Relation between Asymptomatic Proteinase 3 Antibodies and Future Granulomatosis with Polyangiitis

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Summary

Background and objectives The subclinical pathogenesis of granulomatosis with polyangiitis (GPA) has not been completely elucidated. Proteinase 3 (PR3) antibodies are strongly associated with GPA, but have not been evaluated before disease presentation.

Design, setting, participants, & measurements This was a retrospective case-control serum bank study in which PR3 antibodies and C-reactive protein (CRP) in up to three longitudinal serum samples for 27 GPA patients before diagnosis (1 day–19 years) were compared with 27 controls whose serum samples were matched for age, sex, and race. This study analyzed all patients with American College of Rheumatology criteria–confirmed disease identified in the Department of Defense electronic medical records between 1990 and 2008.

Results A greater percentage of GPA patients had at least one elevated PR3 antibody level (≥6 U/ml) as well as at least one detectable PR3 antibody level (>1 U/ml) before diagnosis compared with matching controls (63% [17 of 27] versus 0% [0 of 27], $P<0.001$; and 85% [23 of 27] versus 4% [1 of 27], $P<0.001$, respectively). A greater percentage of GPA patients had a >1 U/ml per year rate of increase in PR3 antibody level compared with matching controls (62% [21 of 26] versus 0% [0 of 26], $P<0.001$). PR3 antibody more frequently became elevated before CRP (67% [12 of 18] versus 33% [6 of 18], $P=0.04$).

Conclusions Subclinical PR3 antibody presence, trajectory, and temporal relationship to CRP associates with the future diagnosis of GPA. This data set further elucidates the pathogenesis of GPA.

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Introduction

The pathophysiology of granulomatosis with polyangiitis (GPA) has become better understood over the past 3 decades (1–4). Proteinase-3 antibodies (PR3-ANCA) are associated with GPA at diagnosis. However, the exact role of PR3-ANCA in the increasingly complex pathophysiology of GPA is less clearly defined (5–7). Animal, *in vitro*, and clinical evidence support direct PR3-ANCA pathogenicity. PR3-ANCA was recently shown to induce vasculitis in a rat model (8). PR3-ANCA stimulates neutrophils *in vitro* to both release inflammatory mediators and to damage endothelial cells (1,3,9,10). Some studies showed that an increase in remission PR3-ANCA levels associates with future relapse (11). However, PR3-ANCA could simply be a passive marker or just one of multiple culprits of disease. Healthy controls with detectable PR3-ANCA, patients with "seronegative" GPA, and patients with persistent asymptomatic PR3-ANCA in post-therapeutic clinical remission have all been reported (7,11,12).

In vivo production of PR3-ANCA before GPA diagnosis has not been previously investigated. C-reactive protein (CRP), previously shown to be associated with GPA at diagnosis, has also not been evaluated before disease (13,14). More importantly, the timing of PR3-ANCA production has not been compared with the timing of CRP elevation, with CRP elevation functioning as a nonspecific surrogate for early asymptomatic subclinical disease. Our objective was to describe the prediagnostic trajectory and temporal relationship of PR3-ANCA and CRP using the Department of Defense serum repository (DoDSR). We hypothesized that PR3-ANCA precedes both clinical and subclinical evidence of GPA, thus supporting the direct contribution of PR3-ANCA to GPA pathogenicity.

Materials and Methods

Patients

We performed a retrospective matched case-control DoDSR study of 27 patients with GPA disease. The DoDSR contains >50 million military serum samples banked from biennial HIV and deployment screenings. Specimens are linked to demographic, occupational, and medical information. The index sample is banked at the time of entry into the military when recruits are cleared for service with a standardized medical examination.

We identified 58 patients initially by International Classification of Diseases, Ninth Revision (ICD-9) code 446.40 ("Wegener's Granulomatosis") in the military electronic medical record and DoDSR databases between January 1990 and October 2008. Twenty-five patients had sufficient electronic medical records to confirm GPA diagnosis by meeting at least two of four American College of Rheumatology (ACR) criteria (15). One patient was excluded due to myeloperoxidase (MPO)-ANCA predominant disease. Six additional patients did not have serum in the DoDSR. A total of 18 patients with accessible electronic medical records remained. There were 33 patients identified in the DoDSR that did not have electronic medical records for review. These patients could have been diagnosed by civilian subspecialists if they were not located near a major military medical center and still had banked serum. However, it is possible that these patients were erroneously coded during an ultimately negative diagnostic evaluation. In addition to an ICD-9 code for GPA, patients were required to meet "modified" ACR criteria by having at least two additional systemic ICD-9 codes for pulmonary hemorrhage (786.3 "Hemoptysis" or "Pulmonary Hemorrhage"), renal involvement (580–589), or sinus involvement (461.9 and 473.9) to maximize patient specificity. Only 9 of 33 patients met these criteria, resulting in a combined total of 27 study participants.

The DoDSR provided a maximum of three 0.5-ml serum samples per patient to include the earliest index, the second to last, and the last samples before GPA diagnosis. In addition, the DoDSR identified one healthy control for each study participant matched for age (within 1 year), sex, race, and age of serum sample (within 90 days). Healthy controls were defined by the absence of ICD-9 codes for any chronic infectious, inflammatory, or malignant disease process in the DoDSR database.

Laboratory Assays

The DoDSR sent the serum samples to Quest Diagnostics Nichols Institute (Chantilly, VA). PR3-ANCA assays were performed with Varelisa PR3 ANCA enzyme immunoassay kits (Phadia GmbH, Freiburg, Germany). Briefly, 100 μl of diluted patient serum (1:101) was dispensed into wells coated with human PR3 antigen and prepared with wash buffer. After 30 minutes of incubation, the serum was removed and the wells were washed 3 times with wash buffer. This process was repeated with 100 μl of the enzyme-labeled second antibody (conjugate) followed by 100 μl of substrate 3,3′,5,5"-tetramethylbenzidine (TMB) (incubated in the dark). After removal of the substrate TMB, 50 μl of stop solution was added to the well. After no more than 30 minutes, absorbance (OD) was read at 450 nm with a reference wavelength of 620 nm. An individual calibration was performed for each run. The mean and median results of 432 apparently healthy participants were 0.7 U/ml and 0.6 U/ml, respectively. The average intra-assay and interassay variability were 5.5% and 5.0%, respectively (16). Clinical assays for CRP were performed with the COBA Integra C-Reactive Protein (Latex) cassette on a COBAS INTEGRA analyzer (Test CRPL2, 0-293; Roche Diagnostics, Indianapolis, IN). Quest Diagnostics was blinded as to whether the samples were from patients or controls. PR3-ANCA levels ≥6 U/ml and CRP levels >0.8 mg/dl were elevated.

Statistical Analyses

The two-tailed Fisher's exact test was used to compare all categorical values, specifically the number of study participants versus the number of control participants above various threshold PR3-ANCA values. Thresholds of >0, >1, >2, and ≥6 U/ml for PR3-ANCA were used to compare GPA patients with matched healthy controls at <1 year, 1–5 years, >5 years, and all time periods before diagnosis. Change in PR3-ANCA over time was analyzed using thresholds of >0 U/ml, >1 U/ml, and >2 U/ml per year, as well as 0%, 20%, and 200% per year. Absolute PR3-ANCA change per year was calculated by dividing the difference between last PR3-ANCA (PR3-ANCA-L) minus the index PR3-ANCA (PR3-ANCA-I) by the difference in days (T) between the two samples (TL − TI) and multiplying the total by 365 days per year, as follows: (PR3-ANCA-L − PR3-ANCA-I)/(TL − TI) × 365. Percentage change per year was calculated by dividing PR3-ANCA-L by PR3-ANCA-I, subtracting 1, and then multiplying by 100. This value was then divided by difference in days between the two samples (TL − TI) and multiplied by 365 days per year ((PR3-ANCA-L/PR3-ANCA-I) × 100/TL − TI) × 365. Infinite odds ratio values were estimated by adding 1 to the numerator (if 0 controls were positive) or denominator (if all study participants were positive) of both the disease and control groups.

This study was approved by the Human Use Committee at Walter Reed National Military Medical Center and informed consent was waived.

Results

Demographics

The GPA patients consisted of predominantly Caucasian men aged <40 years. Pulmonary and renal involvement was most common (Table 1).

PR3 Antibody

A greater percentage of GPA patients had at least one elevated PR3-ANCA (≥6 U/ml) compared with matching controls at any time before diagnosis (63% [17 of 27] versus 0% [0 of 27]; $P<0.001$) and <1 year before diagnosis (74% [14 of 19] versus 0% [0 of 19], $P<0.001$) (Table 2).

In addition, a greater percentage of GPA patients had at least one PR3-ANCA level above the threshold of 1 U/ml versus matching controls at any time before diagnosis (85% [23 of 27] versus 4% [1 of 27], $P<0.001$), <1 year before diagnosis (79% [15 of 19] versus 0% [0 of 19], $P<0.001$), and 1–5 years before diagnosis (69% [9 of 13] versus 0% [0 of 13], $P<0.001$) (Table 2).

Time Course of Antibody Development

A greater percentage of GPA patients had a rate of increase in PR3-ANCA of >1 U/ml per year compared with matching controls (62% versus 0%, $P<0.001$) (Table 3). In addition, a greater percentage of GPA patients had a rate of increase in PR3-ANCA of >20% per year compared with matching controls (77% versus 4%, $P<0.001$) (Table 3).

The mean and median number of days between the first elevation of PR3-ANCA and diagnosis of GPA were 269 and 239 days, respectively. Our results showed that 96%

Table 1. GPA study cohort

Characteristic	Value
Age (yr)	33 (18–60)
Race	
Caucasian	78
African American	7
Other	15
Meets ACR criteria for diagnosis	100 (27/27)
Male sex	89 (24/27)
Sinus involvement	48 (13/27)
Pulmonary involvement	82 (22/27)
Hemoptysis/respiratory failure	37 (10/27)
Renal involvement	85 (23/27)
ARF	33 (9/27)
Preceding CKD	0
Hypertension (chronic)	41 (11/27)
Biopsy evidence of vasculitis (total)	66 (12/18)
Kidney	22 (4/18)
Lung	33 (6/18)
Sinus	11 (2/18)
Skin	28 (5/18)
PR3-ANCA level (U/ml) (n=9)	61 (10–113); 1:640 (1:160–1:1280)
C-reactive protein (n=7)	7.8 (2.1–19.4)
Therapeutic intervention (cyclophosphamide, steroids, rituximab, methotrexate, azathioprine)	100 (18/18)
Abnormal chest CT scan or radiograph	92 (11/12)
Abnormal sinus CT scan	80 (4/5)
Abnormal chest or sinus imaging scan	100 (12/12)
Concurrent anti-GBM disease	0
Other antibody (ANA, dsDNA, Smith, RNP, Ro, La, SCL-70, Jo-1, GBM antibodies)	0 (0/8)

Data are presented as the median (range) or percentage (proportion). Comorbidity percentages may be underestimated due to incomplete background information from patients diagnosed at civilian hospitals. International Classification of Diseases, Ninth Revision codes cannot fully compensate for this limitation, because sometimes the final diagnosis is the only code listed when billing outside the military is not required. GPA, granulomatosis with polyangiitis; ACR, American College of Rheumatology; PR3, proteinase-3; CT, computed tomography; GBM, glomerular basement membrane; ANA, antinuclear antibody; dsDNA, double-stranded deoxyribonucleic acid; RNP, ribonucleoprotein; SLC-70, topoisomerase I.

of patients with an elevated PR3-ANCA became elevated <1.5 years before diagnosis, with one outlier at 932 days.

Time Course of Increase in Antibody versus CRP

Of GPA patients with a clear antecedent event, 67% had an elevated PR3-ANCA before an elevated CRP (Table 4). The most extreme example was a patient with a PR3-ANCA of 148 U/ml and a CRP of 0.05 mg/dl 241 days before diagnosis. Thirty-three percent of disease patients had an elevated CRP before an elevated PR3-ANCA (Table 4). The most extreme example was a patient with a PR3-ANCA of 1 U/ml and a CRP of 20.1 mg/dl 195 days before diagnosis.

Discussion

Our description of the natural history of PR3-ANCA before the diagnosis of GPA is consistent with the previously proposed complex pathophysiology of GPA (1–7). Specifically, our results support direct PR3 antibody pathogenicity in a subset of GPA patients. PR3-ANCA was elevated in 63% of disease patients predominantly <1.5 years before GPA diagnosis. Elevated prediagnostic PR3-ANCA levels with concurrent normal CRP levels in 67% of GPA patients suggest that PR3-ANCA levels cannot only elevate before overt clinical disease, but also before even subclinical serologic evidence of systemic inflammatory disease. Although there is no previous description of PR3-ANCA before GPA diagnosis, there are reports that PR3-ANCA precedes 33%–81% of postremission GPA relapses (17–20). CRP is known to be elevated before GPA relapse (21), but there has been no previous evaluation of CRP or the temporal relationship between CRP and PR3-ANCA elevation before initial diagnosis. Passive elevation of PR3-ANCA after another neutrophil activating event and before CRP elevation cannot be ruled out.

There are many explanations for why only 63% of GPA patients had elevated PR3-ANCA before diagnosis. First, the 10 study participants without an elevated PR3-ANCA could have developed elevated PR3-ANCA during the average 1038 days between the last serum sample and diagnosis. The maximum time between last sample and diagnosis was 4234 days, with three patients' last sample >6 years before diagnosis. Second, previously high PR3-ANCA could have degraded to normal levels during storage. Third, PR3-ANCA and alternate neutrophil activating mechanisms could both separately initiate GPA. Thirty-three percent of GPA patients first manifest an elevated CRP before an elevated PR3-ANCA supporting an alternate neutrophil activating mechanism. Elevated antibodies (cPR3, plasminogen, lysosomal-associated membrane protein 2, cathepsin G, lactoferrin, azurocidin, and elastase), elevated cytokines (TNF-α, IL-17, and IL-23), reduced regulatory T cell to effective T cell ratio, alterations in CD8+ T cell transcription, dysfunctional neutrophil apoptosis, and disrupted epigenetic silencing are all possible contributors to internal neutrophil activation (1,3,5,7,22). Environmental exposures (silica and asbestos), pathogens (*Staphylococcus aureus*, Ross virus, and *Entamoeba histolytica*), or medications (propylthiouracil and hydralazine) could all provide an external stimulus for neutrophil activation (2,3,23–28).

Our novel findings that GPA patients have higher levels of PR3-ANCA within the normal range than healthy controls are consistent with previously proposed multiple hit mechanisms of disease (1–7). The PR3-ANCA threshold of ≥6 U/ml was established based on the evaluation of patients with active clinical disease. It is possible that abnormal PR3-ANCA levels in the preclinical setting may occur in the currently accepted normal range. We previously demonstrated that a significantly larger percentage of patients with anti-glomerular basement membrane

Table 2. Comparison of the percentage of study patients with at least one serum sample with PR3-ANCA above the thresholds >0, >1, >2, and ≥6 U/ml compared with matched healthy controls

PR3 Antibody, U/ml (yr)	Patients (%)	Controls (%)	Odds Ratio	95% Confidence Interval	*P* Value (Fisher's Exact Test)
>/6					
All	63 (17/27)	0 (0/27)	48.6[a]	5.7 to 413[a]	<0.001
<1	74 (14/19)	0 (0/19)	57[a]	6.0 to 73[a]	<0.001
1–5	31 (4/13)	0 (0/13)	7.2[a]	0.72 to 72.7[a]	0.1
>5	0 (0/15)	0 (0/15)	1.0[a]	0.1 to 17.5[a]	1.0
> 2					
All	78 (21/27)	0 (0/27)	99[a]	11.1 to 885[a]	<0.001
<1	79 (15/19)	0 (0/19)	76[a]	7.7 to 751[a]	<0.001
1–5	39 (5/13)	0 (0/13)	9.8[a]	1.0 to 97[a]	0.03
>5	13 (2/15)	0 (0/13)	3.5[a]	0.3 to 37.5[a]	0.48
>1					
All	85 (23/27)	4 (1/27)	150	15.6 to 1436	<0.001
<1	79 (15/19)	0 (0/19)	76[a]	7.7 to 751[a]	<0.001
1–5	69 (9/13)	0 (0/13)	32.5[a]	3.1 to 338[a]	<0.001
>5	13 (2/15)	7 (1/15)	2.2	0.2 to 26.7	0.5
> 0					
All	96 (26/27)	56 (15/27)	20.8	2.5 to 176	<0.001
<1	95 (18/19)	37 (7/19)	30.9	3.4 to 284	<0.001
1–5	80 (10/13)	46 (6/13)	3.9	0.72 to 21.1	0.23
>5	73 (11/15)	40 (6/15)	4.1	0.9 to 19.3	0.14

Not all patients had samples available for each time period. If multiple serum samples were present for a patient in a specific subgroup analysis time period, the highest antibody level dictated group assignment. For example, the one control with PR3-ANCA >1 U/ml occurred in the >5-year time period. A higher percentage of patients had an elevated PR3-ANCA <1 year before diagnosis than over all time periods because the majority of patients without a banked sample a year before diagnosis did not have an elevated level. PR3, proteinase-3.
[a]Estimated due to actual infinite value.

Table 3. A comparison of the absolute and percent PR3-ANCA increase per year in GPA patients versus matching healthy controls

PR3 Antibody Elevation	Patients (%)	Controls (%)	Odds Ratio	95% Confidence Interval	*P* Value (Fisher's Exact Test)
U/ml per year					
>0	81 (21/26)	15 (8/26)	23.1	5.5 to 98	<0.001
>1	62 (16/26)	0 (0/26)	44.2[a]	5.2 to 377[a]	<0.001
>2	50 (13/26)	0 (0/26)	28[a]	3.3 to 237[a]	<0.001
>10	27 (7/26)	0 (0/26)	11.0[a]	1.3 to 95[a]	0.01
% per year					
>0	81 (21/26)	15 (4/26)	23.1	5.5 to 98	<0.001
>20	77 (20/26)	4 (1/26)	83	9.3 to 750	<0.001
>200	50 (13/26)	0 (0/26)	28[a]	3.3 to 237[a]	<0.001
>1000	23 (6/26)	0 (0/26)	9.1[a]	1.0 to 80[a]	0.02

Of the 27 study patients, 26 had multiple serum samples available for calculation. Calculations of change in percentage per year better adjust for patient variable serum sample time intervals before diagnosis. PR3, proteinase-3; GPA, granulomatosis with polyangiitis.
[a]Estimated due to actual infinite value.

disease have subclinical anti-glomerular basement membrane antibody above thresholds within the normal clinical range before diagnosis compared with matching controls (29). Neutrophil plasma membrane PR3 antigen burden is one explanation for higher baseline PR3-ANCA within the detectable but normal range. GPA patients have higher membrane PR3 antigen levels likely secondary to unique gene polymorphisms (30,31). Additional insults or hits, to include, but not limited to, Silica or pathogen exposures, may increase production of previously low-level asymptomatic PR3-ANCA to manifest clinical disease (28).

Finally, we demonstrated that PR3-ANCA levels increase over time in a statistically significant number of disease patients versus healthy controls. Previous literature describes the percentage of GPA relapses preceded by increased PR3-ANCA production as well as the potential

Table 4. Temporal relationship between surpassed PR3-ANCA thresholds and elevated CRP levels in GPA patients

PR3 Antibody (U/ml)	Before CRP (>0.8 mg/dl) (%)	After CRP (>0.8 mg/dl) (%)	Odds Ratio	95% Confidence Interval	*P* Value (Fisher's Exact)
>/6	67 (12/18)	33 (6/18)	4.0	1.0 to 16	0.04
>2	80 (16/20)	20 (4/20)	16	3.4 to 75	<0.001
>1	86 (18/21)	14 (3/21)	36	6.4 to 203	<0.001
>0	96 (23/24)	4 (1/24)	529	31.2 to 8977	<0.001

Antecedent increase in PR3-ANCA or CRP was established for patients with a serum sample that had only one biomarker above the designated threshold value. If both became elevated in the same sample or if neither was elevated in any sample, there was no antecedent elevation determined. Because subclinical PR3-ANCA levels above the threshold of 0 U/ml, 1 U/ml, and 2 U/ml within the normal clinical range were associated with future GPA, we also compared the percentage of GPA patients with an antecedent antibody above these thresholds to the percentage of patients that first had an elevated CRP. PR3, proteinase-3; GPA, granulomatosis with polyangiitis; CRP, C-reactive protein.

increased relapse risk with an ANCA four times normal, but not the rate of PR3-ANCA increase before relapse or before initial diagnosis (17–19,32).

One explanation for the data set as a whole is that GPA patients have a baseline genetic disease propensity, or first hit, reflected by persistent subclinical PR3-ANCA production. Subsequent internal or external hit(s) can either pathologically activate neutrophils or PR3-ANCA production. Activated neutrophils/monocytes increase membrane display and/or release of PR3 antigen and cytokines. PR3-ANCA and cytokines can activate neutrophils. With this evidence, it is reasonable to conclude that there could be more than one entry point into a common deleterious feedback cycle (2–4,7).

The natural history of subclinical PR3-ANCA better defines GPA pathophysiology. However, it is not realistic to screen an entire population for a disease with a low pretest probability. However, future genetic and epigenetic analysis may define a subpopulation at increased risk for disease (33,34). Polymorphisms in the PR3 promoter region, Fc-γ IIa and IIIa receptors, CD-18, C3, C4, IL-10, and the α-1 antitrypsin genes were found to be associated with GPA (3,33–35). PR3-ANCA vasculitis was specifically shown to be associated with HLA-DP, SERPINA1, and PRTN3 (33). However, at-risk genotypes do not guarantee clinical disease. Subclinical PR3-ANCA trends described in our study could provide diagnostic synergy with future high-risk genomes, epigenetic profiles, or proteomes. Evaluation of the prediagnostic serum levels of cPR3-ANCA, matrix metalloproteinases (*e.g.*, matrix metalloproteinase-3), CD8+ T cell transcript signatures, fibrinogen, and lysosomal-associated membrane protein 2 would help to even better define the subclinical pathophysiology of GPA (1,35). In addition, further evaluation of potential IgG subclass switching of PR3-ANCA would also have potential diagnostic value (36).

Our study has unique limitations. Although we erred on having overly specific criteria for confirmation of patients diagnosed *via* ICD-9 code, we cannot completely rule out the inclusion of a patient without GPA in the study group. This, in addition to degradation of antibody in stored serum, would only reduce the statistical significance of our findings by underestimating the number of study patients with detectable or elevated antibody. Although all patients were assigned a diagnostic code for Wegener's granulomatosis, most patients did not have confirmatory granulomas noted on histopathology even when present for review. It is possible that the study cohort included patients of microscopic polyangiitis based on strict Chapel Hill classification criteria. As used previously, small vessel vasculitis would be a reasonable alternate description of the study cohort (28). Due to de-identification requirements, serum bank results could not be linked to specific patient diagnostic data even when present, which prevents more robust secondary and subgroup analyses. We were also not able to reliably compare the timing of antibody elevation to the earliest patient symptoms. Our GPA study cohort skewed younger and more male than GPA diagnosed in the general population, most likely because it was derived from a current or recent active duty military population. Due to the retrospective evaluation of the prospectively compiled serum samples, the time from last sample to diagnosis and the intervals between samples vary between study participants. Although time-matched controls compensate for general comparisons, this makes the evaluation of specific time periods before diagnosis more challenging because of limited power in some subgroups. In addition, percentage and unit-per-milliliter rate of change per year assumes a linear rate of PR3-ANCA increase over time before diagnosis. This has not been proven. CRP is nonspecific and the results could have been confounded by undocumented infection. However, an initial CRP elevation remained elevated before GPA diagnosis in 100% of patients. In addition, 95% of all patients demonstrated an increase in CRP from their index to last sample, even within the normal range. This suggests, but does not prove, that the CRP elevations were not due to isolated acute infections. The study cohort included only PR3-ANCA GPA patients. Omission of MPO-ANCA patients was thought to be appropriate because they have genetically distinct associations compared with PR3-ANCA patients and are postulated to represent a different autoimmune syndrome (33).

The DoDSR, in conjunction with the large heterogeneous military population, provides the only known resource to analyze the subclinical trajectory of PR3-ANCA and CRP before GPA diagnosis. It is possible that our data could be incorporated with future genomic, epigenetic, and

subclinical biomarker data to formulate predictive diagnostic models for GPA that would require prospective confirmation.

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Disclosures

None.

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