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Cell-mediated autoimmunity in patients with Wegener's granulomatosis (WG)

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SUMMARY

*Despite the well described infiltration of cells of the cellular immune system in vasculitic lesions and the granuloma formation in patients with WG, the role of T cell-mediated autoimmunity in WG is not clear. Reports of T cell proliferation in response to neutrophil azurophilic granule proteins are contradictory. In this study we have assessed the proliferation of T cells of WG patients to purified proteinase 3 (PR3) and to total azurophilic granule proteins in two different assays. In addition to the classical proliferation assay with isolated peripheral blood mononuclear cells, we have used a whole blood proliferation assay. In both assays we found proliferative responses to PR3 in patients with WG. The number of patients reacting to the azurophilic granule extract was higher than the patients reacting to the purified PR3, suggesting that other autoantigens may also be involved. We have identified epitopes of PR3 that may be potential targets of class I-restricted T cell responses in the context of HLA-A*0201, the most common MHC class I molecule. These epitopes were determined by the binding of synthetic PR3 peptides to HLA-A*0201 on the antigen-processing defective cell line, T2. In addition, T cell lines were established from tissue biopsies, obtained from WG patients, and assessed for cytolytic reactivity against T2 cells, preloaded with synthetic PR3 peptides. We conclude that T lymphocytes of WG patients have increased proliferative responses to purified PR3 and to a larger extent to non-fractionated proteins of azurophilic granules of polymorphonuclear neutrophilic leucocytes (PMN).*

Keywords proteinase 3 T cells vasculitis HLA-A*0201

INTRODUCTION

WG is characterized by a systemic necrotizing vasculitis of small and medium arteries in combination with crescentic necrotizing glomerulonephritis and development of granulomas in the upper airways, lungs and kidneys. The most frequent clinical manifestations are granulomatous lesions of the lower and upper respiratory tract and a rapidly progressive glomerulonephritis [1,2]. The pathogenesis of WG is largely unknown, but a mechanism has been suggested for the endothelial cell damage and vasculitis in WG patients [3–6]. This model comprises intravascular activation of cytokine-primed neutrophils by anti-neutrophil cytoplasmic antibodies (ANCA) followed by degranulation of the neutrophil and release of lysosomal enzymes and reactive oxygen radicals. ANCA are autoantibodies, found in the circulation of most WG patients, directed against several constituents of the azurophilic granules of neutrophils [7]. Based on the fluorescence pattern on ethanol-fixed granulocytes, ANCA can be divided in C-ANCA (cytoplasmic pattern), P-ANCA (perinuclear pattern) and aspecific ANCA. The most important target antigens are proteinase 3 (PR3) for C-ANCA and myeloperoxidase (MPO) for P-ANCA [7]. These antigens are expressed on the surface of cytokine-primed polymorphonuclear neutrophilic leucocytes (PMN) and are secreted upon cellular degranulation [8,9]. In addition to activating PMN, C-ANCA may interfere with the enzymatic activity of PR3 and with the inhibition of PR3 by α_1 proteinase inhibitor [10]. Although levels of ANCA in the circulation tend to correlate with disease activity [7], not all aspects of the pathophysiology of WG can be attributed to the presence of ANCA. Massive cellular infiltrates in the renal interstitium, the nasal mucosa and (peri)vascular tissue of the lung, mainly consist of T cells, B cells and macrophages [11–14]. Extensive granuloma formation [14,15] suggests an important role of cell-mediated immune reactions in the pathogenesis of this disease. Furthermore, levels of soluble CD25 are elevated in patients with active WG, suggesting activation of the cellular immune system [16]. Moreover, a correlation was found between the number of T

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lymphocytes in renal interstitium and renal function of patients with rapid progressive glomerulonephritis [12]. Lymphocyte proliferation induced by azurophilic granule proteins [13,17] is still controversial. In other autoimmune diseases, T cell responses against the target antigens of autoantibodies have been described, and have also been implicated in the pathogenesis of the disease, such as type 1 diabetes, autoimmune thyroiditis and myasthenia gravis [18–21].

We have investigated the cellular immune response to purified PR3 using the classical lymphocyte transformation test (LTT) and an adaptation of a whole-blood proliferation assay. This whole-blood assay requires only limited quantities of blood and no *in vitro* separation of blood cells [22]. Proliferative responses against exogenous antigens are generally MHC class II-restricted. However, since PR3 is also synthesized by endothelial cells [23–25], it may be presented in the context of MHC class I on endothelial cells and therefore be a target for autoreactive $CD8^+$ T cells. Cytotoxic T cells have been implicated in the pathogenesis of several autoimmune diseases [26–28]. We have identified epitopes on PR3 that fit into the peptide-binding groove of HLA-A*0201 by determining binding of synthetic PR3 peptides to HLA-A*0201 on the processing-defective T2 cell line [29]. This strategy has been successful for determining cytotoxic T lymphocyte (CTL) epitopes on viral peptides and self-antigens that may act as targets for tumour-directed T cells [30–32]. We have attempted to use the potential epitopes of PR3 to detect PR3-specific HLA-A*0201-restricted T cells in T cell lines isolated from biopsies of inflamed tissues of WG patients.

PATIENTS AND METHODS

Materials

Azurophilic granules were prepared by subcellular fractionation of freshly isolated neutrophils [33] and the granules were extracted as described by Goldschmeding *et al.* [34]. PR3 was isolated from azurophilic granules as described in Leid *et al.* [35]. Both preparations were enzymatically active as determined by the cleavage of the synthetic substrate t-Boc-Ala-ONp (Sigma B5126). Enzymatic activity of PR3 and azurophilic granule extract was no longer detectable in the presence of 10% normal human serum.

The Q66–9 CTL clone specific for an influenza peptide was a kind gift of Dr H. Spits (Department of Immunology, Dutch Cancer Institute, Amsterdam, The Netherlands). Q66–9 is specific for the peptide GILGFVFTL of the influenza matrix protein in the context of HLA-A*0201.

The WG patients who were selected for this study received limited or no immunosuppressive therapy, or had a stimulation index (SI) higher than 2 in the corresponding proliferation assay upon stimulation with a recall antigen (tetanus toxoid (TT)). All patients were positive in the ANCA fluorescence assay, titres $\geqslant 64$. Mean age of the patients was 52 years (range 28–87 years) and all patients had histology-proven WG. Healthy laboratory workers were used as controls.

The culture medium was Iscove's modified Dulbecco's medium supplemented with penicillin/streptomycin, glutamine, β-mercaptoethanol (IMDM). IMDM containing 10% pooled human serum (IMDM+) was used for most cell cultures.

Lymphocyte transformation test

Peripheral blood mononuclear cells (PBMC) were isolated from fresh heparinized blood by Ficoll amidotrizoate density separation. The cells were washed three times with PBS to remove platelets and washed once with IMDM+. The cells were resuspended in IMDM+ to a concentration of 1×10^6 cells/ml and 100 μl were transferred to each well of 96-well U-bottomed plates (Costar, Badhoevedorp, The Netherlands). One hundred microlitres of PR3 (32 μg/ml), Lymphocult T (20%) or phytohaemagglutinin (PHA; 2 μg/ml) were added per well. The cells were incubated in a humidified incubator with 5% CO_2 at 37°C for 5 days, and 16 h before harvesting, ^{3}H-thymidine was added (1 μCi/well; Amersham, Den Bosch, The Netherlands). Cells were harvested in a Skatron (Lier, Norway) cell harvester and ^{3}H-thymidine incorporation was assessed using a LKB scintillation counter and Packard opti-fluor scintillation fluid. All experiments were performed in triplicate and background proliferation was usually less than 2000 ct/min with medium alone. Assays with higher background proliferation were not included in the results.

Whole-blood proliferation assay

This method was adapted from Bloemena *et al.* [22]. Heparinized peripheral blood of humans or rats was diluted 1:10 in IMDM and transferred in 150-μl volumes per well to a 96-well flat-bottomed plate (Greiner B.V., Alphen a/d Rijn, The Netherlands). Fifty microlitres of PR3 (4, 16 or 64 μg/ml) in IMDM were added per well and as a control 50 μl of Lymfocult T (40% in IMDM) or rat T cell growth factor (TCGF; 40% in IMDM) or IMDM alone were added. Incubation and harvesting were performed as described for the LTT. All experiments were performed in triplicate and background proliferation with medium alone was usually less than 200 ct/min.

Immunization of the rats with PR3

Five male Wistar rats (180–200 g) were immunized subcutaneously with 20 μg of PR3 in Freund's complete adjuvant (FCA). After 14 days the rats were boosted with 20 μg of PR3 in Freund's incomplete adjuvant (FIA). Venous blood (1·5 ml) was taken from the tail vein by incision 6 days after booster immunization. The blood samples were collected in sterile vials containing 75 U of heparin (Organon Technika, Boxtel, The Netherlands).

Selection of synthetic peptides of PR3

Nonamer, 10-mer and 11-mer sequences of PR3 were selected based on the consensus sequences of HLA-A*0201-binding peptides using two different computer programs. The consensus sequence used by the first program [30] is based on the sequence of peptides eluted from HLA-A*0201 [36,37]. The second program uses a consensus sequence based on the reported binding of synthetic peptides to HLA-A*0201 *in vitro* [48]. The peptides finally synthesized were those selected from the list of peptides generated by both programs. This selection was based on the rankings of the peptides in the list and on the hydrophobicity of the peptide. Peptides were synthesized using Fmoc chemistry on an Abimed AMS 422 automated multiple peptide synthesizer. Purity of peptides was more than 90% as determined by reverse-phase high performance liquid chromatography (HPLC) analysis. Extremely hydrophobic peptides were excluded due to their insolubility. A list of the 28 synthetic peptides synthesized and tested for *in vitro* binding to

Table 1. Overview of the synthesized proteinase 3 (PR3) peptides that were tested for binding to HLA-A*0201

Amino acid no.	Sequence	Peptide no.	Maximal FI	FI at 25 μg/ml ⩾ 1·0
38	FVLTAAHCL	23	1·81	+
41	TAAHCLRDI	27	–	
45	CLRDIPQRL	1	0·11	
45	CLRDIPQRLV	14	0·63	
52	RLVNVVLGA	5	0·30	
77	QVFLNNYDA	25	0·35	
79	FLNNYDAENK	9	1·36	
84	DAENKLNDV	6	0·47	
88	KLNDVLLIQL	12	2·16	
92	VLLIQLSSPA	15	0·89	
95	IQLSSPANL	28	–	
102	NLSASVATV	18	1·43	+
111	QLPQQDQPV	3	1·26	+
118	PVPHGTQCL	7	0·51	
140	AQVLQELNV	26	–	
141	QVLQELNVT	24	0·38	
142	VLQELNVTV	4	0·80	+
143	LQELNVTVV	22	0·75	
159	NICTFVPRRKA	10	0·90	
180	LICDGIIQGI	13	4·18	+
181	ICDGIIQGI	21	1·01	
184	GIIQGIDSFV	16	1·42	
184	GIIQGIDSFVI	11	1·09	
185	IIQGIDSFVI	17	0·80	
185	IIQGIDSFV	19	0·15	
212	YVDWIRSTL	2	0·95	
215	WIRSTLRRV	8	0·35	
219	TLRRVEAKG	20	–	

The maximal fluorescence index (FI) induced by the peptide is indicated in the fourth column (– indicates FI ⩽ 0). A + in the last column indicates that at a concentration of 25 μg/ml or lower an FI of at least 1 was induced by the peptide. The (putative) anchor residues are underlined.

HLA-A*0201 is given in Table 1. The selected peptides were dissolved in DMSO and diluted to a concentration of 4 mg/ml with PBS and stored in a –80°C freezer. (The concentration of DMSO never exceeded 0·2% in the final peptide solution.) Binding of the peptides to HLA-A*0201 was tested in two series (peptides 1–8 and 9–28).

*Assessment of peptide binding to HLA-A*0201 on T2 cells*

Binding of the selected peptides to HLA-A*0201 was assessed as described by Nijman *et al.* [30]. In short, 174CEM.T2 cells (T2 cells) were incubated overnight with serial dilutions of the peptides in IMDM, starting at a concentration of 100 μg/ml, and the next day the cells were incubated with a MoAb against HLA-A2 (BB7.2) and subsequently with goat anti-mouse IgG–FITC (Becton Dickinson, Etten-Leur, The Netherlands). Mean fluorescence of peptide-incubated T2 cells was compared with PBS-incubated T2 cells on a FACScan flow cytometer (Becton Dickinson). The fluorescence index (FI) was calculated for each concentration of peptide:

$$FI = \frac{(\text{mean fluorescence with peptide} - \text{mean background fluorescence})}{\text{mean background fluorescence}}$$

The maximal FI induced by a peptide and the maximal dilution of a peptide resulting in an FI higher than 1 were used as criteria for the binding of a peptide (Table 1). Maximal FI was usually found at 100 μg/ml of peptide, although for some peptides the FI was maximal at 50 μg/ml.

T cell lines from biopsies

Needle biopsies from kidneys and biopsies from lung, skin, nasal mucosa or salivary gland were cut into very small pieces and transferred to 24-well culture plates containing 1 ml of IMDM + /10% Lymphocult T (LcT; Biotest). After 10–14 days of culture the proliferating T cells were restimulated with irradiated pooled human lymphocytes and 1 μg/ml of PHA in IMDM + /10% LcT. After 12 days of culture portions of the T cells were frozen in liquid nitrogen. The rest of the T cells were restimulated as described and tested in a cytotoxicity assay 7 days after restimulation.

Cytotoxicity assay

T2 cells (2×10^6) or Epstein–Barr virus (EBV)-transformed B cells were labelled with 150 μCi of sodium ^{51}chromate for 1 h at 37°C, washed twice with IMDM and resuspended in IMDM+ at a concentration of 4×10^4 cells/ml. Sufficient numbers of labelled cells were incubated with 20 μg/ml (final concentration) of peptide for 15 min at 37°C or without peptide and then transferred to 96-well plates (2000 cells/well). Various numbers of T cells were added to the wells, resulting in effector : target ratios of 100, 50, 25 and 12·5 and the plates were centrifuged for 5 min at 1400 rev/min. As controls 50 μl of IMDM + (spontaneous release) or 2% Triton X-100 (maximal release) were added instead of T cells. Supernatant (50 μl) was harvested after 4 h of incubation at 37°C and counted in a Packard gamma counter. The percentage of ^{51}Cr release was calculated as follows:

$$\frac{\text{ct/min experimental well} - \text{ct/min spontaneous release}}{\text{ct/min maximal release} - \text{ct/min spontaneous release}} \times 100\%$$

Statistical analysis

Student's *t*-test for paired data was used to calculate *P* values of differences between rats before and after immunization. The upper limit of the normal controls was calculated as follows: mean SI ± 2 × s.d. Using this limit, *P* values were calculated by χ^2 Fisher's exact test.

RESULTS

To find out whether circulating PR3-specific T cells were present in WG patients we tested PBMC of 21 patients and eight healthy controls in an LTT for proliferative responses against PR3. In the patient group the SI of five patients exceeded the upper limit of the controls (mean SI ± 2 × s.d. = 2·48) and therefore should be considered positive (Fig. 1). The mean SI of the total patient group in response to 16 μg/ml PR3 was slightly higher than the mean SI of the controls (mean ± s.d. 1·66 ± 0·90 and 1·14 ± 0·67, respectively), but these differences were not statistically significant ($P = 0{\cdot}16$). Lower concentrations of PR3 resulted in lower responses in those patients who responded to PR3. No significant difference in proliferation was found between patients with active disease (●) or with non-active disease (○).

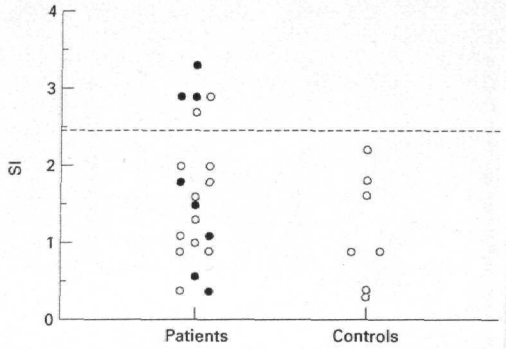

Fig. 1. Proliferation of lymphocytes from patients and controls against 16 μg/ml proteinase 3 (PR3) in a lymphocyte transformation test (LTT). Proliferation is indicated as stimulation index (SI) (ct/min with PR3/ct/min with medium alone). The upper limit of the controls (mean ± 2 × s.d.) is shown as a dashed line. ●, Patients with active disease.

To test a larger group of patients, an alternative whole-blood proliferation assay requiring only limited amounts of blood was used. This assay utilizes non-fractionated peripheral blood diluted in culture medium, without addition of serum proteins. First, to test the assay conditions, we set up an animal model for the T cell responses to PR3 in rats. We performed whole-blood assays comparable to the human assay with PR3-immunized Wistar rats ($n = 5$). All rats responded to PR3 (Fig. 2a) and a significant proliferation against PR3 was found with 1, 4 and 16 μg/ml PR3 (mean ct/min ± s.d. medium, 109 ± 10; PR3, 2190 ± 625 and 3892 ± 1054 and 5931 ± 2048, respectively, $P \leqslant 0{\cdot}05$ (paired t-test) for each concentration of PR3). Some rats responded even to 0·05 μg/ml of PR3 (data not shown). PR3 seemed to have a slight mitogenic effect at 16 μg/ml in non-immunized rats, but this was statistically not significant (ct/min of medium 120 ± 18; PR3 16 μg/ml, 152 ± 3 (mean ± s.e.m.; $P = 0{\cdot}129$). Sera of all rats diluted up to 1 : 640 were positive in an ELISA using purified PR3 (Fig. 2b).

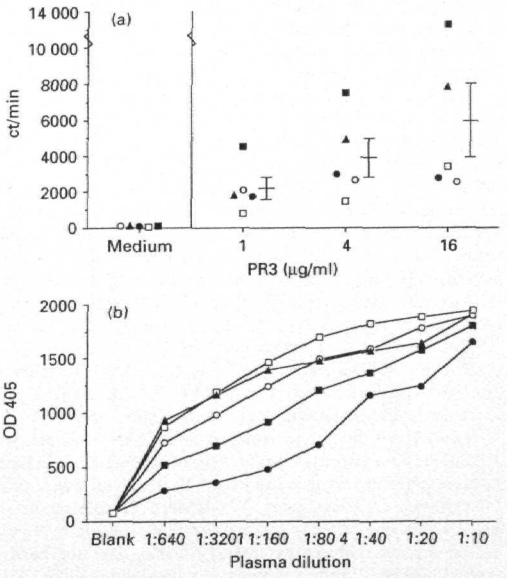

Fig. 2. (a) Proliferation of lymphocytes from rats (previously immunized with purified proteinase 3 (PR3)) in a whole blood assay against various concentrations of PR3 (ct/min). Mean ± s.e.m. of five rats is indicated. (b) Reactivity of serial dilutions of plasma from rats (previously immunized with purified PR3) in an ELISA using purified PR3 (OD 405). Corresponding symbols in a and b refer to identical animals. Plasma was derived from the samples used in the whole-blood assay.

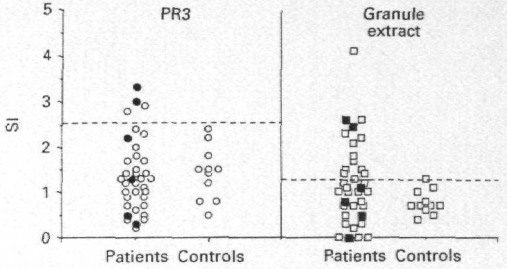

Fig. 3. Proliferation of lymphocytes from WG patients and controls against 16 μg/ml proteinase 3 (PR3) or 64 μg/ml of an azurophilic granule extract in a whole-blood assay. Proliferation is indicated as stimulation index (SI; ct/min with PR3/ct/min with medium alone). The upper limit of the controls (mean ± 2 × s.d.) is shown as a dashed line. Patients with active disease are represented by filled symbols.

Having shown the potential use of this assay in rats, we tested 36 patients and 11 healthy controls in whole-blood proliferation assays (Fig. 3). Cellular proliferation against 16 μg/ml PR3 and 80 μg/ml of a non-fractionated extract of azurophilic granules (granule extract) was tested. Four patients out of 36 had an SI against PR3 exceeding the upper limit of the controls (mean + 2 × s.d. = 1·42 + 2 × 0·58 = 2·58) and were considered positive. For the whole patient group, differences in SI between patients and controls were not significant ($P = 0{\cdot}33$).

An almost significant difference between patients and controls was found for granule extract-induced proliferation (1·19 ± 0·90 and 0·77 ± 0·26, respectively (mean ± s.d.), upper limit of controls = 1·29; $P \approx 0{\cdot}063$). Fourteen patients had an SI against the granule extract exceeding the upper limit of the controls. Lower concentrations of antigen generally resulted in lower responses of those patients who responded to PR3 or the azurophilic granule extract. No significant correlation could be found between the reaction against PR3 and against the granule extract. However, three of the four patients with positive response against PR3 also had a positive response against the granule extract. No significant differences in proliferation between patients with active disease (filled symbols) and non-active disease (open symbols) were found. Correlations between the responses to PR3 in the LTT and in the whole-blood assay could not be calculated since patient groups of both assays were not related and only a few patients were tested in both assays at the same time.

Since the 95% CI of the mean of the controls (1·03–1·81,

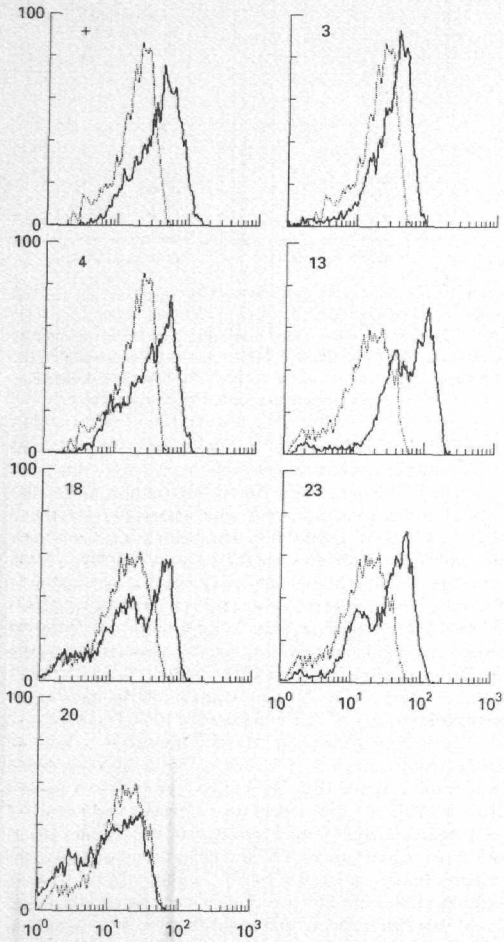

Fig. 4. FACS histograms of the up-regulated HLA-A2 expression mediated by the indicated peptides (as mentioned in Table 1, + = positive control, 20 = non-binding peptide). Dotted lines indicate the background fluorescence without peptide. Solid lines indicate the up-regulated fluorescence mediated by the peptide.

mean = 1·42) does not equal 1, it can be concluded that PR3 had a slight but significant mitogenic effect ($P \leqslant 0{\cdot}05$). Similarly, it can be concluded that azurophilic granule extract had a slight inhibitory effect on the proliferation of control lymphocytes (mean SI = 0·77, 95% CI of the mean = 0·61–0·94) ($P \leqslant 0{\cdot}05$). In this assay, an SI in response to TT of almost 50 could be found in some patients, indicating the efficiency of the assay. No correlation was found between the responses against TT and PR3 or granule extract (data not shown).

Since PR3 is synthesized by endothelial cells after stimulation with tumour necrosis factor-alpha (TNF-α) [24], we investigated whether cytotoxic T cell responses to PR3 in the context of class I MHC were detectable in T cell lines of WG patients.

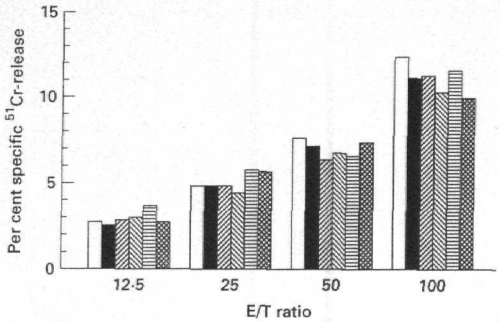

Fig. 5. Percentage specific ^{51}Cr-release of 2000 T2 cells at various E:T ratios induced by a T cell line grown from a lung biopsy of a WG patient. T2 cells were preincubated either with medium (□) or with proteinase 3 (PR3) peptides 3 (■), 4 (▨), 13 (▧), 18 (▤) or 23 (▩).

We selected and synthesized 28 synthetic peptides of 9–11 amino acids (Table 1), based on two computer models predicting the binding of peptides to HLA-A*0201 ([30], unpublished results). Actual binding of these peptides to HLA-A*0201 was tested using the processing-defective T2 cell line. Peptides 3, 4, 13, 18 and 23 induced at least two-fold up-regulation of the mean fluorescence at concentrations of 25 μg/ml and higher (FI $\geqslant$ 1). Therefore these peptides are considered to be effectively binding to HLA-A*0201 (Fig. 4). Peptides 9, 11, 12 and 16 also induced a maximal FI higher than 1·0 at concentrations of 50–100 μg/ml. This up-regulation was no longer detectable at concentrations of 25 μg/ml and lower, and these peptides were therefore not used in the cytotoxicity assays (Table 1).

Peptides 3, 4, 13, 18 and 23 were used to study the cytotoxic potential of biopsy-derived T cells of HLA-A*0201-positive WG patients. HLA-A2 frequencies are not different in WG patients compared with the normal population [38–40]. HLA-A*0201 is the major subtype of A2 and is found at high frequencies in all human races [41]. The presence of HLA-A*0201 on patient lymphocytes was detected with a cytotoxicity assay using EBV-immortalized B cells of HLA-A2-positive WG patients. After incubation of the B cells with a HLA-A*0201-binding peptide of the influenza matrix protein, these cells served as targets for the influenza-specific HLA-A*0201-restricted CTL clone Q66–9 [31]. Of the five A2-positive B cell lines tested, four were HLA-A*0201-positive and were efficiently lysed by the Q66–9 CTL clone after incubation with the influenza peptide (data not shown).

Four T cell lines were obtained from biopsies of HLA-A*0201-positive patients taken at sites of inflammation (nasal mucosa, lung, salivary gland and skin). Each cell line was tested for cytotoxic capacity against ^{51}Cr-labelled T2 cells preincubated with each of the five selected peptides or medium. None of the peptides induced cytotoxicity higher than the background lysis of T2 cells preincubated with medium alone. All T cell lines contained both $CD4^+$ and $CD8^+$ T cells. A representative experiment is shown (Fig. 5).

DISCUSSION

T cell-mediated autoimmune reactions have been suggested as

an important factor in the pathogenesis of WG and other vasculitides, not only because of the influx of T lymphocytes in inflamed tissues, but also because of the formation of granulomas in WG patients [14,22]. Secondary evidence for T cell-mediated inflammatory processes in WG is found in the enhanced levels of sCD25 in the circulation of patients with active disease [16]. Although contradictory descriptions of the proliferative responses of PBMC of WG patients to PR3 and other constituents of the azurophilic granules of neutrophils have been published [17,42], the positive results suggest that T cell reactivity against target antigens of ANCA could be important in WG. Furthermore, the predominance of IgG4–C-ANCA in sera of WG patients suggests repeated stimulation of PR3-specific B cells by (IL-4-producing) PR3-specific, T helper cells [43,44].

In the present study, we tested the proliferative capacity of T cells of WG patients against PR3 and an extract of azurophilic granules from PMN. In a classical proliferation assay with isolated PBMC we observed that five patients from a panel of 20 specifically proliferated in the presence of PR3. To test a larger group of patients we used a modified proliferation assay that used whole blood instead of isolated PBMC. Using PR3-immunized rats, it was found that the optimal PR3 concentration (16 μg/ml) induced strong proliferative responses in whole blood of immunized rats without mitogenic effects in non-immunized animals. Similar results were found with TT in TT-immunized rats (van Breda-Vriesman, unpublished data). Using the optimal PR3 concentration we tested a larger group of patients for PR3-specific proliferation in the whole blood assay. As in the LTT, a limited group of patients had a significant proliferative response to 16 μg/ml PR3. Surprisingly, a larger percentage of the patients tested (14/36), reacted to a non-fractionated azurophilic granule extract. This finding suggests that other constituents of azurophilic granules may be targets of autoimmune responses. These antigens may include myeloperoxidase, cathepsin G and elastase, all of which have been described as target antigens for ANCA [7]. Our findings confirm the proliferative response of lymphocytes from WG patients to azurophilic granule extract, as shown by Rasmussen & Petersen [13]. Other investigators have not found specific proliferation of patient lymphocytes using a total cytoplasmic extract of neutrophils [17]. This latter finding may reflect the method of antigen preparation (acid extraction). Another possible explanation for the higher response to azurophilic granule extract may be found in the observation that autoreactive $CD4^+$ T cell clones from lupus-prone mice specifically respond to nucleosomes, complexes of DNA and histones, while purified histones or DNA alone do not stimulate these clones [45]. The authors suggest that, during processing and presentation of these complexes, certain epitopes are protected against proteolysis, whereas these epitopes are degraded during processing of the purified histones. Similarly, PR3 may be present in azurophilic granules in the form of multimeres or complexes with other molecules. During isolation of PR3 these complexes may be disrupted by the Triton X-100 used for the extraction of the granules, while the non-fractionated azurophilic granule extract used in the proliferation assays is not treated with detergent.

Another protein that can be a potential target for T cell-mediated autoimmune responses is α-enolase, a neutrophil cytosolic protein related to heat shock proteins. Antibodies against this enzyme have been described in the sera of both WG and systemic lupus erythematosus (SLE) patients with renal involvement [46]. T cell responses to heat shock proteins have been described in various autoimmune diseases, such as rheumatoid arthritis [47].

Since PR3 is synthesized by cytokine-activated endothelial cells, PR3 peptides may be presented in the context of MHC class I and thus be a target for autoreactive cytotoxic T cells. We have identified several peptides of PR3 that may be presented in the context of the common MHC class I molecule HLA-A*0201. The programs used to select the peptides to be synthesized and tested for binding to HLA-A*0201 were either based on the sequences found in peptides eluted from HLA-A*0201 [31,36] or derived from the consensus sequence of an extended set of synthetic peptides known to bind to HLA-A*0201 *in vitro* [48]. Peptides 4, 13 and 18 all fulfilled the motif defined by Rammensee [31] (leucine or isoleucine at position 2 and valine or isoleucine at position 9 or 10), while peptide 4 was strongly selected by both programs. Peptide 3 has the correct anchor residues, but the other residues do not match with the residues found in naturally processed peptides, and therefore peptide 3 was not selected by the first program. Peptide 23 (FVLTAAHCL) has valine as anchor on position 2, which is not found in naturally processed peptides on HLA-A*0201 [36]. However, valine at position 2 is incorporated in the consensus sequence used by the second program, as it supports *in vitro* binding of synthetic peptides to HLA-A*0201. Although it is not clear whether such peptides are processed naturally, synthetic peptides of the human papillomavirus type 16 (HPV-16) oncogenes E6-E7, selected by binding to class I, can be effective for the induction of peptide-specific CTL, that lyse tumour cells *in vitro* [49]. Attempts to detect CTL activity against the HLA-A*0201-binding PR3 peptides in T cell lines derived from biopsies of WG patients were uniformly unsuccessful. This failure might be due to the low frequencies of peptide-specific CTL in the biopsy tissue or to the culture conditions that do not favour outgrowth of CTL. It may be necessary to add IL-12, a potent stimulator of the cellular immune response [50], to generate T cell lines with higher frequencies of PR3-specific CTL. Attempts to generate PR3 peptide-specific T cell clones out of biopsy T cell lines, using peptide-loaded autologous EBV-transformed B cells as stimulator cells, were not successful either. Generation of peptide-specific T cell lines from peripheral blood of WG patients by stimulation with peptide-loaded T2 cells (as described previously [31]) may provide a different way to detect the presence of PR3-specific CTL.

Not much is known about the activation of autoreactive T cells in autoimmunity. In chronic active hepatitis it was found that liver-derived T cell clones of both the CD4 and CD8 subset are stimulated by class II or class I matched hepatocytes, respectively, but not by autologous PBMC [51]. This suggests that autoantigens produced by hepatocytes are presented not only in the context of class I but also in the context of class II. This presentation is confirmed by studies that show that cytosolic proteins can be presented in the context of class II [52]. Since PR3 is synthesized by human endothelial cells and murine endothelial cells are capable of processing and presenting exogenous antigens to T cells in the context of class II after stimulation with interferon-gamma (IFN-γ) [53], it seems possible that $CD4^+$ and $CD8^+$

PR3-specific T cells could be activated by vascular endothelial cells.

Endothelial cells presenting PR3 peptides in the context of class I or class II molecules may also be a direct target of cytotoxic activity of either $CD8^+$ or $CD4^+$ autoreactive T cells. It has been described that hepatocyte-specific T cells of both the CD4 and the CD8 phenotype possess cytotoxic capacity [51]. Cytotoxic autoreactive T cells have been described in type I diabetes mellitus [54] and CTL specific for autologous biliary epithelial cells have been described in primary biliary cirrhosis patients [28].

In conclusion, we have demonstrated that PR3-specific T cells are present in the circulation of some WG patients. Furthermore, the proliferation to azurophilic granule extract suggests that T cell recognition of other azurophilic granule proteins occurs. T cell lines and clones against PR3 or other granule antigens are needed for further analysis of both the cytokine pattern and fine specificity of autoreactive T cells in WG. Furthermore, PR3 induces strong proliferative responses in whole blood assays using blood of PR3-immunized rats. We conclude that this whole blood assay is suitable for the longitudinal study of cellular (auto)immune responses in laboratory animals, and it may therefore serve as a potent tool for the study of animal models of vasculitis or glomerulonephritis.

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REFERENCES

1 Wegener F. Uber eine eigenartige rhinogene Granulomatose mit besonderer Beteiligung des Arteriensystem und der Nieren. Beitr Pathol Anat 1939; **102**:36.

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

3 Keogan MT, Esnault VL, Green AJ, Lockwood CM, Brown DL. Activation of normal neutrophils by anti-neutrophil cytoplasm antibodies. Clin Exp Immunol 1992; **90**:228–34.

4 Charles LA, Caldas ML, Falk RJ, Terrell RS, Jennette JC. Antibodies against granule proteins activate neutrophils *in vitro*. J Leukoc Biol 1991; **50**:539–46.

5 Ewert BH, Jennette JC, Falk RJ. Anti-myeloperoxidase antibodies stimulate neutrophils to damage human endothelial cells. Kidney Int 1992; **41**:375–83.

6 Savage CO, Pottinger BE, Gaskin G, Pusey CD, Pearson JD. Autoantibodies developing to myeloperoxidase and proteinase 3 in systemic vasculitis stimulate neutrophil cytotoxicity toward cultured endothelial cells. Am J Pathol 1992; **141**:335–42.

7 Hagen EC, Ballieux BEPB, van Es LA, Daha MR, van der Woude FJ. Anti-neutrophil cytoplasmic autoantibodies (ANCA). A review of the antigens involved, the assays, the clinical and possible pathogenetic consequences. *Blood 1993*; **81**:1996.

8 Mulder AHL, Heeringa P, Brouwer E, Limburg PC, Kallenberg CGM. Activation of granulocytes by anti-neutrophil cytoplasmic antibodies: a Fc-gamma-RII-dependent process. Clin Exp Immunol 1994; **98**:270–8.

9 Ludemann J, Utecht B, Gross WL. Detection and quantitation of anti-neutrophil cytoplasm antibodies in wegener's granulomatosis by ELISA using affinity-purified antigen. J Immunol Methods 1988; **114**:167–74.

10 van de Wiel BA, Dolman KM, van der Meer-Gerritsen CH, Hack CE, von dem Borne AEGK, Goldschmeding R. Interference of Wegener's granulomatosis autoantibodies with neutrophil proteinase-3 activity. Clin Exp Immunol 1992; **90**:409–14.

11 Bolton WK, Innes DJ, Sturgill BC, Kaiser DL. T cells and macrophages in rapidly progressive glomerulonephritis: clinicopathologic correlations. Kidney Int 1987; **32**:869–76.

12 *Hooke DH, Gee DC, Atkins RC. Leukocyte analysis using monoclonal antibodies in human glomerulo-nephritis.* Kidney Int 1987; **31**:961–72.

13 Rasmussen N, Petersen J. Cellular immune responses and pathogenesis in c-ANCA positive vasculitides. J Autoimmun 1993; **6**:227–36.

14 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.

15 Churg J. Nomenclature of vasculitic syndromes: a historical perspective. Am J Kidney Dis 1991; **18**:148–53.

16 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.

17 Mathieson PW, Lockwood CM, Oliveira DB. T and B cell responses to neutrophil cytoplasmic antigens in systemic vasculitis. Clin Immunol Immunopathol 1992; **63**:135–41.

18 Roep BO, Kallan AK, Hazenbos WL *et al.* T cell reactivity to a 38kDa insulin-secretory-granule protein in patients with recent-onset type 1 diabetes. Lancet 1991; **337**:1439–41.

19 Ewins DL, Barnett PS, Ratanachaiyavong S *et al.* Antigen-specific T cell recognition of affinity-purified and recombinant thyroid peroxidase in autoimmune thyroid disease. Clin Exp Immunol *1992*; **90**:93–98.

20 Champion BR, Page KR, Parish N *et al.* Identification of a thyroxine-containing self-epitope of thyroglobulin which triggers thyroid autoreactive T cells. J Exp Med 1991; **174**:363–70.

21 Berrih-Aknin B, Cohen-Kaminsky S, Lepage V, Neumann D, Bach JF, Fuchs S. T-cell antigenic sites involved in myasthenia gravis: correlations with antibody titre and disease severity. J Autoimmun 1991; **4**:137–53.

22 Bloemena E, Roos MTL, Van Heijst JLAM, Vossen JMJJ, Schellekens PTA. Whole-blood lymphocyte cultures. J Immunol Methods 1989; **1224**:161–7.

23 Mayet WJ, Meyer zum Büschenfelde KH. Membrane expression of proteinase 3 in human endothelial cells under the influence of different cytokines. Clin Exp Immunol 1993; **93** (s1):17 (Abstr.).

24 Mayet WJ, Csernok E, Szymkowiak C, Gross WL, Meyer zum Büschenfelde KH. Human endothelial cells express proteinase 3, the target antigen of anticytoplasmic antibodies in Wegener's granulomatosis. Blood 1993; **82**:1221–9.

25 Mayet WJ, *Hermann EM, Csernok E, Gross WL, Meyer zum* Büschenfelde KH. *In vitro* interactions of c-ANCA (antibodies to proteinase 3) with human endothelial cells. Adv Exp Med Biol 1993; **336**:109–13.

26 Hashimoto E, Lindor KD, Homburger HA *et al.* Immunohistochemical characterization of hepatic lymphocytes in primary biliary cirrhosis in comparison with primary sclerosing cholangitis and autoimmune chronic active hepatitis [see comments]. Mayo Clin Proc 1993; **68**:1049–55.

27 Itoh N, Hanafusa T, Miyazaki A *et al.* Mononuclear cell infiltration and its relation to the expression of major histocompatibility complex antigens and adhesion molecules in pancreas biopsy specimens from newly diagnosed insulin-dependent diabetes mellitus patients. J Clin Invest 1993; **92**:2313–22.

28 Onishi S, Saibara T, Nakata S *et al.* Cytotoxic activity of spleen-derived T lymphocytes against autologous biliary epithelial cells in autopsy patients with primary biliary cirrhosis. Liver 1993; **13**: 188–92.

29 Wei ML, Cresswell P. HLA-A2 molecules in an antigen-processing mutant-cell contain signal sequence-derived peptides. Nature 1992; **356**:443–6.

30 Nijman HW, Houbiers JGA, Vierboom MPM *et al.* Identification of peptide sequences that potentially trigger HLA-A2.1 restricted cytotoxic T lymphocytes. Eur J Immunol 1993; **23**:1215–9.

31 Nijman HW, Van der Burg SH, Vierboom MPM, Houbiers JGA, Kast WM, Melief CJM. p53, a potential target for tumor-directed T cells. Immunol Letters 1994; **40**:171–8.

32 Kast WM, Brandt RM, Drijfhout JW, Melief CJM. Human leukocyte antigen-A2.1 restricted candidate cytotoxic T lymphocyte epitopes of human papillomavirus type 16 E6 and E7 proteins identified by using the processing-defective human cell line T2. J Immunother 1993; **14**:115–20.

33 Borregaard N, Heiple JM, Simons ER, Clark RA. Subcellular localization of the b-cytochrome component of the human neutrophil microbicidal oxidase: translocation during activation. J Cell Biol 1983; **97**:52–61.

34 Goldschmeding R, van der Schoot CE, ten Bokkel Huinink D *et al.* Wegener's granulomatosis autoantibodies identify a novel diisopropylfluorophosphate-binding protein in the lysosomes of normal human neutrophils. J Clin Invest 1989; **84**:1577–87.

35 Leid RWes, Van der Heijden I, Ballieux BEPB *et al.* Cleavage and inactivation of human C1-inhibitor by the human leukocyte proteinase, proteinase-3. Eur J Immunol 1993; **23**:2939–45.

36 Rammensee HG, Falk K, Rötzschke O. Peptides naturally presented by MHC class I molecules. Ann Rev Immunol 1993; **11**:213–44.

37 Falk K, Rötzschke O, Stevanovic S, Jung G, Rammensee HG. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature 1991; **351**:290–6.

38 Katz BP, Alling DW, Haynes BF, Fauci AS. Association of Wegener's granulomatosis with HLA-B8. Clin Immunol Immunopathol 1979; **14**:268–70.

39 Papiha SS, Murty GE, Ad Hia A, Mains BT, Venning M. Association of Wegener's granulomatosis with HLA antigens and other genetic markers. Ann Rheum Dis 1992; **51**:246–8.

40 Murty GE, Mains BT, Middleton D, Maxwell AP, Savage DA. Hla antigen frequencies and Wegener's granulomatosis. Clin Otolaryngol 1991; **16**:448–51.

41 The databook of the 11th International Histocompatibility Workshop (Yokohama, Japan) 1991; **2**:807.

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

43 Brouwer E, Cohen Tervaert JW, Horst G *et al.* 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.

44 Spiegelberg HL. Biological role of different antibody classes. Int Arch Allergy Appl Immunol 1990; **90**:22.

45 Mohan C, Adams S, Stanik V, Datta SK. Nucleosome: a major immunogen for pathogenic autoantibody-inducing T cells of lupus. J Exp Med 1993; **177**:1367–81.

46 Moodie FDL, Leaker B, Cambridge G, Totty NF, Segal AW. Alpha-enolasea novel cytosolic autoantigen in ANCA positive vasculitis. Kidney Int 1993; **43**:675–81.

47 Res PCM, Breedveld FC, van Embden JDA *et al.* Synovial fluid T cell reactivity against 65 kD heat shock protein of mycobacteria in early chronic arthritis. Lancet 1988; **ii**:478–80.

48 Dryfhout JW, Brandt RMP, D'Amaro J, Kast WM, Melief CJM. Detailed motifs for peptide binding to HLA-*0201, derived from large random sets of peptides using a cellular binding assay. Hum Immunol 1995; (in press).

49 Feltkamp MCW, Smits HL, Vierboom MPM *et al.* Vaccination with cytotoxic T lymphocyte epitope-containing peptide protects against a tumor induced by human papillomavirus type 16-transformed cells. Eur J Immunol 1993; **23**:2242–9.

50 Clerici M, Lucey DR, Berzofsky JA *et al.* Restoration of HIV-specific cell-mediated immune responses by interleukin-12 *in vitro*. Science 1993; **262**:1721–4.

51 Franco A, Barnaba V, Ruberti G, Benvenuto R, Balsano C, Musca A. Liver-derived T cell clones in autoimmune chronic active hepatitis: accessory cell function of hepatocytes expressing class II major histocompatibility complex molecules. Clin Immunol Immunopathol 1990; **54**:382–94.

52 Malnati MS, Marti M, LaVaute T *et al.* Processing pathways for presentation of cytosolic antigen to MHC class II-restricted T cells. Nature 1992; **357**:702–4.

53 St. Louis JD, Lederer JA, Lichtman AH. Costimulatory deficient antigen presentation by an endothelial cell line induces non-proliferative T cell activation response without anergy. J Exp Med 1993; **178**:1597–605.

54 Roep BO, Kallan AK, De Vries RRP. b-Cell antigen-specific lysis of macrophages by CD4 T-cell clones from newly diagnosed IDDM patient. Diabetes 1992; **41**:1380–4.