ANCA: SEROLOGY IN WEGENER'S GRANULOMATOSIS

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ABSTRACT

BACKGROUND AND OBJECTIVES *Wegener's granulomatosis (WG) is being increasingly diagnosed in India, which exists in two forms, the 'limited Wegener's granulomatosis' (LWG) having upper respiratory tract (URT) and lower respiratory tract (LRT) involvement and the 'classical Wegener's granulomatosis' (CWG), with the triad of URT, LRT involvement along with kidney involvement. Cytoplasmic ANCA (C-ANCA) or anti-Proteinase3 (anti-PR3), which is highly diagnostic for WG, rarely perinuclear ANCA (P-ANCA) may exist. Aims To detect anti-neutrophil cytoplasmic antibodies (ANCA) and correlate it with serological, hematological parameters, and the Birmingham Vasculitis Activity Score (BVAS). SETTINGS AND DESIGN Twenty-three clinically and histopathologically proven WG (16 CWG, 7 LWG) were studied. MATERIAL AND METHODS C-ANCA and P-ANCA patterns were identified by immunofluorescence and specificities were confirmed by 'α granule' enzyme linked immunosorbent assay (ELISA), anti-PR3, anti-MPO (myeloperoxidase) and anti-Lactoferrin (anti-LF) by ELISA. RESULTS LRT involvement was seen in 91.3%, URT in 78.3%, and renal manifestations in 69.6% cases. The BVAS in CWG was significantly higher than BVAS in the LWG. Decreased hemoglobin, increased WBC counts, ESR, CRP and Creatinine were seen in CWG as compared to LWG. The C-ANCA was present in 65.2% patients and P-ANCA in 13% cases. Anti-PR3 was seen in 69.6% patients and anti-LF in 17.4% cases. Severity of disease and ANCA was higher in CWG than in LWG.*

Conclusions *Vasculitis syndromes are known to overlap and many go undetected; therefore ANCA testing, along with the clinical and histopathological observations may be helpful in early detection and management of WG cases.*

KEY WORDS: *anti-neutrophil cytoplasmic antibodies; anti-Proteinase3; cytoplasmic ANCA; enzyme linked immunosorbent assay; indirect immunofluorescence; Wegener's granulomatosis.*

Wegener's granulomatosis (WG), a systemic necrotizing granulomatous vasculitis, has upper and lower respiratory tract (LRT) involvement manifesting with fatal pulmonary hemorrhage to sinusitis and accompanying renal involvement. If untreated, the disease carries a high mortality and delay in diagnosis could lead to death of the patients.[1],[2] In 1985, van der Woude et al. first reported that IgG autoantibodies against cytoplasmic components of neutrophils, granulocytes, and monocytes have a immunodiagnostic potential for WG. The titers of anti-neutrophil

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cytoplasmic antibodies (ANCA) too, often correlated well with the activity of the disease.[3]–[5]

The methods used in the demonstration of ANCA are indirect immunofluorescence (IIF) test or enzyme-linked immunosorbent assays (ELISAs), either using a purified neutrophil cytoplasmic extract called as 'α granules (G)' or using purified neutrophil cytoplasmic granules like myeloperoxidase (MPO) or Proteinase3 (PR3). Immunofluorescence is still the method of choice for ANCA detection,[6],[7] and is the most commonly used technique where perinuclear ANCA (P-ANCA), cytoplasmic ANCA (C-ANCA), and atypical ANCA (X-ANCA) patterns could be well distinguished. Cytoplasmic ANCA pattern is mostly associated with WG and is less often seen in other conditions, and its major target antigen is PR3.[8]

An increase in C-ANCA titer often precedes relapse of WG while a decrease in C-ANCA is seen with remission. Treatment, based on changes in serum C-ANCA titers, prevents disease relapses.[9] Monitoring of C-ANCA, in most cases, would therefore be of great value in distinguishing between changes due to WG and symptoms caused by other diseases. The present study was undertaken to identify the specificities and strength of ANCA and its subtypes in classical and limited WG cases by IIF and ELISA, and to correlate them with systemic or organ involvement and with other laboratory parameters.[10],[11]

MATERIAL AND METHODS

Twenty-three patients with an established diagnosis of WG were studied for ANCA serology. The diagnosis of WG was established according to the clinical and histopathological observations.[3] All these patients were classified according to the American College of Rheumatologists (ACR) criteria.[12] The clinical and laboratory findings were carefully noted on the specially designed proforma. Systemic necrotizing vasculitis scores were noted in all cases on the basis of the Birmingham Vasculitis Activity Scores (BVAS).[13] This prospective study was carried out over a period of 4 years (2000–2004) after obtaining the requisite Ethics committee permission and informed consents from patients. Patients' selection bias was avoided by selecting cases only after they were diagnosed by clinicians and the blinding was achieved, as they were unaware of the laboratory findings. The patients having the ages of above 18 years were included in the study and the exclusion criteria was HIV and HbsAg positivity, as well as the pregnant and lactating women. Confirmation of the diagnosis of renal vasculitis, necessitated renal biopsies examined by light microscopy and also by immunofluorescent microscopy using anti-IgG, anti-IgM, anti-IgA, anti-C3, anti-C4, and anti-fibrinogen FITC conjugates (Sigma, USA). Blood (4–5 ml) was aseptically collected from each patient and the separated serum was divided into two parts and frozen in 1 ml aliquots at -80°C until use.

Anti-neutrophil cytoplasmic antibodies were detected using human neutrophils (PMN) by indirect IIF technique.[9] Human neutrophils were used to prepare a cytospun substrate using Hettich Univaersal 16A cytocentrifuge and some slides were fixed with 96% ethanol

and others with formalin. After reacting with test serum, the slides were probed using fluorescein isothiocynate (FITC) tagged polyvalent anti-human globulin serum and observed under a fluorescent microscope, Nikon Optiphot II, Japan. Microphotography was also done using an automated photography system, Nikon AFX II A, Japan. The test was considered positive when P-ANCA, C-ANCA or any unusual X-ANCA patterns were noted. All the positive sera were diluted further by double dilution technique and the same test protocol was followed to know the end point titration i.e. the highest dilution of test serum showing a positive result. Ten control sera having anti-MPO and anti-PR3 antibodies were used as required. A cut off for positivity was 1 : 10 dilution of test serum for ANCA testing. Anti-neutrophil cytoplasmic antibodies were also detected by a rapid ELISA using ultrasonicated neutrophil cytoplasm extract called as (αG).[14],[15] A cutoff of normal human serum (NHS) ± 2SD was considered as positive. The specificity of the antibodies was also confirmed by antigen binding ELISAs for anti-myeloperoxidase (anti-MPO) and anti-Proteinase3 (anti-PR3) using kits from Genesis (UK). A value <3.0 m/ml was negative, 3–5 m/ml equivocal and >5 m/ml was considered as positive. Anti-Lactoferrin (anti-LF) ELISA was developed in the laboratory using purified Lactoferrin from Sigma, USA and the assay was standardized.[16]

Anti-nuclear antibodies (ANA) were qualitatively and quantitatively tested by IIF test using HEp-2 cells obtained from Enterovirus Research Center, ICMR, Mumbai. Cells were maintained in a continuous culture and harvested at log phase of growth. The results were interpreted in terms of titers, that is, test sera giving positivity for IIF at its highest dilution. A cut off for positivity was 1 : 20 dilution of test serum for ANA testing.[17],[18] All ANA positives were discriminated from true ANCA positives as perinuclear or P-ANCA shows a IIF rim pattern just around or outside the nucleus of the PMN on ethanol fixed preparations, while ANA shows IIF of the nuclear components inside the nucleus. Anti-double stranded antibodies (anti-dsDNA) antibodies were detected by ELISA.[19]

RESULTS

The 23 patients included in this study with an established diagnosis of WG, were divided into two groups. One group consisted of 16 patients (69.6%) of classical Wegener's granulomatosis (CWG) having clinical manifestations of upper respiratory tract (URT), LRT and renal involvement. The other group comprised of seven patients (30.4%) with the limited or nonrenal WG (LWG). All had LRT manifestations, whereas URT involvement was seen in six patients. In both groups, a few cases also showed joint, skin, GI tract, CNS, and ophthalmic involvement as shown in Table 1. Considering both groups together, the involvement of LRT was seen in 91.3%, URT in 78.3%, and renal involvement in 69.6% cases. A slightly higher incidence of GI tract involvement in 30.4% of cases was seen with low ophthalmic involvement (8.7%) [Table 1]. The mean BVAS in patients with CWG was 28.3 (range: 16–48), whereas in LWG group it was just 6.6 (range: 6–8).

Among the CWG, 14 patients (87.5%) had an active form of the disease at presentation and

Table 1: Organ involvement as per BVAS in classical and limited WG

Type	*Systemic*	*Cutaneous*	*Mucous membrane /ophthalmic*	*ENT*	*Chest*	*Cardio vascular*	*GI*	*Renal*	*CNS*
CWG (16)	3	4	1	12	14	1	5	16	4
LWG (7)	0	1	1	6	7	1	2	0	1
Total (23)	3*	5	2	18	21	2	7	16	5
Positivity (%)	13	21.7	8.7	78.3	91.3	8.7	30.4	69.6	21.7

CWG, Classical Wegener's granulomatosis; ENT, Ear, Nose, throat; LWG, Limited Wegener's granulomatosis; GI, Gastro-Intestinal; BVAS, Birmingham Vasculitis Activity score; CNS, Central Nervous System.

* Joint involvement was seen in three patients with Rheumatoid Factor positivity.

two patients (12.5%) were in remission, whereas seven patients in the LWG group had active disease. The male : female ratio varied in CWG being 2.2 : 1, whereas in LWG it was 1 : 6 [Table 2]. Some of the hematological findings of interest, as given in Table 3, were the low Hb values, increased WBC counts, and increased ESR values in CWG as compared to LWG. The CRP and creatinine levels, too, were found to be significantly increased in CWG group. This indicated that the CWG group of cases has more disease severity than the LWG group.

Table 2: Demographic features and BVAS in CWG and LWG cases

Features	*CWG*	*LWG*
Number tested	16 (69.6%)	7 (30.4%)
Age1		
Range	19–55 years	20–64 years
(Mean ± SD)	(29.6 ± 10.9)	(31.7 ± 15.5)
Disease status		
Active	14	7
Remission	2	0
Male/female ratio	2.2 : 1	1 : 6
BVAS		
Mean	28.3	6.6
Range	16–48	6–8

CWG, Classical Wegener's granulomatosis; SD, Standard Deviation; LWG, Limited Wegener's granulomatosis; BVAS, Birmingham Vasculitis Activity score.

Table 3: Laboratory findings in cases of WG

Laboratory parameters	*CWG (16)*	*LWG (7)*
Creatinine (mg) (%)	3.5 (1.2–8.5)	(0.9–1.2)
CRP (mg/l)	20 (5–100)	5 (3–25)
WBC count (´10^9/l)	10.5 (5.2–12.5)	7.5 (4.5–10.0)
Hemoglobin (g) (%)	8.5 (5.5–12.0)	12.5 (9.8–13.8)
ESR (mm/h)	65 (30–90)	25 (20–70)

CWG, Classical Wegener's granulomatosis; CRP, C reactive protein; LWG, Limited Wegener's granulomatosis; WBC, White Blood Cells; ESR, Erythrocyte Sedimentation Rate.

The serology of ANCA in WG showed interesting results. Totally 18 of 23 cases (78.3%) were ANCA positive by IIF and all of them were also detected by the broad spectrum PMN aG ELISA [Table 4]. In CWG, 13 cases (81.2%) showed ANCA positivity by IIF, of which 11 displayed the classical C-ANCA pattern and two had P-ANCA pattern. When these sera were further tested by ELISA for ANCA specificities, 12 in CWG (75%) had anti-PR3, one patient had anti-MPO and three had anti-LF antibodies. The two patients who were in remission after treatment did not show ANCA positivity, although earlier, during the active phase of disease, they were ANCA positive and had anti-PR3 antibodies. In the LWG group, five cases (71.4%) were ANCA positive by IIF, of which four cases showed C-ANCA pattern and one P-ANCA. When tested by ELISA, four cases in LWG (57.1%) had anti-PR3 and one patient had anti-LF. As

Table 4: ANCA serology in CWG and LWG cases

WG type	*Immunofluorescence C-ANCA*		*P-ANCA*		*ELISA αG*	*Anti-PR3*		*Anti-MPO*		*Anti-LF*
	No. Pos	*Titer*	*No. Pos*	*Titer*		*No. Pos*	*U/ml*	*No. Pos*	*U/ml*	
CWG (16)										
RPGN with crescents (8)*	6	80–640	1	320	7	6	25–56	1	25	1
FPGN with crescents (2)	2	160	0	–	2	2	23–35	0	–	1
MPGN with crescents (4)	1	80	1	80	2	2	25–30	0	–	1
Gran. Int. Nephritis (2)	2	80–160	0	–	2	2	25–35	0	–	0
LWG (7)	4	80–160	1	80	5	4	22–40	0	–	1
Total (23)	15	80–640	3	80–320	18	16	22–56	1	25	4
Percentage of positivity										
18/23 (78.3%)	65.2	13		78.3	69.6		4.3		17.4	

ANCA, anti-neutrophil cytoplasmic antibodies; CWG, Classical Wegener's granulomatosis; RPGN, Rapidly progressive glomerulonephritis; LWG, Limited Wegener's granulomatosis; FPGN, Focal proliferative glomerulonephritis; C-ANCA, Cytoplasmic ANCA; MPGN, Membrano proliferative glomerulonephritis; P-ANCA, Perinuclear ANCA; anti-PR3, anti-Proteinase3 antibodies; anti-MPO, Anti-Myeloperoxidase antibodies; anti-LF, anti-Lactoferrin antibodies; aG, α granules .

* One case underwent kidney transplant, and another, which was also Hepatitis B antigen (HbsAg) positive.

shown in Table 4, serological specificity tests indicated a higher preponderance of anti-PR3 antibodies in both the WG groups.

When all 16 CWG cases were subdivided according to their clinical manifestations and histopathological findings, eight had rapidly progressive glomerulonephritis (RPGN) with crescents, two had focal proliferative glomerulonephritis (FPGN) with crescents, four had membranoproliferative glomerulonephritis (MPGN) with crescents and two had granulomatous interstitial nephritis. It was interesting to note that in one of the patients having RPGN with crescents, the P-ANCA immunofluorescence pattern with a high titer of 1 : 320 was seen which by ELISA showed specificity for anti-MPO [Table 4], whereas most of the other positive cases had the C-ANCA pattern with titers ranging from 1 : 80—1 : 160. It was observed that the mean ± SD value for anti-PR3 was raised (39.8 ± 11.8 units/ml) in CWG group as compared to (33 ± 7.7 units/ml) in the LWG group. Other autoantibodies like ANA, anti-dsDNA were found to be absent.

DISCUSSION

Wegener's granulomatosis is an autoimmune disease of unknown aetiology where the initial phase is characterized by necrotizing granulomatous lesions, which usually affects the upper and lower airways. If undiagnosed and untreated, the kidneys may also be affected and the limited form would lead to a classical vasculitis form of WG and its accompanying necrotizing crescentic glomerulonephritis.[20],[21] The involvement of other organs like skin, GI tract, ophthalmic and CNS as seen by us have also been noted by other workers.[22]–[24] An earlier clinical study of WG cases from India,[25] had reported that, among 25 patients studied, 84% had nose/paranasal, sinus involvement, 84% had lung involvement, 40% had ear manifestations, whereas kidney involvement was present in 72% patients and other symptoms such as joint and skin manifestations were present in 44 and 32%, respectively. Also the BVAS in

the CWG group was higher than in LWG group. A male preponderance in CWG cases has been observed, which has also been reported by Kaushal et al., 1986 in an Indian study.[26]

Cytoplasmic ANCA is a serological marker for WG and C-ANCA titers mirror the disease activity, and relapses are often preceded by recurrence or increase in titer of C-ANCA.[27] Rarely, P-ANCA with anti-MPO specificity has been reported in WG.[28],[29] Edgar et al.[29] had observed that titration of ANCA by IIF were clinically useful for monitoring disease activity in WG. van der Woude et al.[4] observed that 92.6% cases with active WG had high titer ANCA, while only 12.5% of patients in remission had ANCA in low titers and concluded that the detection of ANCA is a valuable tool for early diagnosis and estimation of disease activity. Bambery et al.,[24] has pointed out that a delay in diagnosis may lead to a higher mortality in patients with WG due to extensive vasculitis and renal syndromes. Cytoplasmic ANCA positivity was found in 88% of the cases and is considered as a sensitive marker of active WG.[30] Jennings et al.[15] noted that anti-PR3 antibodies were found in 77% of the active WG cases studied. Earlier, Haubitz et al., Ludemann and Gross too, had acknowledged the diagnostic value of ANCA in monitoring the clinical activity of WG.[31],[32] Savige et al.,[22] have reported that in patients with 'limited' WG about 60% are ANCA positive. Limited Wegener's granulomatosis is recognized with increasing frequency, but, probably most of the patients go on to develop renal involvement, indicating that LWG and CWG are part of a continuum and the long-term follow up of these cases is essential. In the Indian scenario,[23]–[25] patients with LWG, have been mistakenly diagnosed to have tuberculosis, and drug therapies have been continued, despite the patients deteriorating. In such instances, ANCA testing would surely have helped. In the present study too, we have encountered three cases on anti-TB treatment since the last 6 months to 1 year, but have experienced no benefits.

This study shows a high incidence and strong titres of C-ANCA, along with anti-PR3 antibodies by ELISA in the active form of WG, whereas the two patients in remission after treatment, did not show ANCA positivity. We have also come across an unusual case of CWG with high titers of P-ANCA and having anti-MPO antibodies by ELISA, though P-ANCA is usually associated with a diverse disease spectrum, and is rarely seen in WG cases.[33] In a study, P-ANCA positivity with anti-MPO antibodies was reported in 20% of the WG cases studied.[28] Schonermarck et al.,[34] however, had reported that fewer than 5% cases of WG have anti-MPO antibodies and also have a milder form of disease. The present study has also shown that the ANCA titers seen by IIF, correlated well with the ELISA values for anti-PR3 antibodies in WG cases, as has been reported in a larger study.[32]

Early detection of ANCA which shows a good correlation with disease activity is important because current treatment protocols can help to achieve remission in a large number of patients; however, it is difficult to establish a diagnosis of WG, especially in the early stages or in the limited forms of the disease where kidney involvement is not seen. Most of the

vasculitic syndromes are known to overlap and many go undetected, and therefore the ANCA serology along with clinical and histopathological support may be helpful in early detection and management of WG cases. Also, from the immunopathological point of view, the CWG and LWG groups in WG may be a part of a disease spectrum with possible conversion of LWG to CWG, a more severe disease, which could be seen in follow-up studies.

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