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T cell reactivity to proteinase 3 and myeloperoxidase in patients with Wegener's granulomatosis (WG)

E. BROUWER, C. A. STEGEMAN*, M. G. HUITEMA, P. C. LIMBURG† & C. G. M. KALLENBERG
*Departments of Clinical Immunology, *Nephrology and †Rheumatology, University of Groningen, Groningen, The Netherlands*

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SUMMARY

T cell-mediated immunity is hypothesized to play an important role in the pathogenesis of granulomatous inflammation and vasculitis as found in patients with WG. The antigenic specificities of those T cells remain, however, unknown. Anti-neutrophil cytoplasmic antibodies (ANCA) present in patients with WG are directed to proteinase 3 (PR3) and myeloperoxidase (MPO). In the present study we investigated the proliferative capacity of peripheral blood mononuclear cells (PBMC) from patients with WG and age- and sex-matched controls in response to the WG autoantigens PR3 and MPO. Possible mitogenic effects of active PR3 and toxic effects of active MPO were excluded by using heat-inactivated PR3 and MPO. Antigen-specific stimulation induced by these autoantigens was studied by using processed PR3 and MPO in the lymphocyte stimulation test (LST). Proliferation induced by processed antigen correlated with that by heat-inactivated free antigen. The general capacity to proliferate in response to mitogens and recall antigens did not differ between patients and controls. However, patients with WG who were or had been positive for PR3-ANCA ($n = 17$) responded more strongly to PR3 than to MPO and showed higher responses to PR3 compared with controls ($n = 13$). Within the PR3-ANCA group T cell proliferation did not correlate with ANCA titre. In a small group of patients with MPO-ANCA ($n = 5$) no differences were observed compared with controls for MPO-specific proliferation. The data presented demonstrate that autoreactive PR3-specific T cells are present in patients with WG. Their fine specificity and possible role in the pathogenesis of WG have to be defined in further studies.

Keywords Wegener's granulomatosis ANCA anti-proteinase 3 T cells anti-myeloperoxidase

INTRODUCTION

WG is characterized by granulomatous inflammation of the respiratory tract, vasculitis, and necrotizing crescentic glomerulonephritis [1,2]. Inflammatory lesions of lung and kidney in WG are characterized by large mononuclear infiltrates containing monocytes and T cells, with a predominance of $CD4^+$ cells [3–5]. The presence of these large cellular infiltrates, in contrast to the scanty deposits of immunoglobulins [6,7], suggests that direct cell-mediated immunity is involved in the pathogenesis of WG [8]. In agreement with this hypothesis, elevated levels of soluble IL-2 receptors (sIL-2R) and increases in levels of sIL-2R preceding major relapses have been reported in patients with WG, and may indicate the presence and involvement of activated T cells [9,10]. The target antigens of those activated T cells are, however, not known. Proteinase 3 (PR3) and myeloperoxidase (MPO), both constituents of the neutrophil, are relevant autoantigens in WG. Anti-neutrophil cytoplasmic antibodies (ANCA) directed to PR3 or MPO are strongly associated with WG, and changes in levels of ANCA correlate closely with changes in disease activity [11–13]. Preliminary data showed that lymphocytes isolated from patients with WG proliferate in response to crude neutrophil extracts containing PR3 [14,15].

In the present study we investigated the reactivity of peripheral blood mononuclear cells (PBMC) isolated from patients with WG to the purified autoantigens PR3 and MPO. PR3 is an elastinolytic enzyme [16–20] and MPO converts hydrogen peroxide (H_2O_2) to toxic oxygen metabolites and hypochlorous acid [21]. Enzymatically active elastinolytic enzymes possess mitogenic activity and induce proliferation of lymphocytes [22]. MPO and its products, on the other hand, are toxic for PBMC [23,24]. Reactivity to PR3 and MPO was tested in a lymphocyte stimulation test (LST) with inactivated PR3 and MPO in order to prevent mitogenic activity of PR3 and toxicity of MPO. In order to test whether T cell proliferation in

Correspondence: Professor Dr C. G. M. Kallenberg, Department of Clinical Immunology, University Hospital, Ooostersingel 59, 9713 EZ Groningen, The Netherlands.

this assay results from stimulation of the T cell receptor (TCR) complex by PR3 or MPO in the context of class II MHC antigens on antigen-presenting cells (APC), results of LST were compared with those obtained by a test in which APC presenting PR3 and MPO were added to freshly isolated PBMC. In addition, we investigated the relationship between the proliferative capacity of PBMC to PR3 and MPO and the ANCA titre, in order to assess whether possible autoreactive T cells could be related to specific autoantibody production.

PATIENTS AND METHODS

Patients and controls

PBMC were isolated from patients diagnosed as having WG according to clinical and/or histological criteria [2] and fulfilling criteria for the classification of WG as described by the American College of Rheumatologists [25]. With the exception of two patients who both received prednisolone at a dose of 2·5 mg daily, patients did not receive immunosuppressive medication. In order to circumvent anergy due to immunosuppressive treatment, most of the patients were tested at the time of inactive disease. Initially, 12 patients and 10 controls were tested in preliminary studies performed in order to develop optimal conditions for assessment of T cell proliferation to PR3 and MPO. Next, proliferative responses of PBMC to PR3 and MPO were tested in 22 patients with WG (mean age 64 years (range 27–82 years), nine males and 13 females, mean ANCA titre 40 (range 0–>1 : 640)) and 13 ANCA-negative controls (mean age 57 years (range 30–82 years), three males and 10 females). Twelve patients were positive for PR3-ANCA at the time of the study, and five had been positive before ($n = 5$). Five patients were positive for MPO-ANCA at the time of the study. The control group consisted of 13 ANCA-negative age- and sex-matched controls.

ANCA detection

ANCA were detected in serum samples, drawn simultaneously with the samples used for LST, by indirect immunofluorescence, as described previously [26]. Samples were scored as positive if most neutrophils showed positive fluorescence at a serum dilution of at least 1 : 20. Antigen specificity was determined by antigen-specific ELISAs for antibodies to PR3 and MPO [17,26].

Antigens

Proteinase 3. PR3 was purified from isolated neutrophils disrupted by nitrogen cavitation by dye-ligand affinity chromatography over Matrex Gel Orange A (Amicon Div., Danvers, MA), followed by cation exchange chromatography (Bio-Rex 70; Bio-Rad Labs, Richmond, CA) as described by Kao *et al.* [16]. PR3 was detected by sandwich ELISA, using MoAb 12.8 directed against PR3 and patients' serum containing PR3-ANCA [17], and by alpha-naphtyl acetate reactivity [27] (Aldrich Chemie, Brussels, Belgium). Contamination with elastase and cathepsin G was excluded by ELISA. Purity of the PR3 preparation was further analysed by gel electrophoresis, which showed a 29-kD band specific for PR3.

Myeloperoxidase. The flow through of the Matrix Gel Orange A was absorbed to a concanavalin A (Con A) Sepharose gel (Pharmacia Fine Chemicals AB, Uppsala, Sweden) and eluted with α-methyl-D-mannoside (Sigma Chemical Co., St Louis, MO). Eluted fractions with a ratio between the OD obtained at 428 nm and the OD obtained at 280 nm > 0·7 were pooled and extensively dialysed against sodium acetate buffer pH 4·7 containing 0·05% cetyltrimethyl ammonium bromide (CETAB). This extract was further purified on a Sephadex G150 gel (Pharmacia). Fractions with an OD 428/280 ratio >0·8 were pooled. Contamination with PR3 or human leucocyte elastase (HLE) was ruled out by antigen-specific ELISAs for PR3 and HLE. Purity of the MPO preparation was further analysed by gel electrophoresis which showed specific bands for MPO (at 15, 39, and 58 kD, data not shown) [28]. In addition we also used MPO from Calbiochem (La Jolla, CA) with an OD428/280 ratio larger than 0·7.

Inactivation of proteinase 3 and myeloperoxidase

PR3 and MPO were inactivated by heating for 15 min at 100°C. Effectiveness of the procedure was tested using the guaiacol assay which specifically measures MPO activity for MPO [29] and using MeO-Suc-Ala-Ala-Pro-Val-pNA (M4765; Sigma) as a substrate for PR3 [20]. Heat inactivation inhibited enzyme activity of both PR3 and MPO completely (results not shown).

Antigen-presenting cell assay

The APC assay used was a modified version of the test developed by Ota *et al.* [30]. PBMC were isolated on Lymphoprep from freshly drawn heparinized blood. PBMC (1×10^7/ml) were incubated at 37°C in 1 ml medium (RPMI supplemented with 15% pool serum and gentamycin 60 μg/ml) containing the antigen. During the incubation time the antigen is taken up by APC present in PBMC, processed and presented on the surface of the cells. The PBMC thus obtained were designated as APC. APC were irradiated for 6 min with 6 Gy/min (36 Gy in total) so that the cells remained vital but lost their capacity to proliferate. Effectiveness of irradiation was tested by culturing APC alone. After irradiation APC were extensively washed. Next, those APC were cultured with freshly isolated autologous PBMC. Optimal conditions were determined using various antigen concentrations and different incubation times for pulsing APC, and different ratios of APC to PBMC as well as different culture conditions in the final culture. We found that culturing 50 μl of APC, at a concentration of 1×10^6/ml, that had been incubated for 1 h at 37°C with identical concentrations of PR3 or MPO as used in the LST assay, with 50 μl PBMC, at a concentration of 2×10^6/ml, in 96-well plates for 5 days resulted in optimal proliferation of PBMC. Background proliferation was assessed by culturing PBMC with APC pulsed with medium alone.

Lymphocyte stimulation test

PBMC were isolated on Lymphoprep from freshly drawn heparinized blood. PBMC proliferation assays were performed in sterile round-bottomed 96-well plates (Costar Europe, Badhoevedorp, The Netherlands). PBMC (50 μl/well of 2×10^6 cells/ml) were stimulated with PR3 and MPO in RPMI (Gibco, Paisley, UK) containing 15% pooled human serum and gentamycin 60 μg/ml, at concentrations of 0·025, 0·1, 0·5, and 2 μg/ml. Comparable concentrations have been shown to be optimal for stimulation of PBMC with other autoantigens [31]. In order to assess mitogenic influences of enzymatically active PR3 and toxicity of active MPO we compared proliferation of PBMC to active PR3 and MPO

with proliferation to heat-inactivated PR3 and MPO. Background proliferation was assessed by incubating cells with medium alone.

Con A, pokeweed mitogen (PWM), and an antigen cocktail containing recall antigens (Candida, *Corynebacterium diphtheria*, tetanus toxoid (TT), and purified protein derivative (PPD)) were added as positive controls for the proliferative capacity of isolated PBMC. PBMC were cultured for 3 days with mitogens at a concentration of 6×10^5 PBMC/ml and for 5 days with antigens at a concentration of 2×10^6 PBMC/ml. Proliferation was assessed by tritiated thymidine incorporation, 25 μl/well of 0·5 μCi/ml, during the last 16 h of incubation. All assays were performed in triplicate, and the results were expressed either as desintegrations per second (d.p.s.) (mean ± s.e.m.) or as stimulation index (SI) calculated by the ratio of test values to background. The LST was considered positive for a specific antigen when values exceeded 20·3 d.p.s. (mean + 2 s.d. of background proliferation) or when the SI was higher than 3. LST results with high background values (>20·3 d.p.s. (mean + 2 s.d.)) were discarded.

Statistical analysis

For comparison between paired results or between two groups a paired or unpaired Wilcoxon test, respectively, was used or a χ^2 test when two groups were tested for data scored as absent or present. A two-tailed P value <0·05 was considered significant. Correlations were studied with Spearman's rank test.

RESULTS

Optimization of the proliferation assay

Twelve patients and 10 controls were tested in preliminary studies comparing stimulation with active PR3 and MPO *versus* inactive PR3 and MPO, and free PR3 and MPO *versus* processed PR3 and MPO, in order to optimize the proliferation assay.

A significant correlation ($P < 0·01$) was found between stimulation with active *versus* inactive PR3 in patients and controls. Stimulation with active PR3, however, resulted in 22% of the patients and controls in higher responses than stimulation with inactive PR3 (data not shown). This could be due to mitogenic influences of active PR3 [22]. For this reason all subsequent tests were performed with inactive PR3.

Active MPO inhibited the response of PBMC to Con A, PWM, and a cocktail containing recall antigens in a dose-dependent way (Table 1). Heat inactivation of MPO reduced its toxicity to a great extent (Table 1). Thus, heat-inactivated MPO was used in subsequent testing.

PBMC isolated from patients and controls were cultured with free inactivated PR3 and MPO and with autologous APC presenting PR3 and MPO also, in order to exclude mitogenic or toxic influences of PR3 and MPO, and to test whether proliferation of PBMC is based on antigen recognition in the context of class II molecules. We tested reactivity of patients and controls to processed PR3 and MPO, and compared the results with those from LST performed with simultaneously isolated PBMC using identical concentrations (2·5, 1·0 and 0·1 μg/ml, respectively) of inactive PR3 or MPO. A significant correlation between the two assays was observed for PR3 at 1 and 2·5 μg/ml ($r = 0·505$ and $r = 0·576$; $P < 0·05$), whereas proliferation to MPO at 1 μg/ml tended to correlate ($r = 0·429$) between the APC and LST assay. Based on these results it was decided to use the LST with heat-inactivated PR3 and MPO to assess the proliferative capacity of PBMC in a larger group of patients with WG.

Proliferation in response to mitogens and common recall antigens

First, we tested the spontaneous proliferation and reactivity of isolated PBMC from WG patients ($n = 22$) and controls ($n = 13$) to the mitogens Con A and PWM and to an antigen cocktail containing common recall antigens (candida, *Corynebacterium diphtheria*, PPD and TT). Patients did show a lower response to PWM compared with controls ($P = 0·017$), while no significant differences in the capacity to react to Con A and recall antigens were found between patients with WG in an inactive phase of their disease and age- and sex-matched healthy controls (Fig. 1).

Proliferative responses to PR3 and MPO

Next, proliferation of isolated PBMC from patients and controls was tested to PR3 and MPO in LST. Stimulation of PBMC in the PR3-ANCA-positive group ($n = 17$) with PR3 at a concentration of 0·1 μg/ml led to a significantly higher response compared with controls ($n = 13$) (Fig. 2). At a concentration of 0·1 μg/ml the response to PR3 was higher than to MPO in the PR3-ANCA-positive patients ($P = 0·0004$). For individual patients and controls a dose-response curve could be observed for PR3. Thirty-five per cent of the patients (6/17) reacted with all four PR3 concentra-

Table 1. Dose-dependent inhibition of proliferative responses to concanavalin A (Con A), pokeweed mitogen (PWM), and antigen cocktail by active myeloperoxidase (MPO)

	Con A 10 μg/ml*	PWM 10 μg/ml†	Antigen cocktail‡
Without MPO	174 ± 20	355 ± 81	692 ± 225
MPO, 0·01 μg/ml	116 ± 10	378 ± 74	523 ± 91
MPO, 1 μg/ml	48 ± 9	285 ± 22	249 ± 183
MPO, 3 μg/ml	6 ± 0·3	134 ± 13	40 ± 28
Heat-inactivated MPO, 3 μg/ml	63 ± 4	344 ± 30	159 ± 35

*† Peripheral blood mononuclear cells (PBMC) cultured for 3 days with Con A or PWM.

‡ PBMC cultured for 5 days with an antigen cocktail containing diphtheria toxoid 10 LF/ml, tetanus 10 LF/ml, purified protein derivative (PPD) 3 μg/ml, and candida extract 15 μg/ml.

Results are expressed as disintegrations per second; numbers represent mean ± s.d. of assays done in triplicate.

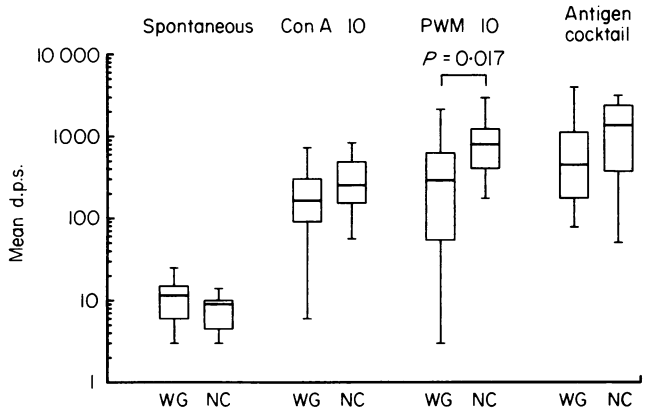

Fig. 1. Box plots indicating ranges (error bars), 25–75% intervals (box), and median value (horizontal line) of proliferation of peripheral blood mononuclear cells (PBMC) measured as disintegrations per second (d.p.s., log scale), spontaneously and in response to concanavalin A (Con A), pokeweed mitogen (PWM), and common recall antigens. PBMC were isolated from patients with WG ($n = 22$) and normal controls ($n = 13$).

tions. For stimulation with PR3 at 0·025, 0·1, 0·5, and 2 μg/ml percentages of positive responses as expressed in d.p.s. were 50%, 65%, 35% and 50% in the PR3-ANCA group, and 33%, 23%, 33% and 33% in the control group, respectively. Also, when proliferation expressed both in d.p.s. and in SI had to be positive, more PR3-ANCA patients than controls were found to be positive: 12%, 35%, 18% and 18% *versus* 0%, 8%, 8% and 8% for stimulation with PR3 at 0·025, 0·1, 0·5 and 2 μg/ml, respectively. Within the PR3-ANCA-positive group no correlation could be found between the PR3-ANCA titre and the capacity to proliferate to PR3.

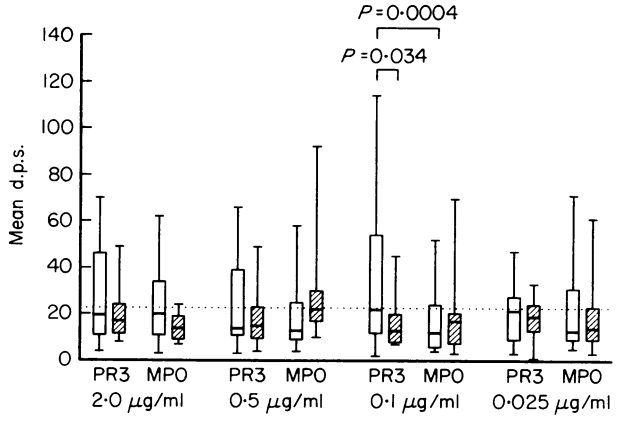

Fig. 2. Proliferative capacity of isolated peripheral blood mononuclear cells (PBMC) to proteinase 3 (PR3) and myeloperoxidase (MPO) at different concentrations (abscissa) measured as mean disintegrations per second (d.p.s.) as calculated from triplicate cultures in the lymphocyte stimulation test (ordinate) in patients with WG presently or previously positive for PR3-ANCA (open boxes, $n = 17$) and in ANCA-negative age- and sex-matched normal healthy controls (hatched boxes, $n = 13$). Box plots indicate range (error bars), 25–75% interval (box), and median value (horizontal line). The horizontal line denotes the upper limit of spontaneous proliferation of PBMC (mean ± 2 s.d.).

For MPO we found that only one out of the five tested MPO-ANCA-positive patients showed a significant proliferation of PBMC to inactive MPO (data not shown). None of the MPO-ANCA patients had an SI larger than 3. Of the controls, 16–58%, dependent on the concentration of MPO used, reacted with MPO. The percentage of controls with a positive SI varied from 8% to 39%, dependent on the concentration of MPO used in the LST assay.

DISCUSSION

Since autoreactive T cells are hypothesized to play an important role in the pathogenesis of WG, we tested the capacity of PBMC isolated from patients with WG to respond to the highly purified autoantigens PR3 and MPO.

We found that PBMC from some of the WG patients and controls responded better to active than inactive PR3. Since elastinolytic enzymes, such as elastase and cathepsin G, possess mitogenic activity this might also be the case for the third serine proteinase PR3 [22]. In order to exclude a possible mitogenic effect of PR3, all subsequent tests were performed with heat-inactivated PR3. Active MPO was found to be very toxic for PBMC [23,24]. MPO inhibited proliferation of PBMC to Con A, PWM, and recall antigens dose-dependently. This effect could, however, be reduced to a great extent by inactivating MPO.

Next, we compared proliferation of PBMC to inactivated PR3 or MPO with their proliferation in response to processed PR3 or MPO in order to test whether T cells recognize a complex ligand composed of PR3- or MPO-derived peptides in association with MHC molecules. No differences were observed between the two assays, suggesting that proliferation of PBMC results from TCR recognition of PR3 and MPO in the context of class II antigens on APC. The proliferative capacity of PBMC to mitogens and common antigens was tested in patients and controls. No significant differences in reactivity to Con A and to recall antigens (candida, *Corynebacterium diphtheria*, PPD and TT) were found between PBMC isolated from WG patients and controls, whereas only the response to PWM was lower in the patients. Thus, the general lymphoproliferative capacity of the patients was not severely disturbed despite periods of previous disease activity and immunosuppressive treatment.

Stimulation of PBMC with PR3 led to a significantly higher response rate in the PR3-ANCA group compared with controls. Also, a higher percentage of patients were stimulated positively with PR3 in the PR3-ANCA group. This suggests that precursor T cells specific for PR3 are present at a higher frequency in patients than in controls. These PR3-reactive T cells could be T helper cells involved in ANCA production as well as effector cells playing a direct role in the pathogenesis of WG. Within the PR3-ANCA group, however, no correlation was observed between ANCA titre and proliferation to PR3. Previous studies in which the proliferative capacity of PBMC to crude preparations containing PR3 was tested reported that only PR3-ANCA-positive WG patients, in contrast to ANCA-negative healthy controls, reacted with PR3 [14,15]. Both studies, however, tested only small groups of patients and controls. A third study, in which PBMC were also stimulated with a crude extract from neutrophil granules, reported no differences between patients and controls [32]. In all three studies PBMC were stimulated with active enzymes, so mito-

genic as well as toxic influences could have influenced the results. For MPO we found that only one of the five tested MPO-ANCA-positive patients reacted with inactive MPO. All patients, however, had a positive MPO-ANCA titre at the time of testing. The observed discrepancy between antibody titre to PR3 or MPO and T cell reactivity to those antigens has also been reported for other autoantigens such as thyroglobulin (Tg) and thyroid peroxidase (TPO) [33], and adrenal antigens [34]. ANCA predominantly belong to the IgG1 and IgG4 subclass [35]. Especially the presence of IgG4 subclass antibodies, known to be a T cell-dependent isotype, indicates that T cells are involved in ANCA production. Since T cell epitopes are linear [36] and B cells tend to recognize conformational determinants, it might well be that most of the epitopes recognized by T and B cells are different, and that common reactivity depends on which parts of PR3 and MPO are presented by APC. Indeed, Brocke *et al.* [37] reported simultaneous reactivity of T and B cells to only a few of the tested synthetic acetylcholine-receptor peptides (AChR). Also, the fact that we used PBMC as APC, and not dendritic cells or other cells capable of presenting antigens, might have influenced T cell responsiveness [33]. On the other hand we cannot exclude that ANCA production in WG might also be due to polyclonal activation of B cells.

The observed reactivity of PBMC to PR3 and MPO in controls has also been reported in studies investigating reactivity to other autoantigens like myelin basic protein (MBP), Tg, TPO, AChR, and adrenal antigens [33,34,37,38]. At the B cell level V-domain antibody fragments specific for neutrophil cytoplasmic antigens were found within the normal B cell repertoire [39]. One explanation for positive reactivity in controls could be that autoreactive T cell populations present in normal healthy individuals are specific for minor or non-dominant antigens which have failed to induce tolerance during early development [40,41]. Another explanation might be that parts of PR3 and MPO are also present in exogenous antigens to which the subject has previously been exposed [42]. Epitope mapping and cloning of autoreactive T cells should give further information on the question, whether recognized epitopes differ between patients and controls [30,37,43], and whether reactivity to these epitopes is related to ANCA production and/or disease activity.

In conclusion, we found that T cells reactive to PR3 are present in higher percentages in patients with WG compared with controls. Their involvement in ANCA production and in the pathogenesis of the granulomatous lesions in WG needs further investigation. Therefore studies need to be done with synthetic PR3 peptides in order to unravel the fine specificity of those PR3-reactive T cells that contribute to ANCA production and/or granuloma formation.

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REFERENCES

1 Godman GC, Churg J. Wegener's granulomatosis; pathology and a review of the literature. Arch Pathol 1954; **58**:533–53.

2 Fauci AS, Haynes BF, Katz P, Wolff SM. Wegener's granulomatosis; prospective clinical and therapeutic experience with 85 patients for 21 years. Ann Int Med 1983; **98**:76–85.

3 Gephardt GN, Ahmad M, Tubbs RR. Pulmonary vasculitis (Wegener's granulomatosis); immunohistochemical study of T and B cell markers. Am J Med 1983; **74**:700–4.

4 Berge RJMten, Wilmink JM, Meyer ChrJLM, Surachno S, Veen JH ten, Balk AG, Schellekens PThA. Clinical and immunological follow-up in patients with severe renal disease in Wegener's granulomatosis. Am J Nephrol 1985; **5**:21–29.

5 Brouwer E, Cohen Tervaert JW, Weening JJ, Kallenberg CGM. Immunohistopathology of renal biopsies in Wegener's granulomatosis (WG): clues for its pathogenesis? Am J Kidney Dis 1990; **1**:558.

6 Stilmant MM, Bolton WK, Sturgill BG, Schmitt GW, Couser WG. Crescentic glomerulonephritis without immune deposits; clinicopathologic features. Kidney Int 1979; **15**:184–95.

7 Ronco P, Verroust P, Mignon F *et al.* Immunopathological studies of polyarteritis nodosa and Wegener's granulomatosis: a report of 43 patients with 51 renal biopsies. Quart J Med 1983; **206**:212–23.

8 Kallenberg CGM, Tervaert JW, Woude FJ van der, Goldschmeding R, Borne AEGKr von dem, Weening JJ. Autoimmunity to lysosomal enzymes: new clues to vasculitis and glomerulonephritis? Immunol Today 1991; **12**:61–64.

9 Schmitt WH, Heesen C, Csernok E. Elevated serum levels of soluble interleukin-2-receptor (sI1-2R) in Wegener's granulomatosis (WG): association with disease activity. Arthritis Rheum 1992; **35**:108–10.

10 Stegeman CA, Cohen Tervaert JW, Huitema MG, Kallenberg CGM. Serum markers of T cell activation in relapses of Wegener's granulomatosis. Clin Exp Immunol 1993; **91**:415–20.

11 Cohen Tervaert JW, Woude FJ van der, Fauci AS *et al.* Association between active Wegener's granulomatosis and anticytoplasmic antibodies. Arch Int Med 1989; **149**:2461–5.

12 Jayne D, Heaton A, Brownlee A, Evans DB, Lockwood C. Sequential antineutrophil cytoplasmic antibody titres in the management of systemic vasculitis. Nephrol Dial Transplant 1990; **5**:309–10.

13 Egner W, Chapel HM. Titration of antibodies against neutrophil cytoplasmic antigens is useful in monitoring disease activity in systemic vasculitides. Clin Exp Immunol 1990; **82**:244–9.

14 Woude FJ van der, Es LA van, Daha MR. The role of the c-ANCA antigen in the pathogenesis of Wegener's granulomatosis. A hypothesis based on both humoral and cellular mechanisms. Neth J Med 1990; **36**:169–71.

15 Petersen J, Rasmussen N, Szpirt W, Hermann E, Mayet W. T lymphocyte proliferation to neutrophil cytoplasmic antigen(s) in Wegener's granulomatosis (WG). Am J Kidney Dis 1991; **18**:205.

16 Kao RC, Wehner NG, Skubitz KM, Gray BH, Hoidal JR. Proteinase 3; a distinct human polymorphonuclear leukocyte proteinase that produces emphysema in hamsters. J Clin Invest 1988; **82**:1963–73.

17 Goldschmeding R, Schoot CE van der, Bokkel-huinink D ten, Hack CE, Ende CE van den, Kallenberg CGM, Borne AEGKr von dem. Wegener's granulomatosis autoantibodies identify a novel diisopropylfluorophosphate-binding protein in the lysosomes of normal human neutrophils. J Clin Invest 1989; **84**:1577–87.

18 Lüdemann J, Utecht B, Gross WL. Anti-neutrophil cytoplasm antibodies in Wegener's granulomatosis recognize an elastinolytic enzyme. J Exp Med 1990; **171**:357–62.

19 Niles JL, McCluskey RT, Ahmad MF, Arnaout MA. Wegener's granulomatosis autoantigen is a novel neutrophil serine protease. Blood 1989; **74**:1888–93.

20 Rao NV, Wehner NG, Marshall BC, Gray WR, Gray BH, Hoidal JR. Characterization of proteinase 3 (PR-3), a neutrophil serine proteinase. Structural and functional properties. J Biol Chem 1991; **266**:9540–8.

21 Weiss SJ. Tissue destruction by neutrophils. N Engl J Med 1989; **320**:365–76.

22 Vischer TL, Bretz U, Baggiolini M. *In vitro* stimulation of lymphocytes by neutral proteinases from human polymorphonuclear leukocyte granules. J Exp Med 1976; **144**:863–72.
23 Clark RA, Klebanoff SJ. Myeloperoxidase-H_2O_2-halide system cytotoxic effect on human blood leukocytes. Blood 1977; **50**:65–70.
24 El-hag A, Lipsky PE, Bennett M, Clark RA. Immunomodulation by neutrophil myeloperoxidase and hydrogenperoxide: differential susceptibility of human lymphocyte functions. J Immunol 1986; **136**:3420–6.
25 Laevitt RY, Fauci AS, Bloch DA *et al.* The American College of Rheumatology 1990 criteria for the classification of Wegener's granulomatosis. Arthritis Rheum 1990; **33**:1101–7.
26 Cohen Tervaert JW, Goldschmeding R, Elema JD *et al.* Autoantibodies against myeloid lysosomal enzymes in crescentic glomerulonephritis. Kidney Int 1990; **37**:799–806.
27 Wiel BA van de, Dolman KM, Meer-Gerritsen CH van der, Hack CE, Borne AEGKr von dem, Goldschmeding R. Interference of Wegener's granulomatosis autoantibodies with neutrophil proteinase 3 activity. Clin Exp Immunol 1992; **90**:409–14.
28 Olsen RL, Little C. Purification and some properties of myeloperoxidase and eosinophil peroxidase from human blood. Biochem J 1983; **209**:781–7.
29 Klebanoff SJ, Waltersdorph AM, Rosen H. Antimicrobial activity of myeloperoxidase. Meth Enzym 1984; **105**:399–403.
30 Ota K, Matsui M, Milford EL, Mackin GA, Weiner HL, Hafler DA. T-cell recognition of an immunodominant myelin basic protein epitope in multiple sclerosis. Nature 1990; **346**:183–7.
31 Ewins DL, Barnett PS, Ratanachaiyavong S, Sharrock C, Lanchbury J, McGregor AM, Banga JP. Antigen-specific T cell recognition of affinity-purified and recombinant thyroid peroxidase in autoimmune thyroid disease. Clin Exp Immunol 1992; **90**:93–98.
32 Mathieson PW, Lockwood CM, Oliveria DBG. T and B cell responses to neutrophil cytoplasmic antigens in systemic vasculitis. Clin Immunol Immunopathol 1992; **63**:135–41.
33 Fukuma N, Mclachlan SM, Rapoport B *et al.* Thyroid autoantigens and human T cell responses. Clin Exp Immunol 1990; **82**:275–83.
34 Freeman M, Weetman AP. T and B cell reactivity to adrenal antigens in autoimmune Addison's disease. Clin Exp Immunol 1992; **88**:275–9.
35 Brouwer E, Cohen Tervaert JW, Horst G, Huitema MG, Giessen M van der, Limburg PC, Kallenberg CGM. Predominance of IgG1 and IgG4 subclasses of anti-neutrophil cytoplasmic autoantibodies (ANCA) in patients with Wegener's granulomatosis and clinically related disorders. Clin Exp Immunol 1991; **83**:379–86.
36 Rothbard JB, Taylor NR. A sequence pattern common to T-cell epitopes. EMBO J 1988; **1**:93.
37 Brocke S, Brautbar C, Steinman L *et al. In vitro* proliferative responses and antibody titers specific to human acetylcholine receptor synthetic peptides in patients with myasthenia gravis and relation to HLA class II genes. J Clin Invest 1988; **82**:1894–900.
38 Olsson T, Zhi WW, Höjeberg B *et al.* Autoreactive T lymphocytes in multiple sclerosis determined by antigen-induced secretion of interferon-γ. J Clin Invest 1990; **86**:981–5.
39 Finnern R, Lockwood CM, Ouwehand W. Anti-neutrophil cytoplasm autoantibody (ANCA) fragments from a human V-gene library. Clin Exp Immunol 1993; **93**:21.
40 Gammon G, Sercarz E. How some T-cells escape tolerance induction. Nature 1989; **342**:183–5.
41 Gammon G, Sercarz E, Benichou G. The dominant self and the cryptic self: shaping the autoreactive T-cell repertoire. Immunol Today 1991; **12**:193–5.
42 Harcourt G, Jermy A. Mapping the autoimmunising epitopes on acetylcholine receptors. Immunol Today 1987; **8**:319–22.
43 Tandon N, Freeman M, Weetman AP. T cell responses to synthetic thyroid peroxidase peptides in autoimmune thyroid disease. Clin Exp Immunol 1991; **86**:56–60.