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Analysis of T cell responses to the autoantigen in Goodpasture's disease

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SUMMARY

Goodpasture's disease is a rare form of glomerulonephritis characterized by the production of autoantibodies to the glomerular basement membrane (GBM). In order to understand the development of autoimmunity to the GBM, it is important to examine mechanisms underlying T cell responses to the autoantigen. A MoAb P1, with the same specificity as patients' autoantibodies, was used to affinity-purify the antigen from collagenase-digested human GBM. This material was enriched in the NC1 domain of the α3 chain of type IV collagen (α3(IV)NC1), known to be the principal target of anti-GBM antibodies, but also contained lower quantities of α4(IV)NC1. In proliferation assays, T cells from 11/14 patients with Goodpasture's disease showed significant responses (SI $\geqslant$ 2·0) to affinity-purified human GBM. Peak responses were demonstrated at 7 or 10 days at antigen concentrations of 10–30 μg/ml. As in other autoimmune disorders, the presence of autoantigen-reactive T cells was also demonstrated in 5/10 healthy volunteers. Tissue typing revealed that all patients possessed HLA-DR2 and/or -DR4 alleles, while normal individuals whose T cells responded possessed DR2 and/or DR7 alleles. The specificity of the T cell response in Goodpasture's disease was further investigated using monomeric components of human GBM purified by gel filtration and reverse phase high performance liquid chromatography (HPLC). Two antigenic monomer pools were obtained, which were shown by amino-terminal sequence analysis to contain α3(IV)NC1 and α4(IV)NC1, respectively. In all patients tested, significant T cell proliferation was observed in response to one or both of these α(IV)NC1 domains. These results demonstrate that patients with Goodpasture's disease possess T cells reactive with autoantigens known to be recognized by anti-GBM antibodies.

Keywords T cell proliferation autoimmunity glomerular basement membrane type IV collagen Goodpasture's disease

INTRODUCTION

Goodpasture's disease is characterized by rapidly progressive glomerulonephritis (RPGN), often accompanied by pulmonary haemorrhage, and associated with the presence of autoantibodies to the glomerular basement membrane (GBM) [1]. Passive transfer studies demonstrated the pathogenicity of these antibodies [2], and this finding was supported by observations that the level of circulating autoantibodies correlated with the severity of nephritis [3]. Current treatment is based on removal of anti-GBM antibodies by plasma exchange and the use of immunosuppressive agents to control their production [3]. The autoantigen has been localized to the non-collagenous domain of the α3 chain of type IV collagen (α3(IV)NC1) [4,5], and two-dimensional Western blotting studies have shown that the autoantibody response is largely restricted to this molecule in all patients [6]. There is a strong association with the MHC class II alleles DRB1*1501 and *1502 (DR2) and DRB1*04 (DR4), and it has been proposed that environmental factors may trigger the disease in genetically susceptible individuals [7,8]. The highly restricted antibody response, and strong MHC associations, suggest that the autoimmune process is likely to be T cell-dependent.

In addition to the probable role of T cells in the production of anti-GBM antibodies, they may also be directly involved in tissue injury. T cells and macrophages have been demonstrated in the inflamed glomerulus, and maximum cell numbers are present in the early stages of glomerulonephritis, with T cell influx preceding macrophage accumulation [9]. In an antigen-induced rat model of anti-GBM disease, experimental autoimmune glomerulonephritis (EAG), $CD4^+$ T cells infiltrate the glomerulus before monocyte influx (J. Reynolds, unpublished observations), and animals with EAG demonstrate DTH to

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GBM antigens [10]. In addition, it is possible to prime naive recipients for the disease using positively selected $CD4^+$ T cells, but not B cells, from donors with EAG [11], and treatment with a MoAb to CD4 is effective in prevention of the disease [12].

Autoantigen-specific T cells have been demonstrated in several human autoimmune diseases, including the autoantibody-mediated disorders myasthenia gravis [13] and Graves' disease [14,15]. However, little work has been carried out on the specificity of T cells in renal disease, mainly due to the lack of identification of target antigens. Two reports from the early 1970s showed that T cells from patients with Goodpasture's disease produced migration inhibitory factor in response to GBM [16,17]. Early experiments in our laboratory, using crude collagenase digests of human GBM (hGBM) and patients' peripheral blood mononuclear cells (PBMC) in proliferation assays, suggested that it was possible to demonstrate GBM-reactive T cells (A.J. Rees, unpublished observations).

This study represents the first demonstration of autoreactive T cells specific for affinity-purified Goodpasture antigen in patients with Goodpasture's disease and normal individuals, and provides further data on the specificity of the T cell response in patients.

PATIENTS AND METHODS

Patients

Fifteen patients with Goodpasture's disease, all with RPGN and some with pulmonary haemorrhage, were studied (Table 1). Circulating anti-GBM antibodies were demonstrated by solid-phase radioimmunoassay (RIA) in all cases [18], and there was linear staining for IgG on the GBM in all available kidney biopsies. T cells were collected from patients before they had undergone prolonged treatment with immunosuppressive drugs. Approximately 50 ml of buffy coat were obtained from 12 patients at the time of their first plasma exchange, while 50 ml of peripheral blood were obtained from the remaining three patients and from 10 healthy laboratory volunteers.

Isolation of PBMC

PBMC were purified from blood and buffy coat by density gradient centrifugation using Lymphoprep (Nycomed, Oslo, Norway), and resuspended in freezing medium (40% RPMI, 50% fetal calf serum (FCS) and 10% dimethyl sulphoxide) for storage in liquid nitrogen.

Preparation of GBM

Normal human kidneys were obtained at less than 24 h post-mortem and stored at −70°C until use. Glomeruli were extracted from the cortex by differential sieving, and soluble hGBM (containing α(IV)NC1 domains) was prepared by sonication, lyophilization and collagenase digestion [18].

Affinity purification of α(IV)NC1 domains

The hybridoma producing the MoAb P1 was grown under standard tissue culture conditions and the antibody purified by sodium sulphate precipitation [19]. Monoclonal P1 was bound to a cyanogen bromide-activated Sepharose 4B column (Pharmacia, Uppsala, Sweden) at 5 mg/ml, and used to affinity purify hGBM under non-dissociating conditions. Collagenase-digested hGBM (10 mg) was allowed to recirculate for 2 h, before washing unbound material through the column with PBS. Approximately 500 μg of bound material were eluted with 3 M potassium thiocyanate and dialysed against distilled water to remove excess salt. Affinity-purified preparations of α(IV)NC1 domains were analysed by inhibition RIA, and by two-dimensional electrophoresis and Western blotting [6]. Samples of affinity-purified hGBM were lyophilized in micro-

Table 1. Patient characteristics and T cell responses to affinity-purified human glomerular basement membrane (hGBM) and α(IV)NC1 domains in patients with Goodpasture's disease

Patients	Age	Sex	Anti-GBM antibody %*	DR type	SI to AP-hGBM†	SI to α3(IV)NC1†	SI to α4(IV)NC1†
1	71	M	89	2,2	10·9	ND	ND
2	57	M	26	2,6	9·8	10·7	3·3
3	48	M	57	2,9	8·1	2·3	2·9
4	27	M	66	2,4	4·6	2·1	2·0
5	19	M	33	3,4	4·3	1·4	3·3
6	21	M	103	2,3	3·7	ND	ND
7	44	F	63	2,3	3·3	ND	ND
8	24	M	37	2,4	3·2	1·9	5·2
9	63	F	47	2,2	3·1	ND	ND
10	42	F	16	2,2	3·1	ND	ND
11	47	F	57	4,11	2·8	ND	ND
12	53	F	76	2,6	1·8	7·6	2·9
13	66	M	60	4,?	1·8	ND	ND
14	54	F	35	2,6	1·4	4·8	2·7
15	46	F	78	2,4	ND	7·7	1·8

* Anti-GBM antibody concentration (% of standard positive control) as determined by radioimmunoassay (RIA) at the time of collection of T cells.

† Peak SI obtained with the range of concentrations and time points used.

ND, Not done.

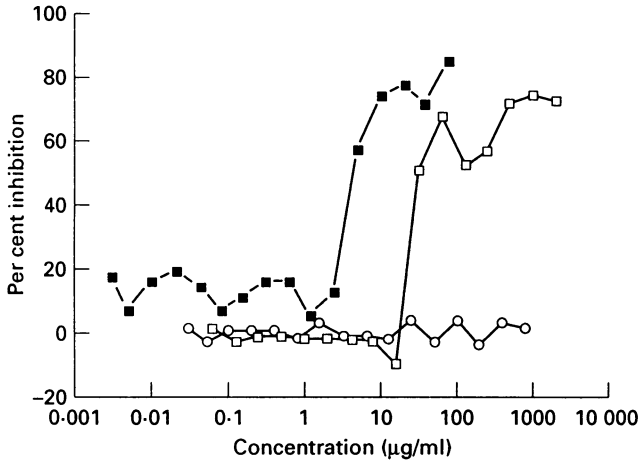


Fig. 1. Analysis of affinity-purified human glomerular basement membrane (hGBM) by inhibition radioimmunoassay (RIA). The antigenicity of the different preparations of hGBM is shown as a measure of their ability to inhibit the binding of monoclonal P1 in an inhibition RIA. Fifty percent inhibition of binding was observed with a concentration of affinity-purified hGBM that was approximately 1 log less than that of unpurified hGBM. Unbound GBM did not contain detectable amounts of antigenic material. □, Unpurified hGBM; ■, purified hGBM; ○, unbound hGBM.

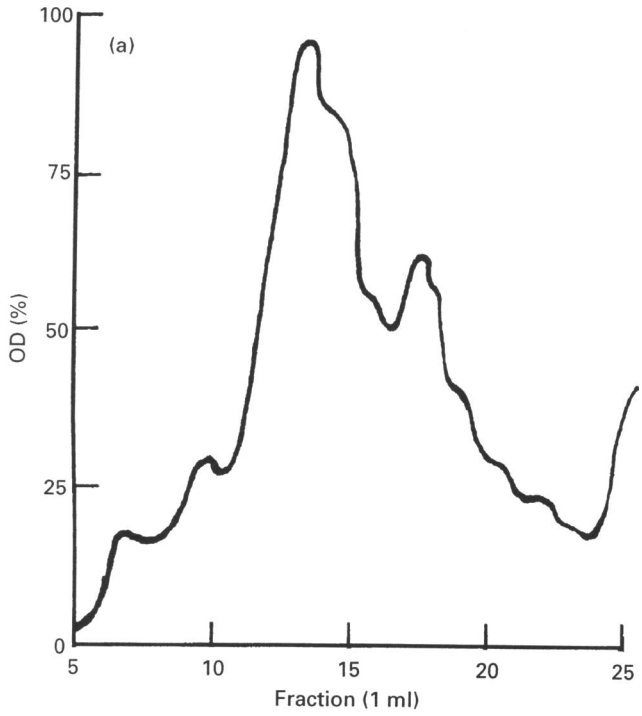
fuge tubes to give approximately 100 μg/tube. Any remaining salt was removed by acetone precipitation overnight at −70°C.

Inhibition RIA

Affinity-purified hGBM, at doubling dilutions from neat to 1 : 16 384, was incubated with MoAb P1 [19]. P1 was used at a dilution (1 : 100) that produced 50% of the maximal binding (of a neat sodium sulphate precipitate) in an RIA for anti-GBM reactivity [18]. These samples were left for 1 h at 37°C in glass vials. Each sample was then applied in triplicate to microtitre plates pre-coated with hGBM at 10 μg/ml. Bound P1 was detected with ^{125}I-labelled anti-mouse IgG (Capel). Inhibition of P1 binding was expressed as a percentage of the binding of the 1 : 100 dilution of P1 with no antigen added (Fig. 1).

Purification of antigenic 28-kD monomers

Human GBM α(IV)NC1 monomers were prepared by gel filtration followed by reverse phase high performance liquid chromatography (HPLC) [5]. Approximately 25 mg of collagenase-digested hGBM were lyophilized, resuspended in 1 ml of 6 M guanidine hydrochoride in 50 mM Tris HCl pH 7·0, and applied to a 17 × 900 mm Sephacryl S-200 column (Pharmacia). Size-separated material was eluted at 1 ml/min and 2-ml fractions were collected. Optical densities were monitored at 280 nm. Alternate fractions (100 μl) of the monomer peak were ethanol precipitated and analysed on 12·5% SDS–PAGE Phast gels (Pharmacia). Fractions that contained only monomers were pooled and applied to a C18 reverse phase HPLC column (Dynamax; 1 × 10 cm, 12-nm particles, 30-nm pores; Anachem, Luton, UK) that was equilibrated with 0·1% trifluoroacetic acid (TFA) in water (Gilson HPLC system). Bound material was eluted with a gradient of acetonitrile from 25% to 40%, plus 0·08% TFA, at a flow rate of 1 ml/min. Aliquots of alternate fractions were lyophilized, resuspended in water and analysed on SDS–PAGE Phast gels (Pharmacia)



(b)

α3(IV)NC1	G	L	K	G	K	R	G	D	S	G
Fraction 17–19	–	–	X	–	–	–	–	–	–	–
α4(IV)NC1	G	P	K	G	F	G	P	G	Y	L
Fraction 13–15	–	–	–	–	–	X	–	–	–	–

Fig. 2. Purification of the Goodpasture antigen. (a) Reverse phase high performance liquid chromatography (HPLC) elution profile, with silver stain of monomer fractions aligned to profile and Western blots of the same fractions (using a patient's autoantibodies and monoclonal P1). (b) Sequences of purified monomers compared with known sequences of α(IV)NC1 domains [5,20]. —, Identity with α(IV) chain amino acid; X, unidentified residues. Fractions 17–19 contain the majority of the antigenic monomer corresponding to α3(IV)NC1, while fractions 13–15 are less antigenic and contain α4(IV)NC1.

(Fig. 2). The remainder of each fraction was lyophilized and stored at −20°C. Amino-terminal sequence analysis was used to determine the α(IV) NC1 domain present in each fraction. Amino acid sequences are shown in Fig. 2, aligned to the

known sequences of type IV collagen α chains [5,20]. Fractions containing uncontaminated antigenic monomers were pooled for use in T cell proliferation assays.

T cell proliferation assays

T cell proliferation assays were set up in flat-bottomed 96-well plates (Flow, Irvine, UK) with 2×10^5 PBMC per well. PBMC were suspended in RPMI 1640 medium (Flow) containing 10% AB^+ human serum (Blood Transfusion Laboratories, Edgware, UK), 100 U/ml penicillin, 100 μg/ml streptomycin (GIBCO, Paisley, UK), 4 mM glutamine (Flow), and 0·2% sodium bicarbonate (GIBCO). All PBMC samples were analysed in triplicate at a final volume of 200 μl/well. Stimulation with the mitogen phytohaemagglutinin (PHA), at 2 μg/ml, was used as a non-specific indicator of the ability of cells to proliferate. Antigen-specific T cell proliferation was tested with *Mycobacterium tuberculosis* soluble extract (MTSE). Affinity-purified antigen preparations were tested over a range of log dilutions from 0·3 to 30 μg/ml. The concentrations of HPLC-purified material used were estimated from the amount of starting material, allowing for losses in the purification procedure.

T cell proliferation was tested at various time points using a thymidine incorporation assay. Initial experiments were set up for 4, 5, 6, 7, and 10 days to determine the optimal time point for detection of T cell proliferation. In later experiments using affinity-purified hGBM, 7- and 10-day proliferation assays were carried out, and for the α(IV)NC1 monomers an extra time point of 5 days was included. Tritiated thymidine (Amersham, Aylesbury, UK) was added at 1 μCi/well 16 h before harvesting of T cells onto glass fibre filters using a cell harvester (Pharmacia LKB). Thymidine incorporation was measured using an automated β counter (Pharmacia LKB). Results were expressed as ct/min or stimulation indices (SI). The SI was calculated by dividing the ct/min in wells with antigen by the ct/min in wells with no antigen.

RESULTS

Analysis of affinity-purified hGBM

Human GBM was purified on a P1 affinity column and the resulting material analysed by inhibition RIA and two-dimensional electrophoresis. Fifty percent inhibition of P1 binding was achieved in the inhibition RIA by a concentration of affinity-purified hGBM that was approximately 1 log less than that of unpurified hGBM required to achieve the same inhibition (Fig. 1). The unbound material did not inhibit the binding of P1 even when concentrated. Two-dimensional electrophoresis demonstrated that the affinity-purified hGBM was enriched in cationic dimers and monomers, known to contain α3(IV)NC1, and to a lesser extent more neutral components corresponding to α4(IV)NC1 [6,21] (data not shown).

Purification of α3(IV) and α4(IV) NC1 domains

Reverse phase HPLC-purified hGBM was analysed by SDS–PAGE. On silver staining most of the protein was present in fractions 13–15. However, by Western blotting with patients' serum and P1, fractions 17–19 and to a lesser extent 13–15 contained the most antigen. Amino terminal sequence analysis identified the α3(IV)NC1 domain in fractions 17–19 and α4(IV)NC1 in fractions 13–15 (Fig. 2).

T cell responses to affinity-purified hGBM

In initial experiments, no significant T cell proliferation was observed at less than 7 days, and therefore all subsequent assays were set up for 7 and 10 days. Patients' T cells often showed a peak response at 10 days, at an antigen concentration of 10 μg/ml, e.g. patient 2 (Fig. 3), although in some patients a peak response was observed at 7 days at a higher antigen concentration of 30 μg/ml, e.g. patient 1 (Fig. 3). T cells from half the normals studied demonstrated similar responses, often with peak SI values at day 10 at 10 μg/ml, e.g. normal 1 (Fig. 3).

All patients' PBMC were tested in T cell proliferation assays at 7 and 10 days. The highest SI observed with affinity-purified hGBM at one of these time points is given in Table 1. T cells from 11/14 patients and 5/10 normal individuals tested showed an SI of $\geqslant$2·0 (Fig. 4). There was no statistically significant difference in the frequency of positive T cell responses between patients and normals (Fisher's exact test). Similarly, the mean SI for patients' T cell responses (4·4) was not statistically higher than that for normals (3·4) (Wilcoxon matched pairs test). All PBMC proliferated in response to PHA (SI range 25–150), and most responded to MTSE (SI $\geqslant$ 2·0) (data not shown).

T cells from some individuals were tested several times, and for these the values given in Table 1 are the mean values. The assay was relatively reproducible, with the same individual responding consistently, even though different batches of affinity-purified antigen were used. For example, patient 2 was tested twice with peak SI values of 9·8 and 10·4, and normal 1 was tested four times, with peak SI values of 4·9, 5·2, 7·6 and 8·3.

Analysis of patients' circulating anti-GBM antibody levels, as determined by RIA on sera taken at the same time as the T cells, revealed no clear relationship with the level of T cell proliferation (Table 1). HLA types were available for most subjects studied. All patients that were tissue typed possessed HLA-DR2 and/or -DR4 (Table 1). In normal individuals tested, significant T cell proliferation was observed in those who possessed HLA-DR2 and/or -DR7, but not -DR4 (Table 2).

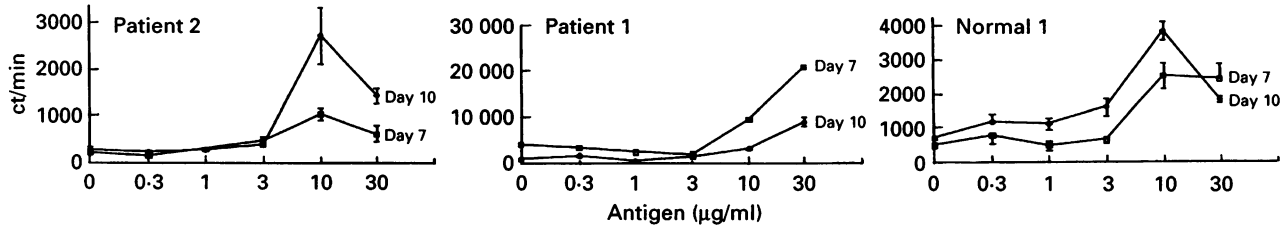


Fig. 3. Representative examples of T cell proliferation to affinity-purified human glomerular basement membrane (hGBM) (± s.e.m.).

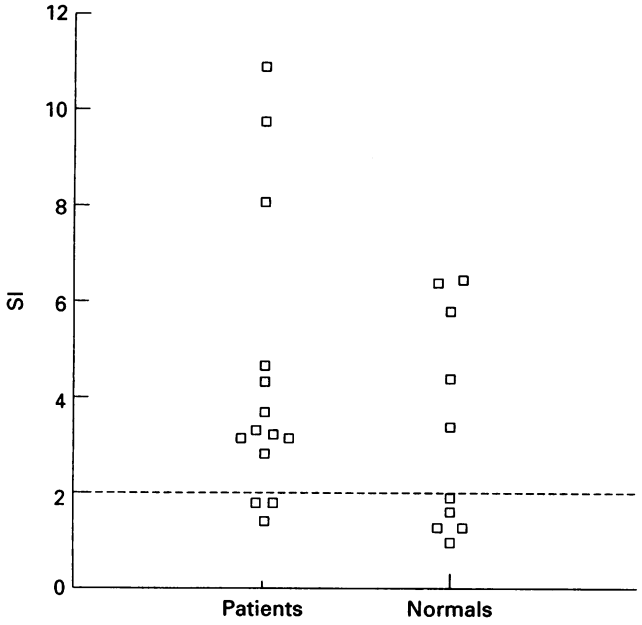


Fig. 4. T cell responses to affinity-purified human glomerular basement membrane (hGBM) in patients and normal individuals.

T cell responses to antigenic 28-kD monomers

The response of T cells to HPLC-purified NC1 domains of α3(IV) and α4(IV) chains was tested in a number of patients at three time points, 5, 7 and 10 days. The earlier time point was included as pilot experiments showed a response in some patients at 5 days, unlike the response to affinity-purified antigen. Proliferative responses were demonstrated in 6/8 patients to α3(IV)NC1, in 7/8 to α4(IV)NC1 and in 5/8 to both (Table 1). The concentration of these preparations of antigen is difficult to measure, because they are eluted from the reverse phase HPLC column in acetonitrile. However, in comparison with the same amount of starting material, similar SIs were achieved with approximately 20 times less of the purified α(IV)NC1 monomers than affinity-purified hGBM. In addition, a significant response was observed at the earlier time point of 5 days in 5/8 patients to α3(IV)NC1 and in 4/8 patients to α4(IV)NC1.

DISCUSSION

The initial step in investigating the role of T cells in autoimmune diseases is to demonstrate the presence of autoantigen-specific T cells. This study represents the first attempt to identify and characterize autoreactive T cells in a number of patients with Goodpasture's disease. Although this is a rare disorder, making it difficult to examine T cells from a large number of patients, the results obtained here are similar to those reported at early stages of the work in more common autoimmune diseases, such as myasthenia gravis [13] and thyroiditis [14,15]. Subsequent research in these disorders has led to the isolation of autoreactive T cell clones, and to progress in identifying T cell epitopes and defining functional HLA restriction [13–15]. The work described here should provide the foundation for similar studies in Goodpasture's disease, with the ultimate aim of understanding the mechanisms of autoreactive T cell activation sufficiently well to design specific immunotherapy.

Table 2. T cell responses to affinity-purified human glomerular basement membrane (hGBM) in normal individuals

Normals	Age	Sex	DR type	SI to AP-hGBM*
1	26	F	2,3	6·5
2	34	M	2,7	6·4
3	43	M	3,7	5·8
4	38	F	2,7	4·4
5	34	M	5,7	3·4
6	34	M	3,6	1·9
7	32	M	6,8	1·6
8	28	M	1,4	1·3
9	45	M	3,4	1·2
10	35	M	4,4	1·0

* Peak SI obtained with the range of concentrations and time points used.

In the present study, we have shown that T cells from 11/14 patients with Goodpasture's disease responded to affinity-purified hGBM. This material is enriched for the α3(IV)NC1 domain, but contains smaller amounts of the α4(IV)NC1 domain and traces of other α(IV)NC1 domains [6]. As in studies of other autoimmune diseases, a T cell response was taken to be significant if SI values were $\geqslant 2{\cdot}0$. Even taking an SI of 2·5 as significant, 11/14 patients and 5/10 normal individuals showed a significant T cell response. T cells from three patients did not show proliferative responses to affinity-purified antigen, and this may be due in part to the preparation of antigen used. However, it is possible that antigen-specific T cells are sequestered in the glomerulus or lung, and that the frequency of autoreactive T cells in the periphery is low, as reported in other autoimmune diseases [22,23]. Thus, the inability of the proliferation assays used here to detect low numbers of antigen-specific cells may explain the lack of detectable responses in some patients [24,25].

It was not surprising that T cells from 5/10 normal individuals also demonstrated proliferative responses to affinity-purified hGBM. Such responses to autoantigens are commonly reported in studies of other autoimmune diseases, including myasthenia gravis [24], thyroiditis [26], and multiple sclerosis [27]. The precursor frequency of autoreactive T cells may be higher in patients than in controls [28], and this is being investigated by limiting dilution analysis in our patients. Furthermore, the development of disease could depend upon several other factors, including the autoantigenic epitopes recognized, the concentration and route of presentation of the autoantigen, and the phenotype of the responding T cells [29,30]. Further work is required to address these possibilities.

The HLA-DR associations observed in autoimmune diseases are most easily explained by classical Ir gene effects, whereby a particular DR molecule (or DQ molecule in linkage disequilibrium) binds an autoantigenic peptide more strongly than other alleles. This could influence antigen presentation to mature T cells, or selection of the T cell repertoire [31]. Although all the patients whose HLA type was known

possessed DR2 and/or DR4 as previously reported in Goodpasture's disease [7,8], there was variation in the HLA types of the controls. Numbers are small, but of the five controls who responded to affinity-purified hGBM, a response was associated with DR2 or DR7. The presence of other HLA types, including DR4, was not associated with a response. However, the relevance of these observations needs to be investigated in functional studies of the HLA restriction of T cell clones specific for different peptides from the Goodpasture antigen.

In order to define further the specificity of autoreactive T cells in Goodpasture's disease, we investigated antigenic α(IV)NC1 monomers purified by HPLC. Human kidneys are scarce and the monomers are technically difficult to prepare, thus limiting the number of assays that could be performed. However, we were able to demonstrate T cell responses in patients towards the NC1 domains of one or both of the 'novel' α(IV) chains, α3(IV) and α4(IV). Studies from this laboratory and others have shown that the autoantibody response is directed principally against α3(IV)NC1 [4,5], although many patients' autoantibodies also detect α4(IV)NC1 on two-dimensional Western blotting, albeit to a lesser degree [6]. Furthermore, in the same study we found little reactivity of anti-GBM antibodies with the 'classical' α1(IV) and α2(IV)NC1 domains, which were present in much higher quantities in the two-dimensional gels [6]. Interestingly, α3(IV) and α4(IV) chains are absent from many basement membranes where α1(IV) and α2(IV) can be detected, but are co-expressed in the GBM and alveolar basement membranes, which are the main sites of antibody binding and tissue injury in Goodpasture's disease [32]. Thus, both α3(IV) and α4(IV)NC1 domains could contain epitopes recognized by autoreactive B and T cells. This possibility could be investigated by examining T cell help for autoantibody production *in vitro*, as performed in experimental myasthenia gravis [33]. However, it remains theoretically possible that T cells reactive with other molecules physically linked to α3(IV) chains in the GBM could provide help for autoantibody synthesis. Two other α(IV) chains, α5(IV) [34] and α6(IV) [35], have recently been identified, but these are present in low quantities and are not apparently recognized by conventional anti-GBM antibodies.

Further research on T cell responses in Goodpasture's disease is largely dependent on the production of autoantigen-specific T cell clones. This is hampered by the rarity of the disease and the lack of suitable antigenic material. In myasthenia gravis, T cell proliferation was initially observed in response to affinity-purified acetyl choline receptor (AChR) [13], but the isolation of AChR-specific T cell clones was only achieved after the expression of recombinant AChR α subunits [13,24,36]. The cloning of cDNA for α3(IV)NC1 [5,37] and the subsequent production of immunoreactive recombinant human α3(IV)NC1 in our laboratory should allow similar work in Goodpasture's disease [38]. Our preliminary results (unpublished), and those of Merkel *et al.* [39], suggest that it is possible to isolate antigen-specific T cell clones from PBMC of patients with Goodpasture's disease. Analysis of the specificity of autoreactive T cell lines or clones using recombinant α(IV)NC1 domains and synthetic peptides will allow further characterization of the autoimmune response in Goodpasture's disease, and this knowledge may lead to the development of specific anti-T cell therapy in the future [40].

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