2009). The IC_{50} of the PCRS was previously shown to be 10.3 molecules of GMAb per molecule of GM-CSF (Sakagami et al., 2010), similar to values reported for GMAb from a single patient (10.6), GMAb from serum pooled from 11 patients (10.3) (Uchida et al., 2007), or the mean value for GMAb from 11 patients determined individually (7.05 ± 3.81) (Uchida et al., 2004). In the GMAb ELISA, optical absorbance increased as a smooth, slightly curved function of PCRS concentration over a range from 0.781 to 50 ng/ml (Fig. 3B). Quadratic regression analysis of optical absorbance versus PCRS concentration yielded an outstanding correlation coefficient and gave a lower percent deviation for repeated measurements over the entire PCRS concentration range than did either linear or log regression analysis (Fig. 3C).

Evaluation of the initial serum aliquot volume used to prepare the standard serum dilutions for measurement in the GMAb ELISA revealed an important source of assay variability. The mean serum GMAb concentration was slightly higher with 10 μl initial serum aliquots than with 100 μl aliquots but did not reach statistical significance (Fig. 4A). However, the percent deviation was significantly greater for smaller aliquots (Fig. 4B). The total amount of serum transferred to the first dilution tube includes that intended for measurement (i.e., serum inside the pipet tip) plus unintentionally included serum (i.e., serum adherent to the outside and end of the pipet tip), these findings suggest that unintentionally included serum comprised a greater fraction of the total amount transferred when the initial serum aliquot was smaller. The net effect was a tendency to overestimate GMAb concentration and markedly increased variability of repeated measurements.

Since dilution is required to reduce the optical absorbance of PAP patient serum samples on the ELISA plate into a readable range and experience has shown that the serum GMAb concentration determined can vary with the dilution used to make the measurement, we evaluated the effect of the serum dilution used for determining GMAb concentration on variability of the results for both PAP patients and healthy people. As expected, the percent deviation of GMAb concentration was higher for diluted samples with optical absorbance values falling outside the range of reference standard values ('out-of-range') (Fig. 5A) at all dilutions (Fig. 5B) in both PAP patients and healthy controls. In contrast, the percent deviation was lowest and similar at all dilutions for PAP patient serum when the absorbance was 'in-range' (Fig. 5B). Most PAP patients had an in-range absorbance value at a dilution of 1/3000or 1/6000, with slightly fewer at

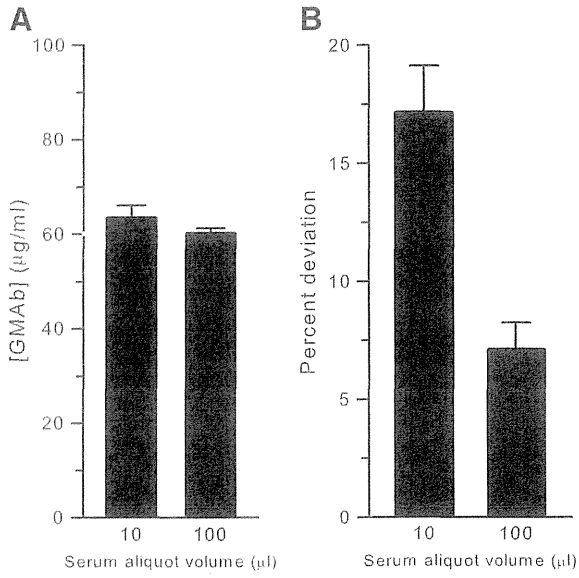
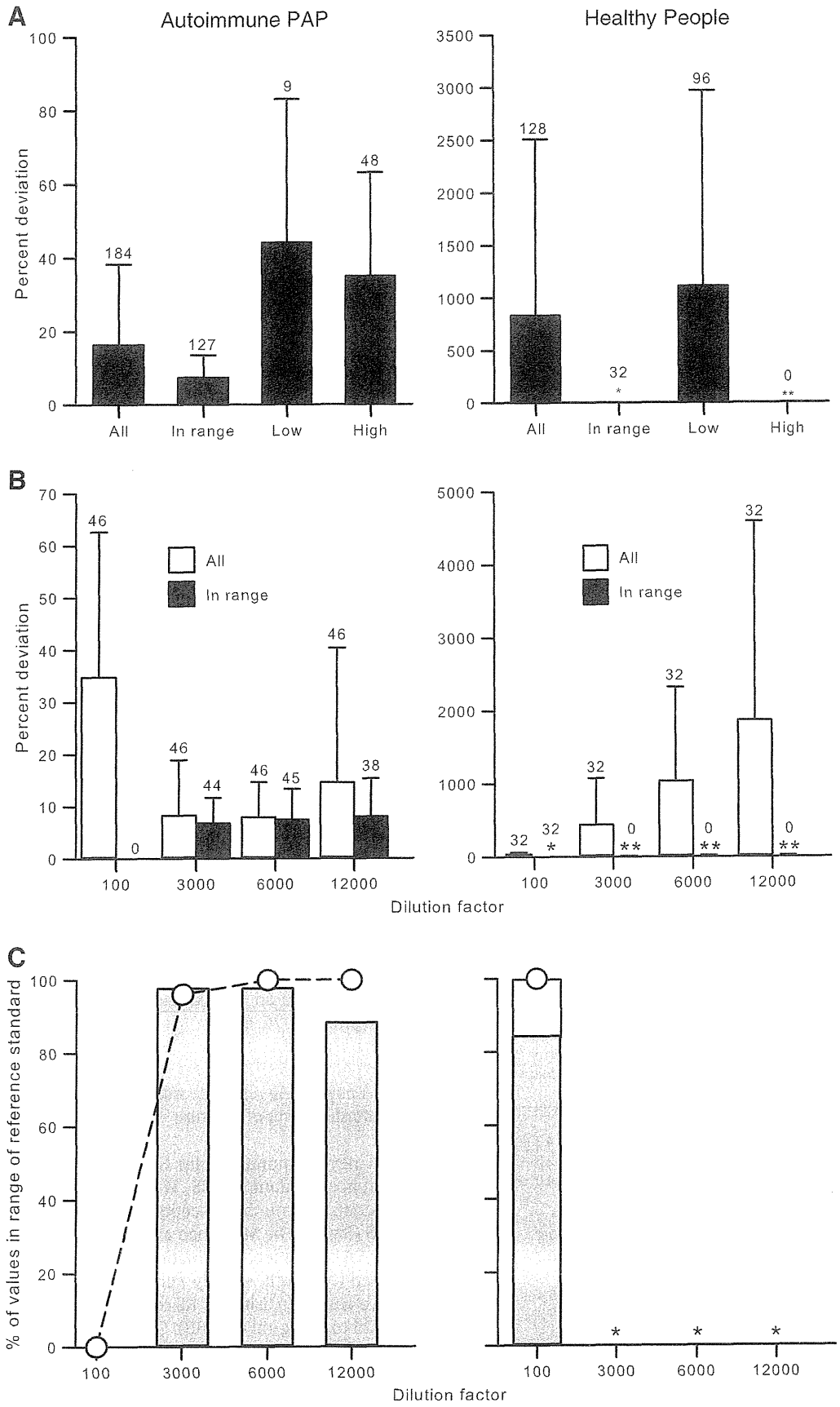


Fig. 4. Effect of serum aliquot volume on GMAb ELISA performance. A. Effect on serum GMAb concentration. An initial volume of serum either 10 or 100 μl was used to measure serum GMAb concentration with the GMAb ELISA. Bars represent the mean (±SD) of 20 independent determinations using the same autoimmune patient serum. The gray line represents the mean for the data obtained using the 100 μl aliquots of serum. The difference in median (IQR) GMAb concentration for the assay performed using 10 vs 100 μl initial serum aliquots did not reach statistical significance (64.9 [52.6–73.4] vs 59.3 [56.0–64.8] μg/mL; P = 0.525, n = 20 samples; Mann–Whitney Rank Sum Test). B. Effect on percent deviation of the serum GMAb concentration. Data from the experiment shown in panel A were used to calculate the percent deviation of the 20 determinations. The mean (±SD) percent deviation for the assay performed when using 10 μl initial serum aliquots was markedly higher than with 100 μl aliquots (17.1 ± 8.9 vs 7.1 ± 5.2; P < 0.001; n = 20 samples; Student's *t* test).

1/12,000 and none at 1/100 (Fig. 5B–C). In healthy people, all dilutions greater than 1/100 had an out-of-range absorbance with the percent deviation increasing in proportion to the dilution (Fig. 5B) and all had an in-range or undetectable value at a dilution of 1/100 (Fig. 5C). These results indicate that any dilution can be used to determine serum GMAb concentration as long as the optical absorbance of the diluted sample is within the range of the reference standard values, or below the level of detection as in some healthy people.

The specificity of GMAb detection by the GMAb ELISA was evaluated using PAP patient serum before and after GMAb

Fig. 5. Effect of the choice of serum dilution on determination of GMAb concentration. A. Effect of the sample absorbance to that of the reference standard range. Separate series of standard dilutions (1/100, 1/3000, 1/6000, 1/12,000) were prepared from serum from 46 autoimmune PAP patients and 32 healthy people and GMAb concentration were measured for each dilution (184 for autoimmune PAP and 128 for healthy controls) using the GMAb ELISA. Individual measurements were defined as 'in-range' if the optical absorbance for the dilution was within the range of the optical absorbance values for the reference standards, 'low' (and out of range) if the optical absorbance was less than that of the lowest reference standard, and 'high' (and out of range) if the optical absorbance was greater than that of the highest reference standard. Only 'in-range' values were used to calculate the mean GMAb concentration. In healthy people, the percent deviation for 'in range' values was small (*) and no measurements were greater than the highest reference standard (**). Bars represent the mean (±SD) and the number of determinations is shown above the error bars. B. Effect of serum dilution factor on percent deviation. Data are derived from the experiments shown in panel A but shown here as function of the specimen dilution. In healthy people, the percent deviation for 'in-range' values was small at the 1/101 dilution (*) and no determinations were in-range at higher dilutions (**). Bars represent the mean (±SD) and the number of determinations is shown above the error bars. C. Effect of serum dilution on the frequency of obtaining 'in range' measurements. Data are derived from the experiments shown in panels A and B. The percentage of determinations that are 'in-range' for each of the standard dilutions (1/100, 1/3000, 1/6000, and 1/12,000) is shown (bars). Gray bar represent measurements that were 'in-range' and the clear bar represents measurements in which GMAbs were undetectable. The cumulative percent of determinations that are 'in-range' at each successively greater dilution is shown (open symbols). In healthy people, GMAb values below the LLOQ were accepted as normal and counted as being in-range.


A
Autoimmune PAP
Healthy People
Percent deviation
All
In range
Low
High
184
127
9
48
128
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100
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% of values in range of reference standard
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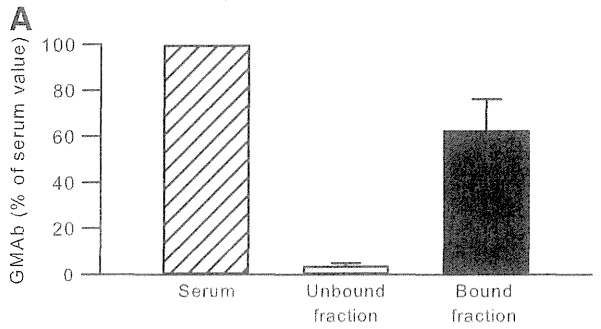


Fig. 6. Specificity of the GMAb ELISA. Serum from autoimmune PAP patients ($n = 3$) was subjected to GM-CSF affinity chromatography and the unbound fraction (flow-through) and bound fraction (after elution from the column) from each was collected as previously described (Uchida et al., 2004). The GMAb concentration was measured in serum aliquots taken before chromatography and the unbound and bound fractions. All data was normalized to the serum GMAb concentration for each patient, which was set to 100%. Bars represent the mean (±SD) amount of GMAb present in serum (hatched bar), or recovered in the Unbound (open bar) and Bound (solid bar) fractions.

depletion by GM-CSF affinity chromatography performed as previously described (Uchida et al., 2004). GMAb was readily detected in serum before depletion, essentially undetectable in the unbound fraction (i.e., column flow-through), and approximately 60% of the serum value was detected in the bound fraction (i.e., bound to the column) recovered after elution with acidic buffer and neutralization (Fig. 6). These results indicate that the GMAb ELISA is highly specific for detection of GMAb in serum.

3.2. GMAb ELISA performance

The accuracy of the GMAb ELISA was evaluated using the PCRS with 'spiked' samples containing known concentrations of exogenously added, purified GMAbs. The standard error for measurements of GMAb concentration in serial dilutions of the PCRS ranging from 50 ng/ml to 1.563 ng/ml (plate concentration) was less than 15% (Table 1) in accordance with FDA guidance regarding diagnostic assay performance (Anonymous, 2001). The standard error of the measurement at a GMAb PCRS concentration of 0.78125 ng/ml in the well was 16.7 ± 12.1% (Table 1), which is less than the limit of 20% specified by FDA guidance to define the LLOQ for the GMAb ELISA. The standard error of measurement of GMAb concentration in 'spiked' human serum samples containing 10, 20, 40 ng/ml GMAb revealed a standard error of less than 7% (Table 1) in accordance with FDA guidance criteria for assay accuracy (Anonymous, 2001).

Table 1
Concentration response of the calibration curve and accuracy of measuring GMAb concentration in human serum using the GMAb ELISA.

Expected concentration, ng/ml	Measured concentration, ng/ml (Mean ± SD)	Coefficient of variation (100 × SD / Mean)	Standard error (%) (Mean ± SD)
Calibration curve[a]			
50	51.9 ± 2.3	4.39	4.3 ± 3.9
25	24.4 ± 0.7	2.79	2.5 ± 2.7
12.5	12.2 ± 0.4	5.07	4.0 ± 3.5
6.25	5.8 ± 0.4	6.09	7.7 ± 5.2
3.125	3.1 ± 0.2	6.35	5.3 ± 2.5
1.5625	1.3 ± 0.1	10.58	14.8 ± 7.0
0.78125	0.8 ± 0.2	19.41	16.7 ± 12.1
Spiked samples[b]			
10	10.1 ± 0.6	6.32	5.4 ± 2.9
20	19.0 ± 1.1	5.89	6.2 ± 4.0
40	41.8 ± 1.7	4.18	4.5 ± 4.2

[a] Prepared using the autoimmune PAP-patient derived PCRS by serial dilution in sample dilution buffer.
[b] Spiked samples were dilution buffers spiked with known concentration of purified GMAb.

The precision of GMAb ELISA measurements with respect to well-to-well variability, plate-to-plate variability, day-to-day variability was evaluated using the three samples from PAP patients. The coefficient of variation for measurement of GMAb concentration in patient sera was less than 15% in accordance with FDA guidance criteria for assay precision (Anonymous, 2001). The coefficient of variation for measurement of GMAb concentration in spiked samples was also less than 15% (not shown). The GMAb concentrations in PAP patient serum measured repeatedly using the GMAb ELISA by two different operators were similar (Table 2).

Repeated cycles of freezing and thawing of serum had no effect on measurement of GMAb concentration at low or high GMAb concentrations (Table 3). Storage of serum samples at room temperature for 6 h or at −80 °C for three months had no detectable effect on measurement of GMAb concentration (Table 3). Storage of the PCRS at room temperature for 6 h had no detectable effect on measurement of GMAb concentration (Table 3).

The optimized GMAb ELISA was used to measure serum GMAb concentration in people previously diagnosed with autoimmune PAP and in healthy, asymptomatic people. The serum GMAb concentration in patients with autoimmune PAP (94.13 [34.64–158.80] μg/ml; n = 44) was markedly higher than in healthy people (0.28 [0.20–0.51] μg/ml; n = 38) (Fig. 7A). Serum GMAb concentration was skewed towards higher concentrations in PAP patients but there was a clear separation between the two groups (Fig. 7B). ROC curve analysis in this limited group of patients and controls showed a good sensitivity and specificity for a diagnosis of autoimmune PAP and an optimal cut off value for serum GMAb of 5.0 μg/ml (Fig. 7C, D).

3.3. A new GMAb reference standard as the basis for a standardized unit of measure

A new immunoglobulin G_1 subclass, monoclonal GMAb reference standard (MCRS) was prepared as described in the Materials and methods section and characterized as for the PCRS (Fig. 3). The MCRS had a molecular mass similar to IgG1 (Fig. 8A). Functional evaluation in the GMAb ELISA demonstrated a smooth, slightly curved increase in optical absorbance with increasing MCRS concentration over a range from 0.3125 to 20 ng/ml (Fig. 8B). Quadratic regression analysis of the optical absorbance versus MCRS concentration yielded an outstanding correlation coefficient and gave a lower percent error over the entire MCRS concentration range than did linear or logarithmic regression analysis (Fig. 8C).

Table 2
Precision of measuring GMAb concentration in human serum.[a]

	Coefficient of variation[a]			Operator to operator[b] (Mean ± SD) (mcg/ml)		
Serum samples[c]	Well-to-well	Plate-to-plate	Day-to-day	Operator 1	Operator 2	P value
PAP patient 1	10.3	11.7	11.6	100.4 ± 4.5	97.8 ± 7.0	0.27
PAP patient 2	9.1	8.3	5.4	142.1 ± 12.9	138.4 ± 11.1	0.61
PAP patient 3	4.5	10.0	7.2	24.0 ± 2.5	25.5 ± 2.1	0.47

[a] Coefficient of variation was calculated as defined in the methods. Standard deviation × 100 / mean.
[b] GMAb concentration of three serum samples were evaluated by two different operators. Data are expressed as mean.
[c] GMAb concentration of three serum samples were evaluated to validate precision of the assay.

To determine the relationship between GMAb concentration determined using the PCRS ($[GMAb]_{PCRS}$) with that determined using the MCRS ($[GMAb]_{MCRS}$), standard dilutions of sera from PAP patients and healthy people were measured with both reference standards included on the same plate. $[GMAb]_{MCRS}$ values were ~5-fold less than corresponding $[GMAb]_{PCRS}$ values; the ratio varied slightly with concentration resulting in a nonlinear relationship (Fig. 9A). Regression analysis using linear, quadratic, and cubic fit equations was done to define the relationship between $[GMAb]_{PCRS}$ and $[GMAb]_{MCRS}$. Cubic regression gave the best fit (Fig. 9A). To confirm this, $[GMAb]_{PCRS}$ values were converted to values equivalent to $[GMAb]_{MCRS}$ using each set of regression parameters just described and the percent error was determined for each measurement. Conversion using cubic equation parameters gave the lowest for percent error, which was negligible at all GMAb concentrations >0.2 ng/ml in diluted serum samples (Fig. 9B). Thus, the relationship between $[GMAb]_{PCRS}$ and $[GMAb]_{MCRS}$ was best described as cubic polynomial. To establish the parameters needed to routinely convert $[GMAb]_{PCRS}$ values into values equivalent of $[GMAb]_{MCRS}$, six independent experiments similar to and including the one shown above (Panel A) were evaluated simultaneously, which yielded an excellent correlation coefficient (Fig. 9C). Thus, the relationship between the GMAb concentration determined with these two reference standards is given by the following equation:

$$F(x) = 0.00002 \cdot x^3 - 0.0028 \cdot x^2 + 0.3026 \cdot x - 0.0575;$$

where x is the GMAb concentration in diluted serum in units of ng/ml determined with the PCRS (i.e., $[GMAb]_{PCRS}$) and F(x) is the GMAb concentration in ng/ml determined with the MCRS (i.e., $[GMAb]_{MCRS}$). To standardize reporting, one international unit (IU) of GMAb is hereby defined as a GMAb concentration equivalent to 1 μg/ml measured using the GMAb ELISA as described in the Materials and methods section with the MCRS as the reference standard.

To validate the mathematical approach for conversion of $[GMAb]_{PCRS}$ data to international units equivalent to values determined with the MCRS, serum GMAb was measured in 12 additional, separate autoimmune PAP patients using the PCRS (described above) and simultaneously using the MCRS. A plot of $[GMAb]_{PCRS}$ after conversion to IU against $[GMAb]_{MCRS}$ revealed a linear relationship with an outstanding correlation (Fig. 10A). Bland and Altman analysis confirmed this agreement for the two values with a difference of less than 5 IU at all concentrations below 60 IU (Fig. 10B).

To determine the range of normal and abnormal values for GMAb concentration in international units, serum GMAb in 56 autoimmune PAP patients and 38 healthy individuals were measured using the GMAb ELISA with the PCRS and converted to international units using the equation parameters identified (Fig. 11).

Table 3
Stability of samples and reagents used in GM-CSF autoantibody assay.

Condition/sample[a]	Concentration (Mean ± SD) μg/ml		P value[b]
	Before	After	
Freeze–thaw (×3) [c]			
Low	9.398 ± 0.9	10.46 ± 0.67	0.17
High	37.20 ± 2.44	37.66 ± 1.61	0.77
Short term sample storage[d]			
Low	9.398 ± 0.9	9.82 ± 0.69	0.56
High	37.20 ± 2.44	38.64 ± 2.18	0.49
Long term sample storage[e]			
Low	9.398 ± 0.9	9.75 ± 0.05	0.54
High	37.20 ± 2.44	38.19 ± 1.10	0.55
Short term reagent storage[f]			
Diluted PCRS, low concentration	8.45 ± 0.56	7.82 ± 0.75	0.31
Diluted PCRS, high concentration	27.85 ± 1.21	27.79 ± 1.09	0.95

[a] GMAb concentration was measured in separate serum aliquots (n = 3) from two patients.
[b] P values were determined by comparison of before and after values using Student's *t*-test.
[c] GMAb concentration was measured in serum samples subjected to freezing to −80 °C and thawing at room temperature three times.
[d] GMAb concentration was measured in serum samples before and after keeping at temperature for 6 h.
[e] GMAb concentration was measured in serum samples before and after keeping in −80 °C freezer for 3 months.
[f] GMAb concentration was measured in aliquots of PCRS diluted to low or high concentration (indicated) before or 6 h after being kept on the counter at room temperature for 6 h.

4. Discussion

In this study, we optimized a GMAb ELISA (Schoch et al., 2002) and evaluated its accuracy, precision, and reliability for measuring serum GMAb concentration. The assay performed very well in distinguishing patients with autoimmune PAP from healthy people. A monoclonal GMAb reference standard (MCRS) was developed as the basis for a new proposed international unit of measure for GMAb concentration and a method for

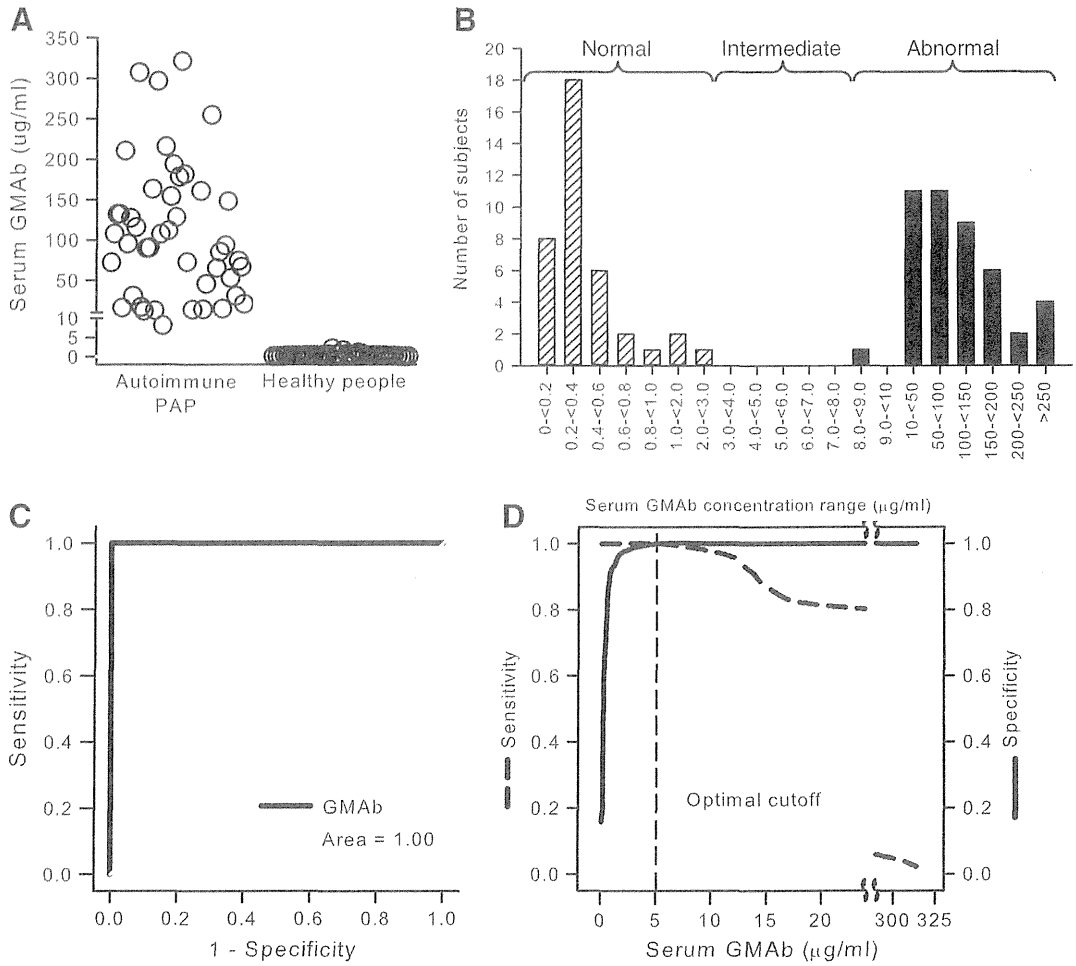


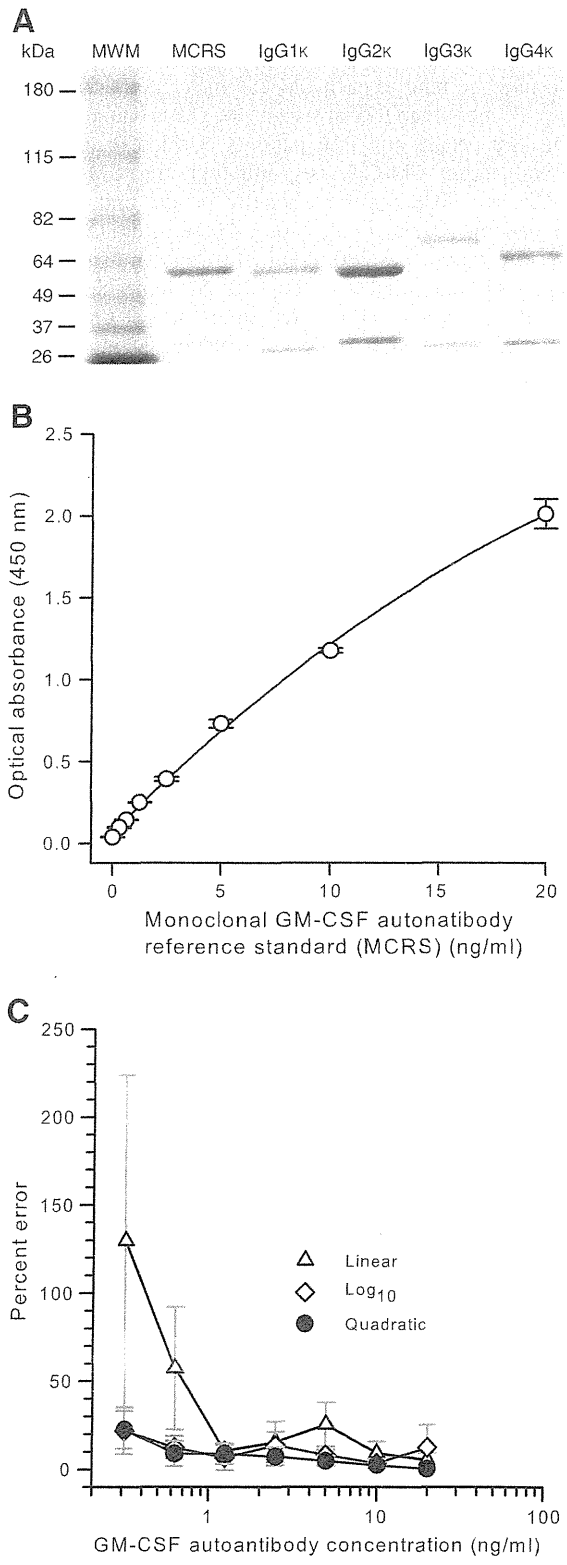
Fig. 7. Measurement and ROC analysis of serum GMAb concentration in autoimmune PAP patients and healthy controls. A. Serum GMAb levels in autoimmune PAP patients and healthy people. Serum GMAb concentration was measured using the GMAb ELISA with the PCRS in 44 autoimmune PAP patients and 38 healthy controls as described in the text. B. Histogram of the distribution of serum GMAb concentrations in autoimmune PAP patients and healthy people. Data represented as a frequency distribution of serum GMAb concentrations in autoimmune PAP patients (filled bars) and healthy people (hatched bars). C.–D. Receiver operating characteristic (ROC) curve analysis of serum GMAb ELISA test results for 44 autoimmune PAP patients and 38 healthy people. Standard ROC characteristic analysis was performed to determine the sensitivity and specificity for the data shown in panel A. The area under the curve was 1.0 (C), and, at a cut off value for GMAb of 5.0 μg/ml determined by the software, the sensitivity and specificity of the GMAb ELISA were both 100% (D).

converting GMAb data obtained with the PCRS into international units was developed. These results facilitate the comparison of serum GMAb concentration testing obtained in different laboratories thereby facilitating research on this rare disease.

Several modifications to the original GMAb ELISA were important in improving assay performance. First, identification of a capture antigen preparation with low baseline optical absorbance minimized non-specific background. Second, use of an increased initial serum aliquot volume to prepare specimen dilutions improved precision. Third, regression of reference standard data with a quadratic equation resulted in a better fit than with linear or logarithmic equations, which have been used previously. Fourth, restriction of absorbance

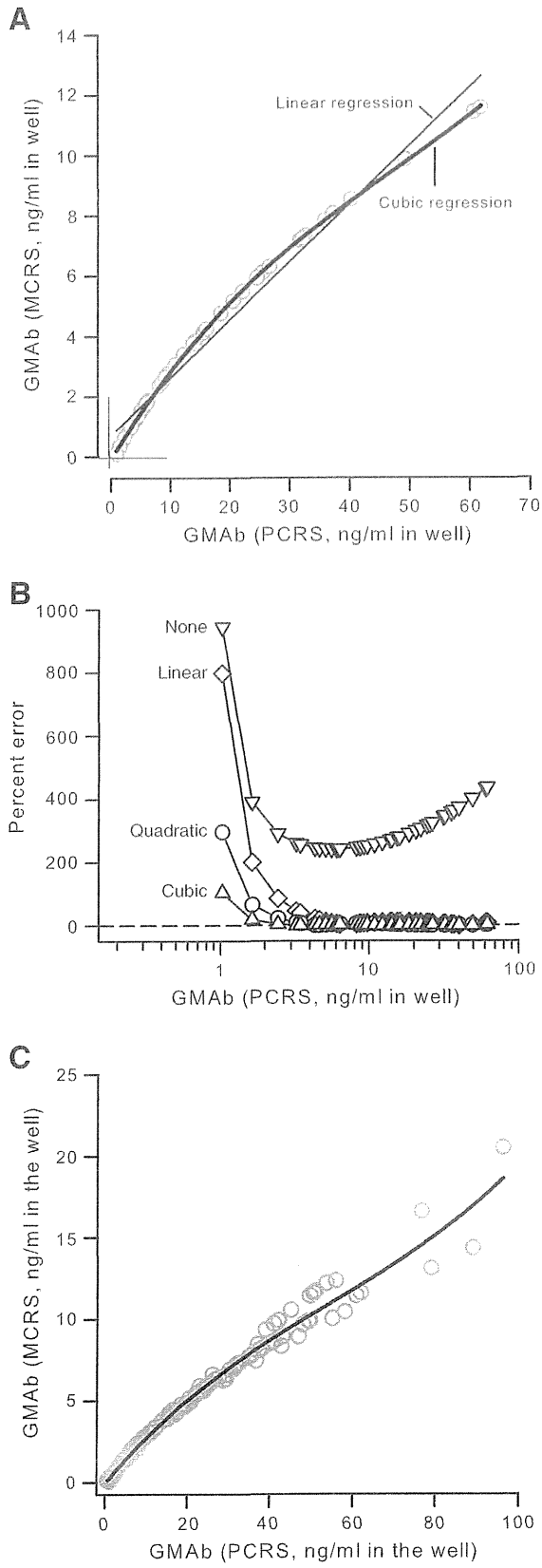
Fig. 8. Characterization and performance of pharmaceutical-grade, GMAb monoclonal reference standard (MCRS). A. Purity of the MCRS. The MCRS was prepared as described in the Materials and methods section. MCRS, commercially available IgG heavy chain isotype standards (IgGκ 1, 2, 3, or 4), or molecular weight markers (MWM) were subjected to polyacrylamide gel electrophoresis under reducing conditions, Coomassie blue staining, and photography as described in the Materials and methods section. B. Optical absorbance of the MCRS as a function of concentration. The MCRS was serially diluted and evaluated as the standard in the GMAb ELISA as described in the Materials and methods section. Optical absorbance increased smoothly in proportion with MCRS concentration. Regression analysis using a quadratic equation yielded a correlation coefficient (R^2) of 0.999. C. Effect of regression method used on percent error of the MCRS curve fit. Results from 6 independent, simultaneously conducted experiments determining the optical absorbance of serial dilutions of the MCRS were subjected to linear, quadratic, or logarithmic regression analysis and the percent deviation at each concentration was determined. The percent error of the $[GMAb]_{PCRS}$ measurement was calculated as $[GMAb]_{PCRS}$ minus $[GMAb]_{MCRS}$ divided by $[GMAb]_{MCRS}$ multiplied by 100; where $[GMAb]_{PCRS}$ are the unconverted values (None) or values after conversion using the linear, quadratic, or cubic regression equation parameters (indicated) and $[GMAb]_{MCRS}$ is the value actually determined using the MCRS (assumed to be the true value). The mean (±SD) correlation coefficients for regression analysis of 6 separate experiments (not shown) were 0.999 ± 0.0004 (quadratic), 0.990 ± 0.008 (log_{10}), and 0.982 ± 0.0168 (linear).

values used for calculating serum GMAb concentration to those within range of the reference standards improved accuracy. Further, any sample dilution with an 'in-range' absorbance value was acceptable for determining the GMAb concentration. In contrast, some variables had little influence on GMAb ELISA performance, e.g., glycosylation of the capture antigen and use of an $F(ab')_2$ fragment or intact IgG as the anti-human IgG detection antibody.



These results help establish a basis for the routine clinical use of the GMAb ELISA for diagnosis of autoimmune PAP. First, the accuracy, precision, and reliability of the GMAb ELISA were within performance parameter guidelines established by the United States Food and Drug Administration (Anonymous, 2001). Second, the ranges of serum GMAb concentrations in autoimmune PAP patients and healthy people were similar to prior reports utilizing the GMAb ELISA with a PCRS (Trapnell et al., 2003; Uchida et al., 2004, 2007, 2009). Third, the optimal cut off value of 5 μg/ml for the upper limit of normal determined by ROC curve analysis in this study is consistent with passive immunization studies in non-human primates identifying a serum GMAb concentration of 5 μg/ml as the critical threshold above which GM-CSF bioactivity was completely neutralized and the risk of autoimmune PAP in passively immunized animals is increased (Sakagami et al., 2009, 2010). At this cut off value, the sensitivity and specificity of the assay were both 100%, which is improved compared to a prior report utilizing the assay prior to optimization (Presneill et al., 2004). This is important given the low prevalence of autoimmune PAP (Inoue et al., 2008). Notwithstanding, the present study was not designed to establish the range of normal and abnormal serum GMAb levels or the sensitivity and specificity of the GMAb ELISA for a diagnosis of autoimmune PAP. It is also necessary to establish and validate the cutoff values used to identify autoimmune PAP and to validate the use of GMAb ELISA testing and these parameters for the diagnosis of autoimmune PAP. To this end, a study designed for this purpose involving subjects from Germany, Italy, Japan, and the United States (the MICEPAP study) has been undertaken and will be subsequently reported elsewhere.

One limitation of the GMAb ELISA is that it measures both neutralizing and non-neutralizing GMAbs. This should not be a problem when the total serum GMAb levels is high as in most autoimmune PAP patients but could be for values near the cutoff as can occur in some healthy people (Uchida et al., 2009). In such cases, the measurement of serum GM-CSF neutralizing capacity using a functional assay may help determine if such increases are functionally important, i.e., disrupt GM-CSF signaling. Several cell-based assays are useful for this including inhibition of GM-CSF-dependent TF1 cell growth (Uchida et al., 2004, 2009) or the GM-CSF-stimulated increase in whole blood leukocyte surface CD11b levels (Uchida et al., 2007) or GM-CSF stimulated increase in signal inducer of transcription 5 (STAT5) phosphorylation in blood leukocytes (Suzuki et al., 2008). Another limitation relates to small increases in serum GMAb observed in diseases not associated with development of PAP (Bendtzen et al., 2007; Han et al., 2009). For example, in 272 pediatric and 88 adult patients with Crohn's disease who did not have PAP, the median serum GMAb concentrations were 2.4 and 11.7 μg/ml, respectively (Han et al., 2009). Functional testing was helpful and indicated that GM-CSF signaling was reduced but not abolished in these patients. Since the clinical symptoms of autoimmune PAP do not occur in patients without significant radiographic findings, combining GMAb testing with routine



chest computed tomography will likely resolve any discrepancy potentially arising from intermediate GMAb values close to the cutoff. Further, in a typical clinical setting, GMAb testing would likely be considered after radiographic evaluation had suggested a diagnosis of PAP. Another limitation is the GMAb ELISA only detects free GMAb and not GMAb bound to GM-CSF, which could underestimate the GMAb concentration at the very low levels in individuals without PAP. This is illustrated by considering the following: 1) up to 7.8 GMAb molecules can bind to each GM-CSF molecule (Uchida et al., 2004); 2) more than 99% of serum GM-CSF is bound to GMAb in healthy people and PAP patients (Uchida et al., 2009); 3) total serum GM-CSF, i.e., unbound and GMAb-bound GM-CSF, is ~3048 pg/ml in healthy people and ~2360 in aPAP patients (Uchida et al., 2009); 4) the serum GMAb level in healthy people was 280 ng/ml (this report). Assuming 7.8 GMAb molecules bind to each GM-CSF molecule and the molecular mass for GMAb is ten times that of GM-CSF, the amount of GMAb bound to GM-CSF would be 7.8 multiplied by 3048 multiplied 10, or ~237 ng/ml. Thus, in healthy people, the percentage of GMAb detected by the ELISA would equal unbound GMAb (280 ng/ml) divided by total GMAb (unbound and GM-CSF-bound; 280 + 237 ng/ml) multiplied by 100 (conversion to percent) or approximately 54% of total GMAb. In autoimmune PAP patients, by similar calculations, ~99.8% of GMAb would be unbound and therefore detected by the GMAb ELISA. From above calculations, it is anticipated that GM-CSF bound to GMAb minimally interfere with the diagnostic threshold. Further, the lower limit of detection (0.78 ng/ml) is well below the cutoff for a positive result (5 μg/ml).

The observation that optical absorbance was greater for the MCRS than for the PCRS can be interpreted in terms of

Fig. 9. Conversion of GMAb concentration data into International units. A. Comparison of Serum GMAb concentration determined with the PCRS and MCRS. Separate sets of standard dilutions (1/100, 1/3000, 1/6000, 1/12,000) were prepared from serum from 14 autoimmune PAP patients and 1 healthy person. All standard dilutions (60 total) were used to measure GMAb concentration with the PCRS ($[GMAb]_{PCRS}$) and MCRS ($[GMAb]_{MCRS}$) as described in the legends in Figs. 3 and 7, respectively, in parallel on the same plate. All in-range values (n = 41; defined in the legend to Fig. 5) are shown and were used for analysis. The relationship between GMAb concentration determined using each of the two reference standards was evaluated by linear, quadratic (not shown to improve readability), or cubic regression analysis. The correlation coefficients for regression analysis were 0.980 (linear), 0.998 (quadratic), and 1.00 (cubic). B. Effect of regression method and GMAb concentration range on the accuracy of conversion of $[GMAb]_{PCRS}$ to international units. Using the data and regression equation parameters determined from the linear, quadric, or cubic regression analysis described above (panel A), the values for GMAb concentration determined with the PCRS ($[GMAb]_{PCRS}$) were converted mathematically to values that would have been obtained if the MCRS had been used (i.e., equivalent to $[GMAb]_{MCRS}$). The percent error of the $[GMAb]_{PCRS}$ measurement was calculated as $[GMAb]_{PCRS}$ minus $[GMAb]_{MCRS}$ divided by $[GMAb]_{MCRS}$ multiplied by 100; where $[GMAb]_{PCRS}$ are the unconverted values (None) or values after conversion using the linear, quadratic, or cubic regression equation parameters (indicated) and $[GMAb]_{MCRS}$ is the value actually determined using the MCRS (assumed to be the true value). The dashed line represents a percent error of zero. C. Determining the equation to convert $[GMAb]_{PCRS}$ data to international units. Data from six independent experiments similar to and including the one shown in panel A were combined and cubic regression analysis was done to determine the equation needed to convert data for GMAb concentration determined using the PCRS into units equivalent to that determined using the MCRS. In total, serum samples from 55 autoimmune PAP patients and 12 healthy controls were used for determining the equation. The correlation coefficient (R^2) was 0.98343.

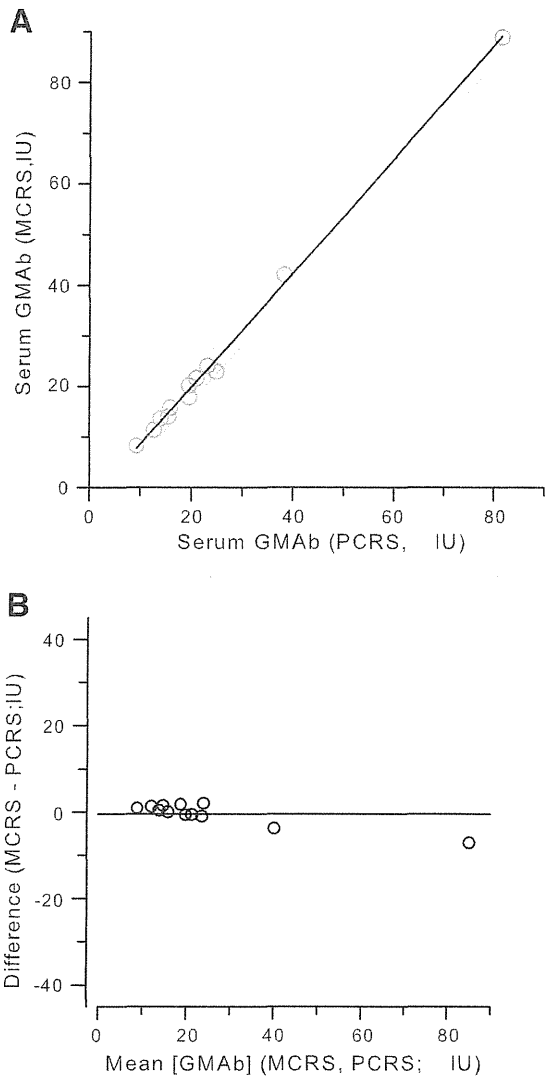


Fig. 10. Validation of the equation to convert $[GMAb]_{PCRS}$ data to international units. A. Comparison of $[GMAb]_{PCRS}$ data before and after conversion to international units with data determined directly with the MCRS. The converted PCRS-based data described above was plotted against the MCRS-based data and evaluated by linear regression analysis. The correlation coefficient was 0.984. See text for further details. B. Bland and Altman analysis. The data shown in panel A were used to evaluate the error for the comparison between $[GMAb]_{PCRS}$ converted to international units and $[GMAb]_{MCRS}$ by Bland and Altman analysis as described in the Materials and methods section.

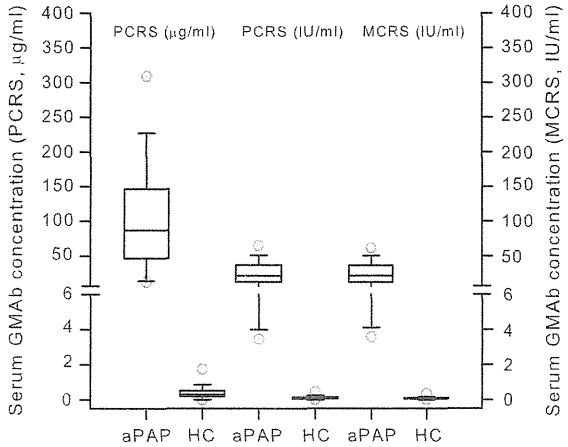


Fig. 11. Normal and abnormal ranges for GMAb concentration expressed in units of the PCRS, MCRS, and international units. Serum GMAb concentration in 56 autoimmune PAP patients and 38 healthy people was measured using the GMAb ELISA with the PCRS and converted into international units using the equation parameters determined by the analysis shown in Fig. 9C and was also simultaneously measured with the MCRS on the same plate. Box plots show the median (line) and IQR (box top and bottom), and 5th, 95 CI (circles) for patients with autoimmune 56 PAP patients (aPAP), and 38 healthy people (HP).

epitope differences: the PCRS has polyclonal epitopes distributed throughout the entire GM-CSF molecule while the MCRS targets a single epitope. In the GMAb ELISA, a lower optical absorbance indicates reduced secondary (detection) antibody binding, which could be caused by either 1) reduced binding of GMAb to capture antigen or 2) reduced binding of the detection antibody to captured GMAb (i.e., fewer 'targets for detection or reduced efficiency of target detection). Assuming that GMAb capture has reached equilibrium, theoretically, reduced binding of PCRS in the GMAb ELISA (compared to MCRS) could be explained by 'epitope hiding', perhaps of less-avid GMAb species, or by reduced access to GM-CSF adjacent to bulkier complexes of polyvalent PCRS. Neither of these mechanisms seems likely since capture antigen is in molar excess of GMAb. Alternatively, since the binding ratio can be as high as 7.8 molecules of GMAb per GM-CSF molecule for the PCRS and only one for MCRS, reduced binding of the detection antibody could be explained by increased steric hinderance for the polyvalent PCRS–antigen complexes than for monovalent MCRS–antigen complexes. While we believe the latter is most likely, these mechanisms are not mutually exclusive and additional studies would be needed to elucidate the mechanism.

The availability of a high-quality, monoclonal GMAb reference standard (e.g., MCRS) should facilitate research on autoimmune PAP, which is challenged by low prevalence like many rare diseases. Specifically, the use of a common international unit of measure for GMAb concentration based on the MCRS would improve comparability of test results from different laboratories. To this end, efforts are underway to establish the GMAb ELISA in multiple reference laboratories in Japan, Europe, Asia, and the United States. The MCRS and a common set of serum specimens will be used to calibrate serum GMAb test results between laboratories. Conceivably, these reagents could also allow GMAb data obtained by other methods, e.g., serial dilution GMAb titer data (Bonfield et al., 2002), to be expressed in a common unit of measure. Finally, the availability of a validated method for converting data obtained with other GMAb reference standards to international units may facilitate meta-analysis of published data from small cases series, which are common in rare diseases.

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ORIGINAL ARTICLE

CYFRA 21-1 as a disease severity marker for autoimmune pulmonary alveolar proteinosis

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ABSTRACT

Background and objective: **Serum markers, including Krebs von den Lungen (KL-6), surfactant protein (SP)-D, SP-A and carcinoembryonic antigen (CEA), are reported to reflect autoimmune pulmonary alveolar proteinosis (APAP) disease severity. We evaluated serum CYFRA21-1 levels as a marker of APAP.**

Methods: **In addition to KL-6, SP-D and CEA, we prospectively measured serum CYFRA 21-1 levels in 48 patients with APAP, consecutively diagnosed between 2002 and 2010. Diagnostic usefulness of CYFRA 21-1 was determined from 68 patients with interstitial lung diseases by receiver operator characteristic curve analysis. We evaluated the association between these serum markers and other disease severity markers, including pulmonary function parameters, alveolar-arterial oxygen gradient, British Medical Research Council score reflecting shortness of breath, and disease severity score. CYFRA 21-1 localization in the lung was examined by immunohistochemistry.**

Results: **Receiver operator characteristic curve demonstrated that CYFRA 21-1 effectively identified APAP. Serum CYFRA 21-1 levels at diagnosis were significantly associated with the measured disease severity parameters. Following whole lung lavage (n = 10) and granulocyte-macrophage colony-stimulating factor (GM-CSF) inhalation (n = 20), serum CYFRA 21-1 levels were significantly decreased. Responders (n = 11) to GM-CSF inhalation revealed significantly higher serum CYFRA 21-1 levels than non-responders (n = 9). Serum CYFRA 21-1 appeared to be a significant predictor of effectiveness of GM-CSF based on regression analysis. Immunohistochemistry showed that CYFRA 21-1 was localized on hyperplastic alveolar type II cells and lipoproteinaceous substances in alveoli.**

Conclusions: **Serum CYFRA 21-1 is a sensitive and useful serum marker for diagnosis and evaluation of disease severity of APAP, and may predict the response to GM-CSF inhalation.**

SUMMARY AT A GLANCE

Serum CYFRA 21-1 is useful for the diagnosis of autoimmune pulmonary alveolar proteinosis (APAP) by receiver operating characteristic curve analysis and significantly correlated with other disease severity markers of APAP. Serum CYFRA 21-1 might be a predictor of responsiveness to GM-CSF inhalation therapy in APAP.

Key words: alveolar epithelial hyperplasia, granulocyte-macrophage colony-stimulating factor, inhalation therapy, rare lung disease, whole lung lavage.

Abbreviations: APAP, autoimmune pulmonary alveolar proteinosis; CEA, carcinoembryonic antigen; GM-CSF, granulocyte-macrophage colony-stimulating factor; KL-6, Krebs von den Lungen; PaO_2, partial pressure of oxygen; SP, surfactant protein; WLL, whole lung lavage.

INTRODUCTION

Pulmonary alveolar proteinosis is characterized by the accumulation of phospholipids and surfactant protein (SP) in alveolar spaces.[1] Impaired alveolar macrophage function underlies the pathogenesis of idiopathic pulmonary alveolar proteinosis, which is caused by a deficiency of the granulocyte-macrophage colony-stimulating factor (GM-CSF) in the presence of anti-GM-CSF autoantibodies.[2,3] Hence, in recent years, idiopathic pulmonary alveolar proteinosis has been referred to as autoimmune pulmonary alveolar proteinosis (APAP) when anti-GM-CSF autoantibodies are detected in the blood.[4] The levels of SP-A,[5] SP-B,[6] SP-D,[7] carcinoembryonic antigen (CEA),[8] Krebs von den Lungen (KL-6)[9] and lactate dehydrogenase[10] are elevated in the sera of idiopathic pulmonary alveolar proteinosis patients. Previously, Seymour and Presneill showed that SP-A and lactate dehydrogenase levels correlated with idiopathic pulmonary alveolar proteinosis disease severity.[10] Moreover, the levels of CEA[11] and KL-6[12] in

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idiopathic pulmonary alveolar proteinosis have been shown to decrease following whole lung lavage (WLL).[13] We demonstrated for the first time that serum KL-6, SP-A, SP-D, CEA and lactate dehydrogenase levels are elevated and are significantly correlated with disease severity in patients with APAP.[4]

Cytokeratins are the main protein components of the cell cytoskeleton and belong to the family of intermediate filaments. Cytokeratin 19 is a low-molecular weight (40 kD) protein and is expressed in simple epithelia, including bronchial epithelial cells.[14] A fragment of cytokeratin 19, called CYFRA 21-1, can be measured in serum and has been evaluated as a useful tumour marker for non-small cell lung cancer.[14] Moreover, it is also known to be elevated in various types of non-malignant lung disorders,[15] such as bacterial pneumonia, tuberculosis, bronchiectasis and interstitial pneumonia, particularly acute interstitial pneumonia.[16] In addition, increased serum CYFRA 21-1 levels and its decrease after WLL have been reported in pulmonary alveolar proteinosis patients.[17] We have previously reported in a pulmonary alveolar proteinosis patient that high levels of serum CYFRA 21-1 declined following successful GM-CSF inhalation therapy, accompanied by an improvement in arterial blood gas levels and the diffusing capacity of carbon monoxide (DLco).[18] Thus, serum CYFRA 21-1 levels may be related to pulmonary alveolar proteinosis severity.

To assess the value of serum CYFRA 21-1 as a biomarker of APAP reflecting disease severity, we measured serum CYFRA 21-1 levels in APAP patients, and examined the relationship between serum CYFRA 21-1 levels and disease severity using respiratory function tests and arterial blood gas analysis. This is the most comprehensive and largest study to assess the role of serum CYFRA 21-1 as biomarker of APAP.

Table 1 Patient characteristics at the time of diagnosis of APAP

	Mean ± SE or number of cases
Number of patients	48
Age (years)	52.9 ± 1.9
Gender (M/F)	31/17
Smoking history (NS/ES/CS)	17/20/11
Dust exposure (Yes/No)	18/29
MRC (0/1/2/3/4)	9/18/18/3/0
DSS (I/II/III/IV/V)	4/20/8/9/6
PaO_2 (Torr)	66.5 ± 2.0
$AaDO_2$ (Torr)	36.3 ± 2.1
VC, %predicted	87.5 ± 2.9
DLco, %predicted	54.9 ± 2.9
CYFRA 21-1 (ng/mL)	9.03 ± 1.64
KL-6 (U/mL)	7320 ± 1224
SP-D (ng/mL)	224 ± 24
CEA (ng/mL)	10.0 ± 1.3
Anti-GM-CSF autoantibody (ng/mL)	48.5 ± 4.7

Normal serum ranges were CYFRA 21-1 (<2.8 ng/mL), KL-6 (<500 U/mL), SP-D (<110 ng/mL) and CEA (<5.0 ng/mL). The serum CYFRA 21-1 levels of 8 cases out of the total 48 cases were within the normal range. The 8 cases were mild or moderate diseases categorized in DSS 1, 2 and 3, and complaint of mild dyspnoea (MRC 0 and 1).

$AaDO_2$, alveolar-arterial oxygen gradient; APAP, autoimmune pulmonary alveolar proteinosis; CEA, carcinoembryonic antigen; CS, current smoker; DLco, diffusing capacity of carbon monoxide; DSS, disease severity score; ES, ex-smoker; GM-CSF, granulocyte-macrophage colony-stimulating factor; KL-6, Krebs von den Lungen-6; MRC, British Medical Research Council score for shortness of breath upon exertion; NS, non-smoker; PaO_2, partial pressure of oxygen; SP-D, surfactant protein-D; VC, vital capacity.

METHODS

Subjects

Forty-eight consecutive patients diagnosed with APAP at the Kinki-Chuo Chest Medical Center between 2002 and 2010 were enrolled in the study. The diagnosis of APAP was confirmed based on transbronchial lung biopsy, bronchoalveolar lavage, radiological findings and the presence of anti-GM-CSF autoantibody (Table 1). Serum levels of anti-GM-CSF autoantibody were measured at diagnosis by enzyme-linked immunosorbent assay as previously reported with minor modification,[4,19] and its cut-off level to diagnose APAP was 0.5 μg/mL.

Serum CYFRA 21-1 levels of APAP were compared with that of 68 patients with interstitial lung diseases diagnosed at the Kinki-Chuo Chest Medical Center, including idiopathic pulmonary fibrosis ($n = 25$), non-specific interstitial pneumonia ($n = 6$), collagen vascular disease associated interstitial pneumonia ($n = 15$), chronic hypersensitivity pneumonia ($n = 13$) and sarcoidosis ($n = 9$) (Table S1 in the online supporting information).

WLL was performed in 10 patients, and inhaled GM-CSF was administered to 20 patients (Table S3 in the online supporting information). A positive response to the therapy was defined as improvement of alveolar-arterial oxygen gradient ($AaDO_2$) by 10 Torr.

Comprehensive informed consents for measurements of serum biomarkers and comparison with clinical measures were obtained from all subjects. Additional informed consents were obtained on GM-CSF inhalation therapy. The review board of Kinki-Chuo Chest Medical Center approved this prospective study (Approval Number 73, 99).

Procedure of WLL

WLL was performed as previously described by Ramirez,[20] with slight modifications. The patient was in supine position, and a left-sided double-lumen tube was placed, after which one-lung ventilation with an inspiratory oxygen fraction of 1.0 was performed. Saline, warmed to body temperature, was delivered by gravity, and the lavage fluid was also drained by gravity after chest-wall percussion. Lavage

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was completed when the appearance of the lavage fluid turned from milky to clear, and the total saline delivered usually reached 15–20 L for a single lung. WLL for the other side was performed about 2 weeks after the first WLL. Response to WLL was evaluated 1 month after the first WLL.

GM-CSF inhalation

Twenty patients were treated with GM-CSF inhalation. Three protocols for GM-CSF inhalation were employed in 15 patients, as described by Tazawa *et al.*[21] One patient from our first pilot study[18] was treated with a daily inhalation of 250 μg of GM-CSF every second week for 24 weeks starting on the first week. Two patients from our second pilot study received 125 μg of GM-CSF inhalation daily during the first 6 weeks, and an additional 125 μg of GM-CSF inhalation daily during the second 6 weeks if the change in $AaDO_2$ was ≥10 Torr, or an additional 250 μg of GM-CSF inhalation daily during the second 6 weeks if the change in $AaDO_2$ was <10 Torr. Twelve patients were from a phase II trial.[21] The remaining five APAP patients were treated with 50 μg of GM-CSF inhalation twice on days 1–8 and no GM-CSF inhalation on days 9–14 for 12 weeks, and 50 μg once on days 1–4 and no GM-CSF inhalation on days 5–14 for the next 12 weeks.

Evaluation of disease severity

Disease severity of the patients were evaluated at the time of diagnosis, and before and after WLL or GM-CSF inhalation using the following parameters: $AaDO_2$, partial pressure of oxygen (PaO_2), percentage of predicted DLco (%DLco), percentage of predicted vital capacity, the British Medical Research Council score for shortness of breath upon exertion and the disease severity score of APAP, as defined by Inoue *et al.*[4] The severity was classified into five grades: grade 1, $PaO_2 \geq 70$ Torr without respiratory symptoms; grade 2, $PaO_2 \geq 70$ Torr with respiratory symptoms; grade 3, 70 Torr > $PaO_2 \geq 60$ Torr; grade 4, 60 Torr > $PaO_2 \geq 50$ Torr; and grade 5, $PaO_2 < 50$ Torr. Arterial blood gas analyses were performed on samples obtained with the patient breathing room air at rest in the supine position for at least 15 min.

Measurement of CYFRA 21-1 and other serum markers

Serum CYFRA 21-1, KL-6, SP-D and CEA levels were measured by commercial enzyme-linked immunosorbent assay kits (CYFRA, Boehringer Mannheim, Tokyo, Japan; KL-6, Eizai, Tokyo, Japan; SP-D, Kyowa Medex, Tokyo, Japan; CEA, Abbott Japan, Tokyo, Japan). These markers were measured at diagnosis, and before and after therapy.

Immunohistochemistry

To detect the location of CYFRA 21-1 in the lung, we performed immunohistochemistry using the modified method of Nakayama *et al.*[15] Immunohistochemistry was performed by the avidin-biotin peroxidase complex method using the VECTASTAIN ABC mouse IgG Kit (Vector Laboratories, Burlingame, CA, USA) and a murine monoclonal anti-cytokeratin-19 antibody, Ks 19.1 (Progen, Biotechnik GmbH, Heidelberg, Germany), which is used for the measurement of serum CYFRA 21-1, at the concentration of 5 μg/mL. As a negative control, mouse IgG was used at the same concentration.

Statistical analysis

Each parameter was presented as mean ± SE. Correlations between serum CYFRA 21-1 and parameters of disease severity were evaluated by Spearman's rank correlation analysis. The differences in serum CYFRA 21-1 levels before and after GM-CSF inhalation or after WLL were determined by the paired *t*-test. Serum CYFRA 21-1 levels of GM-CSF responders and non-responders were compared by the Student *t*-test. Receiver operator characteristic curve analysis was performed to evaluate serum markers as diagnostic tests for APAP. The correlation between the change in serum CYFRA 21-1 and the change in $AaDO_2$ was assessed using the Pearson correlation analysis. A *P*-value of <0.05 was considered statistically significant. All statistical calculations were performed using the JMP version 8.0.2 for Macintosh (SAS Institute Inc., Cary, NC, USA).

RESULTS

Cut-off level of CYFRA 21-1 for diagnosis of APAP

Serum CYFRA 21-1 levels were significantly elevated in APAP (9.03 ± 1.64 ng/mL) (Table 1) as compared with interstitial lung diseases (2.96 ± 0.22 ng/mL) (Table S1). Cut-off level of serum CYFRA 21-1 to diagnose APAP was 3.80 ng/mL by receiver operator characteristic analysis (Fig. 1). Area under receiver operator characteristic curve of CYFRA 21-1 was similar to that of KL-6 (Fig. 1, Table S2 in the online supporting information).

Correlation between serum CYFRA 21-1 levels and other parameters reflecting disease severity

Serum CYFRA 21-1 levels were significantly correlated with British Medical Research Council score, disease severity score, $AaDO_2$ and %DLco, but not with percentage of predicted vital capacity. Serum KL-6 and SP-D were significantly correlated with all five parameters (Table 2).

Change in serum CYFRA 21-1 and $AaDO_2$ levels 1 month after WLL

In all 10 patients treated with WLL, serum CYFRA levels significantly decreased 1 month after WLL (Fig. 2a). $AaDO_2$ before WLL and 1 month after WLL was evaluated in nine patients, except for one patient whose disease was too severe to evaluate $AaDO_2$ under room air conditions. The decrease in $AaDO_2$ in

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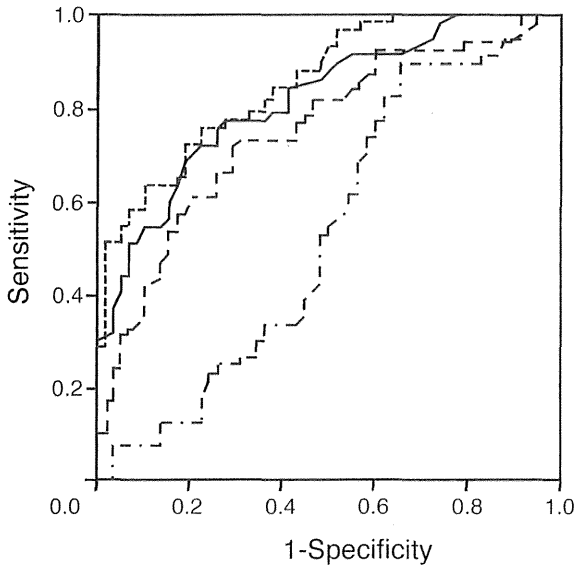

Figure 1 Receiver operator characteristic (ROC) curves showing comparison of serum markers including KL-6, surfactant protein-D (SP-D), carcinoembryonic antigen (CEA) and CYFRA 21-1 as diagnostic test for autoimmune pulmonary alveolar proteinosis (APAP). Levels of serum markers were measured in 68 patients with interstitial lung diseases (ILD), including idiopathic pulmonary fibrosis; 25 patients, non-specific interstitial pneumonia; 6 patients, collagen vascular disease associated interstitial pneumonia; 15 patients, hypersensitivity pneumonia; 13 patients and sarcoidosis; 9 patients, and 48 patients of APAP. Area under ROC curve of CYFRA 21-1, KL-6, CEA and SP-D was 0.8171, 0.8516, 0.7505 and 0.4951, respectively. Cut-off level of serum CYFRA 21-1 to diagnose APAP was 3.80 ng/mL. Details of serum markers in ILD (Table S1) and results of ROC curve analysis (Table S2) were described in the online supporting information. ——, CYFRA 21-1; ·····, KL-6; – – –, SP-D; - – -, CEA.

Table 2 Relationship (ρ) between serum markers and disease severity markers in APAP patients

	CYFRA 21-1	KL-6	SP-D	CEA
MRC	0.6127*	0.6113*	0.5117**	0.5909*
DSS	0.6441*	0.6991*	0.5044*	0.5635*
$AaDO_2$	0.7474*	0.7239*	0.6091*	0.5914*
%DLco	−0.6793*	−0.7309*	−0.6878*	−0.4356***
%VC	−0.2326	−0.5097**	−0.5222*	−0.2403

* $P < 0.0001$; ** $P < 0.001$; *** $P < 0.01$.

The relationship between serum markers and disease severity markers was evaluated by ρ using the Spearman rank correlation analysis.

$AaDO_2$, alveolar-arterial oxygen gradient; APAP, autoimmune pulmonary alveolar proteinosis; CEA, carcinoembryonic antigen; DLco, diffusing capacity of carbon monoxide; DSS, disease severity score; KL-6, Krebs von den Lungen-6; MRC, British Medical Research Council score for shortness of breath upon exertion; SP-D, surfactant protein-D; VC, vital capacity.

nine patients following WLL ($\Delta AaDO_2$) was significantly correlated with the decrease in CYFRA 21-1 after WLL (ΔCYFRA 21-1) (Fig. 2b; $n = 9$, $r = 0.7621$, $P = 0.0170$), but not with ΔKL-6 ($n = 9$, $r = 0.4378$, $P = 0.2386$), ΔSP-D ($n = 9$, $r = 0.4686$, $P = 0.2033$) and ΔCEA ($n = 9$, $r = 0.3948$, $P = 0.2930$).

Change in serum CYFRA 21-1 and $AaDO_2$ following GM-CSF inhalation therapy

In GM-CSF-effective cases ($n = 11$), serum CYFRA 21-1 levels diminished significantly after GM-CSF inhalation therapy ($P = 0.002$); however, in GM-CSF-ineffective cases ($n = 9$), serum CYFRA 21-1 levels did not change significantly (Fig. 3). Although the characteristics of responders were not different from that of non-responders, except for age (Table S3), the serum CYFRA 21-1 levels in responders were significantly higher compared with non-responders before GM-CSF inhalation therapy (Fig. 4). There was no significant difference in serum KL-6, SP-D and CEA between responders and non-responders (Table S3). Multivariate logistic regression analysis with a stepwise method to predict effectiveness of GM-CSF inhalation revealed that serum CYFRA level (ng/mL) is a significant predictive factor; however, other factors, including the other serum markers, were insignificant (Table 3, Table S4 in the online supporting information).

Immunohistochemistry for CYFRA 21-1 detection

Immunohistochemical analysis of the transbronchial lung biopsy specimens obtained from one patient was performed. CYFRA 21-1-positivity was detected in the proteinaceous material in the alveolar spaces and in the hyperplastic alveolar epithelial cells (Fig. 5).

DISCUSSION

Receiver operator characteristic curve analysis revealed that CYFRA 21-1 is a diagnostic marker of APAP. CYFRA 21-1 is also a sensitive serum marker for APAP reflecting disease severity that is comparable to KL-6 and CEA, two markers that we have previously described.[1,22] In the present study, serum CYFRA 21-1 levels were significantly correlated with other disease severity parameters. Serum CYFRA 21-1 levels significantly decreased in APAP patients in whom $AaDO_2$ improved following GM-CSF inhalation therapy and WLL. Thus, we propose that CYFRA 21-1 is an important serum marker for diagnosis and disease severity of APAP. No significant relationship of CYFRA 21-1 with percentage of predicted vital capacity was found. However, no significant correlation might be a natural consequence of the percentage of predicted vital capacity decreasing below normal levels only in severe APAP, as previously reported.[4]

Elevation of serum markers in APAP patients is associated with impaired metabolism through the dysfunction of alveolar macrophages and through augmented permeability of each marker observed in interstitial lung diseases.[23,24] Increased permeability is necessary for KL-6, a large molecule with a molecular weight of more than 1000 kD,[25] to flow into the blood.

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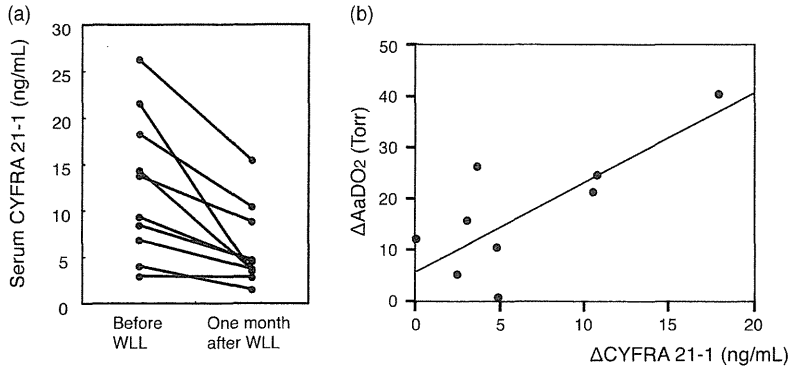

Figure 2 Serum CYFRA 21-1 levels (mean ± SE) in patients treated with whole lung lavage (WLL) ($n = 10$) decreased significantly after the treatment (from 12.53 ± 2.42 ng/mL to 5.90 ± 1.36 ng/mL) ($P = 0.0041$) (a). Alveolar-arterial oxygen gradient ($AaDO_2$) was evaluated before and after the WLL in nine patients. Decrease in CYFRA 21-1 (ΔCYFRA 21-1) significantly correlated with the decrease in $AaDO_2$ ($\Delta AaDO_2$) in nine patients 1 month after WLL ($P = 0.0170$). Statistical analyses were performed by the paired *t*-test (a) and the Pearson correlation analysis (b).

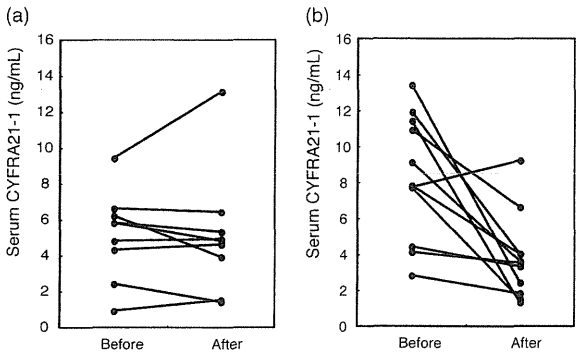

Figure 3 Measurement of serum CYFRA 21-1 levels (mean ± SE) before and after granulocyte-macrophage colony-stimulating factor (GM-CSF) inhalation. In GM-CSF-ineffective cases (non-responders, $n = 9$), there was no significant difference before (5.13 ± 0.82 ng/mL) and after the inhalation therapy (5.10 ± 1.14 ng/mL) (a), and in GM-CSF-effective cases (responders, $n = 11$), the serum CYFRA 21-1 levels significantly decreased from 8.29 ± 1.04 ng/mL to 3.74 ± 0.71 ng/mL after the inhalation therapy ($P = 0.002$) (b). Statistical analysis was performed by the paired *t*-test.

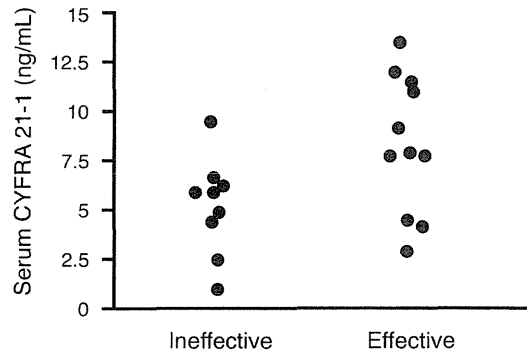

Figure 4 Serum CYFRA 21-1 levels before the inhalation of granulocyte-macrophage colony-stimulating factor (GM-CSF) were significantly higher in GM-CSF-effective cases (responders, $n = 11$, 8.29 ± 1.04 ng/mL) than those in GM-CSF-ineffective cases (non-responders, $n = 9$, 5.13 ± 0.82 ng/mL), as demonstrated by the Student *t*-test ($P = 0.017$). Three GM-CSF-effective cases showed comparatively low levels of serum CYFRA 21-1, although alveolar-arterial oxygen gradient of three patients was more than 40 Torr. Serum levels of Krebs von den Lungen-6, surfactant protein-D and carcinoembryonic antigen were also low in two out of three patients (data not shown) (values were presented as mean ± SE).

Inoue *et al.* reported that the increase in serum KL-6 level was due to increased permeability of the alveolar wall to blood flow in berylliosis.[26] The permeability of the alveolar-airway barrier might be modulated by GM-CSF. GM-CSF increases alveolar epithelial barrier function in vitro[27] and suppresses the apoptosis of alveolar epithelial cells.[28] Thus, it is possible that the deficiency of GM-CSF caused by the presence of anti-GM-CSF autoantibodies in APAP conversely leads to the dysfunction of the alveolar epithelial barrier and increased permeability.

Based on a previous report on non-malignant pulmonary diseases, elevation of serum CYFRA 21-1 is due to epithelial damage and its increased production in the epithelium.[16] CYFRA 21-1 is expressed in hyperplastic type II pneumocytes and metaplastic cells in patients with idiopathic pulmonary fibrosis.[16] Although the pathophysiology is unknown, alveolar epithelial cell hyperplasia is sometimes observed in the lung specimens of APAP patients.[29] In agreement with this finding, we also pointed out the existence of alveolar epithelial cell hyperplasia in the present study. Thus, increased production of CYFRA 21-1 from alveolar epithelial cells may be another reason for the elevation of serum CYFRA 21-1 levels in APAP patients.

Yoshimasu *et al.* calculated the half-life of CYFRA 21-1 to be 1.5 h from the disappearance curve of serum concentration after the resection of lung cancer.[30] The half-lives of SP-D and KL-6 are not known; however, they are predicted to be longer than that of CYFRA 21-1 when considering their higher molecular weights. A short half-life is an important characteristic as a serum marker because levels of a serum marker with a short half-life can change simultaneously with the change in disease activity. To evaluate this point, the disappearance rate of each serum marker 1 month after WLL was examined in the present study; however, there was no significant difference between the markers (data not shown). This is because 1 month is too long an interval to evaluate the disappearance rate of each of the serum markers.

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Table 3 Multivariate logistic regression analysis to predict effectiveness of GM-CSF inhalation in APAP

Parameters	Odds ratio	95% CI	*P*-value
CYFRA 21-1 ≥ 6.4 ng/mL	9.333	1.372–94.188	0.0213

Multivariate logistic regression analysis with a stepwise method to predict effectiveness of GM-CSF inhalation was performed, using serum levels of CYFRA 21-1, carcinoembryonic antigen (CEA), Krebs von den Lungen-6 (KL-6), surfactant protein-D (SP-D), %vital capacity (VC), % diffusing capacity of carbon monoxide (DLco), alveolar-arterial oxygen gradient ($AaDO_2$), disease severity score (DSS), gender, age and British Medical Research Council score for shortness of breath upon exertion (MRC score). Each parameter was classified into two groups by median except for gender: CYFRA 21-1 ≥ 6.4 ng/mL, CEA ≥ 7.3 ng/mL, KL-6 ≥ 4090 U/mL, SP-D ≥ 213 ng/mL, %VC ≥ 89%, %DLco ≥ 47.5%, $AaDO_2$ ≥ 44 Torr, DSS ≥ 4, MRC ≥ 3, age ≥ 53. Univariate analysis revealed that serum CYFRA 21-1 level was only a significant factor to predict good response of GM-CSF inhalation to GM-CSF inhalation (Table S4 in the online support information). Using stepwise method, serum CYFRA 21-1 level was also only a significant predictor for the response of GM-CSF inhalation therapy and other serum factors were insignificant.

APAP, autoimmune pulmonary alveolar proteinosis; CI, confidence interval; GM-CSF, granulocyte-macrophage colony-stimulating factor.

Figure 5 Immunohistochemical analysis of transbronchial lung biopsy specimens obtained from one patient. CYFRA 21-1-positivity was observed in lipoproteinaceous material in the alveolar spaces (arrowhead) and in the hyperplastic alveolar epithelial cells (arrow).

Serum CYFRA 21-1 levels in responders to GM-CSF inhalation were significantly higher compared with non-responders. The same tendency has been reported for serum KL-6;[21] however, there was no difference in the levels of the other serum markers between responders and non-responders in our examination. Serum CYFRA 21-1 was a significant factor to predict effectiveness of GM-CSF inhalation therapy by multivariate logistic regression analysis. Hence, serum CYFRA might be a useful serum marker to predict the effectiveness of GM-CSF inhalation. However, studies of additional APAP patients treated with GM-CSF inhalation are needed to make definite conclusions, and response to GM-CSF itself needs to be judged cautiously considering the possibility of spontaneous regression of some cases of APAP.[1]

It remains unresolved from our investigation which is the best serum marker of APAP. KL-6 is a very good marker, and is highly correlated with both symptoms and pulmonary function tests. However, another important ability requested for a good marker is to predict treatment response and prognosis. We expect that serum CYFRA 21-1 might be able to forecast effectiveness of GM-CSF inhalation shown above and disease severity changes, for example spontaneous regression, due to its short half-life. Future studies are needed to reach definite conclusions.

We conclude that serum CYFRA 21-1 is a sensitive and useful serum marker for diagnosis and evaluation of disease severity of APAP. CYFRA 21-1 levels might predict the response to GM-CSF inhalation.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1 Patient characteristics of interstitial lung diseases

Table S2 Details of ROC curve analysis of serum markers

Table S3 Patient characteristics before therapy

Table S4 Univariate logistic regression analysis to predict effectiveness of GM-CSF inhalation in APAP

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SARCOIDOSIS VASCULITIS AND DIFFUSE LUNG DISEASES 2014; 31; 19-27 © Martioli 1885

THE WASOG SARCOIDOSIS ORGAN ASSESSMENT INSTRUMENT: AN UPDATE OF A PREVIOUS CLINICAL TOOL

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ABSTRACT. *Introduction:* A Case Control Etiology of Sarcoidosis Study (ACCESS) sarcoidosis organ assessment instrument has been used for more than a decade to establish uniform standards for the probability of sarcoidosis organ involvement. The ACCESS instrument has become increasingly outdated as new technologies have been developed. Furthermore, the ACCESS instrument failed to address all possible organs involved with sarcoidosis. For these reasons, the World Association of Sarcoidosis and Other Granulomatous Diseases (WASOG) developed a new sarcoidosis organ assessment instrument. *Methods:* Clinical sarcoidosis experts assessed various clinical manifestations for the probability of sarcoidosis organ involvement. Two criteria were required to apply this assessment: 1) histologic evidence of granulomatous inflammation of unknown cause in an organ that was not being assessed; 2) the clinical manifestation being addressed required that alternative causes other than sarcoidosis had been reasonably excluded. Clinical manifestations were assessed as either: a) highly probable: likelihood of sarcoidosis causing this manifestation of at least 90%.; b) probable: likelihood of sarcoidosis causing this manifestation of between 50 and 90%; c) possible: likelihood of sarcoidosis causing this manifestation of less than 50%. The sarcoidosis experts voted on the likelihood of sarcoidosis causing each manifestation using Delphi study methodology where at least 70% agreement of the experts was needed for consensus. *Results:* Various clinical manifestations were classified as highly probable, at least probable, possible, or indeterminate when no consensus could be reached. *Conclusion:* An instrument was developed by expert opinion that may be useful for the clinician and researcher in establishing criteria for sarcoidosis organ involvement.

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KEY WORDS: sarcoidosis, organ, diagnosis, consensus

Introduction

Sarcoidosis is a multisystem granulomatous disease of unknown cause.[1] Granulomatous inflammation from sarcoidosis may occur in any organ. ACCESS (A Case Control Etiology of Sarcoidosis Study) was a study of sarcoidosis in the 1990's funded by the National Institutes of Health that was primarily aimed at searching for the etiology of sarcoidosis. For the purposes of that effort, the ACCESS investigators were concerned with establishing criteria for sarcoidosis organ involvement. They had noticed that a large number of sarcoidosis clinical trials did not describe rigorous entry criteria for enrollment; other trials that did clearly describe entry criteria for various sarcoidosis organ involvements were not consistent with each other. This issue was the major impetus for the development of the ACCESS Sarcoidosis Organ Assessment Instrument.[2] The instrument assessed various clinical findings of 15 organs in terms of their likelihood of representing sarcoidosis. These clinical findings were graded as "definite," "probable," and "possible" evidence of organ involvement with sarcoidosis. For the purposes of the ACCESS study, "definite" and "probable" involvement was considered to represent organ involvement with sarcoidosis. A prerequisite for using the ACCESS instrument to evaluate the likelihood of a clinical finding of an organ representing sarcoidosis was that at least one additional organ had demonstrated granulomatous inflammation of no alternative cause.

The ACCESS instrument, although useful, suffers from some deficiencies. First, it was developed more than one decade ago, and it is somewhat outdated as new technologies have been developed in the interim for the diagnosis and monitoring of sarcoidosis. Second, the instrument failed to cover all possible organs involved with sarcoidosis. Third, several common and very specific manifestations of sarcoidosis were not addressed. For these reasons, the World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) developed a new sarcoidosis organ assessment instrument. This manuscript will describe this instrument.

Methods

Clinical sarcoidosis experts who were members of WASOG were invited to participate in development of the instrument (Appendix 1). Individuals were invited to serve as "organ group leaders," who would be responsible for developing criteria for a specific organ. Other WASOG members were invited to participate as members of any organ group that was of interest to them. In addition, the organ group leaders were given the authority to invite non-WASOG members to their groups if they believed such individuals had clinical expertise in the assessment of sarcoidosis involvement of specific organs. A list of the organ group leaders and members is shown in Appendix 1.

Various clinical manifestations were assessed for the probability of organ involvement. Two criteria were required to be fulfilled in order to apply this assessment: 1) histologic evidence of granulomatous inflammation of unknown cause needed to be demonstrated in at least one organ that was not being assessed; 2) the clinical manifestation being assessed required that all alternative causes other than sarcoidosis for this clinical manifestation had been reasonably excluded. Provided that these two criteria were fulfilled, clinical manifestations were assessed by assigning them to one of three categories, highly probable, probable, or possible, interpreted as follows. **Highly probable**: such a manifestation is highly specific for sarcoidosis, with a likelihood of sarcoidosis causing this manifestation of at least 90%. In such cases, organ involvement may be assumed without a biopsy. **Probable**: such a manifestation is fairly specific for sarcoidosis, with a likelihood of sarcoidosis causing this manifestation of between 50 and 89%. In general, organ involvement in this category would be adequate to establish a clinical diagnosis of sarcoidosis in that organ. **Possible**: such a manifestation is not specific for sarcoidosis. For all organs, a biopsy showing granulomatous inflammation where alternative causes were reliably excluded was considered "highly probable"; therefore, such a biopsy was not included in this instrument.

Each organ group developed a list of common clinical conditions in their specific organ that could be considered as representing organ involvement with sarcoidosis. The probability of each of these clinical conditions as representing sarcoidosis was determined through organ group discussion that occurred via email and/or conference calls. Although organ group participants were encouraged to use the limited medical evidence available, these criteria were established by expert opinion. Other groups relied on voting of the members. After all organ assessments were developed

by each organ group, each of the clinical conditions was presented to all the organ group leaders and other sarcoidosis experts (all the authors of this manuscript) for a vote as to whether that clinical condition represented "highly probable," "probable," or "possible" involvement of sarcoidosis in that organ (vide supra). The voting group was blinded as to the assessments made by each specific organ group. It was this final vote by which the determination of likelihood of organ involvement on the basis of each clinical condition was based.

In terms of the voting, Delphi study methodology was used to determine that consensus was reached in that at least 70% of the experts needed to agree for consensus.[3] If at least 70% of the experts voted that the clinical condition was "highly probable" to represent sarcoidosis, a consensus was reached. If fewer than 70% of the experts voted that the clinical condition was "highly probable" but at least 70% of the experts voted that it was "highly probable" or "probable," then a consensus was reached that the clinical condition was at least probable. If at least 70% of the experts voted that the clinical condition was only "possible" to represent sarcoidosis, a consensus was reached. Finally, if less than 70% of the experts agreed that the clinical condition was a) "highly probable," b)"highly probable" or "probable" or c)"possible," then a consensus was not reached. In this case, it was unclear, in the opinion of these experts, if such a clinical condition was adequate or inadequate to represent organ involvement with sarcoidosis. After all these expert opinions were rendered, this manuscript was written and was presented to the Executive Committee of WASOG for editing, comments, and approval.

The following organs were evaluated in this instrument: lung, skin, eye, liver, calcium dysregulation, neural tissue, kidney, heart, peripheral lymph node, bone marrow, spleen, bone/joint, ear-nose-throat, parotid/salivary glands, and muscle. In addition, a category of "other organs" was created to encompass all organs involved other than the 15 specific ones listed above.

Results

Table 1 shows the characteristic of the experts. All but one cared for more than 50 sarcoidosis patients yearly. Almost all had participated in at least one clinical sarcoidosis trial and more than three-quarters had published more than 10 manuscripts concerning sarcoidosis.

Table 2 shows the voting results and consensuses reached in terms of the likelihood of various clinical manifestations representing specific organ involvement with sarcoidosis. For all of the 16 organs, the experts reached consensus that at least one clinical condition was as "at least 'probable'" as representing sarcoidosis. Although several of these clinical conditions concerned laboratory testing, several of them concerned physical examination findings or patient symptoms.

Discussion

We have proposed the WASOG Organ Assessment Instrument as an update of the original ACCESS organ assessment instrument[2] based on improvement in diagnostic testing for sarcoidosis and new medical evidence that has occurred over the previous decade. Although this document is based as much as possible on medical evidence, it also incorporates expert opinion of WASOG members and

Table 1. Characteristics of the Experts

Number of years since training completed (N, %):	
<5	2, 8%
5-10	3, 12%
10-20	6, 23%
>20	15, 58%
Number of sarcoidosis patients treated per year, on average:	
0 – 50	1, 4%
51 – 100	6, 23%
101 – 250	11, 42%
>250	8, 31%
Number of sarcoidosis clinical trials that you have participated in:	
0	3, 12%
1-3	9, 35%
4-10	10, 38%
>10	4, 15%
Number of publications authored or co-authored concerning sarcoidosis:	
0	0, 0%
1-10	6, 23%
11-20	4, 15%
>20	16, 62%

Table 2. Histological types of cancer in the "sarcoidosis and cancer" group.

	Highly Probable	At Least Probable	Possible	No Consensus
Lung	CXR: bilateral hilar adenopathy (19-2-0) Chest CT: perilymphatic nodules (18-2-1) Chest CT: symmetrical hilar/mediastinal adenopthy (21-0-0) PET/Gallium-67: mediastinal/hilar enhancement (17-4-0)	CXR: diffuse infiltrates (4-13-3) CXR: upper lobe fibrosis (9-10-2) Chest CT: peribronchial thickening (10-8-3) BAL: lymphocytic alveolitis (6-14-1) BAL: elevated CD4/CD8 ratio (11-9-1) PET/Gallium-67: diffuse parenchymal lung enhancement (9-8-4) TBNA: lymphoid aggregates/giant cells (7-8-5)	CXR: localized infiltrate (1-2-18) PFT: obstruction (1-2-17)	PFT: restriction (2-6-12) PFT: isolated reduction in diffusing capacity (2-6-12)
Skin	Lupus pernio (16-2-0)	Subcutaneous nodules or plaques (3-14-1) Inflammatory papules within a scar or tattoo (7-8-2) Violaceous or erythematous annular lesions (2-15-3) Violaceous or erythematous macular, papular lesions around the eyes, nose, or mouth (11-3-3)	Atypical lesions: ulcerative, erythrodermic, alopecic, ichthyosiform (0-2-14)	Verrucous/scaly papules or plaques (3-7-7) Hypo- or Hyperpigmented macules or patches (2-8-7)
Liver		Abdominal imaging demonstrating hepatomegaly (1-12-5) Abdominal imaging demonstrating hepatic nodules (3-13-1)		Hepatomegaly on physical examination (0-10-8) Serum alkaline Phosphate > 3X the upper limit of normal (3-8-6)
Eye	uveitis (16-1-0) optic neuritis (13-2-2) mutton fat keratic precipitates (12-1-3) iris nodules (13-3-0) snowball/string of pearls (pars planitis) (10-3-1)	lacrimal gland swelling (10-4-3) trabecular meshwork nodules (9-6-0) retinitis (5-9-0) scleritis (5-7-2) multiple chorioretinal peripheral lesions (6-8-1) adnexal nodularity (8-5-2) candle wax drippings (11-2-3)	cataract (0-1-15) glaucoma (1-3-12) red eye (0-3-14)	blindness (0-7-10) painful eye (0-5-10) cystoid macular edema (2-7-5)
Spleen		Low attenuation nodules on CT (7-11-1) PET/gallium-67 uptake in splenic nodules (7-11-1) Splenomegaly on imaging or physical examination (4-11-4)		
Salivary Gland	Positive gallium-67 scan ("Panda sign") (14-3-0) Positive PET scan of the parotid glands (12-5-0)	Symmetrical parotitis with syndrome of mumps (10-5-2) Enlarged salivary glands (2-10-5)	Dry mouth (2-3-12)	

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