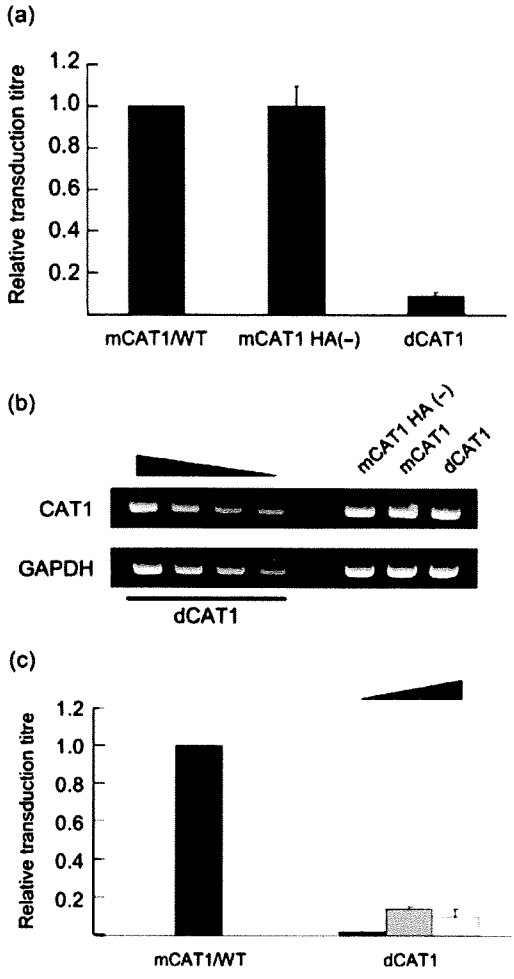

Fig. 2. Relative susceptibilities of mCAT1- and dCAT1-expressing cells. (a) NP2 cell pools expressing the C-terminally HA-tagged mCAT1 (mCAT1/WT), untagged WT mCAT1 [mCAT1 HA(−)] and dCAT1 were inoculated with the Mo-MLV vector. Transduction titres are relative to those in mCAT1/WT-expressing cells. This experiment was repeated three times and results are shown as means ± SD. (b) Expression levels of mCAT1/WT, mCAT1 HA(−) and dCAT1 were analysed by semi-quantitative RT-PCR. To confirm the quantitativeness of the RT-PCR, first-strand cDNA prepared from mCAT1/WT-expressing cells was serially diluted twofold and PCR was performed using the diluted cDNA as template. The PCR products were subjected to agarose gel electrophoresis. (c) Transduction titres in tunicamycin-treated mCAT1/WT- and dCAT1-expressing cells. Cells were treated with 0 (open bar), 400 (shaded bar) or 800 (filled bar) ng tunicamycin ml^{-1} for 24 h. Transduction titres relative to those in untreated mCAT1/WT-expressing cells are indicated. This experiment was repeated three times and results are shown as means ± SD.

To identify the residues that restrict the ability of dCAT1 to function as a Mo-MLV receptor, we constructed HA-tagged mCAT1 mutants and tested each for receptor function following their expression in human glioma NP2 cells (Soda *et al.*, 1999). The mutants mCAT1/I214V, mCAT1/insG, mCAT1/N373D and mCAT1/T590A were constructed (Fig. 1a and b) and their sequences confirmed by sequencing. We engineered the expression of each mCAT1 mutant in NP2 cells using a previously described procedure (Kubo *et al.*, 2004).

mCAT1/insG-expressing cells are resistant to Mo-MLV

Wild-type mCAT1 (mCAT1/WT)-, mCAT1/insG- and mCAT1/I214V-expressing cells were pretreated with tunicamycin (0, 400 or 800 ng ml^{-1}) for 24 h to measure the effects of receptor glycosylation on Mo-MLV vector infection. Relative transduction titres compared with those obtained on untreated mCAT1-expressing cells are shown in Fig. 3(a). Transduction titres of the Mo-MLV vectors in mCAT1/insG-expressing cells were tenfold lower than those in mCAT1/WT-expressing cells ($P<0.001$), as with

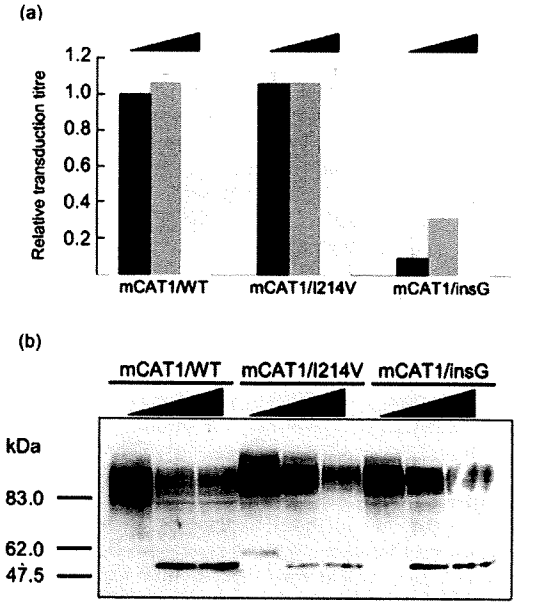

Fig. 3. Relative susceptibility of each mCAT1 mutant-expressing cell pool to Mo-MLV infection and expression of C-terminally HA-tagged mCAT1 mutants in NP2 cells. (a) mCAT1/WT-, mCAT1/I214V- and mCAT1/insG-expressing NP2 cell pools were treated with tunicamycin for 24 h. Transduction titres are shown for untreated cells (filled bars) and for cells treated with 400 ng (shaded bars) or 800 ng (open bars) tunicamycin ml^{-1} and are relative to those in untreated mCAT1/WT-expressing cells. This experiment was repeated three times and results are shown as means ± SD. (b) Cell lysates were prepared from tunicamycin-treated (400 or 800 ng ml^{-1}) and untreated HA-tagged mCAT1 mutant-expressing cell pools. Cell lysates were electrophoresed on 7.5 % polyacrylamide gels and Western blotting was performed using an anti-HA antibody. Molecular mass standards are indicated on the left.

dCAT1-expressing cells (Fig. 2), even though the expression levels of the mCAT1/WT and mCAT1/insG proteins were similar (Fig. 3b). The titres of the Mo-MLV vectors in mCAT1/insG-expressing cells were increased following treatment with tunicamycin in a dose-dependent manner ($P<0.05$), as with dCAT1-expressing cells (Fig. · 2). Transduction efficiency of the Mo-MLV vectors in mCAT1/I214V-expressing cells was comparable to those in mCAT1/WT-expressing cells. Transduction efficiencies of the Mo-MLV vectors in mCAT1/I214V-expressing cells were unaffected by tunicamycin treatment, as was found for mCAT1/WT-expressing cells. Protein expression levels of the mCAT1 mutants were detected at levels similar to those of mCAT1/WT as assessed by Western blotting using an anti-HA antibody (Fig. 3b). Tunicamycin treatment decreased the apparent molecular mass of the CAT1 proteins (~50 kDa), confirming that tunicamycin treatment inhibited CAT1 protein glycosylation. These results indicated that inhibition of Mo-MLV infection by the Gly insertion resulted at least in part from receptor glycosylation, as transduction levels of tunicamycin-treated mCAT1/insG-expressing cells were lower than those of mCAT1-expressing cells.

mCAT1/N373D- and mCAT1/T590A-expressing NP2 cells were as susceptible to Mo-MLV infection as were mCAT/WT-expressing cells (data not shown). Expression levels of both the mCAT1/N373D and mCAT1/T590A proteins were comparable to those of mCAT1/WT, as determined by Western blotting using an anti-HA antibody. These results indicated that the Asp and Ala residues at positions 373 and 590, respectively, as well as the Val residue at position 214 in the dCAT1 protein, are not associated with the reduced susceptibility to Mo-MLV of cells expressing dCAT1.

Glycosylation of dCAT1 inhibits Mo-MLV infection

To assess further the glycosylation-dependent reduction in Mo-MLV titre in cells expressing dCAT1, we constructed the mCAT1/M3insG mutant (Fig. 1a). Two conserved *N*-linked glycosylation sites (Asn 223 and Asn 229) are present in the ECL3 of both mCAT1 and dCAT1. The mCAT mutant mCAT1/M3, lacking both of these *N*-linked glycosylation sites, was provided by Dr D. Kabat (Wang *et al.*, 1996). Site-directed mutagenesis was performed to insert a codon encoding Gly at position 236 of mCAT1/M3. Cells expressing mCAT1/WT were as susceptible to Mo-MLV vector transduction as cells expressing mCAT1/M3 (Fig. 4a). The transduction efficiencies of Mo-MLV vectors determined in cells expressing mCAT1/M3insG were two- to threefold higher than those found in mCAT1/insG-expressing cells, yielding results similar to those seen after tunicamycin treatment of mCAT1/insG-expressing cells ($P<0.05$). This finding confirmed the observation that glycosylation of the mCAT1/insG protein inhibits Mo-MLV infection, but that glycosylation of mCAT1/WT does not. Therefore, glycosylation of the receptors from different

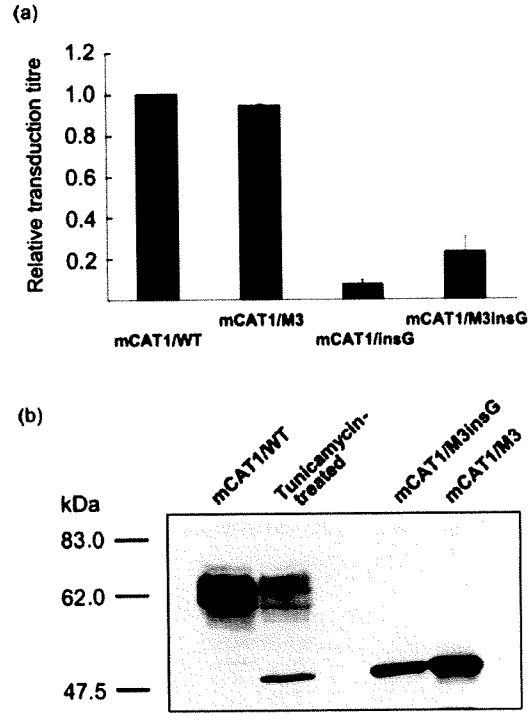

Fig. 4. Effect of amino acid substitutions at the *N*-linked glycosylation sites of CAT1 on Mo-MLV infection and on the molecular masses of HA-tagged mCAT1 mutant proteins. (a) Transduction titres in the mCAT1 mutant-expressing cells relative to mCAT1/WT-expressing cells are indicated. The mCAT1/M3insG mutant has no glycosylation site. This experiment was repeated three times and results are shown as means ± SD. (b) Cell lysates were prepared from untreated mCAT1/WT-, mCAT1/M3- and mCAT1/M3insG-expressing cells and from tunicamycin-treated mCAT1/WT-expressing cells. The cell lysates were electrophoresed on 7.5% polyacrylamide gels and Western blotting performed using an anti-HA antibody.

animals per se does not always diminish receptor function. The molecular mass of the mCAT1/M3insG protein was lower than that of either the mCAT1/WT or mCAT1/insG protein, and that of the unglycosylated mCAT1/WT detected in tunicamycin-treated cells was similar to those of the mCAT1/M3 and mCAT1/M3insG (Fig. 4b). Presumably, this was attributable to elimination of the *N*-linked glycosylation sites in these proteins.

Binding of Mo-MLV to mCAT1/insG is impaired

mCAT1/M3insG-expressing and tunicamycin-treated mCAT1/insG-expressing cells were less susceptible to Mo-MLV infection than mCAT1/WT-expressing cells (Figs 3 and 4). This suggested that the mCAT1/insG protein has an additional factor that inhibits Mo-MLV infection, in addition to receptor glycosylation. As the Gly residue at position 236 has been inserted into the virus-binding motif in the receptor (Albritton *et al.*, 1993;

Yoshimoto *et al.*, 1993), we tested the ability of Mo-MLV vectors to bind to mCAT1 mutant-expressing NP2 cells (Lavillette *et al.*, 2000). Cultured supernatants of either Mo-MLV Env-expressing TELCeB6 cells (Kubo & Amanuma, 2003) or parental TELCeB6 cells (Cosset *et al.*, 1995) (no Env virus, negative control) were exposed to target cells at 4 C. mCAT1/WT-, mCAT1/I214V- and mCAT1/M3-expressing cells, which are all equally susceptible to Mo-MLV infection, showed efficient binding of the virus (Fig. 5a). mCAT1/insG-, mCAT1/M3insG- (Fig. 5a) and dCAT1-expressing cells (Fig. 5b), which were more resistant to Mo-MLV infection than mCAT1/WT-expressing cells, showed much lower binding of the virus than mCAT1/WT-expressing cells. This result suggested that binding of the Mo-MLV vectors to both mCAT1/insG and mCAT1/M3insG was impaired, indicating that the Gly insertion inhibits vector binding to the receptor independently of receptor glycosylation status.

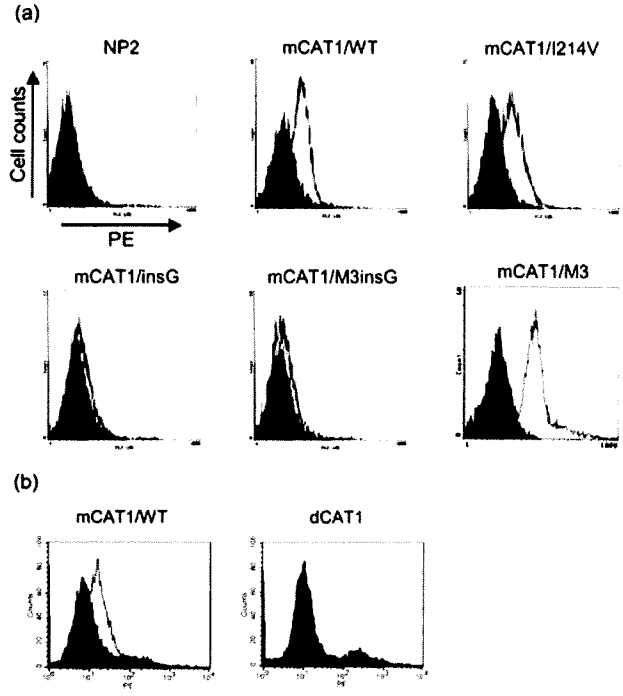

Fig. 5. Specific binding of the Mo-MLV Env glycoprotein to the CAT1 protein. mCAT1 mutant-expressing cells were incubated with Mo-MLV particles, followed by goat anti-Env surface glycoprotein and finally with PE-conjugated donkey anti-goat antiserum, each for 1 h at 4 °C. The fluorescence intensities of the cells were analysed by a flow cytometer from Coulter (a) or Becton Dickinson (b). Filled and open areas indicate mCAT1 mutant-expressing cells incubated with Env-free viral particles (no Env) as a negative control and with Mo-MLV Env-containing particles, respectively.

Cell-surface expression of mCAT1 mutants

To exclude the possibility that altered cellular localization of the mCAT1 mutants affected susceptibility to Mo-MLV infection, we analysed the cellular distribution of the mutant proteins by immunofluorescence microscopy using an anti-HA antibody. Each mCAT1 mutant was detected on the cell surface and in the cytoplasm, as seen for mCAT1/WT-expressing cells (Fig. 6). In addition, the cellular localization of each mCAT1 mutant was unaffected by tunicamycin treatment (400 ng ml^{-1}), indicating that neither the mutations nor tunicamycin treatment had any effect on mCAT1 cellular localization.

DISCUSSION

Mechanism of glycosylation-mediated loss of receptor function in MDTF cells

Human CAT1 does not function as a Mo-MLV receptor, presumably because this receptor lacks a critical motif that is part of the virus-binding domain (^{235}YGE^{237} in the ECL3) (Albritton *et al.*, 1993; Yoshimoto *et al.*, 1993). Susceptibility of hCAT1 to Eco-MLV infection is completely suppressed by a sugar-chain linkage to the receptor, although the virus-binding domain is preserved (Wilson & Eiden, 1991). Eco-MLV infection is suppressed in part by a sugar-chain linkage in rCAT1 (Kubo *et al.*, 2002). Although mCAT1 is glycosylated, as are both hCAT1 and rCAT1, it does not inhibit Eco-MLV infection. We previously reported that the extra amino acids in rCAT1 (compared with mCAT1) were associated with inhibition of Eco-MLV infection by glycosylation at these residues (Kubo *et al.*, 2004). As the hCAT1 orthologue contains the extra amino acids that are a resident part of rCAT1 (Fig. 1c), a similar glycosylation mechanism may also inhibit viral infection in both hCAT1 and rCAT1.

The MDTF cell line expresses a paralogue of mCAT1, dCAT, and glycosylation of dCAT1 inhibits Mo-MLV infection (Eiden *et al.*, 1993, 1994). The dCAT1 protein does not have the extra amino acids of rCAT1, but possesses a single amino acid insertion and a single amino acid substitution in the ECL3 compared with mCAT1 (Fig. 1b and c). The mechanism of inhibition of Mo-MLV infection by dCAT1 glycosylation appears to be different from those used by both rCAT1 and hCAT1. Thus, we attempted to clarify the mechanisms of viral infection control by glycosylation of dCAT1.

In this study, we constructed several mCAT1 mutants (Fig. 1b). Transduction titres of the Mo-MLV vectors in mCAT1/I214V-expressing cells were comparable to those expressing mCAT1/WT. mCAT1/insG-expressing cells were more resistant to Mo-MLV than those expressing mCAT1/WT (Fig. 3a). Protein expression levels and cellular localization of the mCAT1 mutants were similar to those of mCAT1/WT (Figs 3b and 6). These results suggested that the Gly residue at position 236 in the ECL3

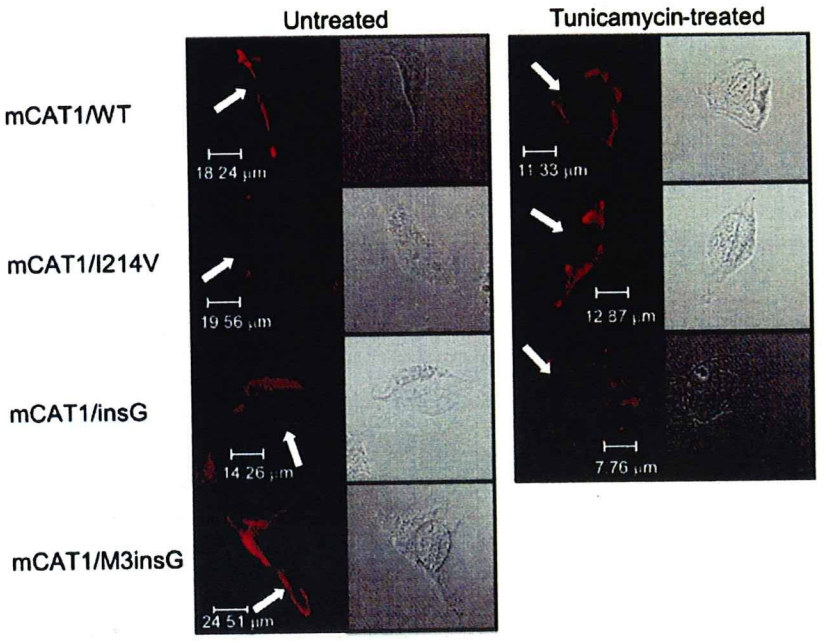

Fig. 6. Cell-surface expression of mCAT1/WT and mCAT1 mutant proteins. mCAT1-expressing cells were cultured on chamber slides, fixed with cold methanol and treated with mouse anti-HA antibody, followed by treatment with Cy3-conjugated anti-mouse IgG antibody. Cells were observed by confocal fluorescence microscopy. Fluorescence and differential interference contrast images of the same fields are shown in the left and right panels, respectively.

of dCAT1 is responsible for the failure of dCAT1 to function efficiently as a receptor for Mo-MLV. It has been reported that the Val residue of dCAT1 is a determinant for the lower susceptibility to Mo-MLV infection (Eiden *et al.*, 1994). However, in our study, mCAT1/I214V-expressing cells were as susceptible as mCAT1/WT-expressing cells, indicating that the Val residue did not affect Mo-MLV infection. Further analysis is required to determine why these inconsistent results have been observed.

Titres of Mo-MLV vectors in mCAT1/insG-expressing cells increased following treatment with tunicamycin (Fig. 3a). The transduction titres of the Mo-MLV vectors in the mCAT1/M3insG-expressing cells were two- to threefold higher than cells expressing mCAT1/insG (Fig. 4a). These results suggested that the Gly residue introduced at position 236 induces a conformational change in the dCAT1 protein that inhibits viral infection by glycosylation.

mCAT1/insG- and mCAT1/M3insG-expressing cells showed much lower binding of virus than mCAT1/WT- and mCAT1/M3-expressing cells. The dCAT1 protein has a Gly residue within the putative virus-binding site of mCAT1. As viral entry is initiated by binding of the viral envelope protein to the receptor at the target cell surface, this result indicated that the inserted Gly residue inhibits Mo-MLV infection by impairing the interaction between the Mo-MLV envelope and the CAT1 receptor. It has been reported previously that the Gly insertion in dCAT1 is responsible for the lower susceptibility of MDTF cells to Mo-MLV infection (Bae *et al.*, 2006). This study determined the mechanism by which the Gly insertion into position 236 of mCAT1 makes it less susceptible to Mo-MLV infection: the glycosylation-mediated loss of receptor function and the inhibition of Env protein binding to the receptor protein.

The Mo-MLV vector particles bound equally to mCAT1/insG- and mCAT1/M3insG-expressing cells (Fig. 5). This result suggested that dCAT1 glycosylation does not suppress the Env–dCAT1 interaction, but affects the folding of the mCAT1/insG protein as the Eco-MLV receptor (Helenius & Aebi, 2001, 2004). However, mCAT1/M3insG-expressing cells were two to three times more susceptible to Mo-MLV infection than mCAT1/insG-expressing cells (Fig. 4a), and this extent of difference in Env–receptor interaction could be undetectable by this binding assay. Therefore, we cannot exclude the possibility that the sugar chain linked to dCAT1 physically prevents efficient interaction of Mo-MLV Env and dCAT1.

Speculation on the co-evolution of Eco-MLV and receptor CAT1

Based on both the present results and previous reports, we suggest a hypothesis of co-evolution of Eco-MLV and CAT1. rCAT1 is a less efficient receptor for Eco-MLV than mCAT1 (Kubo *et al.*, 2002; Takase-Yoden & Watanabe, 1999). We hypothesize that the rCAT1 protein might, over a number of years, have gained extra residues for protection from Eco-MLV infection (Kubo *et al.*, 2004). Hamster cells are completely resistant to Mo-MLV infection as a result of CAT1 glycosylation (Wilson & Eiden, 1991). hCAT1 has extra amino acids as does rCAT1, and is also 4 aa longer than rCAT1 (Fig. 1c). Therefore, the gain of these residues in hCAT1 accompanies a loss of Mo-MLV receptor function. Although hCAT1 is completely resistant to Mo-MLV infection, it has been reported that

neuropathogenic and hamster-adapted variants of ecotropic Friend MLV infect hamster cells efficiently (Ishimoto, 1985; Jung *et al.*, 2004; Masuda *et al.*, 1996). These findings indicate that certain ecotropic murine virus variants have also evolved to counteract the host defences that are mediated by receptor glycosylation.

The six additional residues in the ECL3 are present in both human and mink CAT1s (Kubo *et al.*, 2002; Yoshimoto *et al.*, 1991) (Fig. 1c). Although dCAT1 does not have the extra residues present in rCAT1 and hCAT1, it is resistant to Mo-MLV. It appears that dCAT1 has evolved from a mCAT1-type receptor independently of both rCAT1 and hCAT1, whereas human and mink CAT1 proteins have evolved to combine features of both hamster and *M. dunni* receptors. Finally, human and mink CAT1 proteins have acquired complete resistance to infection by all ecotropic murine retroviral variants as a consequence of the acquired mutations in the virus-binding domain.

It is likely that a viral receptor protein directly acquires resistance to viral infection by mutations in its virus-binding domain. However, the CAT1 protein shows a complex evolutionary pathway. CAT1 consists of multiple membrane-spanning proteins and its expression requires an unknown complex mechanism, as many of the CAT1 mutants are not expressed (Kubo *et al.*, 2004). It has been reported that knockout of the *cat1* gene in mice is lethal, indicating a critical role for CAT1 in growth control during mouse development (Perkins *et al.*, 1997). These characteristics of the CAT1 protein may decrease the speed of evolution and cause a more complex evolutionary pathway. One of the reasons why most retroviral receptors have multiple membrane-spanning proteins such as mCAT1 (Sommerfelt, 1999) might be the reduced evolutionary rate of mutation among transporter proteins.

These investigations into glycosylation-mediated inactivation of ecotropic receptor provide novel insights into the co-evolution of host–virus interactions. All of the gammaretrovirus receptors identified to date employ glycosylated carrier facilitator proteins as receptors (Tailor *et al.*, 2003); therefore, it is not surprising that the cell-specific addition of *N*-linked sugars to receptor proteins has evolved as a method for restricting efficient infection by gammaretroviruses (Overbaugh *et al.*, 2001; Tailor *et al.*, 2000). The ASCT1, Pit1 and Pit2 receptors have all been reported to be inactivated by glycosylation in some types of cell and hence fail to facilitate entry by the feline endogenous retrovirus RD114, gibbon ape leukemia virus and amphotropic MLV, respectively. Thus, glycosylation-mediated blocks in receptor function extend not only to other orthologues of CAT1 such as rCAT1 (Kubo *et al.*, 2004) but also to other gammaretroviral receptors.

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Characteristics of a Large Cohort of Patients with Autoimmune Pulmonary Alveolar Proteinosis in Japan

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Rationale: Acquired pulmonary alveolar proteinosis (PAP) is a syndrome characterized by pulmonary surfactant accumulation occurring in association with granulocyte/macrophage colony-stimulating factor autoantibodies (autoimmune PAP) or as a consequence of another disease (secondary PAP). Because PAP is rare, prior reports were based on limited patient numbers or a synthesis of historical data.

Objectives: To describe the epidemiologic, clinical, physiologic, and laboratory features of autoimmune PAP in a large, contemporaneous cohort of patients with PAP.

Methods: Over 6 years, 248 patients with PAP were enrolled in a Japanese national registry, including 223 with autoimmune PAP.

Measurements and Main Results: Autoimmune PAP represented 89.9% of cases and had a minimum incidence and prevalence of 0.49 and 6.2 per million, respectively. The male to female ratio was 2.1:1, and the median age at diagnosis was 51 years. A history of smoking occurred in 56%, and dust exposure occurred in 23%; instances of familial onset did not occur. Dyspnea was the most common presenting symptom, occurring in 54.3%. Importantly, 31.8% of patients were asymptomatic and were identified by health screening. Intercurrent illnesses, including infections, were infrequent. A disease severity score reflecting the presence of symptoms and degree of hypoxemia correlated well with carbon monoxide diffusing capacity and serum biomarkers, less well with pulmonary function, and not with granulocyte/macrophage colony-stimulating factor autoantibody levels or duration of disease.

Conclusions: Autoimmune PAP had an incidence and prevalence higher than previously reported and was not strongly linked to smoking, occupational exposure, or other illnesses. The disease severity score and biomarkers provide novel and potentially useful outcome measures in PAP.

Keywords: epidemiology; serum biomarkers; disease severity score; granulocyte/macrophage colony-stimulating factor; autoantibody

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AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Acquired pulmonary alveolar proteinosis (PAP) is a syndrome characterized by pulmonary surfactant accumulation occurring in association with granulocyte/macrophage colony-stimulating factor autoantibodies (autoimmune PAP) or as a consequence of another disease (secondary PAP). Because PAP is rare, prior reports were based on limited patient numbers or a synthesis of historical data.

What This Study Adds to the Field

Autoimmune PAP had an incidence and prevalence higher than previously reported and was not strongly linked to smoking, occupational exposure, or other illnesses.

Pulmonary alveolar proteinosis (PAP), first described in 1958 (1), is a rare syndrome characterized by the intraalveolar accumulation of surfactant lipids and proteins impairing gas exchange and resulting in progressive respiratory insufficiency. PAP, which has been reported in the medical literature under various terms (alveolar proteinosis, alveolar lipoproteinosis, alveolar phospholipidosis, pulmonary alveolar lipoproteinosis, and pulmonary alveolar phospholipoproteinosis), is recognized to occur in three distinct clinical forms: primary, secondary, and congenital (2, 3). Primary (idiopathic) PAP is a disorder of unknown etiology thought to represent approximately 90% of PAP cases. Secondary PAP occurs as a consequence of any one of a heterogeneous group of underlying clinical conditions (hematologic malignancies; inhalation of toxic dust, fumes, or gases; infectious or pharmacologic immunosuppression; or lysinuric protein intolerance) that impairs alveolar macrophage function, resulting in surfactant accumulation (4). Congenital PAP is a heterogeneous collection of disorders caused by homozygous mutation of the genes encoding surfactant protein (SP)-B, SP-C, and the ABCA3 transporter or by the absence of granulocyte/macrophage colony-stimulating factor (GM-CSF) receptor (5).

Our understanding of PAP pathogenesis has advanced rapidly over the past decade due to a series of contributions from basic, clinical, and translational research. The first real pathogenic clue was provided by the discovery that mice deficient in GM-CSF develop a lung phenotype biochemically, histologically, physiologically, and ultrastructurally indistinguishable from primary PAP (6, 7). Surfactant accumulation in these mice is not due to

increased production but rather to impaired catabolism of surfactant lipids and proteins by alveolar macrophages (8). This and numerous other alveolar macrophage defects were shown to be due to impaired terminal differentiation in mice caused by reduced levels of the GM-CSF–dependent transcription factor, PU.1 (9). GM-CSF also regulates PU.1 levels in human alveolar macrophages (10), which in patients with PAP have defects similar to those of GM-CSF–deficient mice (11). The observation of PAP in GM-CSF–deficient mice was quickly followed by the evaluation of GM-CSF therapy, first in single patient (12) and then in several small series (13–15). GM-CSF deficiency has not been reported in humans (3, 16). However, a second vital pathogenic clue was the observation that primary PAP is specifically and strongly associated with very high levels of GM-CSF autoantibodies (17) that eliminate GM-CSF bioactivity *in vivo* (18). Several methods for measuring GM-CSF autoantibodies have been developed, and an ELISA (19) has demonstrated excellent results (20).

Notwithstanding recent advances, current knowledge about PAP is based on small series and individual case reports. Although data from these studies have been synthesized into a comprehensive review (2), these data represent cases spanning nearly half a century. The incidence, prevalence, or the natural history of primary PAP have not been assessed in a large contemporaneous population. Furthermore, although diagnostic methods have evolved significantly over this period, the strong association of GM-CSF autoantibodies with primary PAP provides a potentially powerful new approach. Novel biomarkers of PAP lung disease also provide additional and potentially useful outcome measures for use in clinical trials to evaluate new therapies.

In July 1999, a national PAP registry was established to more accurately characterize the demographic, clinical, physiologic, radiologic, and serologic features of individuals with PAP in Japan. The present study was not designed to answer questions related to the natural history or responses to therapy; nor was it intended to report on secondary or congenital PAP. Rather, it is intended to provide a cross-sectional evaluation outlining the epidemiology and baseline characteristics of a large contemporaneous group of individuals with primary PAP in Japan at the time of enrollment into the registry. Some of the results of these studies have been previously reported in the form of an abstract (21) and in the proceedings of an international scientific meeting on PAP (22).

METHODS

Study Design

This study was conducted by the Japanese PAP Research Network, which includes nine primary clinical research centers and secondary clinical sites comprising a referral base encompassing the entirety of Japan, a steering committee comprised of the principal investigators at each of the nine primary clinical research centers, and a data collection and analysis center (National Hospital Organization Kinki-Chuo Chest Medical Center, Osaka, Japan). Primary clinical centers included Aichi Medical University, Chiba University, Hokkaido University, Kitazato University, National Kinki-Chuo Chest Medical Center, National Hospital Organization Sanyo Hospital, Nagasaki University, Niigata University Medical and Dental Hospital, and Tohoku University; secondary sites are listed in the APPENDIX. Some investigators in this research network (Y.I., K.N.) are also members of the Rare Lung Diseases Consortium supported by the United States National Institutes of Health. This trial was conducted in three phases: (*1*) study design and establishment of the Japanese PAP Research Network, (*2*) participant recruitment and data collection, and (*3*) data analysis. Three principle components of project were undertaken, including a cross-sectional study (reported here), a natural history study (ongoing), and an evaluation of GM-CSF inhalation therapy (to be reported elsewhere). An independent statistical evaluation of all data was done by the Data and Technology Coordinating Center of the Rare Diseases Clinical Research Network in the United States. The registry protocol was approved by the institutional review boards of each participating institution, and all participants gave informed consent. At enrollment, the treating physician for each participant completed a case report form and obtained a serum sample, both of which were sent to the data coordinating center for analysis.

Study Participants

Recruitment. Between July 1999 and July 2006, individuals diagnosed with PAP were recruited through the existing physician referral networks at primary or secondary clinical centers and pulmonary clinics and through letters to Japanese pulmonary physicians. Registration into the study was defined as the time of serum collection and completion of the case report form by the referring physician. All patient-related historical, physical examination, and other data were collected within 2 months of registration. To ensure the inclusion of individuals diagnosed with PAP independent of disease severity, patients with PAP were enrolled regardless of whether they were symptomatic. Asymptomatic individuals initially identified by a compatible chest radiograph obtained via mandatory annual health screening programs (students, employees of the government, and registered corporations) in whom a diagnosis of PAP was subsequently confirmed were included. As controls for the measurement of serum GM-CSF autoantibody levels, 24 individuals with other lung diseases (including idiopathic interstitial pneumonias [n = 10], sarcoidosis [n = 9], and collagen vascular disease [n = 5]) and 13 healthy control subjects were recruited into the study from the Kinki-Chuo Chest Medical Center in Osaka.

To evaluate recruitment efficiency, a secondary "intensive screening" was performed in the Niigata prefecture that took advantage of the close relationship among regional pulmonary physicians, 98% of whom trained at and remained affiliated with Niigata University and received weekly departmental communications. All physicians received a recruitment letter and follow-up phone calls as necessary to ensure participation.

Inclusion. All patients with a proven diagnosis of PAP were offered the opportunity to participate in the study regardless of whether they had been previously diagnosed and followed, were currently being followed, or were newly referred. Thus, prevalent and incident cases were enrolled. An acceptable PAP diagnosis was based on histopathologic findings of specimens obtained by open lung biopsy or transbronchial biopsy or on cytologic findings in BAL samples.

Exclusion. Individuals were excluded if they did not have a tissue-proven diagnosis of PAP or if serum was unavailable for analysis of GM-CSF autoantibody levels.

Composition of the study population. Two hundred forty-eight individuals with PAP were enrolled in the study; 11 cases of secondary PAP diagnosed at autopsy were identified during the study period but were excluded from the analysis because serum was unavailable. Of the participants enrolled, 223 had an elevated serum level of GM-CSF autoantibody and no underlying clinical condition known to cause PAP. Twenty-five individuals did not have an elevated serum level of GM-CSF autoantibody (*see below*) but had a condition known to cause PAP secondarily. One individual had neither elevated GM-CSF autoantibody levels nor an underlying explanation for the presence of PAP. These three groups of patients were categorized as autoimmune, secondary, and unclassified PAP, respectively. This report includes the data and analysis for individuals with autoimmune PAP. Data and results for secondary PAP will be reported elsewhere.

Diagnostic Criteria

The diagnosis of PAP was established by the presence of characteristic findings from high-resolution computed tomography (HRCT) of the chest (23) and pathologic and/or cytologic specimens obtained by video-assisted thoracoscopic lung biopsy (n = 16), transbronchial lung biopsy (n = 86), or bronchoalveolar lavage fluid (BALF)/cell cytology (n = 218) (24). The diagnosis of PAP was based on HRCT and bronchoalveolar lavage (BAL) cytology in 58.7% of patients; HRCT, BALF, and transbronchial lung biopsy (TBLB) in 34.1% of patients; and BAL cytology or TBLB and video-assisted thoracoscopic surgery (VATS) in 7.2% of patients. The radiologic features characteristic of PAP on HRCT used here included the presence of a diffuse, patchy, "geographic" pattern of ground glass opacification superimposed on interlobular septal thicken-

ing in multiple lobes (25). The diagnostic features of PAP in pathologic specimens included intraalveolar eosinophilic, periodic acid-Schiff–positive material and in cytology specimens included turbid, periodic acid-Schiff–positive, eosinophilic BALF, and intracellular surfactant inclusion bodies in alveolar macrophages (26, 27). Patients with PAP were enrolled irrespective of whether they were symptomatic at the time of registration.

Epidemiology

Because determining the incidence and prevalence of rare diseases is difficult, strict criteria were used. Only patients enrolled in full years of the study were included in calculations to avoid potential seasonal ascertainment bias. Incidence was determined for each full year of the study (2000–2006) and calculated by dividing the number of individuals newly diagnosed with PAP in a given year by the size of the Japanese population (129,180,548) taken from the governmental census report (WHLW Statistical Database, 2006, Ministry of Health, Labor and Welfare). Prevalence was calculated by dividing the total number of patients with PAP registered during all full years of the study period by the size of the Japanese population.

Pulmonary Function Methodology

Pulmonary function testing was performed by certified pulmonary function technicians. The data collected included FEV_1/FVC, FVC, VC, single-breath diffusing capacity of carbon monoxide (D_{LCO}), and arterial blood gases. Acceptability and reproducibility criteria from the American Thoracic Society recommendations for standardization were used to judge the validity of each testing session (28). Pulmonary function data are presented as the percentage of predicted values. Arterial blood measurements were performed on samples obtained with the patient breathing room air at rest in the supine position.

Disease Severity Score

Participants were assigned a PAP disease severity score (DSS) based on the presence of symptoms and degree of reduction in Pa_{O_2} (both determined at registration) determined with the individual breathing room air in the supine position as previously described (22). The categories included DSS 1 = no symptoms and $Pa_{O_2} \geq 70$ mm Hg; DSS 2 = symptomatic and $Pa_{O_2} \geq 70$ mm Hg; DSS 3 = 60 mm Hg $\leq Pa_{O_2} < 70$ mm Hg; DSS 4 = 50 mm Hg $\leq Pa_{O_2} < 60$ mm Hg; DSS 5 = $Pa_{O_2} < 50$ mm Hg. Qualifying symptoms include dyspnea or cough related to PAP. Pa_{O_2} data were available for 215 of 223 registrants with autoimmune PAP. In the eight registrants in whom Pa_{O_2} was unavailable, oxygen saturation was used to estimate the Pa_{O_2} as follows: oxygen saturation values of 94%, 90%, and 85% were used as the values representing cutoff values of Pa_{O_2} of 70, 60, and 50 mm Hg, respectively.

GM-CSF Autoantibody Measurement

Sera from each participant (patients with PAP or control subjects) were stored frozen (−20°C or −80°C), and GM-CSF autoantibody concentration was measured by ELISA essentially as previously reported (19) with minor modifications as described in the online supplement. Standard antibody was kindly provided by Dr. K. Takada, Hokkaido University.

Serum Biomarker Measurements

Serum biomarkers were evaluated in duplicate samples in blinded fashion in a central laboratory (Y.I.). Serum KL-6, SP-A, and SP-D were measured by ELISA using commercial kits (ED046; Eizai Co. Ltd., Tokyo, Japan; SP-A Test Kokusai-F, International Reagents Co. Kobe, Japan; SP-D ELISA; Yamasa Co., Tokyo, Japan; respectively) as described previously (29–32). Serum carcinoembryonic antigen (CEA) level was measured by radioimmunoassay using monoclonal antibodies (Dainabott, Tokyo, Japan) as described previously (33). Normal serum ranges were KL-6 (<500 U/ml), SP-A (<43.8 ng/ml), SP-D (<110 ng/ml), and CEA (<2.5 ng/ml) (30–34). The normal range for lactate dehydrogenase (LDH) in the clinical laboratory used was <230 IU/L.

Statistics

Numeric data were evaluated for a normal distribution using the Kolmogorov-Smirnov test and for equal variance using the Levene median test. Parametric data are presented as means (±SE), and nonparametric data are presented as medians and interquartile ranges. Categorical data are presented as a percentage of the total or numerically, as appropriate. Statistical comparisons of parametric numeric data were made with Student's t test for two group comparisons if the assumption of equality of variance was satisfied or with the Scatterwaite test if not. Nonparametric numeric data were compared with the Wilcoxon test. Comparisons of categorical data were made with chi-square or Fisher's exact test. The correlation coefficient was obtained using Spearman's correlation method for all data. All tests were two sided, and $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Demographics

Of the 248 patients with histologically and/or cytologically proven PAP enrolled in the Japanese PAP registry, 223 (89.9%) had a positive GM-CSF autoantibody test and were considered to have autoimmune PAP (Figure 1). Patients with a negative GM-CSF autoantibody test had an underlying illness known to cause PAP and were considered to have secondary PAP (9.7%) or were unclassified (0.4%). This article focuses on patients with autoimmune PAP; the features of the latter two groups will be reported elsewhere.

Two-thirds of the patients with autoimmune PAP were men (Table 1). The median age at diagnosis was 51 years, and the mean duration of symptoms at enrollment was 10 months; neither differed according to gender. Patients younger than 10 years of age were rare (Figure 2). Two-thirds of the patients were symptomatic at enrollment, and the proportion did not differ by gender. The age at the time of diagnosis was skewed toward younger individuals due to skewing of the data for men (skewness = 0.0775; $P = 0.03$) but not for women, for whom it was distributed normally (skewness = −0.422; $P = 0.068$) (Figure 2). The bimodal distribution of age at diagnosis for women with PAP, with peaks at ages 25 and 40 years, as previously reported (2), was not observed in this cohort.

Although smoking is a suspected risk factor for PAP, at registration, more than one third of the patients (43%) were never-smokers, a proportion that differed significantly according to gender (83% of women and 24% of men were never-smokers; $P < 0.001$; Table 1). Exposure to dust inhalation, another suspected risk factor for acquired PAP, was present in the

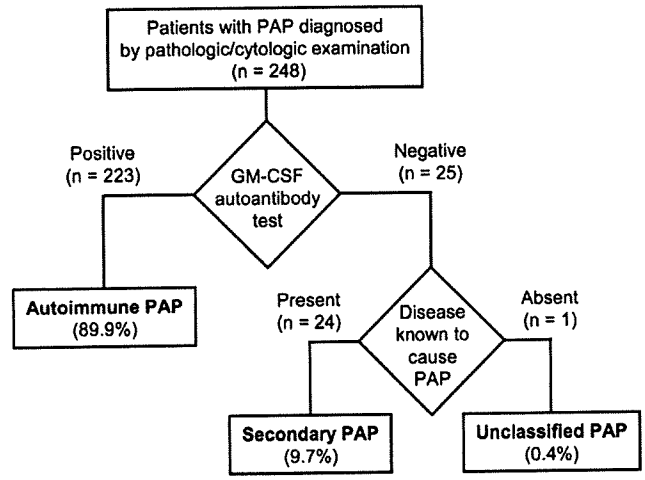

Figure 1. Disposition of the patients with pulmonary alveolar proteinosis (PAP) enrolled into the study. Participants were stratified according to the presence or absence of granulocyte/macrophage colony-stimulating factor (GM-CSF) autoantibodies and then by the presence or absence of an underlying disorder known to cause PAP.

TABLE 1. DEMOGRAPHICS AND DISEASE FEATURES OF PATIENTS WITH AUTOIMMUNE PULMONARY ALVEOLAR PROTEINOSIS AT ENROLLMENT ACCORDING TO GENDER

Characteristic	All Patients (*n* = 223) n	%	Median (IQR)* or Mean ± SD	Male (*n* = 151) n	%	Median (IQR)* or Mean ± SD	Female (*n* = 72) n	%	Median (IQR)* or Mean ± SD	*P* Value
Age, yr	223		53 (44–61)	151		52 (44–58)	72		55 (43.5–63)	0.08†
Age at diagnosis, yr	223		51 (41–58)	151		50 (15–89)	72		52 (9–85)	0.064†
Duration of symptoms CXR changes, mo	223		10 (4–36)	151		11 (4–48)	72		9 (4–24)	0.301†
Symptoms	220			147			70			0.754‡
Yes	150	68.4		103	69.6		47	65.3		
No	70	31.8		45	30.4		25	34.7		
Smoking status	217			147			70			<0.001‡
Current smoker	62	28.5		56	37		6	8.5		
Ex-smoker	62	28.5		56	37		6	8.5		
Never-smoker	93	43		35	24		58	83		
Dust exposure	199			137			62			0.001‡
Yes	52	26		44	32		8	13		
No	147	74		93	68		54	87		
Pulmonary function, mean ± SD										
FVC, % predicted	150		88.0 ± 18.9	101		90.2 ± 18.1	49		83.5 ± 19.9	0.04§
FEV_1/FVC	186		84.3 ± 11.2	128		84.7 ± 9.5	58		83.5 ± 14.4	0.57§
VC, % predicted	187		89.4 ± 19.3	129		90.4 ± 19.4	58		87.3 ± 19.1	0.31§
D_{LCO}, % predicted	154		68.6 ± 26.6	106		70.6 ± 29.0	48		64.3 ± 19.5	0.12§
Pa_{CO_2}, mm Hg	213		39.0 ± 3.91	145		39.2 ± 3.8	68		38.6 ± 4.2	0.31§
Pa_{O_2}, mm Hg	213		71.8 ± 13.5	144		71.6 ± 13.2	69		72.1 ± 14.1	0.83§
$[A\text{–}a]Do_2$, mm Hg	211		31.14 ± 15.1	144		31.0 ± 14.9	67		31.3 ± 15.6	0.90§
GM-CSF autoantibody, μg/ml	223		15.29 (7.96–26.76)	151		14.91 (7.42–27.03)	72		15.58 (9.34–26.15)	0.51†

Definition of abbreviations: $(A\text{–}a)Do_2$ = alveolar–arterial oxygen gradient; CXR = chest X-ray; D_{LCO} = carbon dioxide diffusing capacity; IQR = interquartile range; PAP = pulmonary alveolar proteinosis.

* Interquartile range is the range from the 25th to the 75th percentiles of the distribution.

† Calculated using the Wilcoxon rank sum test.

‡ Calculated using the chi-square test.

§ Calculated using Student's *t* test.

histories of only one-quarter (26%) of the patients and differed according to gender (32% of men and 13% of women had a history of exposure; n = 199; $P < 0.001$). No instances of autoimmune PAP were familial among any of the 223 individuals, consistent with the absence of a genetic predisposition.

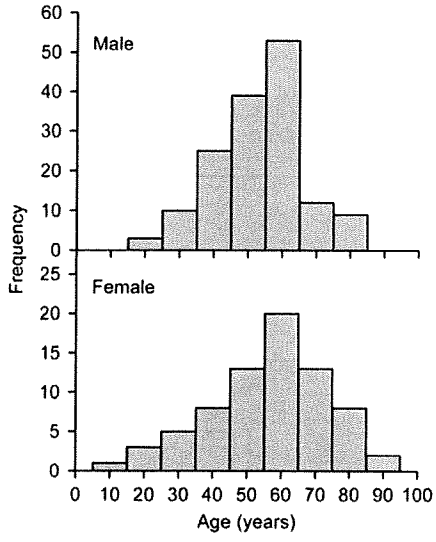

Figure 2. Histogram of the age at diagnosis of autoimmune pulmonary alveolar proteinosis in male (*top*) and female patients (*bottom*). Data are grouped into 10-year intervals. The age distribution in female subjects, but not male subjects, was normally distributed ($P = 0.03$ and 0.68, respectively; Kolmogorov-Smirnov test). The median age at diagnosis was similar in male and in female subjects (*see* Table 1).

Of the pulmonary functions evaluated in patients with autoimmune PAP, the median values for lung volumes (FVC % predicted, VC % predicted) and airflow (FEV_1/FVC) were within the normal range, and only gas transfer (D_{LCO} % predicted) was abnormal (Table 1). Arterial blood gas measurements revealed a similar degree of hypoxemia and elevation of alveolar-arterial oxygen gradient in male and female patients with autoimmune PAP (Table 1).

GM-CSF Autoantibody Levels

GM-CSF autoantibodies levels in the serum were elevated to a similar extent in male and female patients with autoimmune PAP ($P = 0.51$) and were below the level of detection in individuals with secondary PAP; individuals with other lung diseases; disease-free, healthy control subjects (Figure 3); individuals with congenital PAP (n = 5, not shown); or individuals with unclassified PAP (n = 1, not shown). GM-CSF autoantibody concentrations were skewed toward higher values (skewness = 2.03; $P < 0.001$), a pattern that was similar in men and women (Figure 4). Serum autoantibody concentrations were less than 35 μg/ml in most patients, a proportion that did not differ by gender (86% for men, 85% for women).

Levels of total serum immunoglobulins were not increased in autoimmune PAP; median (interquartile range [n]) values (in mg/dl) were IgG: 1,323 (1,091–1,468 [83]); IgA: 232 (178–284 [79]); IgM: 101 (71–149 [79]); IgE: 106 (27–219 [60]) mg/dl. In five of these patients, the level of serum IgG exceeded 2,000 mg/dl. Of these, three had intercurrent pulmonary aspergillosis, one had hepatitis C, and one had polymyalgia rheumatica.

Epidemiology

The incidence and prevalence of autoimmune PAP in Japan were evaluated using two approaches, one encompassing nine

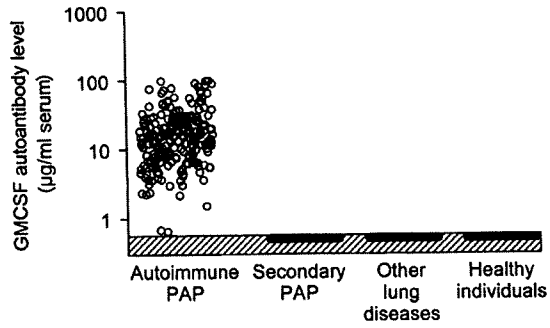

Figure 3. Concentration of granulocyte/macrophage colony-stimulating factor (GM-CSF) autoantibodies in the serum at enrollment of individuals with autoimmune pulmonary alveolar proteinosis (PAP) (n = 223), secondary PAP (n = 24), other lung diseases (n = 24), or healthy individuals (n = 14). In one individual with unclassified PAP, serum GM-CSF autoantibodies were undetectable (not shown).

nonoverlapping regions representing the entirety of Japan and a second focused to the Niigata prefecture.

In the first approach, incidence was estimated by enumerating individuals registered from all regions of Japan receiving a diagnosis of autoimmune PAP for each full year of the study from 2000 through 2005 (Table 2). Using the Japanese population size in 2005 (129,180,548) taken from the governmental census report (WHLW Statistical Database, 2006, Ministry of Health, Labor and Welfare), the mean (±SE) incidence of autoimmune PAP was 0.24 ± 0.03 cases per million population. The total number of patients with autoimmune PAP registered from each of the nine nonoverlapping regions (Hokkaido, Tohoku, Kanto, Hokushinetsu, Tokai, Kinki, Chugoku, Shikoku, and Kyushu) correlated closely with the size of the regional population (Figure 5). The mean (±SE) prevalence across all regions was 2.04 ± 0.31 cases per million. These geographic regions range in climate from temperate (Kyushu) to subarctic (Hokkaido), suggesting that climate may not influence the occurrence of autoimmune PAP.

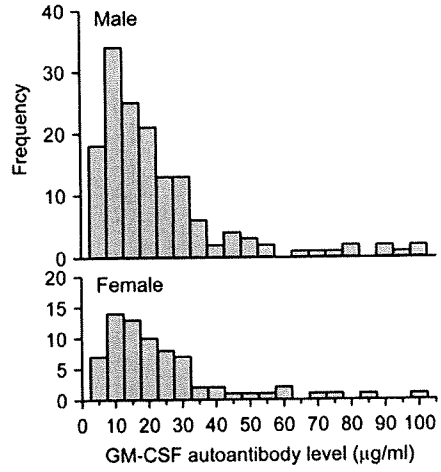

Figure 4. Histogram of serum granulocyte/macrophage colony-stimulating factor (GM-CSF) autoantibody concentrations in male (*top*) and female (*bottom*) patients with autoimmune pulmonary alveolar proteinosis. The concentrations were not normally distributed and were significantly skewed toward high values ($P < 0.001$ for both, Kolmogorov-Smirnov test). The distribution of serum GM-CSF autoantibody concentrations was similar in males and female subjects (*see* Table 1).

TABLE 2. ANNUAL INCIDENCE OF AUTOIMMUNE PULMONARY ALVEOLAR PROTEINOSIS AND ENROLLMENT DURING THE STUDY PERIOD

	Diagnosed		
Year*	Japan†	Niigata Prefecture‡	Enrolled§
1999	10	4	52
2000	30	2	15
2001	14	2	18
2002	26	1	23
2003	43	1	41
2004	43	1	34
2005	31	0	18
2006	22	4	22
Total	219	15‖	223

* Partial years of enrollment include 1999 (July 1 through December 31) and 2006 (January 1 through June 30).

† Includes patients diagnosed in all regions of Japan, the population of which was 129,180,548 as determined in the 2005 Japanese Census report.

‡ Includes patients diagnosed only in Niigata Prefecture, the population of which was 2,410,000 in 2005.

§ Enrollment in 1999 includes prevalent cases diagnosed before 1999.

‖ Includes 11 cases identified in the Japan-wide screen and four additional cases identified only in the intensive screen conducted in the Niigata prefecture.

A second approach used in the Niigata Prefecture took advantage of the especially close relationship and communication among regional pulmonary physicians, 98% of whom received their training at the Niigata University. Intensive screening in this region (population = 2.41 million) resulted in identification of 15 patients with autoimmune PAP during the study period (Table 2). This approach resulted in a mean (±SE) incidence of 0.49 ± 0.13 per year (in all full years of the study (2000–2005) and a prevalence of 6.2 cases per million of autoimmune PAP.

Clinical Characteristics

At the time of registration in the study, most patients with autoimmune PAP (69%) were symptomatic, with exertional dyspnea being most common (39%), followed by dyspnea and cough (11%) and cough only (10%) (Table 3). Fever was present in two cases, and weight loss was present in one case. Of the patients with autoimmune PAP, 31% were asymptomatic and were identified by mandatory or voluntary annual health screening programs.

Most patients with autoimmune PAP (65%) had no other intercurrent medical illnesses (Table 4). Among those that did, hypertension was most common (8.5%) and was commensurate with the frequency of hypertension in the Japanese population. Other autoimmune diseases were diagnosed in only three individuals and included polymyalgia rheumatica, hemolytic anemia, and Wegner's granulomatosis. Infections occurred in 12 individuals and included pulmonary aspergillosis in four, atypical mycobacteria in three, mycobacterial tuberculosis in two, pneumonia, hepatitis C, and tinea corporus. The proportion of individuals with intercurrent illness did not vary according to gender. COPD and asthma were underrepresented in our study population (Table 4).

Correlation of Disease Severity with Demographic, Clinical, and Biomarker Data

All patients with autoimmune PAP were stratified by disease severity score (DSS) categories at enrollment as defined in the methods from least severe (DSS-1) to most severe (DSS-5). Roughly one quarter of the patients fell into in each of DSS categories 1, 2, and 3, and one quarter were split between DSS categories 4 and 5 (Table 5).

Pulmonary gas transfer (D_{LCO}%) correlated well with DSS, showing a marked decrease at all successive DSS categories

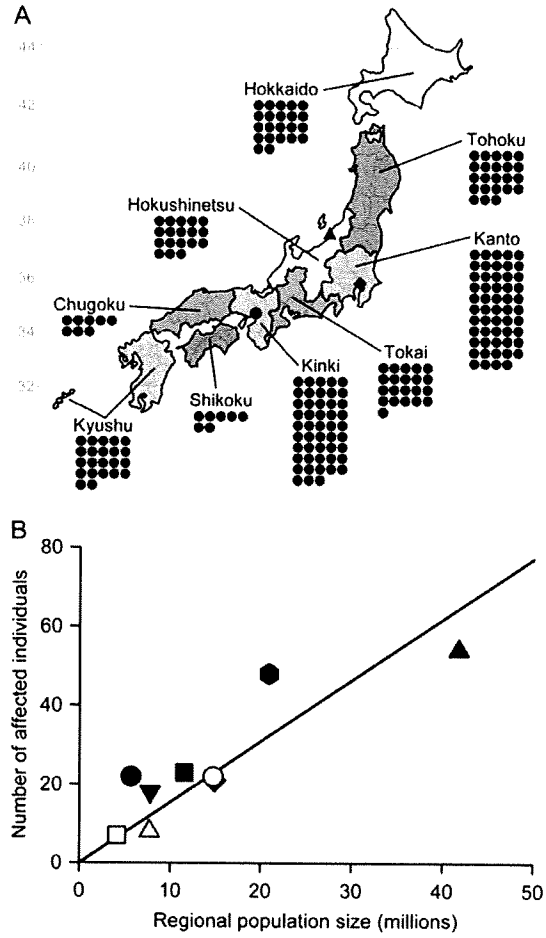

Figure 5. Distribution and regional prevalence of autoimmune pulmonary alveolar proteinosis (PAP) in Japan at the time of enrollment into the study. (*A*) Geographic locations of the patients. Shown are the nine separate regions (regional population size from the 2005 Japanese census report) representing all of Japan, including Hokkaido, (5,653,548), Tohoku (11,568,884), Kanto (41,760,805), Hokushinetsu (7,781,254), Tokai (14,894,128), Kinki (20,930,730), Chugoku (7,705,615), Shikoku (4,128,260), and Kyushu (14,757,324). Each *solid dot* represents one autoimmune PAP patient in the identified region. The Niigata prefecture, in which a second intensive screening for PAP cases was performed, is located within the Hokushinetsu region. The cities of Niigata (*triangle*), Osaka (*circle*) and Tokyo (*diamond*) and the global latitude (in degrees) for each region are indicated for orientation. (*B*) Correlation of the prevalence of autoimmune PAP cases and regional population size. Hokkaido (*solid circle*), Tohoku (*solid square*), Kanto (*solid triangle*), Hokushinetsu (*inverted solid triangle*), Tokai (*solid diamond*), Kinki (*solid hexagon*), Chugoku (*open triangle*), Shikoku (*open square*), Kyushu (*open circle*).

(Figure 6). Although PAP is often described as a lung disorder with restrictive physiology, the lung volumes (FVC% and VC%) were in the normal range in all patients except those with the most severe disease (DSS-5) (Table 5). Notwithstanding, the DSS correlated weakly with the mild reduction in lung volumes (FVC and VC). The DSS did not correlate with airflow limitation (FEV_1/FVC).

Infections were equally distributed in each of DSS categories 1 through 4, but none occurred in DSS-5. Thus, infections were infrequent in our population and did not correlate with DSS or the degree of impairment in pulmonary function (Table 5).

TABLE 3. SYMPTOMS OF THE PATIENTS WITH AUTOIMMUNE PULMONARY ALVEOLAR PROTEINOSIS*

Symptom	Number	%
Asymptomatic	70	31.4
Dyspnea only	87	39.0
Cough only	22	9.9
Sputum only	0	0
Dyspnea + cough	24	10.8
Dyspnea + sputum	3	1.3
Dyspnea + cough + sputum	5	2.2
Other†	9	4.0
Not available	3	1.3

* Complete information was available for 220 individuals.
† Dyspnea and weight loss, or fever, or cough and fever.

The DSS correlated well with selected serum biomarkers, including LDH, SP-A, SP-D, KL-6, and CEA, the strongest of which were the latter two (Table 5). No correlation was observed between DSS and serum GM-CSF autoantibody titer or with antinuclear antibody titer. The DSS was only weakly correlated with age (Table 5) and not with gender, smoking status, history of occupational dust inhalation, or intercurrent medical illness (not shown).

Disease Severity and Progression

Although the present study was cross-sectional in design, data on disease severity and progression since disease onset were collected by the primary treating physician at the time of regis-

TABLE 4. INTERCURRENT MEDICAL ILLNESSES IN INDIVIDUALS WITH AUTOIMMUNE PULMONARY ALVEOLAR PROTEINOSIS

Intercurrent Medical Illnesses	Total (*n* = 212)	Male (*n* = 143)	Female (*n* = 69)	*P* Value
None, n (%)	137 (65)	91 (63.6)	46 (66.7)	0.78
Hypertension, n (%)	18 (8.5)	11 (7.7)	7 (10.1)	0.74
Infections, n (%)*	12 (5.7)	7 (4.9)	5 (7.2)	ND
Hyperlipidemia, n (%)	9 (4.2)	6 (4.2)	3 (4.3)	ND
Diabetes mellitus, n (%)	8 (3.8)	6 (4.2)	2 (2.9)	ND
Inflammatory disorders of the gastrointestinal tract, n (%)‡‡	7 (3.3)	5 (3.5)	2 (2.9)	ND
Liver disease, n (%)§	7 (3.3)	6 (4.2)	1 (1.5)	ND
Asthma, n (%)	5 (2.4)	1 (0.1)	4 (5.8)	ND
Airflow limitation, n (%)‖	5 (2.4)	2 (1.4)	3 (2.1)	ND
Allergic rhinitis, n (%)	3 (1.4)	3 (2.1)	0 (0)	ND
Cancer, n (%)¶	4 (1.9)	4 (2.8)	0 (0)	ND
Autoimmune disorders, n (%)**	3 (1.4)	1 (0.1)	2 (2.9)	ND
Interstitial lung disease, n (%)††	3 (1.4)	3 (2.1)	0 (0)	ND
Stroke, n (%)	3 (1.4)	3 (2.1)	0 (0)	ND
Schizophrenia, n (%)	3 (1.4)	2 (1.4)	1 (1.5)	ND
Other disorders‡‡	(<1)	(<1)	(<1)	ND

Definition of abbreviation: ND = not determined.
* Diseases include pulmonary aspergillosis (n = 4), atypical mycobacteria (n = 3), tuberculosis (n = 2), pneumonia (n = 1), hepatitis C (n = 1), and tinea corporus (n = 1).
† Values shown are calculated using the chi-square test. For some diseases, the number of expected values was too low to be evaluated statistically.
‡ Diseases include oral ulceration (n = 1), esophagitis (n = 2), gastritis (n = 1), gastric ulcer (n = 1), duodenal ulcer (n = 1), and ulcerative colitis (n = 1).
§ Diseases include cirrhosis (n = 3), fatty liver (n = 3), and liver dysfunction (n = 1).
‖ Defined as individuals with FEV_1/FVC < 0.7.
¶ Diseases include cancer of the lung (n = 1), colon (n = 1), prostate (n = 1), and thyroid (n = 1).
** Diseases include polymyalgia rheumatica (n = 1), hemolytic anemia (n = 1), and Wegner's granulomatosis (n = 1).
†† Diseases include pulmonary fibrosis (n = 2) and interstitial pneumonitis (n = 1).
‡‡ Diseases include heart failure (n = 2), Alzheimer's (n = 1), obstructive sleep apnea (n = 1), monoclonal hypergammaglobulinemia (n = 1), hypothyroidism (n = 1), angina (n = 1), membranous nephropathy (n = 1), and Parkinson's disease (1).

TABLE 5. CORRELATION OF DISEASE SEVERITY SCORE WITH THE AGE AT DIAGNOSIS, SYMPTOMS, INFECTIONS, PULMONARY FUNCTION, AND SERUM BIOMARKERS IN PATIENTS WITH AUTOIMMUNE PULMONARY ALVEOLAR PROTEINOSIS

	Disease Severity Score*						
Characteristic	1 (*n* = 58)	2 (*n* = 60)	3 (*n* = 55)	4 (*n* = 37)	5 (*n* = 12)	*R*†	*P* Value‡
Age at diagnosis, yr	48 (34–55)	46 (36–56)	55 (45–59)	53 (46–59)	58 (53–65)	0.25	<0.001
Symptomatic, %	0	100.00	78.18	94.59	100.00	0.64	<0.001
Infection, n	3	3	3	3	0	0.01	0.887
Aspergillus spp., n	2	0	1	1	0		
Mycobacterium spp., n	1	1	1	2	0		
Other§	0	2	1	0	0		
Pulmonary function							
FVC, % predicted	97.1 ± 17.3	92.3 ± 17.2	81.9 ± 21.4	82.0 ± 13.1	76.4 ± 19.0	−0.36	<0.001
FEV_1/FVC	82.4 ± 7.9	84.8 ± 10.9	86.0 ± 8.3	85.7 ± 10.7	79.3 ± 27.3	0.16	0.034
VC, % predicted	98.8 ± 16.3	91.3 ± 20.6	87.4 ± 19.2	81.5 ± 14.4	76.1 ± 23.7	−0.35	<0.001
D_{LCO}, % predicted	86.8 ± 23.2	72.4 ± 24.5	64.9 ± 24.1	50.4 ± 18.0	43.1 ± 20.4	−0.56	<0.001
Biomarkers							
GM-CSF autoantibody, μg/ml	14.8 (7.78–25.62)	16.3 (7.49–27.03)	12.75 (6.46–27.38)	16.34 (11.82–25.77)	13.21 (7.70–25.88)	0.004	0.947
LDH, IU/ml	233 (199–365)	316 (247–454)	324 (253–445)	361 (266–474)	406 (247–538)	0.29	<0.001
CEA, ng/ml	1.6 (1.0–2.3)	3.0 (1.8–5.7)	4.7 (2.65–6.85)	5.6 (3.9–12)	17.1 (6–23)	0.53	<0.001
SP-A, ng/ml	48.4 (31.0–74.3)	80.1 (51.3–131)	106 (63.3–143)	133 (78.8–237)	162 (85.4–302)	0.45	<0.001
SP-D, ng/ml	126 (75.9–170)	193 (103–298)	211 (121–324)	268 (183–426)	233 (159–253)	0.37	<0.001
KL-6, U/ml	1,120 (739–2,480)	2,676 (1,585–8,200)	4,030 (2,000–8,330)	7,410 (4,290–12,600)	15,100 (2,150–24,400)	0.52	<0.001
ANA, 1/dilution	0 (0–20)	0 (0–20)	0 (0–40)	0 (0–20)	0 (0–40)	0.03	0.73

Definition of abbreviations: ANA = antinuclear antibody; CEA = carcinoembryonic antigen; GM-CSF = granulocyte/macrophage colony-stimulating factor; LDH = lactate dehydrogenase; SP-A = surfactant protein A; SP-D = surfactant protein D.

* Data are for all participants evaluated at enrollment and are presented as median (interquartile range) or mean ± SE or as percent affected or number of cases where indicated.

† Spearman correlation coefficient.

‡ Values calculated using the Spearman correlation test.

§ Includes hepatitis C (disease severity score [DSS]-3), unspecified pneumonia (DSS-2), and ringworm (DSS-2).

tration. To determine if the severity of autoimmune PAP at registration correlated with its duration, patients were stratified into three groups in which the time between onset and registration was as follows: (*1*) ≤1 year, (*2*) >1 year and ≤10 years, or (*3*) >10 years. No differences were observed among these three groups with respect to (*1*) the proportion of symptomatic individuals, (*2*) the proportion of individuals falling into each DSS category, (*3*) pulmonary function (FVC % predicted, VC % predicted, FEV_1/FVC, D_{LCO} % predicted) (Table 6), or (*4*) most serum biomarkers (GM-CSF autoantibody level, CEA, SP-A, KL-6, and antinuclear antibody) (not shown). A weak correlation of LDH and SP-D with the duration of disease in patients with autoimmune PAP was observed (not shown).

In two-thirds of the recently diagnosed individuals (group 1), symptoms were unchanged from the time of onset to registration (Table 6). In those with disease of intermediate duration (group 2), 42.5% had improved, 29.8% had worsened, and 27.7% were unchanged. In those with prolonged disease (group 3), nearly two-thirds had worsened, and one-quarter were unchanged. This result reflects the various and in some cases multiple treatment approaches used in these individuals.

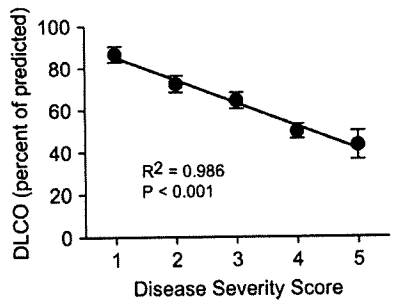

Figure 6. Correlation of the disease severity score with carbon monoxide diffusing capacity (D_{LCO}) in patients with autoimmune pulmonary alveolar proteinosis. Results are shown as mean (±SE) for individuals classified as disease severity score (DSS)-1 (n = 42), DSS-2 (n = 38), DSS-3 (n = 27), DSS-4 (n = 28), or DSS-5 (n = 12).

Our study design also permitted enrollment of asymptomatic individuals with autoimmune PAP. Among those asymptomatic at registration, 60% of those recently diagnosed, 74.1% with an intermediate duration of disease, and 33.3% with prolonged disease were unchanged since onset (Table 6). Despite the absence of whole-lung lavage therapy for PAP, 11 of 39 (28.2%) had subclinical disease or had undergone spontaneous improvement and were asymptomatic at the time of registration.

Correlations with Serum GM-CSF Autoantibody Concentration

To determine if the severity of the pulmonary abnormalities was correlated with the level of GM-CSF autoantibody, patients with autoimmune PAP were grouped into quartiles according to their serum GM-CSF autoantibody level (Q1–Q4 = <7.9, 8–15.2, 15.3–26.8, and >26.8 μg/ml, respectively). GM-CSF autoantibody levels in serum did not correlate with duration of disease, DSS, pulmonary function (FVC%, VC%, FEV_1%, D_{LCO}%), or serum biomarkers (LDH, SP-A, SP-D, CEA, KL-6) (*see* Table E1 in the online supplement). Even in individuals with GM-CSF autoantibody levels above 35 μg/ml (the top 13% of individuals), no correlations were observed in any of these parameters (not shown). The serum GM-CSF autoantibody also did not correlate with age, gender, smoking status, a history of environmental or occupational dust inhalation exposure, or duration of disease (not shown).

DISCUSSION

In this study, we report on the epidemiologic, demographic, clinical, pulmonary function, and serum biomarker data from

TABLE 6. CORRELATION OF DURATION OF DISEASE WITH CLINICAL PROGRESSION AND PULMONARY FUNCTIONS IN PATIENTS WITH AUTOIMMUNE PULMONARY ALVEOLAR PROTEINOSIS

Characteristic	Duration of Disease* ≤1 yr (*n* = 124)	>1 yr, ≤10 yr (*n* = 89)	>10 yr (*n* = 10)	*P* Value†
Disease severity score				0.11
1, % of patients	29.84	20.22	30.00	
2, % of patients	28.23	25.84	20.00	
3, % of patients	22.58	26.97	40.00	
4, % of patients	14.52	20.22	10.00	
5, % of patients	4.84	6.74	0.00	
Pulmonary function				
FVC, % predicted	89.7 ± 18.4	86.6 ± 18.9	80.0 ± 26.2	0.10
FEV_1/FVC	84.5 ± 12.0	84.6 ± 10.6	80.4 ± 8.4	0.12
VC, % predicted	91.0 ± 19.0	87.6 ± 19.4	86.5 ± 23.5	0.08
D_{LCO}, % predicted	71.5 ± 27.7	64.3 ± 24.9	72.1 ± 26.0	0.26
Symptomatic, %	65.0	72.4	70.0	0.28
Disease progression‡				
Symptomatic individuals	(n = 50)	(n = 47)	(n = 8)	0.002
Improved, n	11	20	1	
Worsened, n	6	14	5	
No change, n	33	13	2	
Asymptomatic individuals§	(n = 15)	(n = 21)	(n = 3)	0.99
Improved, n	5	4	2	
Worsened, n	1	2	0	
No change, n	9	15	1	

Definition of abbreviation: D_{LCO} = carbon dioxide diffusing capacity.

* Data are for all participants at enrollment and are presented as percent, median (interquartile range), or mean ± SE.

† Values shown are calculated using the Spearman correlation test.

‡ Treating physician's clinical impression of the severity of pulmonary disease progression from the time of onset of symptoms or an abnormal chest radiograph to the time of enrollment in the study.

§ None of the individuals who were asymptomatic at registration had previously received whole-lung lavage or granulocyte/macrophage colony-stimulating factor aerosol therapy.

the largest contemporaneous cohort of patients with autoimmune PAP assembled to date.

Several lines of evidence support the use of the term "autoimmune PAP" and the stratification of PAP into autoimmune and secondary forms. First, underscoring the importance of GM-CSF in pulmonary surfactant homeostasis, mice deficient in GM-CSF (6, 7) or its receptor (35) develop a pulmonary phenotype biochemically, histologically, physiologically, and ultrastructurally identical to autoimmune PAP in humans (36). Second, GM-CSF autoantibodies seem to be critical to the pathogenesis of autoimmune PAP because high levels are strongly associated with it but are not present in secondary or congenital PAP, other lung diseases, or in healthy individuals (17). Their binding affinity for GM-CSF (~20 pmol/L) is higher than the GM-CSF receptor in its low- (~3,200 pmol/L) or high-affinity (120 pmol/L) binding state (37), and they eliminate GM-CSF bioactivity *in vivo* (18). Third, transfer of purified GM-CSF autoantibodies from patients with PAP into blood from healthy individuals reproduces the myeloid cell abnormalities observed in patients with autoimmune PAP (38). Fourth, anti-murine GM-CSF antibodies reproduce these abnormalities in wild-type mice. Fifth, the courses of autoimmune and secondary PAP are different: Secondary PAP has a far worse outcome (H. Ishii, in preparation).

Important findings of this study are the incidence and prevalence data for autoimmune PAP in Japan. Using an intensive screening approach involving 98% of pulmonary physicians in one region, the highest incidence and prevalence estimates were 0.49 and 6.2 cases per million, respectively. Our prevalence value is higher than reported for Israel (3.7 per million) (39) and lower than for the United States (~10–40 cases per million) (40). One of 15 cases in the former study was congenital PAP, and the latter study included all three clinical forms. If we included all types of PAP identified in Japan during the study period, the prevalence would be 8.7 per million. We did not observe familial clustering in our study, in contrast to the Israeli report in which PAP occurred in two siblings. These likely represent congenital PAP, which occurs secondary to mutations in the genes encoding SP-B (41), surfactant protein C (42), or ABCA3 transporter protein (43). Of the patients with autoimmune PAP in our study, 31% were asymptomatic and were identified by annual medical screening programs. Our observations suggest that the true incidence and prevalence of autoimmune PAP is higher than reported and show that nearly a third of cases are subclinical. Although regional differences in may exist, more studies are needed.

The demographics of Japanese patients with autoimmune PAP differ in several respects from a retrospective meta-analysis done by Seymour and colleagues, which includes most or all cases of PAP reported in the medical literature as of 2002 (2). First, the median age at diagnosis was 51 years and similar in men and women, in contrast to Seymour and colleagues' report in which the median age was 39 years and was different in men and women (39 and 35 years respectively). Second, the age at diagnosis was normally distributed in women and did not have the bimodal pattern previously reported (2). Third, the male:female ratio (2.10:1.0) and, fourth, the proportion of current smokers (28.5%) were lower than previously reported (2.65:1.0 and 72%, respectively) (2). The absence of a male predominance among nonsmokers (never- and ex-smokers) in our study (male:female ratio = 0.60:1.0) is similar to the prior report (0.69:1.0) (2) and is consistent with the possibility that the high proportion of men among patients with PAP may be explained by their higher frequency of tobacco use. However, a high proportion of women in our cohort had no history of smoking (83%) or occupational exposure (87%), suggesting that another factor may be involved in the etiology of autoimmune PAP. Our study did not address potential effects of passive smoke exposure.

It is surprising that COPD was not recognized more commonly in our cohort given that the proportion of current, ex-, and never-smokers was similar to the Japanese population in whom COPD occurs in 8.6% (44, 45). Using the same criterion ($FEV_1/FVC < 0.70$) as Fukuchi and colleagues (44), only five individuals (2.7%) in our cohort had airflow limitation, whereas 24 individuals were expected of having airflow limitation. Of these, two were male (one ex-smoker, aged 55 yr; one never-smoker, aged 71 yr), and three were female (one current smoker, aged 39 yr; one ex-smoker, aged 28 yr; and one never-smoker, aged 44 yr). Although the reason for this is not clear, it is interesting that asthma, another common lung disorder with an inflammatory component of pathogenesis, was also underrepresented in our cohort (observed frequency = 2.4%; expected frequency ~8.2%). PAP may alter the phenotype of disorders with an inflammatory component of the pathogenesis. This is supported by observations that GM-CSF is required for myeloid cell functions in humans (38) and mice (9), where it regulates a number of innate immune responses, including the TLR4 response to lipopolysaccharide (46), and GM-CSF autoantibodies in patients with PAP virtually eliminate GM-CSF bioactivity (18). Thus, GM-CSF autoantibodies may blunt inflammatory responses in patients with PAP, which may affect tissue destruction in COPD and the tendency for exacerbations in asthma.

The DSS (22) provided a useful measure of lung disease severity in symptomatic and asymptomatic autoimmune patients with PAP, which was important because nearly one-third of the patients were asymptomatic and because dyspnea is difficult to quantify in PAP due to the insidious onset. A limitation of this