
Glycosylation of proteinase 3 (PR3) is not required for its reactivity with antineutrophil cytoplasmic antibodies (ANCA) in Wegener's granulomatosis

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ABSTRACT

Objective. *The glycosylation status of autoantigens appears to be crucial for the pathogenesis of some autoimmune diseases, since carbohydrates play a crucial role in the distinction of self from non-self. Proteinase 3 (PR3), the main target antigen for anti-neutrophil cytoplasmic antibodies (ANCA) in patients with Wegener's granulomatosis (WG), contains two Asn-linked glycosylation sites. The present study explores the influence of the glycosylation status of PR3 on the PR3 recognition by ANCA in a well characterized population of patients with WG.*

Methods. *Forty-four patients with WG (459 serum samples) who participated in a multicenter randomized trial, were tested by capture ELISA for ANCA against PR3 and deglycosylated recombinant variants of PR3.*

Results. *The patients were followed for a median of 27 months, and the median number of serum samples per patient was 10. At baseline, the correlation between the levels of ANCA against PR3 and against all the deglycosylated recombinant variants of PR3 were greater than 0.94 ($p < 0.001$ for all the comparisons). Longitudinal analyses comparing the levels of ANCA against PR3 versus all the deglycosylated recombinant variants of PR3, using linear mixed models, showed no significant statistical differences ($\rho \geq 0.90$ in all cases).*

Conclusions. *The glycosylation status of PR3 has no impact on its recognition by ANCA in WG.*

Introduction

Proteinase 3 (PR3), a serine protease that is stored in granules of neutrophils and monocytes, is the main target anti-

gen for anti-neutrophil cytoplasmic antibodies (ANCA) in patients with Wegener's granulomatosis (WG) (1-4). PR3 contains two Asn-linked glycosylation sites, Asn-113 (Asn-Leu-Ser) and Asn-159 (Asn-Val-Thr) (5). In a previous study, we found that glycosylation occurs at both sites in native PR3 as well as in recombinant PR3 (rPR3), and that glycosylation at Asn-113 was critical for thermal stability and for optimal hydrolytic activity of PR3 (6).

Glycosylation is the most common post-translational modification of proteins (about 50 to 70% of human proteins are subject to glycosylation), and important biological information is contained in spatially accessible carbohydrate structures of glycoproteins (7). Importantly, carbohydrates play a crucial role in the distinction of self from non-self (7), and variable post-translational modifications of protein glycosylation affect the recognition of antigens by the immune system (8, 9). Aberrant glycosylation may render target antigens more immunogenic by exposing cryptic epitopes (10, 11), while deglycosylated antigens may be more susceptible to proteases involved in the generation of autoantigenic peptides (11, 12). Furthermore, for some autoimmune diseases, glycosylation of the target antigen seems to be a requirement for its antigenicity (13-15). Since modifications of the PR3 molecule affect the reactivity of ANCA and could provide important insight in the pathogenesis of WG, we conducted the present study to investigate the influence of the PR3 glycosylation status on the recognition of PR3 by ANCA in a well characterized longitudinal population of patients with WG.

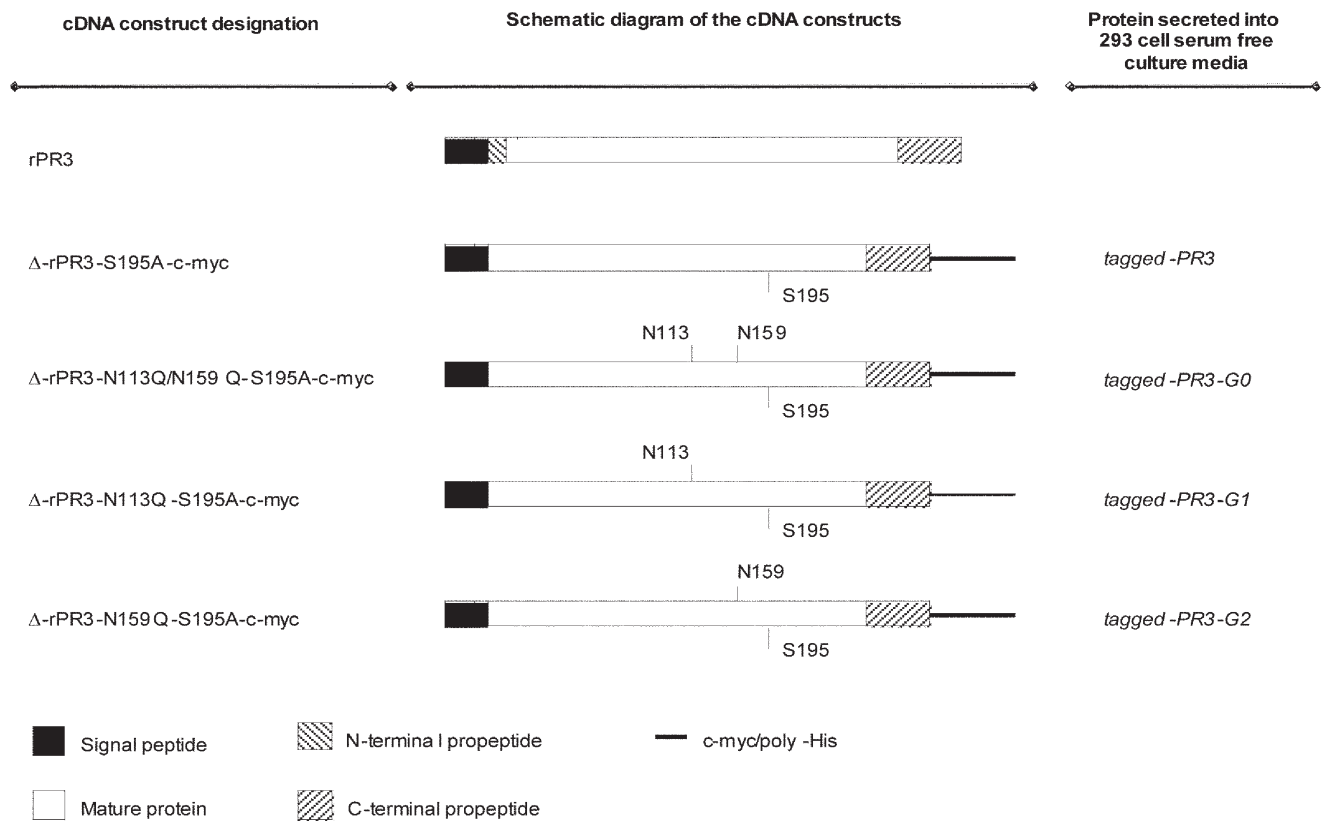


Fig. 1. Proteinase 3 and related cDNA constructs.

The transfection of 293 cells with rPR3 results in the expression of a protein in the serum free media that carries both the amino- and carboxy-terminal propeptides (19, 20), precluding the folding of PR3 into its mature conformation. Therefore, all the cDNA constructs used in this study do not code for the N-terminal propeptide, resulting in the expression of PR3 variants with a mature conformation (20). Additionally, all the constructs carry the mutation S195A, which results in lack of enzymatic activity without affecting their recognition by PR3-ANCA (20). The addition of the c-myc tag to the carboxy-terminus of PR3 also does not affect binding of PR3-ANCA (19). The constructs Δ -rPR3-N113Q-S195A-c-myc, and Δ -rPR3-N159Q-S195A-c-myc carry the mutations N113Q and N159Q coding for glutamine instead of the asparagine in position 113 and 159, respectively. As a result, their corresponding expressed proteins, *tagged-PR3-G1* and *-G2*, do not carry a glycosylation residue in position 113 and 159, respectively. The construct Δ -rPR3-N113Q/N159Q-S195A-c-myc carries both mutations, expressing a protein, *tagged-PR3-G0*, without any Asn-linked sugar moieties. Protein sequences are numbered based on the crystal structure correspondence with chymotrypsinogen A (5, 17).

Material and methods

Unless specified otherwise, all reagents were purchased from Sigma (St. Louis, MO). The 293 (adenovirus type 5 transformed human embryonic kidney) cell line was obtained from ATCC (Rockville, MD). Anti-c-myc coated 96-well ELISA plates (mouse anti-c-myc moAB) were purchased from Sigma (P 2241), and purified human neutrophil PR3 from Athens Research and Technology (Athens, GA, USA). The mouse monoclonal antibody (moAB) MCPR3-2 and the rabbit polyclonal antibody, both against human PR3 have been previously described (16). The mouse moAB against the c-myc polypeptide, was purchased from Invitrogen (San Diego, CA).

c-DNA constructs and cell transfection

Fig. 1 shows the cDNA constructs used. Protein sequences are numbered based on the crystal structure correspondence with chymotrypsinogen A (5, 17).

The construct coding for Δ -rPR3-S195A-c-myc, as well as their expression in serum-free culture media supernatants of stably transfected 293 cells have been previously described (18). Clones of 293 cells transfected with Δ -rPR3-S195A-c-myc express *tagged-PR3*.

The cDNA constructs for the deglycosylated variants, Δ -rPR3-N113Q/N159Q-S195A-c-myc, Δ -rPR3-N113Q-S195A-c-myc, and Δ -rPR3-N159Q-

S195A-c-myc were prepared with the Quick-Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The primers are presented in Table I.

To generate Δ -rPR3-N113Q-S195A-c-myc, the construct rPR3-c-myc was used as template (19), and the primers US181 and US182 were used to produce the mutation of asparagine-113 to glutamine. Then the primers *MactI* and *MactII* were used to produce the mutation of serine-195 at the active site to alanine, and the primers US205 and US206 were used to remove the six nucleotides coding for the amino-terminal propeptide. Adherent 293 cells were transfected using the calcium phosphate precipitation method

Table I. Primers used for the preparation of the cDNA constructs coding for Δ -rPR3-N113Q/N159Q-S195A-c-myc, Δ -rPR3-N113Q-S195A-c-myc, and Δ -rPR3-N159Q-S195A-c-myc.

Name	Sequence	
US181	5'-GCAGCCCAGCC <u>CA</u> ACTCAGTGCC-3'	sense
US182	5'-GGCACTGAGT <u>T</u> GGGCTGGGCTGC-3'	antisense
US183	5'-GCAGGAGCTC <u>C</u> AGGTCACCGTGG-3'	sense
US184	5'-CCACGGTGAC <u>C</u> TGGAGCTCCTGC-3'	antisense
<i>MactI</i>	5'-ATCTGCTTCGGAGACG <u>C</u> CGGTGGCCCCCTGATC-3'	sense
<i>MactII</i>	5'-GATCAGGGGGCCACCG <u>G</u> CGTCTCCGAAGCAGAT-3'	antisense
US205	5'-GCCCGAGCTATCGTCGGC-3'	sense
US206	5'-GCCACGATAGCTCGGGC-3'	antisense

The underlined nucleotides in US181 and US182 convey the mutation asparagine-113 to glutamine. The underlined nucleotides in US183 and US184 convey the mutation asparagine-159 to glutamine. The underlined nucleotides in *MactI* and *MactII* convey the mutation of serine-195 at the active site to alanine. US205 and US206 were used to remove the six nucleotides coding for the amino-terminal propeptide of proteinase 3.

(Stratagene) (20). Stable clones transfected with Δ -rPR3-N113Q-S195A-c-myc were selected in the presence of Zeocin (200 μ g/mL) (Invitrogen, Carlsbad, CA), and express *tagged-PR3-G1*.

Similarly, to generate Δ -rPR3-N159Q-S195A-c-myc, the construct rPR3-c-myc was used as template, and the primers US183 and US184 were used to produce the mutation of asparagine-159 to glutamine. Then the primers *MactI* and *MactII*, and the primers US205 and US206 were used as in the previous construct. Adherent 293 cells were transfected using the calcium phosphate precipitation method (Stratagene) (20). Stable clones transfected with Δ -rPR3-N159Q-S195A-c-myc were selected in the presence of Zeocin (200 μ g/mL) (Invitrogen, Carlsbad, CA), and express *tagged-PR3-G2*.

To generate Δ -rPR3-N113Q/N159Q-S195A-c-myc, the construct, rPR3-c-myc was used as template, and the primers US181 and US182, and US183 and US184 were used to produce the mutation of asparagines-113 and -159 to glutamine, respectively. The primers *MactI* and *MactII*, and the primers US205 and US206 were used as in the previous constructs. Adherent 293 cells were transfected using the calcium phosphate precipitation method (Stratagene) (20). Stable clones transfected with Δ -rPR3-N113Q/N159Q-S195A-c-myc were selected in the presence of Zeocin (200 μ g/mL) (Invitrogen, Carlsbad, CA), and express *tagged-PR3-G0*.

Detection of c-myc tagged proteins

Clones were screened for expression of c-myc tagged PR3 variants by capture ELISA of their serum-free culture media supernatants as previously described (18). Plates coated with mouse anti-c-myc moAB (Sigma P2241) were used as the capturing antibody, and the rabbit polyclonal antibody against human PR3 was used as the detecting antibody.

Immunoblotting

Proteins were precipitated in 55% trichloroacetic acid, separated by SDS-PAGE (12% gels) under reducing conditions, transferred to nitrocellulose membranes, and probed with the mouse anti-c-myc antibody and with the mouse monoclonal antibody against human PR3 (MCPR3-2) for PR3 variants. Bound antibodies were detected using goat anti-mouse and goat anti-rabbit peroxidase conjugated antibodies (BioRad, Hercules, CA, USA) and the ECL chemiluminescence system (Amersham Biosciences, Buckinghamshire, UK).

Serum samples

Samples used in this study were obtained from the Wegener's Granulomatosis Etanercept Trial (WGET), a multi-center, randomized, double-blind, placebo-controlled trial that evaluated etanercept for maintenance of remission in 180 patients with Wegener's granulomatosis (21). Follow-up evaluations occurred at baseline, after 6 and 12 weeks, and then every 3 months until the end

of the trial. Two additional evaluations took place at 3 and 6 months after closeout. During each visit, disease activity was measured using the Birmingham Vasculitis Activity Score for Wegener's granulomatosis (BVAS/WG), and serum samples were obtained, frozen, and stored at -80°C . The WGET protocol was approved by the Institutional Review Board at each participating center, and informed written consent was obtained from all participants. Details of the study design, patient characteristics, and trial results have been published previously (21-23).

ANCA detection methods

Standard indirect immunofluorescence (IF), direct ELISAs for PR3-ANCA and MPO-ANCA using commercially available kits (Scimedex, Corporation, Denville, NJ) and the PR3-ANCA capture ELISA were performed as previously described (16, 20).

For detection of c-myc tagged PR3 variants, a novel anti-c-myc capture ELISA was used as described elsewhere (18). In this assay, plates commercially coated with mouse anti-c-myc moAB (Sigma P2241) are used to capture the c-myc tagged PR3 variants (antigens) present in the serum-free culture media supernatants of 293 cells. Bound antibodies from the patient sera are then detected with an alkaline phosphatase-conjugated goat anti-human IgG. Serum samples are run in singles; results are expressed as the net absorbance, calculated by subtraction of the background values from the values obtained from wells containing captured antigens (18).

Baseline serum samples were initially tested at first thaw by IF, direct ELISA for both PR3- and MPO-ANCA, and capture ELISA for PR3-ANCA, and all the serum samples from the 180 patients were tested for *tagged-PR3* at first thaw (24). Serum samples were then tested for *tagged-PR3-G0*, *-G1*, and *-G2* in parallel at second thaw. To minimize variability, all serum samples from an individual patient were run at once in the same plate, and the same lots of all reagents were used for all assays. All laboratory personnel were blinded to the clinical data.

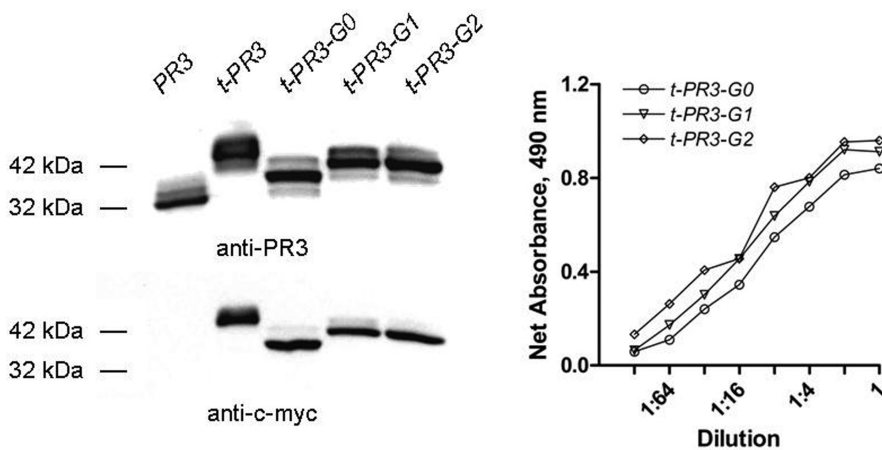


Fig. 2. Expression of c-myc tagged deglycosylated variants of PR3 in 293 cells. All the c-myc tagged deglycosylated variants of PR3 carry indeed the c-myc tag extension, in contrast to purified native PR3. The upper panel shows purified human PR3 and all the c-myc tagged deglycosylated variants of PR3 when probed with MCPR3-2, a mouse monoclonal antibody against human PR3 (anti-PR3). In the lower panel, all c-myc tagged deglycosylated variants of PR3 could be detected when probed with the mouse monoclonal antibody against the c-myc tag polypeptide (anti-c-myc), but not purified human PR3. Proteins were precipitated in 55% trichloroacetic acid and separated by SDS-PAGE (12% gels) under non-reducing conditions. The right panel show the saturation curves of the serum-free culture media supernatants of 293 cells transfected with the c-myc tagged deglycosylated variants of PR3, using plates coated with mouse monoclonal antibody anti-c-myc (Sigma P2241) as the capturing antibody, and using the rabbit polyclonal antibody against human PR3 as detection antibody. PR3: Purified human PR3. *t-PR3*: *tagged-PR3* from Δ -rPR3-S195A-c-myc transfected 293 cells. *t-PR3-G0*: *tagged-PR3-G0* from Δ -rPR3-N113Q/N159Q-S195A-c-myc transfected 293 cells. *t-PR3-G1*: *tagged-PR3-G1* from Δ -rPR3-N113Q-S195A-c-myc transfected 293 cells. *t-PR3-G2*: *tagged-PR3-G2* from Δ -rPR3-N159Q-S195A-c-myc transfected 293 cells.

Table II. Characteristics of the patients at baseline.

Characteristic	Included (n= 44)	Excluded (n= 136)	p-value
Age (years)-median (IQR)	48 (38-62)	52 (41-62)	0.576
Male gender, n. (%)	24 (55)	84 (62)	0.396
Whites, non-Hispanic, n. (%)	39 (89)	127 (93)	0.479
Limited disease, n. (%)	16 (36)	36 (26)	0.208
Disease newly diagnosed at enrolment, n. (%)	14 (32)	66 (49)	0.052
Time since diagnosis (months) - median (IQR)	14 (1-42)	3 (1-32)	0.082
Etarnecept	24 (55)	65 (48)	0.436
BVAS/WG - median (IQR)	6 (4-10)	6 (4-10)	0.302
Organ involvement, n. (%)			
General	32 (73)	97 (71)	0.856
Cutaneous	7 (16)	29 (21)	0.435
Mucous membranes/eyes	15 (34)	32 (24)	0.116
Ear, nose and throat	33 (75)	105 (77)	0.764
Cardiovascular	0 (0)	2 (1)	1.000
Gastrointestinal	0 (0)	2 (1)	1.000
Pulmonary	23 (52)	85 (62)	0.229
Renal	21 (48)	76 (56)	0.346
Nervous system	2 (5)	15 (11)	0.250
Creatinine (mg/dL)-median (IQR)	1.1 (0.8-1.3)	1.1 (0.9-1.8)	0.144
Sedimentation rate (mm)-median (IQR)	22 (10-46)	25 (13-47)	0.479
C-ANCA (IF), n. (%)	32 (73)	104 (76)	0.612
P-ANCA (IF), n. (%)	6 (14)	18 (13)	0.946
PR3-ANCA direct ELISA, n. (%)	32 (73)	101 (74)	0.841
MPO-ANCA direct ELISA, n. (%)	4 (9)	5 (4)	0.225
PR3-ANCA capture ELISA, n. (%)	32 (73)	104 (76)	0.616
<i>tagged-PR3</i> anti-c-myc capture ELISA, n. (%)	35 (80)	113 (83)	0.593

BVAS/WG: Birmingham Vasculitis Score for Wegener's Granulomatosis; IQR: interquartile range.

Statistical methods

All analyses were performed using SAS® (version 9.1; SAS Institute, Inc, Cary, NC). Descriptive data were summarized as mean (standard deviation, SD), median (interquartile range, IQR), or percentages. The baseline characteristics of the patients included in this study were compared to the excluded patients by the Student's *t*-test (or the rank sum test) for continuous variables or by the Chi-square test (or Fisher's exact test) for categorical variables.

Comparisons of baseline anti-c-myc capture ELISA for *tagged-PR3-G0*, *-G1*, and *-G2*, and *tagged-PR3* were assessed using Pearson correlation. Longitudinal analyses were performed using mixed linear models (PROC MIXED) to further assess whether anti-c-myc capture ELISA for *tagged-PR3-G0*, *-G1*, and *-G2* changed differentially over time compared to anti-c-myc capture ELISA for *tagged-PR3*. Since not all patients had data available for all visits, this analysis was performed using all available data and was repeated using data from only the first 24 months and also from only the first 12 months. In all cases, two-tailed *p*-values ≤ 0.05 were considered statistically significant.

Results

Expression of recombinant c-myc tagged PR3 variants in 293 cells

Figure 2 shows the expression of the c-myc tagged PR3 variants by immunoblotting. As expected, because of the c-myc tag incorporation, all these variants have a higher molecular mass compared to purified human PR3 (left upper panel). Probing the blotted proteins with the mouse anti-c-myc antibody confirmed that these variants carry the c-myc tag (left lower panel). Additionally, all c-myc tagged deglycosylated variants could be captured by ELISA using plates coated with anti-c-myc moAb (right panel). This assay was also used to determine the optimal dilution of serum-free culture media supernatant containing c-myc tagged deglycosylated PR3 variants to be used in the c-myc capture ELISA for subsequent ANCA detection. Saturation of the binding capacity of the mouse anti-c-myc moAb

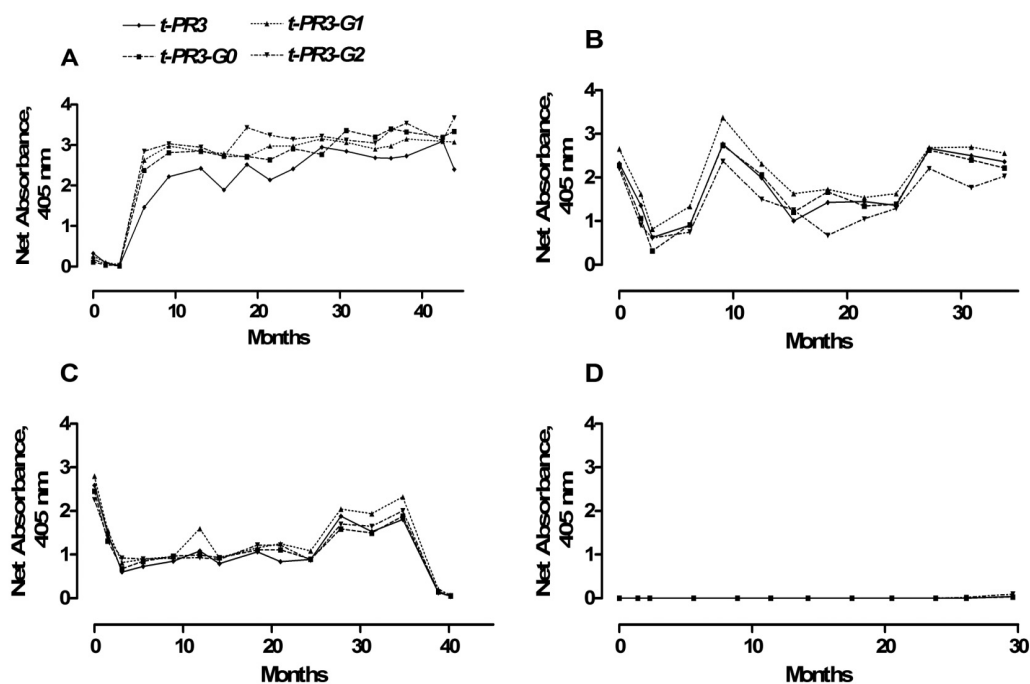


Fig. 3. Examples of patients tested over time for ANCA reactivity with *tagged-PR3* and *tagged deglycosylated variants of PR3*

Each plot shows the levels of *tagged-PR3* and *tagged-PR3-G0*, *-G1*, and *-G2* over time in four different patients. In all the cases, no differences in the pattern of change could be seen among these four determinations. *t-PR3*: *tagged-PR3*. *t-PR3-G0*: *tagged-PR3-G0*. *t-PR3-G1*: *tagged-PR3-G1*. *t-PR3-G2*: *tagged-PR3-G2*.

was achieved with a 1:1.3 dilution of *tagged-PR3-G0* containing media, and 1:2 dilution of both *tagged-PR3-G1* and *tagged-PR3-G2* containing media. The saturation curve for *tagged-PR3* and the dilution (1:4) used in the c-myc capture ELISA have been previously described (18).

Patient characteristics

The original plan was to test all serum samples (1,846) of the 180 patients by anti-c-myc capture ELISA for *tagged-PR3-G0*, *-G1*, and *-G2*, and to compare them to *tagged-PR3*. However, because each serum sample was tested simultaneously for *tagged-PR3-G0*, *-G1*, and *-G2*, it became apparent after completing 44 patients chosen at random (24% of the whole cohort) that no meaningful difference was going to be found among these tests. Given the limited amount of serum available, and the need of serum for future studies, it was decided to limit the study to only these 44 patients.

Demographics, clinical and serological characteristics at baseline of the included patients are presented in Table II. Compared to the excluded patients, there were no significant differences in any of the variables analyzed. These 44 patients were followed for a median (IQR) of 27 (16-34) months, and the

median (IQR) number of serum samples per patient was 10 (6-13). The full WGET cohort has been previously described (21, 23).

ANCA detection by anti-c-myc capture ELISA for *tagged-PR3-G0*, *-G1*, and *-G2*, compared to *tagged-PR3*

All the available 459 samples (25% of all the WGET samples) of the 44 patients included in this study were tested by the anti-c-myc capture ELISA for *tagged-PR3-G0*, *-G1*, and *-G2* and for *tagged-PR3*.

The initial analysis consisted of a comparison of the anti-c-myc capture ELISA for *tagged-PR3-G0*, *-G1*, and *-G2* and the anti-c-myc capture ELISA for *tagged-PR3* in the baseline serum samples. Very strong correlations were found between the anti-c-myc capture ELISAs for *tagged-PR3-G0* and *tagged-PR3* ($r=0.94$, $p<0.001$), between the anti-c-myc capture ELISAs for *tagged-PR3-G1* and *tagged-PR3* ($r=0.96$, $p<0.001$), and between the anti-c-myc capture ELISAs for *tagged-PR3-G2* and *tagged-PR3* ($r=0.95$, $p<0.001$).

Separate comparisons of the longitudinal changes in the ANCA levels obtained by the anti-c-myc capture ELISAs for *tagged-PR3-G0* versus *tagged-PR3*, by the anti-c-myc capture ELISAs for *tagged-PR3-G1* versus *tagged-PR3*, and by the anti-c-myc capture ELISAs

for *tagged-PR3-G2* versus *tagged-PR3* for each patient were performed using linear mixed models. In all these comparisons, no difference in the pattern of change in the ANCA levels was found ($p\geq 0.90$ in all cases). Examples of individual patients are presented in Figure 3.

Discussion

A previous immunoblot study showed that 5 sera from patients with WG, with high titers of PR3-ANCA, bound with similar affinity to neutrophil PR3 and to neutrophil PR3 treated with N-glycanase, which releases all common classes of Asn-linked oligosaccharides (25). The authors concluded that the binding of ANCA to PR3 was independent of the Asn-linked glycosylation of PR3 once it had assumed its disulfide bond constrained conformation. We found however, using a capture ELISA with lysates of human mast cells (HMC-1) expressing rPR3 with both, one or no Asn-linked glycans as antigens, that the binding of ANCA to PR3 was affected by the glycosylation status of the later in 8 of 40 (20%) patients with WG (6). Substantial differences in techniques used in these two studies may account for the apparent discrepancies. Therefore, the present investigation was undertaken to further analyze the clinical relevance of these findings.

In this longitudinal study we found that the glycosylation status of PR3 does not affect the recognition by ANCA. At baseline the correlations between ANCA targeting PR3 and all the deglycosylated variants of PR3 were very strong. Longitudinally, no significant differences were seen in the pattern of change of the levels of ANCA targeting PR3 and all its glycosylation variants. It is possible however, that small differences still exist between ANCA targeting PR3 and ANCA targeting the deglycosylated variants of PR3 (this might be an explanation for the findings of our previous study (6)), but since their levels over time almost overlaps, these differences are unlikely to have any clinical implications.

The present study has several important strengths. The patients included in this study participated in a multi-center randomized trial, had active disease at enrollment, and were treated in a protocolized manner (21). The serum samples were tested in ideal conditions using a newly developed capture ELISA (18). This new assay is based on commercially available covalently coated plates, which eliminates one operator-dependent step from the procedure, improving the inter-assay coefficient of variation compared to standard capture ELISAs (18). This new anti-c-myc capture ELISA is based on the recognition of a tag added to the antigen rather than the recognition of an antigen by a monoclonal antibody, which can compete for an epitope recognized by ANCA, potentially causing a false-negative result (18). We had previously shown that this tag does not change the conformation of PR3 or the binding of PR3-ANCA (19). Consequently, this assay has several advantages over other solid phase assays used commercially for PR3-ANCA detection (26, 27).

A potential limitation of this study is that not all the WGET patients were included. We believe however, that the results would have been the same if the entire cohort would have been included because the 44 analyzed patients were selected at random, without knowledge of their clinical characteristics, treatment assignment or PR3-ANCA reactivity, and additionally the baseline

characteristics of the included and excluded patients were similar.

Our study demonstrates that the glycosylation status of PR3 has no impact on its recognition by ANCA in serum from patient with WG. Furthermore, if PR3-ANCA recognizing different conformational epitopes of PR3 change in individual patients over time, our data indicate that the presence or absence of sugar side chains of PR3 does not affect the conformation of these epitopes.

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Appendix

The WGET Research Group

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