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Characterization of anti-GBM antibodies involved in Goodpasture's syndrome

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Characterization of anti-GBM antibodies involved in Goodpasture's syndrome. Goodpasture's syndrome is a life threatening autoimmune kidney disease. The patients have autoantibodies to the glomerular basement membrane, which are specific for the C-terminal domain of type IV collagen (NC1). The major antigen has been localized to the α3(IV)-chain. We have investigated sera from 44 patients with anti-NC1 antibodies. The quantity of antibodies to four different α(IV)-chains of type IV collagen was measured with direct ELISA. We used affinity chromatography to separate the antibodies and their specificities were studied with ELISA. The results show that about 1% of the patients total IgG are anti-NC1 antibodies and that 90% of these antibodies are specific for the α3(IV)-chain. Antibodies to the other α(IV)-chains were found in 80% of the patients. Furthermore, affinity purified anti-α3(IV) antibodies from one patient were inhibited by antibodies from the other patients, from 4 to 72%. The antibodies, from 39 of the patients, were inhibited by a monoclonal antibody against the α3(IV)-chain. The results indicate that patients with Goodpasture's syndrome can have antibodies to most of the α(IV)-chains, while the majority of anti-NC1 antibodies are restricted to the α3(IV)-chain. Moreover the number of epitopes seems to be limited and the majority of the antibodies from most patients are against one single epitope on the α3(IV)-chain.

Goodpasture's syndrome (GP) is a very severe autoimmune disorder, and it is the classic example of an antibody-mediated glomerulonephritis. It typically affects individuals in the third and in the sixth decades, but may appear at any age [1]. The disease is characterized by rapidly progressive glomerulonephritis, lung hemorrhage and a linear deposit of IgG along the glomerular basement membrane (GBM) [2]. The pulmonary hemorrhage may be severe and life threatening or may be mild and easily overlooked [3, 4]. Still, the rapidly progressive glomerulonephritis is the most common clinical feature of the syndrome.

It is now firmly established that the anti-GBM antibodies in GP are directed to type IV collagen, which is one of the main structural components in basement membranes. The type IV collagen molecule is composed of three α(IV)-chains. In the N-terminal and middle region the three α(IV)-chains are intertwined to a triple helix structure, while in the C-terminal region each chain is folded into a globular domain. This globular domain is called NC1 (non-collagenous). Normally, type IV collagen is composed of two distinct α(IV)-chains, the classical α1(IV) and α2(IV) [5]. However, in GBM three additional α(IV)-chains have been identified, α3(IV), α4(IV) and α5(IV) [6, 7]. The gene for a sixth, α6(IV) has recently been discovered [8]. It has been shown that α3(IV) and α4(IV)-chains co-localize and are only present in specialized basement membranes, for example in the kidney, eye, cochlea, lung and brain, whereas the α1(IV)- and α2(IV)-chains are present in all basement membranes [9]. Primary results show that the α5(IV)-chain also has a limited distribution in different basement membranes. It has also been shown that in bovine GBM, the network consisting of α3(IV) and α4(IV) does interact with the α1(IV) and α2(IV) network via the NC1 domain creating hetrodimers, that is, α1(IV)-α3(IV) and α2(IV)-α4(IV) [10].

All patients with GP have autoantibodies to the NC1 of the α3(IV)-chain [1] and these antibodies are believed to be pathogenetic. Nevertheless, antibodies to the other α(IV)-chains have been reported, but the significance of these antibodies is unknown. Antibodies that are eluted from kidneys from GP patients are shown to have the same specificity as antibodies found in serum, that is, the majority are to the α3(IV) but there are some reactivity to the other α(IV)-chains [11]. We have found a few patients with very mild glomerulonephritis even though they have anti-GBM antibodies. These patients had antibodies to the NC1 domain of the α1(IV)-chain and not to the α3(IV)-chain [12]. Moreover, antibodies from patients with GP show very reduced reactivity if the NC1 is in its hexameric shape, or if it is reduced and the disulphide bonds are broken [13].

It is necessary to know and to characterize all epitopes involved in Goodpasture's syndrome to develop new and better therapeutic and diagnostic methods and to understand the process of disease. Several investigators have earlier shown that patients with GP have antibodies to the α3(IV)-chain of type IV collagen and it has been assumed that the α3(IV)-chain is the major antigen. In this study we have quantified the titer of antibodies in sera from 44 patients with anti-NC1 antibodies and determined the frequency of antibodies to the different α(IV)-chains. We have, furthermore, studied whether all GP patients have antibodies to the same epitope/epitopes on the α3(IV)-chain.

Methods

Patients

Forty sera and four plasma samples from 44 patients with anti-NC1 antibodies, detectable in an ELISA, were used. As negative controls we used sera from healthy blood donors. Serum

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from a patient with anti-GBM antibodies specific for the α1(IV)-chain was used for comparison. This patient is earlier described by Johansson et al [12].

Antibodies

Two monoclonal antibodies were used in this study, one to the α1(IV)-chain (Mab 6) and one to the α3(IV)-chain (Mab 17) of type IV collagen. Both antibodies are described before and well characterized [14]. Additionally, a monoclonal antibody to the α5(IV)-chain (Mab A7) [15] and polyclonal antibodies to the α2(IV) and the α4(IV)-chains, both produced in rabbits [16], were used.

Preparation of antigens

Glomerular basement membrane was prepared from bovine kidneys as previously described [17] with some modifications. Homogenized kidney cortex was extracted overnight at room temperature with 6 M guanidine-HCl buffer (50 mM Tris-HCl, pH 7.4, 6 M guanidine-HCl) with 10 mM EDTA and protease inhibitors (5 mM benzamidine, 25 mM ε-aminocaproic acid, 4 mM N-ethylmaleimide, 1 mM phenylmethanesulfonyl fluoride). It was then centrifuged and the pellet was resuspended in HEPES buffer (50 mM HEPES, pH 7.5 with 10 mM $CaCl_2$ and protease inhibitors). Digestion with bacterial collagenase (Worthington Biochemical Corporation, Freehold, New Jersey, USA) was performed in HEPES buffer at 37°C for 20 hours with continuous stirring. The solubilized basement membrane was ultracentrifuged and the supernatant was dialyzed against 50 mM Tris-HCl, pH 7.5, 2 M urea and then applied to a DEAE-Sephacel ion exchange chromatography column (Pharmacia, Uppsala, Sweden) equilibrated in the same buffer. The NC1 domain, which does not bind to the column, was collected and concentrated in an ultrafiltration cell (YM10 Amicon, Danvers, Massachusetts, USA) and then dialyzed against 6 M guanidine-HCl buffer.

The concentrated sample, containing NC1, was boiled for five minutes, and then applied to a S-200 gel filtration column (Pharmacia) equilibrated in the 6 M guanidine-HCl buffer. Fractions from the major peaks, 7S, dimeric α(IV)-chains and monomeric α(IV)-chains were pooled and the monomeric pool was further separated with reversed-phase HPLC as previously described [18]. This gave four monomer populations containing α1(IV), α2(IV), α3(IV) and α4(IV), respectively. The content of the monomer pools was analyzed by immunoblotting (see below) using antibodies to the α1-5(IV)-chains. Reactivity with the anti-α5(IV) antibody was found in the α1(IV) and in the α2(IV) pool. No antibodies to the α6(IV)-chain were available.

A fraction of the material that went through the DEAE ion exchange column was also separated on a S-200 gel filtration column under non-denaturing conditions (50 mM Tris-HCl, pH 7.3, 0.15 M NaCl, 0.02% wt/vol NaN_3). The major peak, containing NC1, was further separated on an affinity column [10] containing Mab 17, the monoclonal antibody to the α3(IV)NC1 domain. The material that went through the column contained only α1(IV)- and α2(IV)-chains and this fraction was then used for preparing an affinity column (see below).

Affinity chromatography

Three affinity chromatography columns were prepared from 2 ml activated agarose gel, Mini Leak, (Kem-En-Tec IS, Copenhagen, Denmark). Bovine NC1 containing all α(IV)-chains, a fraction of bovine NC1 containing only α1(IV)- and α2(IV)-chains and the monoclonal anti-α3(IV) antibody (Mab 17) were coupled by the procedure described in the product insert. The columns were used as previously described [10]. Briefly, the columns were equilibrated with starting buffer, 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.02% (wt/vol) NaN_3. The samples were applied in starting buffer and eluted with 50 mM sodium citrate, pH 4.0, 6 M urea, 0.02% (wt/vol) NaN_3. The pH of eluted antibodies was immediately adjusted to 7.0 and then dialyzed against starting buffer.

Twenty-five milliliters of sera from two patients and 1 ml sera from five patients were applied to the column containing all α(IV)-chains. The antibodies that bound the column were eluted in 3 ml elution buffer; 1 ml was used for isolation of IgG using a protein G affinity column (Pharmacia). The protein concentrations were estimated by absorbance at 280 nm. Two milliliters were applied to the column containing α1(IV)- and α2(IV)-chains. The unbound fraction was collected in 9 ml and the bound antibodies were eluted in 3 ml. One milliliter of each fraction was used for isolation of IgG.

Electrophoresis

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was done in 10 to 16% gradient gels as described by Laemmli [19]. The gels were stained with silver according to the method of Morrissey [20]. Immunoblot experiments were performed on samples that were separated with SDS-PAGE and transferred to Immobilon™ PVDF membrane (Millipore, Saint-Quentin, France) as described by Burnett [21] using a semi-dry electroblotter as previously described [10].

Enzyme-linked immunosorbent assay (ELISA)

Polystyrene microtiter plates (NUNC Immunoplate, Roskilde, Denmark) were coated with 100 μl of the separated α(IV)-chains from bovine GBM, diluted to 0.2 μg/ml in 50 mM Tris-HCl, pH 7.4, 6 M guanidine-HCl. The plates were incubated overnight at room temperature and then washed three times with 0.15 M NaCl, 0.05% (vol/vol) Tween 20. One hundred microliters of primary antibodies diluted in phosphate buffered saline containing 0.2% (wt/vol) bovine serum albumin, pH 7.3 (BSA-PBS) (1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 0.12 M NaCl, 2.5 mM KCl, 0.05% (wt/vol) NaN_3) were added to each well. The plates were incubated at room temperature for one hour and after washing, alkaline phosphatase-conjugated swine anti-human IgG, diluted 1/500, (Orion Diagnostica AB, Trosa, Sweden) was added and incubated for one hour. P-nitrophenyl phosphate (1 mg/ml; Sigma Chemical Company, St. Louis, Missouri, USA) in substrate buffer (1 M Dietanolamin, 0.5 mM $MgCl_2$, pH 9.8), was used as substrate and color development was measured spectrophotometrically at 405 nm.

For inhibition ELISA affinity-purified antibodies or sera were diluted and preincubated overnight at 4°C in a preincubation plate with different α(IV)-chains, in concentrations from 0.005 to 20 μg/ml. The solutions were then added to microtiter plates coated with α1(IV) or α3(IV) (0.2 μg/ml). Bound antibodies were detected with alkaline phosphatase-conjugated secondary antibodies.

In the competitive ELISA, biotinylated affinity purified anti-α3(IV) antibodies from one patient were used. The antibodies were biotinylated, using NHS-LC-Biotin, according to the instructions provided by the manufacturer (Pierce, Rockford, Illinois,

USA). Biotinylated antibodies, diluted 1/200, were premixed with patient serum or plasma in different dilutions and then added to coated microtiter plates and further treated as above. In this assay bovine α3(IV) was used for coating (0.2 μg/ml) and alkaline phosphatase-conjugated avidin (Pierce) was used as conjugate.

By using the monoclonal antibody to α3(IV), Mab 17, another competitive ELISA was developed. The culture supernatant with the monoclonal antibody, diluted 1/20, were premixed with patient serum or plasma diluted to be equally concentrated, to give an absorbance value of 0.5 in sandwich ELISA. The mixture was added to a microtiter plate, coated with bovine α3(IV) (0.2 μg/ml). Human antibodies, that bound to the coat, were detected with phosphatase-conjugated secondary antibodies.

Results

Antibody specificity measured by direct ELISA

Forty sera and four plasma samples from patients with anti-NC1 antibodies were tested in a sandwich ELISA. Sera from healthy blood donors and serum from a patient with antibodies against α1(IV), but not to α3(IV)-chains, were used as controls. The plates were coated with bovine α1(IV), α2(IV), α3(IV) or α4(IV). To be able to compare the titer of antibodies directed to the different α(IV)-chains the sera were diluted to give 0.5 absorbance units after one hour development time. The number of dilutions that are needed is shown in Table 1. This shows that the majority of the antibodies are directed to α3(IV) and that most patients (80%) have a response to the other α(IV)-chains, even though it is weaker than the response to the α3(IV)-chain. Many patients with elevated levels of anti-α1(IV) also have anti-α4(IV) antibodies, while findings of anti-α2(IV) antibodies seem to be more rare. In some patients there are very small differences between the antibody titers to the different α(IV)-chains. This is, in most cases, due to a low anti-α3(IV) titer rather than a high titer of antibodies to other α(IV)-chains. On average, the samples have to be diluted 14 times more when we are analyzing anti-α3(IV) antibodies, compared to anti-α1(IV) antibodies. This indicates that the majority or more than 90% of the GP antibodies are directed towards the α3(IV)-chain.

Affinity purification of sera

We wanted to find out if the anti-NC1 antibodies were cross reacting or if there were different antibodies to the different α(IV)-chains. To do this, we used two affinity columns to isolate and separate specific anti-NC1 antibodies from seven patients (sample numbers 11, 12, 14, 18, 19, 20 and 26 in Table 1; Fig. 1). IgG, from all fractions, was purified with the protein G column, and the quantity of IgG was measured spectrophotometrically at 280 nm. All antibodies against the NC1 domain of the α(IV)-chains bound the affinity column containing all α(IV)-chains (fraction 2, Fig. 1) and no anti-NC1 antibodies were found in the unbound fraction (fraction 1, Fig. 1). The fraction that bound the first column (fraction 2, Fig. 1), about 1% (that is in average 0.14 mg/ml) of the patients total IgG, was further separated on an affinity column containing only α1(IV)- and α2(IV)-chains. This was used to separate antibodies against α1(IV) and α2(IV) from the antibodies against α3(IV) and α4(IV). Only a minor amount of the anti-NC1 antibodies, 10%, bound to this affinity column (fraction 4, Fig. 1). This fraction did, however, not only react with α1(IV) and α2(IV) but also with α3(IV) and α4(IV) in a sandwich ELISA. These antibodies gave a strong response to α3(IV) and only a weak one against α1(IV) in immunoblotting. Using inhibition ELISA we found two different types of antibodies in this fraction. For antibodies to α1(IV) cross reacting with α2(IV)- and α4(IV)-chains and antibodies specific for α3(IV) but with a low

Table 1. Showing number of dilutions needed to reach 0.5 absorbance units after 1 hour of development time in ELISA with the four different α(IV) chains as antigens

Sample number	Number of dilutions to reach 0.5 Abs units				Inhibition %	
	α1(IV) as antigen	α2(IV) as antigen	α3(IV) as antigen	α4(IV) as antigen	Of biotin-labeled GP antibodies with patient sera	Of patient sera with Mab 17
1	90	40	7200	160	43	100
2	720	60	7100	220	51	63
3	60	—	6300	205	25	66
4	390	50	6200	220	26	61
5	80	30	5500	230	60	45
6	530	70	4300	205	28	44
7	20	—	4000	10	47	68
8	—	—	3500	10	42	72
9	600	250	2921	950	27	50
10	—	—	2818	90	47	100
11	80	80	2022	120	40	21
12	400	—	2000	120	48	81
13	205	30	1909	170	50	53
14	90	60	1848	105	30	30
15	180	10	1750	70	40	45
16	25	—	1636	50	47	100
17	800	50	1636	390	30	87
18	70	—	1235	—	38	41
19	20	10	935	90	66	68
20	25	—	927	50	34	33
21	20	—	870	—	43	87
22	95	—	836	10	52	98
23	20	—	836	—	62	100
24	—	—	818	—	47	90
25	—	—	764	—	61	46
26	30	10	685	50	72	47
27	20	—	674	—	24	56
28	—	10	664	—	41	93
29	—	—	618	—	44	100
30	30	10	545	120	31	100
31	—	10	345	—	31	100
32	70	270	337	205	9	0
33	—	10	333	—	35	51
34	—	10	315	10	15	37
35	105	—	264	80	52	0
36	20	—	190	—	33	54
37	—	—	182	—	49	72
38	—	—	164	—	48	98
39	105	—	142	10	25	0
40	75	130	95	50	18	0
41	—	—	82	—	56	47
42	70	90	82	50	20	40
43	—	—	20	—	47	7
44	—	—	15	—	4	0
NS	—	—	—	—	0	0
A	870	80	—	—	0	0

Samples that do not react with the antigen are indicated with "—". The inhibition in per cent of the affinity purified α3(IV) specified biotin labeled antibodies with sera diluted to give the same response in ELISA with the α3(IV) chain as antigen and the inhibition in per cent of sera diluted to give the same response in ELISA with the monoclonal antibody to α3(IV) (Mab 17). The 44 GP sera are indicated with numbers from 1 to 44, one patient with α1(IV) antibodies is indicated with an A and sera from healthy blood donors are indicated with NS.

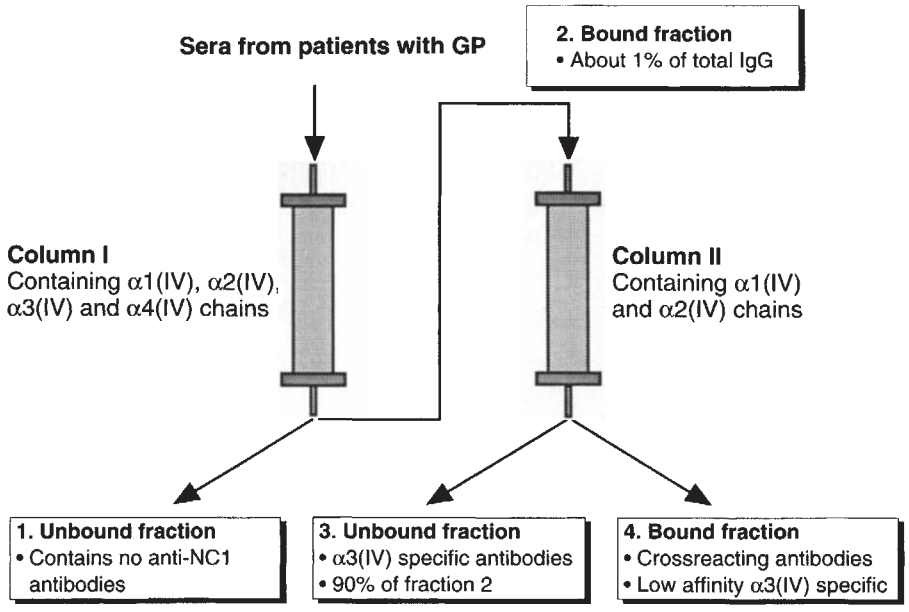

Fig. 1. *The affinity chromatography system used to purify and separate antibodies to the NC1 domain of type IV collagen.* Column I contains all four α(IV)-chains whereas column II only contains α1(IV) and α2(IV)-chains. Of all the anti-NC1 antibodies, 90% were found in fraction 3 and found to be specific for the α3(IV)-chain with a high affinity. In fraction 4 (10% of all anti-NC1 antibodies) we found antibodies to α1(IV), cross reacting with α2(IV) and α4(IV), and anti-α3(IV) antibodies that differ from the antibodies in fraction 3 by a lower affinity.

affinity, 5 μg/ml α3(IV) were needed for 50% inhibition (Fig. 2 A, B). Ninety percent of the anti-NC1 antibodies went through the column containing α1(IV) and α2(IV) (fraction 3, Fig. 1). These antibodies did only bind to α3(IV) in both ELISA and immunoblotting. Furthermore, only 0.5 μg/ml α3(IV) were needed for 50% inhibition in an inhibition ELISA. Thus, they were specific for the α3(IV) and did not cross react with any of the other α(IV)-chains (Figure 2 C). These results, that 90% of the antibodies are anti-α3(IV) specific, confirm the results obtained by the direct ELISA.

We have calculated the quantity of anti-α3(IV) antibodies in sera from the results by direct ELISA. By measuring the absorbance at 280 nm on the affinity purified antibodies from two patients, we found that an affinity-purified anti-α3(IV)-specific antibody concentration of 48 μg/ml corresponds to a dilution of 1000 times to reach 0.5 absorbance units after one hour in a direct ELISA. This gives us, assuming an equal affinity, a mean value of 78 μg/ml anti-α3(IV) antibodies ($N = 44$) which corresponds to a mean value of 0.8% of the total amount of IgG.

Inhibition of affinity-purified biotin-labeled antibodies

We wanted to find out whether all patients with GP have anti-α3(IV) antibodies to the same epitope/s or if different patients react with different parts of the α3(IV)NC1 domain. The affinity-purified anti-α3(IV) specific antibodies (fraction 3 in Fig. 1) from one of the patients (number 12 in Table 1) were labeled with biotin. Serum or plasma from other GP patients, diluted to give the same response in a direct ELISA, were premixed with the biotinylated anti-α3(IV) antibodies and then added to a microtiterplate coated with α3(IV). Forty-four of 44 GP samples could block the biotinylated anti-α3(IV) from 5 to 70% (Table 1) and the mean value was 40%. Serum from the same patient that was biotin labeled did inhibit the reaction to 48%. The relatively low inhibition is explained by how we performed the experiment, that is, the samples were very diluted. This is due to our desire to have all sera equally concentrated, since this result is giving us better possibilities to detect differences between different samples. If all sera were diluted 1/25 instead, we obtained an average inhibition value of 79%, in a range from 49 to 90% (not shown).

Inhibition with a monoclonal anti-α3(IV) antibody

Earlier investigators have shown that their monoclonal antibody to α3(IV) was able to block the binding of GP patient antibodies to α3(IV) [22]. We wanted to investigate if our monoclonal antibody to α3(IV) (Mab 17) also binds to the same epitope as antibodies from GP patients and if all anti-α3(IV) antibodies are to one single epitope that is the same in all patients. The antibody has been characterized previously and primary results (not shown) showed that it is possible to inhibit GP sera from binding to α3(IV). To further investigate this, a competitive ELISA was performed. In this, sera from GP patients were premixed with the monoclonal antibody to α3(IV) and then transferred to a microtiter plate coated with α3(IV). The antibodies from 39 out of 44 patients were inhibited by the monoclonal antibody. Twenty-four of these were inhibited from 50 to 100%, and 13 from 5 to 50%. Besides, there were 5 sera that the monoclonal did not inhibit at all (Fig. 3). None of the samples was inhibited by the monoclonal antibody to the α1(IV)-chain (Mab 6).

Discussion

About 1% of the total IgG in patients with GP are directed to the NC1 domain of type IV collagen. Most of these antibodies, about 90%, are specific for the GP antigen, that is, the NC1 domain of the α3(IV)-chain. Our theory is that this major population of antibodies to the α3(IV)-chain are the pathogenetic antibodies. We found that all patients have antibodies to the α3(IV)-chain and that 20% of them have antibodies to the α3(IV)-chain only, which strongly supports our theory. Almost all patients (80%) have antibodies not only to the α3(IV)-chain but also to the other α(IV)-chains and these antibodies represent about 10% of the anti-NC1 antibodies. In the inhibition ELISA

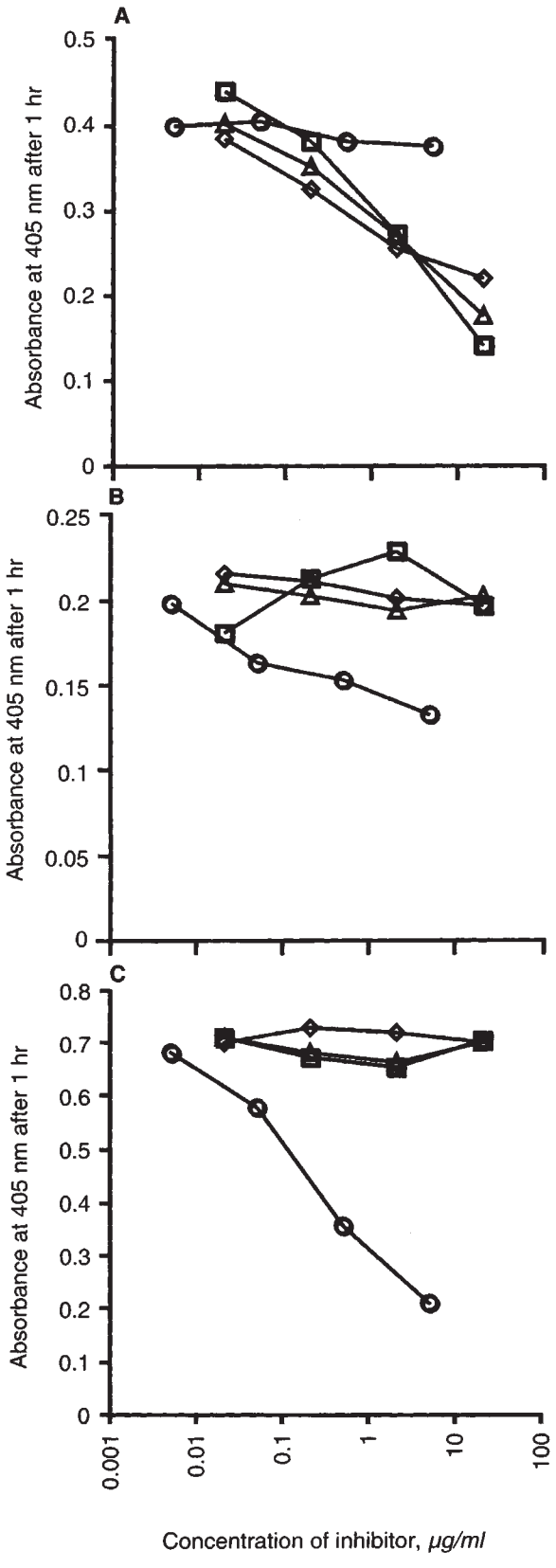

Fig. 2. *Inhibition ELISA where the different fractions from the purification and separation are inhibited with the different α(IV)-chains.* Symbols are: (⊟) inhibition with α1(IV); (◇) inhibition with α2(IV); (⊖) inhibition with α3(IV); (△) inhibition with α4(IV). All 7 purified and separated sera followed the same pattern and one is shown here. **A.** Fraction 4 in Fig. 1, the fraction that bound both columns, was tested in a microtiter plate coated with α1(IV). The antibodies were inhibited by α1(IV), α2(IV) and α4(IV)-chains but not by the α3(IV)-chain. **B.** Fraction 4 was tested in a microtiter plate coated with α3(IV) and only the α3(IV)-chain was able to inhibit the antibodies from binding to the α3(IV)-chain. **C.** Fraction 3 in Fig. 1, the fraction that bound the column containing all α(IV)-chains but not the column containing α1(IV)- and α2(IV)-chains, was tested in microtiter plates coated with the α3(IV)-chain. These antibodies were only inhibited by the α3(IV)-chain. This fraction did not react with any other α(IV)-chain in direct ELISA.

these antibodies were cross reactive, which might reflect a secondary response in which antibodies are produced to other GBM antigens as the disease progresses. Matsukura et al [23] suggested that there are GP antibodies with common epitopes present on α1(IV), α2(IV) and α3(IV). These antibodies could be the same antibodies that we found in the fraction that bound the affinity column containing α1(IV)- and α2(IV)-chains.

The α5(IV) and α6(IV)-chains have not been isolated, but the amounts in GBM seem to be limited. The impurities of α5(IV) in the α1(IV) and α2(IV) monomer pools should not have any influence on the results, since the α3(IV) pool did not contain α5(IV). However, it is likely that there are antibodies to both the α5(IV) and the α6(IV)-chains, as well as we find antibodies to the α1(IV), α2(IV) and α4(IV)-chains.

Neilson et al [24] have shown that the specificity of GP antibodies is to the α3(IV)-chain, by using recombinant α(IV)-chains and Kalluri et al [25] have, by using synthetic peptides, localized the GP epitope to a region of 36 amino acids in the C-terminal region of the α3(IV)-chain. Pusey et al [22] showed that a monoclonal antibody, P1, raised against the NC1 domain of type IV collagen, could block the binding of GP antibodies. In these earlier studies only a few patients have been included. However, we have investigated 44 patients with anti-NC1 antibodies and the results support these earlier studies that have suggested a restricted specificity of the GP-antibodies. Our results show that all patients have antibodies to the same epitopes on the α3(IV)-chain. This was shown by inhibition of biotin labeled GP antibodies. Our monoclonal antibody to the α3(IV)-chain could also inhibit the GP antibodies in 89% of the patients. Thus, the major fraction of the anti-α3(IV) antibodies, in the majority of the patients, is to one single epitope. The facts that 16% of the samples were inhibited to 100% by our monoclonal antibody to the α3(IV) chain and that one of these only had antibodies to the α3(IV)-chain suggest that the monoclonal antibody share one pathogenetic epitope with the GP antibodies. The monoclonal antibody did not inhibit anti-α3(IV) antibodies in five samples and less then 50% in 13. This could be due to different affinities. Another explanation could be that different patients have different, very closely spaced epitopes that both the monoclonal antibody and the patient's antibody could recognize simultaneously. Even though these antibodies do not share exactly the same epitope as the majority of the anti-α3(IV) antibodies, the antibodies could still be pathogenetic. Interestingly, the clinical presentations for two of the patients, whose sera were not inhibited by the monoclonal antibodies, were entirely different

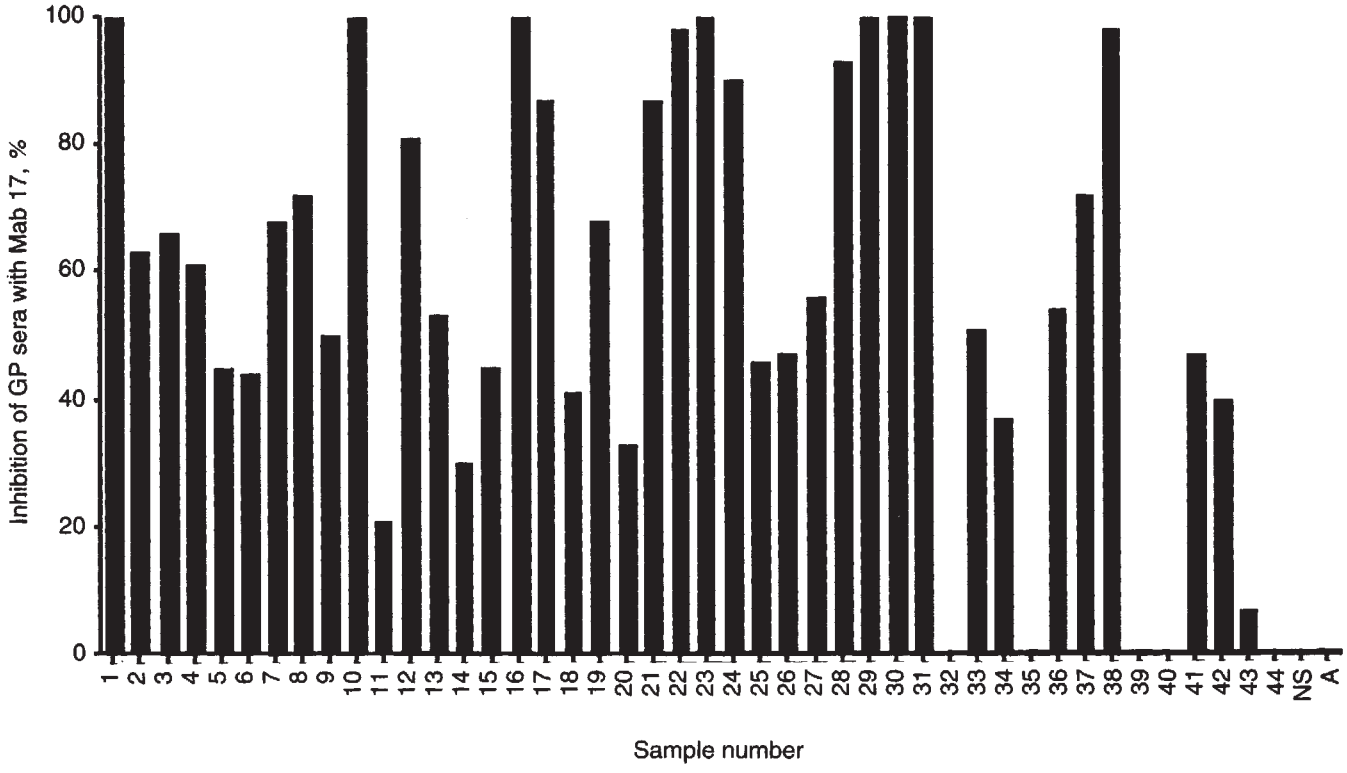

Fig. 3. *Inhibition of sera with our monoclonal antibody to α3(IV) (Mab 17).* All sera were diluted to give the same response in a direct ELISA using α3(IV) as antigen, mixed with Mab 17 diluted 1/20 and then added to a microtiterplate coated with α3(IV)-chains. If the measured absorbance is equal to the absorbance of normal sera, 100% inhibition is reached and if the measured absorbance is equal to the absorbance reached with sera without Mab 17, 0% inhibition is reached. Sample number 1-44 is GP sera, NS is indicating healthy blood donors and A is indicating the patient with anti-α1(IV) antibodies only.

from the typical GP patients. Both patients had a milder disease. The clinical presentation for all patients will be investigated and this might show whether analysis of the fine specificity of antibodies will be a tool to predict the disease prognosis.

The GP epitope has a cryptic conformation and it is dependent on correct disulphide bonds. It is normally hidden and the reaction with GP sera is weak until the epitope is exposed [13]. We have found cross reacting antibodies to the α(IV)-chains as well as antibodies to areas of the α3(IV)-chain other than the GP epitope, and these could explain why short synthetic peptides, which do not contain the GP epitope, still can bind antibodies in sera of GP patients. In future studies of the GP epitope with synthetic peptides and recombinant proteins it is important to use affinity-purified anti-α3(IV) antibodies from patients, whose antibody specificity is well characterized, otherwise it is very likely to find non-pathogenetic or minor epitopes. It is necessary to characterize all epitopes involved in GP and to determine which are pathogenetic, if new and better therapeutic and diagnostic methods will be developed, and to understand the process of the disease.

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