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### A Self T Cell Epitope Induces Autoantibody Response: Mechanism for Production of Antibodies to Diverse Glomerular Basement Membrane Antigens<sup>1</sup> **FREE**

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T Cell Epitope Mimicry in Antiglomerular Basement Membrane Disease

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# A Self T Cell Epitope Induces Autoantibody Response: Mechanism for Production of Antibodies to Diverse Glomerular Basement Membrane Antigens<sup>1</sup>

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The anti-glomerular basement membrane (GBM) Ab has been regarded as a prototypical example of pathogenic autoantibodies. However, the mechanism for elicitation of this Ab remains unknown. In the present paper, we report that the Ab to diverse GBM Ags was induced by a single nephritogenic T cell epitope in a rat model. The T cell epitope pCol<sub>28–40</sub> of noncollagen domain 1 of collagen type IV  $\alpha$ 3 chain not only uniformly induced severe glomerulonephritis but also elicited anti-GBM Ab in 76% of the immunized rats after prominent glomerular injury. Furthermore, we demonstrated that the anti-GBM Ab was not related to the peptidic B cell epitope nested in pCol<sub>28–40</sub>; that is, 1) elimination of the B cell epitope, either by substitution of the critical residues of the B cell epitope or by truncation, failed to abrogate anti-GBM Ab production, and 2) the anti-GBM Ab, eluted from the diseased kidneys, reacted only with native GBM, but not with pCol<sub>28–40</sub>. Confocal microscopy and immunoprecipitation further demonstrated that the eluted anti-GBM Ab recognized conformational B cell epitope(s) of multiple native GBM proteins. We conclude that autoantibody response to diverse native GBM Ags was induced by a single nephritogenic T cell epitope. Thus, anti-GBM Ab may actually be a consequence of T cell-mediated glomerulonephritis. *The Journal of Immunology*, 2004, 172: 4567–4574.

**A**nti-glomerular basement membrane (GBM)<sup>3</sup> glomerulonephritis (GN) (Goodpasture syndrome), a cause of end-stage renal failure worldwide, is characterized by the presence of anti-GBM Ab (1, 2). Historically, anti-GBM GN was among the earliest of recognized human autoimmune diseases (3), and the discovery of the anti-GBM Ab was considered a milestone in autoimmune disease research (4, 5). The anti-GBM Ab has been regarded as a prototypical example for autoantibodies that mediate autoimmune diseases (4, 5).

Numerous studies have been devoted to identification of the Ags/B cell epitopes recognized by the anti-GBM Ab. Several notable studies, using human anti-GBM Abs as probes, identified noncollagen domain 1 of collagen type IV  $\alpha$ 3 chain (Col4 $\alpha$ 3NC1) as the Goodpasture Ag (6, 7). Animal models further demonstrated that immunization with Col4 $\alpha$ 3NC1 induced anti-GBM GN (8–11). In addition to Col4 $\alpha$ 3NC1, diverse GBM proteins, including

different chains of type IV collagen, collagen domains, and the S7 domain of type IV collagens, and other noncollagen components of GBM, have been identified as the Ags recognized by autoantibodies from anti-GBM patients (12–16). Discovery of anti-neutrophil cytoplasmic autoantibody in anti-GBM disease is further evidence for complicity of the Ab response in this disease (17, 18).

A number of investigators further attempted to map the B cell epitopes of identified Goodpasture Ags. Mapping of B cell epitopes by recombinant polypeptides or synthetic peptides has been tried repeatedly (7, 19–24). One report described a linear B cell epitope encoded in the C-terminal 36 residues of Col4 $\alpha$ 3NC1 (19). However, a synthetic peptide encoding the B cell epitope failed to induce GN or anti-GBM Ab (11, 25). Mapping linear epitopes or generating mAbs to native GBM with peptides was not successful, because the Abs reacted only with denatured GBM proteins or did not react with GBM at all (9, 20, 21, 23, 24). Using more sophisticated point mutation and other techniques, several studies demonstrated that the anti-GBM Abs are bound to three-dimensional (3-D) conformation of native Ags (26–29). Thus, autoantibodies of anti-GBM disease react with highly diversified GBM Ags, and the B cell epitopes recognized by the anti-GBM Abs are mainly 3-D conformational, probably due to the complicated quaternary organization of GBM (28).

Despite several decades of study on anti-GBM disease, the investigation of the actual mechanism of the Ab response to diverse B cell epitope/Ags in this disease has been largely ignored. This is a fundamental question for the anti-GBM disease, as well as for many other autoimmune diseases.

We have established a rat model for anti-GBM GN, in which severe disease is induced by transfer of Col4 $\alpha$ 3NC1-specific T cells or a T cell epitope pCol<sub>28–40</sub> derived from this Ag (30–32). Thus, the T cell mechanism alone is sufficient to induce anti-GBM GN. However, it raises the question of whether the GN induced by T cell mechanism is relevant to human disease, because anti-GBM

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<sup>3</sup> Abbreviations used in this paper: GBM, glomerular basement membrane; GN, glomerulonephritis; Col4 $\alpha$ 3NC1, noncollagen domain 1 of collagen type IV  $\alpha$ 3 chain; 2-D, two-dimensional; 3-D, three-dimensional; pCol, synthetic peptide of Col4 $\alpha$ 3NC1; TBM, tubular basement membrane.

Ab, the hallmark for human anti-GBM disease, may not be present. In the present study, we demonstrated that the T cell epitope pCol<sub>28-40</sub> not only induced severe GN but also elicited an auto-antibody response to diverse GBM Ags. Our study suggests that the anti-GBM Ab may actually be a consequence of T cell-mediated glomerular damage. Thus, it is necessary to re-examine classical views of the cause-effect relationship between anti-GBM Ab and GN.

## Materials and Methods

### Ag preparation

Peptides were synthesized on an automatic peptide synthesizer, AMS 422 (Gilson, Middleton, WI), using F-moc chemistry, and were purified by reverse-phase C18 column on a preparative HPLC (Water, Millford, MA). Purified peptides were analyzed by HPLC for purity and mass spectrometry for the correct sequence. Peptides, exceeding 95% purity, were dissolved in milli-Q water (Millipore, Billerica, MA) at a 1 mM concentration, and used for immunization or other investigative purposes.

### Immunization and GN evaluation

Female Wistar Kyoto (WKT) rats (4–6 wk of age) were purchased from Harlan (Indianapolis, IN). The rats were maintained in the animal facility at the University of Texas Health Science Center at Houston and allowed to acclimate for a minimum of 3 days. Rats were immunized by injection of a peptide (0.125  $\mu$ mol) emulsified in CFA at one hindfootpad and the base of tail. Rats injected with CFA alone, or with an irrelevant peptide CP2, served as controls.

Severity of GN was evaluated by proteinuria/albuminuria and renal histopathology. Random urine samples were monitored daily by Multistix (Bayer, Pittsburgh, PA). Urine albumin was semiquantitated by 12% SDS-PAGE (2  $\mu$ l of urine per lane) using BSA as a standard. The experimental animals were sacrificed as indicated. Kidney tissues fixed in Bouin's solution were used for H&E staining. Glomeruli with crescentic lesions, hypercellularity, or no injury were counted, and a glomerular injury score was calculated as follows: ((number of crescentic glomeruli  $\times$  100) + (number of hypercellular glomeruli  $\times$  50))/total glomerular number. A portion of the kidney tissues was snap-frozen in liquid nitrogen for direct immunofluorescence staining for detection of linear IgG binding to GBM. H&E staining and immunofluorescence were examined in a double-blind manner, i.e., only experimental numbers were revealed on the slides.

### Lymphocyte proliferation assay

Lymphocyte proliferation assays were conducted as previously described (31). Briefly, lymphocytes were prepared from immunized rats and CD3<sup>+</sup> T cells were isolated with a T cell enrichment column (RTCC; R&D Systems, Minneapolis, MN). The purity of isolated T cells was determined by flow cytometry analyses (FACSCalibur; BD Biosciences, San Jose, CA) after staining with anti-CD4-PE and anti-rat IgM/G-FITC Abs (BD Pharmingen, San Diego, CA). T cells and irradiated syngeneic thymocytes (1:1) were cultured in 96-well plates at  $4 \times 10^5$  cells/well in 200  $\mu$ l of complete T cell medium. Peptides (0.3–30  $\mu$ M) were added to each well in triplicate, and purified protein derivative was used as positive control. The cells were incubated at 37°C in a humidified, 5% CO<sub>2</sub> atmosphere for 72 h, pulsed with [<sup>3</sup>H]thymidine (0.5  $\mu$ Ci/well) for 18 h (ICN, Costa Mesa, CA), and harvested onto glass fiber filters using a semiautomatic cell harvester (Skatron Instruments, Sterling, VA). The incorporated radioactivity was measured by a liquid scintillation counter (Beckman, Fullerton, CA). The results were expressed as  $\Delta$ cpm (mean triplicate cpm with Ag minus mean triplicate cpm without Ag) or a stimulation index (ratio between cpm with and without Ags).

### Preparation and two-dimensional (2-D) gel electrophoresis of GBM proteins

Normal rat kidneys were used for isolation of glomeruli by a previously described method (33). The isolated glomeruli were sonicated by a Branson ultrasonicator (model W150; Heat Systems Ultrasonics, Plainview, NY) for 3 min at 4°C in a buffer containing 1 M NaCl. The sonicated glomeruli were washed with distilled water and lyophilized. The dried glomeruli were rehydrated at concentration of 5 mg/ml in a Tris-based buffer (pH 7.0), and digested by collagenase (33). The supernatant was collected, heated to 60°C for 10 min, dialyzed against 0.1 M ammonium carbonate, and lyophilized. The digested GBM proteins were kept as powder at –20°C in a desiccator, and used for 2-D gel electrophoresis following a published

method with some modification (30). The lyophilized GBM was rehydrated in sample extraction buffer (Bio-Rad, Hercules, CA), and separated on the first dimension gel using a system from Bio-Rad. The first-dimension gel was soaked in SDS sample buffer containing 2-ME for 30 min and loaded on an SDS-slab gel (12%). After the second electrophoresis, the gel was either stained by silver staining or transferred to nitrocellulose membranes for Western blot.

### Ab elution and preparation

Rats immunized with the peptides were perfused with heparinized PBS through the inferior vena cava, and the kidneys were removed and frozen at –80°C until use. A small portion of the kidney was processed for H&E staining to assess pathology and the perfusion efficiency. Linear binding of IgG to GBM was confirmed by direct immunofluorescence. Only those kidneys graded as “positive” to “strong positive” in linear IgG binding to GBM were selected for Ab elution experiments. Glomeruli were first isolated by an established method (33), washed with cold PBS eight times, and incubated with citric acid/sodium citrate buffer (0.1 M; pH 3.2) for 1 h with constant stirring. The supernatant, which contained anti-GBM Ab, was neutralized by 0.1 M NaOH, dialyzed against ammonium carbonate (pH 8.0; 5 mM), and lyophilized. The powder was rehydrated with PBS, and IgG concentration in the solution was measured by ELISA (32). The solution was kept at 4°C until use.

For preparation of Ab to pCol<sub>28-40</sub>, the peptide was conjugated to cyanogens bromide-activated Sepharose gel (4B) to set up an affinity column (Sigma-Aldrich, St. Louis, MO). The blood samples with high-titer Ab to pCol<sub>28-40</sub> were pooled, and passed through the affinity column twice. The Ab was eluted by citric acid/sodium citrate buffer (0.1 M; pH 3.0), and immediately neutralized by 0.1 M NaOH. The eluate was dialyzed against ammonium carbonate (pH 8.0; 5 mM), and lyophilized. The Ab was finally dissolved in PBS at concentration of 300  $\mu$ g/ml, and kept at 4°C until use.

### Detection of Ab activity

For detecting Ab to peptides, a previously described ELISA was applied (32). Briefly, plates were coated with 50  $\mu$ l of 10  $\mu$ M peptide in carbonate buffer (pH 9.5). Serially diluted serum (100–800) was added to each well in duplicate. The bound rat IgG Abs were detected by HRP-labeled goat anti-rat IgG (1:10,000; Southern Biotechnology, Birmingham, AL) using *O*-phenoldiamine (0.25 mg/ml) as the substrate. The plates were read on an ELISA reader (Molecular Devices, Sunnyvale, CA) at 490 nm.

Western blot was used to detect Ab to the recombinant protein or isolated GBM proteins. Briefly, digested glomerular proteins were separated by 2-D gel electrophoresis and transferred to nitrocellulose membranes. The membranes were incubated with Abs adjusted to 5  $\mu$ g/ml. After washing with PBS containing 0.05% Tween 20, the blots were incubated with HRP-labeled goat anti-rat IgG (1:10,000). The reactants were visualized using an ECL kit (Pierce, Rockford, IL).

Indirect immunofluorescence was conducted for detection of Ab to native GBM (32). The Ab was incubated with frozen sections of SCID mouse kidney, followed by FITC-labeled goat anti-rat IgG or IgM Abs (1:50; Southern Biotechnology, Birmingham, AL). The rat mAb SR13, which reacts with Col4 $\alpha$ 4NC, was used as a positive control (a kind gift from Dr. Y. Sado (Okayama University School of Medicine, Okayama, Japan)). In some cases, sections of normal human kidney obtained from nephrectomy were used for staining. The stained sections were viewed with a fluorescence microscope (BH-2; Olympus, Melville, NY). For two-color staining, the perfused rat kidney was used. The section was first stained by the eluted anti-GBM Ab followed by Texas Red-labeled goat anti-rat IgG. The section was further stained by FITC-labeled SR13 and viewed with a confocal microscope (FV 500; Olympus).

Immunoprecipitation was used to determine Ab activity to native GBM proteins. The eluted Ab or anti-pCol<sub>28-40</sub> Ab was incubated with collagenase-digested GBM for 2 h at room temperature. The anti-rat IgG Ab-bound agarose beads (Rockland, Gilbertsville, PA) were added for incubation for another hour. The beads were collected by centrifugation at 1000 rpm and washed with PBS three times, followed by a brief wash with distilled water. The sample buffer (Bio-Rad) was added to the beads followed by incubation for 10 min to dissociate the Ab-Ag binding. The supernatant, which contained most of precipitated proteins, was added to the first-dimensional gel. The first-dimensional gel was incubated in SDS sample buffer with 2-ME for 30 min before loading onto a 12% gel. The proteins were visualized by a standard silver staining.

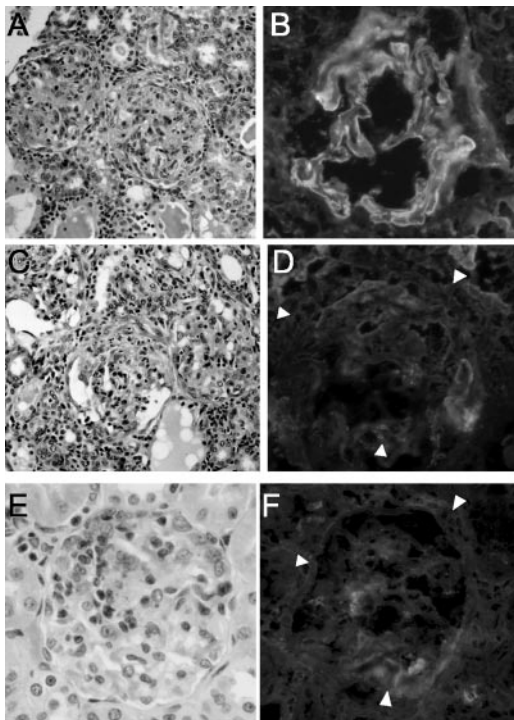


## Results

### Linear binding of IgG to GBM is detected in pCol<sub>28-40</sub>-induced GN

T cell peptide pCol<sub>28-40</sub> of Goodpasture Ag, Col4 $\alpha$ 3NC1, induces extremely severe GN in 100% of Wistar Kyoto rats. Further analyses showed that the peptide also contained a peptide-specific B cell epitope (32). Although pCol<sub>28-40</sub> elicited high titers of circulating Ab to the peptide, the purified peptide-specific Ab did not react with either native or recombinant Col4 $\alpha$ 3NC1 (32). Thus, the T cell epitope in pCol<sub>28-40</sub> was responsible for the induction of GN. However, linear binding of IgG to the GBM, which is the most important diagnostic indicator of human anti-GBM disease, was detected by immunofluorescence in ~76% of the experimental rats (39 of 51 rats) sacrificed around days 40–50 (Fig. 1, A and B). Among the animals showing positive linear IgG binding to their GBM, 9 (11.8%) were described as 3+, 13 (25.5%) as 2+, 11 (21.6%) as +, and 6 (15.7%) as weak positive. The GBM-bound IgG was not associated with severity of GN (Fig. 1, C and D), because the remaining 24% of rats without detectable GBM-bound IgG had similar severe GN with identical pathology features. Similarly, proteinuria was not associated with linear IgG binding to GBM (4012  $\pm$  350 mg/dl for the rats of 3+; 3990  $\pm$  217 mg/dl for those of negative). The lack of linear binding of IgG in remaining experimental rats was not due to absence of GBM in severely damaged glomeruli, because mAb SR13 revealed existence of fragmented GBM in those glomeruli (data not shown).

We determined the time course of linear binding of IgG to GBM in comparison with glomerular injury in the experimental rats



**FIGURE 1.** Renal pathology (H&E stain) (A, C, and E) and linear binding of IgG to GBM (immunofluorescence) (B, D, and F) in rats immunized with pCol<sub>28-40</sub>. A and B, A pair of renal sections from a rat sacrificed at day 45, showing severe glomerular damage (A) and intense linear GBM-bound IgG (B). C and D, A pair of renal sections from a rat also sacrificed at day 45, showing severe glomerular damage (A), but negative in GBM-bound IgG (D). E and F, From a rat sacrificed at day 20, showing glomerular hypercellularity (E), and negative in GBM-bound IgG staining (F). Arrowheads in D and F outline the glomeruli.

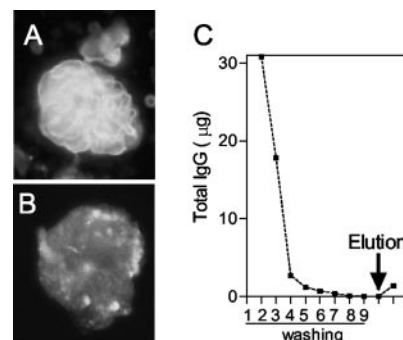
(from day 15 to 25 postimmunization at 2-day intervals). The GBM-bound IgG became detectable by immunofluorescence at approximately day 25, which is ~10 days later than significant proteinuria and 5 days later than prominent GN pathology (Fig. 1, E and F). Thus, GBM-bound IgG appeared only after glomerular injury. Next, we examined the binding of IgM to GBM. Linear binding of IgM to GBM was never demonstrated. However, massive IgM deposition within the crescentic lesion was observed at later stages of the disease (after 30 days). The deposited IgM was obviously trapped in the fibrous tissue of the lesion. Linear binding of IgG to GBM suggested that the IgG was probably anti-GBM Ab.

### GBM-bound IgG is an anti-GBM Ab that does not react with pCol<sub>28-40</sub>

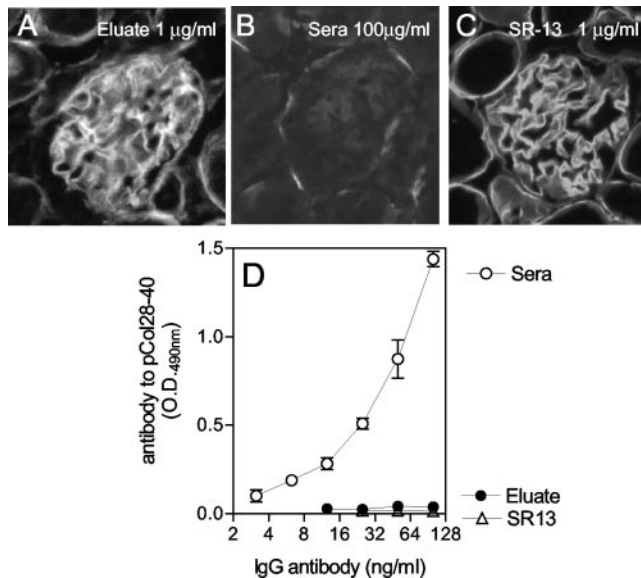
We next investigated whether GBM-bound IgG was the anti-GBM Ab, which is the most important indicator for human anti-GBM disease. The kidneys from the animals immunized with pCol<sub>28-40</sub> were screened for GBM-bound IgG by immunofluorescence, and only those with intense fluorescent staining of IgG (2+ to 3+) were chosen for Ab elution study. Three elution experiments were conducted with four pairs of kidneys for each. First, glomeruli were isolated and GBM-bound IgG was confirmed by immunofluorescence before the elution (Fig. 2A). After the elution, the disappearance of GBM-bound IgG was again checked (Fig. 2B). Eluted IgG was quantified by ELISA and tested for its Ab specificities (Fig. 2C). The eluted IgG, adjusted to a range of 2–0.5  $\mu$ g/ml, reacted strongly with native GBM, and weakly to tubular basement membrane (TBM), as revealed by indirect immunofluorescence on SCID kidney (Fig. 3A). SR13, a control rat mAb to Col4 $\alpha$ 4, reacted with both GBM and TBM at a concentration down to 0.1  $\mu$ g/ml (Fig. 3C). In contrast, purified circulating Ab to pCol<sub>28-40</sub> failed to react with native GBM at a concentration as high as 100  $\mu$ g/ml (Fig. 3B). The Ab reactivity to pCol<sub>28-40</sub> was then tested (Fig. 3D). Neither eluted GBM Ab nor SR13 reacted with pCol<sub>28-40</sub> (Fig. 3D). In contrast, purified pCol<sub>28-40</sub> circulating Ab reacted strongly with the peptide (Fig. 3D). The above experiments demonstrated that the GBM-bound IgG in pCol<sub>28-40</sub> immunized rats was the anti-GBM Ab, which was not related to the B cell epitope in the immunogen pCol<sub>28-40</sub>.

### A modified pCol<sub>28-40</sub> with eliminated B cell epitope induces anti-GBM Ab

We further ruled out the association of the B cell epitope in the pCol<sub>28-40</sub> with anti-GBM Ab by elimination of the B cell epitope. The critical residues for T cell epitope and B cell epitope in

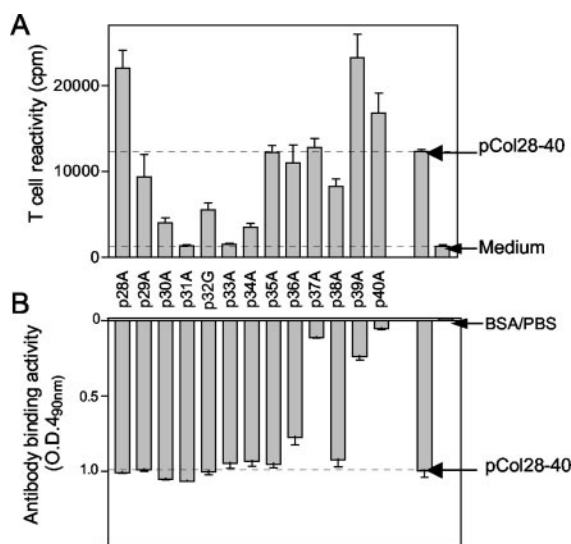


**FIGURE 2.** Elution of GBM-bound IgG. A and B, Shown are a glomerulus with GBM-bound IgG (immunofluorescence) (A), and a glomerulus without GBM-bound IgG after the elution process (immunofluorescence) (B). C, Shown are the changes in total IgG in each washing and in eluate.



**FIGURE 3.** Ag specificity of the Ab eluted from the kidney of pCol<sub>28-40</sub>-immunized rats. A–C, The Ab activity to native GBM, as revealed by indirect immunofluorescence on SCID kidney, is shown. The concentration of Ab is indicated for each Ab. Short curves around Bowman's capsule are background. D, Shown in the reactivity of different Abs to peptide pCol<sub>28-40</sub> measured by ELISA. SR13, a mAb to Col4 $\alpha$ 4, is used as a positive control.

pCol<sub>28-40</sub> were mapped by a set of alanine/glycine substituted peptides. These peptides were designated as p28A to p40A based on the position and the substituted residues (Fig. 4). For example, p40A was the peptide with a single substitution at position 40 with an alanine. First, pCol<sub>28-40</sub>-specific CD4<sup>+</sup> T cells were tested against this set of peptides (Fig. 4A). The pCol<sub>28-40</sub>-specific T cells failed to respond to the peptides p31A, p33A, and p34A, suggesting that the residues 31, 33, and 34 may be critical for T cell epitope. Similarly, purified pCol<sub>28-40</sub>-specific Ab was tested against the substituted peptides (Fig. 4B). The pCol<sub>28-40</sub> Ab did



**FIGURE 4.** Mapping the critical residues for T cell epitope and B cell epitope in pCol<sub>28-40</sub> by a set of peptides with a single substitution at each residue. A, Responses of pCol<sub>28-40</sub>-specific CD4<sup>+</sup> T cells to the peptides, as measured by T cell proliferation assay. T cell responses are expressed by uptake of [<sup>3</sup>H]thymidine. B, Reactivity of pCol<sub>28-40</sub>-specific Ab to the peptides, as determined by ELISA.

not react or greatly reduced its reactivity with the peptides p37A, p39A, and p40A, suggesting that the residues 37, 39, and 40 were important for the B cell epitope in pCol<sub>28-40</sub>.

We selected peptides p33A and p40A as immunogens, because the substitutions in the two might have resulted in defect or alteration of the T and B cell epitope in pCol<sub>28-40</sub>, respectively. Immunization with p33A elicited a strong T cell response to itself (Fig. 5). As we expected, the T cells failed to respond to pCol<sub>28-40</sub>. This result again confirmed that residue 34 is critical for the specificity of pCol<sub>28-40</sub>. Thus, p33A contained a strong T cell epitope that did not have a cross-reactivity with pCol<sub>28-40</sub>. More importantly, p33A also failed to induce any pathological changes in the kidneys (Fig. 5). Although a high titer of circulating Ab to pCol<sub>28-40</sub> was present as revealed by ELISA, linear binding of IgG to GBM was not detected in the immunized rats (Fig. 5). We concluded that p33A failed to induce GN because of the altered specificity of the T cell epitope in p33A. This experiment demonstrated that the intact B cell epitope in this peptide was not able to induce linear binding of IgG to GBM.

In contrast, p40A induced extremely severe proteinuria and GN in all immunized rats. As we expected, p40A no longer elicited Ab response to pCol<sub>28-40</sub>, demonstrating that residue 40 was critical for the B cell epitope (Fig. 5). In contrast, p40A induced a strong T cell response to both pCol<sub>28-40</sub> and p40A (Fig. 5). Thus, pCol<sub>28-40</sub> and p40A shared an identical T cell epitope, which was responsible for GN. More importantly, linear binding of IgG to GBM was detected in 65% of the rats immunized with p40A (Fig. 5). Based on the above results, we concluded that it was the T cell epitope in pCol<sub>28-40</sub>, but not the B cell epitope, that was associated with linear binding of IgG to GBM. Anti-GBM Ab was also eluted from rats immunized with either p40A or pCol<sub>29-39</sub>. Results were consistent with those for pCol<sub>28-40</sub> (data not shown).

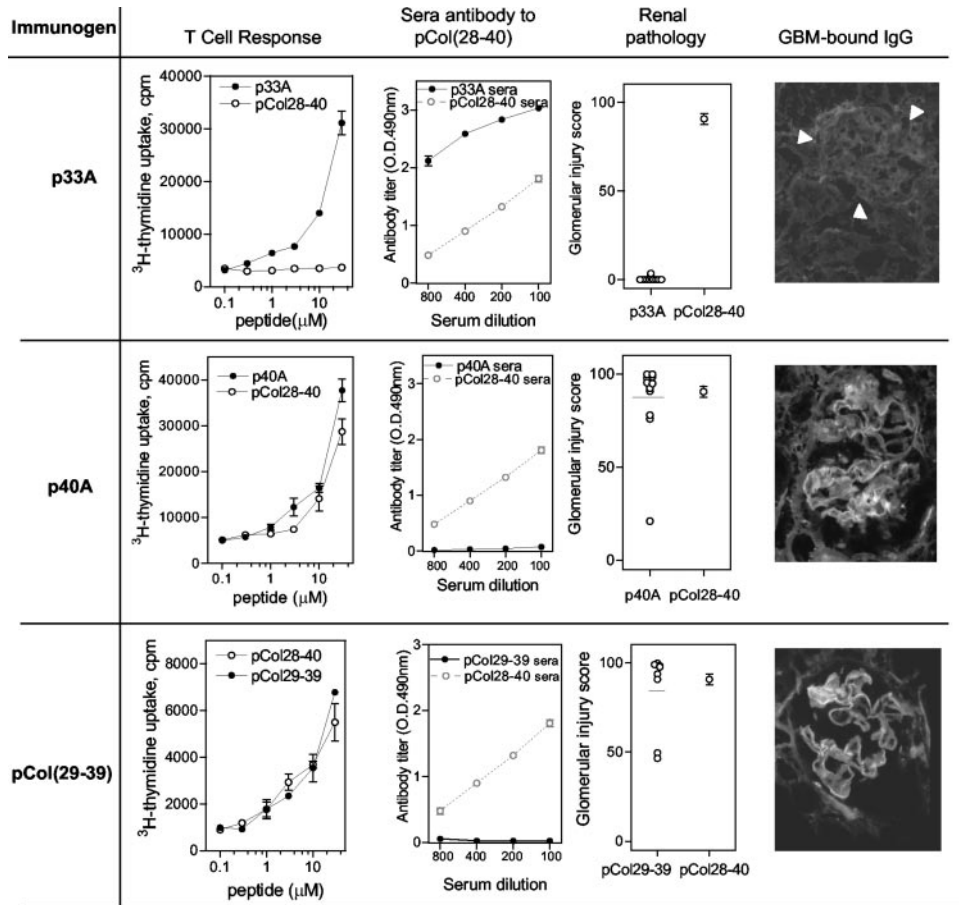
#### A pure T cell epitope pCol<sub>29-39</sub> also induces anti-GBM Ab

The 11-mer peptide pCol<sub>29-39</sub> was mapped to be the core of the T cell epitope encoded within pCol<sub>28-40</sub>. As would be predicted, pCol<sub>29-39</sub> induced severe GN in 14 of 14 immunized animals (Fig. 5). The sera from the animals immunized with pCol<sub>29-39</sub> showed absolutely no circulating Ab reactive with pCol<sub>28-40</sub>, suggesting a lack of a B cell epitope in this peptide (Fig. 5). Thus, pCol<sub>29-39</sub> was a pure nephritogenic T cell epitope, and as we expected, immunofluorescence on the kidneys of the experimental animals revealed intense linear binding of IgG to GBM in 10 of 14 rats (Fig. 5). This experiment once again demonstrated that linear binding of IgG to GBM was associated with the T cell epitope. We concluded that a pure nephritogenic T cell epitope of Goodpasture disease was able to induce IgG-type Ab to GBM.

#### Specificity of anti-GBM Ab induced by the T cell epitope

SR13 evenly stains both GBM and TBM. Two-color immunofluorescence was conducted with FITC-labeled SR13 (green) and Texas Red for eluted GBM Ab (red) on sections of a perfused rat kidney. Although both Abs reacted with GBM, confocal microscopy observations demonstrated different binding patterns between the two. First, eluted GBM Ab reacted strongly with GBM, but much less to TBM (Fig. 6). Second, the binding of eluted GBM Ab to GBM was not completely overlapping with that of SR13. Many areas were stained only by eluted GBM Ab, but not by SR13, or vice versa (Fig. 6A). Third, the eluted Ab showed a fuzzy and broader staining pattern along the GBM, whereas SR13 revealed narrower, sharper linear staining (Fig. 6A). Finally, the eluted Ab showed granular staining in a few areas (Fig. 6A, arrows). These results suggest that the eluted GBM Ab may have reacted with multiple B cell epitopes or multiple components of GBM. We next

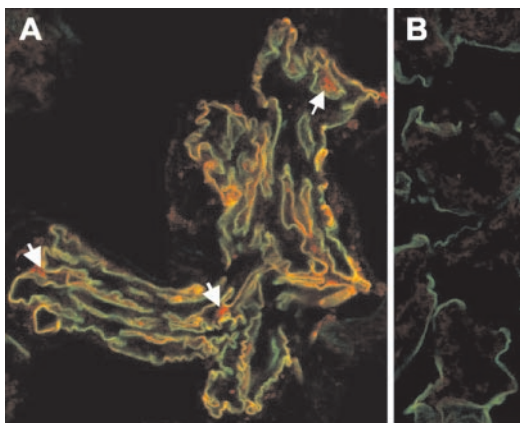
**FIGURE 5.** T cell response, Ab response, renal pathology, and linear binding of IgG to GBM (immunofluorescence) in the rats immunized with peptides p33A, p40A, or pCol<sub>29-39</sub>. Immunogens are indicated at left; T cells isolated from the immunized rats are tested against the immunizing peptide and pCol<sub>28-40</sub>, measured by T cell proliferation assay; circulating Abs are tested against pCol<sub>28-40</sub> by ELISA, while the pCol<sub>28-40</sub>-specific Ab is used as a positive control; renal pathology for each individual is expressed as glomerular injury scores, and their group averages are shown as a short level bar; renal pathology from pCol<sub>28-40</sub>-immunized group is also shown as a control (means ± SE).



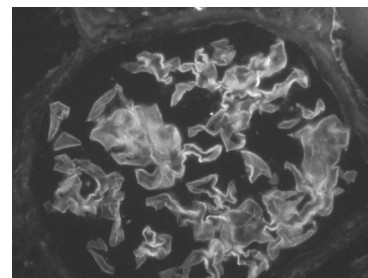
tested whether the eluted Ab reacted with human GBM. The eluted Ab reacted strongly with human GBM, as revealed by indirect immunofluorescence (Fig. 7), whereas SR13 reacted only weakly with human GBM.

Specificity of the eluted Ab was first determined by a 2-D PAGE Western blot using collagenase-digested rat GBM. However, both the eluted Ab and SR13 failed to react with any GBM proteins by Western blot (Fig. 8A). Because proteins were denatured during Western blotting process, we hypothesized that the eluted Ab prob-

ably reacted only with native GBM proteins. We next tested the reactivity of the eluted Ab by immunoprecipitation, in which the antigenic proteins were in their native forms. When the eluted Ab was incubated with digested GBM under neutral conditions, it precipitated multiple GBM proteins. Based on several previously published studies on digested GBM proteins using 2-D PAGE (22), the majority of the proteins were presumably collagen NC dimers of different combinations between various chains (Fig. 8B). However, one group of proteins, with molecular mass higher than the NC dimers, remained unidentified (Fig. 8B). In contrast, serum Ab to pCol<sub>28-40</sub> neither reacted with any denatured GBM proteins in Western blot, nor precipitated any native GBM proteins (Fig. 8C). These experiments demonstrated that the eluted GBM Ab only recognized 3-D conformational B cell epitopes of native GBM proteins, including Col4α3 chain.

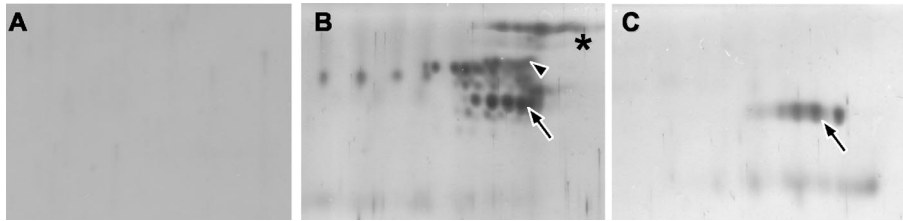


**FIGURE 6.** Confocal microscopic images of two-color immunofluorescence with SR13 (FITC; green) and anti-GBM Ab eluted from kidney of pCol<sub>28-40</sub>-immunized rats (Texas Red). A and B, Shown are a glomerulus and tubules, respectively. Notice that the two colors do not overlap in many areas, indicating their different in Ab specificities. Arrows indicate cytoplasmic staining by the eluted anti-GBM Ab (red).



**FIGURE 7.** The anti-GBM Ab, eluted from the kidney of pCol<sub>28-40</sub>-immunized rats, reacts with human GBM (immunofluorescence).





**FIGURE 8.** Reactivity of the anti-GBM Ab, eluted from pCol<sub>28-40</sub>-immunized rats, with digested GBM proteins. *A*, 2-D PAGE Western blot detection of reactivity of the eluted anti-GBM Ab, showing absence of any reactive proteins. *B* and *C*, 2-D PAGE of GBM proteins immunoprecipitated by the eluted anti-GBM Ab (*B*), or by circulating anti-pCol<sub>28-40</sub> Ab (*C*). A group of H chain proteins of precipitated rat IgG is indicated by arrows. Arrowhead, A serial of NC dimers. \*, Unidentified GBM proteins.

#### *Passive transfer of Ag-specific T cells fails to induce anti-GBM Ab*

Immunization with a pure T cell epitope resulted in not only glomerular damage, but also anti-GBM Ab. We next asked whether Ag-specific CD4<sup>+</sup> T cells were able to transfer the Ab production, because we have shown previously that CD4<sup>+</sup> T cells, specific to Col4 $\alpha$ 3NC1, transferred GN to naive syngeneic recipients (31). We decided to observe anti-GBM Ab response in the rats, which had received pCol<sub>28-40</sub>-specific CD4<sup>+</sup> T cells. However, GBM-bound IgG was not observed in all of the T cell recipients, in which glomerular injury was obvious. We also failed to detect linear binding of IgM to GBM or glomerular C3 deposition in the experimental rats. No circulating Ab to pCol<sub>28-40</sub> was detected.

#### **Discussion**

Our experiments in the present study demonstrated that a single nephritogenic T cell epitope of Goodpasture Ag not only induced severe GN, but also elicited an Ab response to diverse native GBM proteins. The anti-GBM Ab appeared only after prominent glomerular injury. Thus, in our model, the anti-GBM Ab was a consequence of the T cell-mediated glomerular injury. Anti-GBM Ab has been considered the hallmark for human anti-GBM disease. A single T cell epitope of Goodpasture Ag is sufficient to initiate GN with the full clinical spectrum of anti-GBM disease, including anti-GBM Ab.

#### *Anti-GBM Ab is not related with B cell epitope in pCol<sub>28-40</sub>*

Our previous study showed that the T cell epitope pCol<sub>28-40</sub> also contains a B cell epitope (32). It was necessary to carefully determine whether the B cell epitope was associated with anti-GBM Ab in our model. Using multiple methods, we repeatedly demonstrated that the B cell epitope in pCol<sub>28-40</sub> was not associated with the anti-GBM Ab. First, the eluted GBM Ab failed to react with pCol<sub>28-40</sub>. Second, we created a peptide p40A, in which the B cell epitope of pCol<sub>28-40</sub> was eliminated by substitution of a critical residue while leaving the T cell epitope intact. Immunization with p40A induced not only severe GN, but also anti-GBM Ab, despite lacking Ab to pCol<sub>28-40</sub>. In contrast to the peptide p40A, substitution in p33A resulted in an altered T cell epitope specificity, while leaving the B cell epitope identical with pCol<sub>28-40</sub>. It is significant that p33A failed to induce any glomerular injury and anti-GBM Abs, despite the high titer of Ab to pCol<sub>28-40</sub>. Third, a pure T cell epitope pCol<sub>29-39</sub> without a B cell epitope did not induce Ab to pCol<sub>28-40</sub>. However, immunization with pCol<sub>29-39</sub> induced very severe GN, as well as IgG-type anti-GBM Ab. Fourth, our previous study showed that the Ab produced by the B cells from inguinal lymph nodes of immunized animals only reacted with pCol<sub>28-40</sub>, but not GBM (32). Thus, Ab to the peptide pCol<sub>28-40</sub> is not associated with anti-GBM Ab or glomerular injury.

#### *What is the specificity of the anti-GBM Abs in our model?*

First, our study suggests that the anti-GBM Abs may only react with the 3-D conformational B epitope(s) of GBM protein(s) including Col4 $\alpha$ 3NC1. Several pioneering studies on mapping the B cell epitope by the Abs from Goodpasture disease also have shown that the Ab may react with 3-D epitopes of different collagen chains (26–29). Although several groups have found that Goodpasture Ab recognized a linear B cell epitope at the C-terminal of Col3 $\alpha$ 4, the animal models demonstrated that the B cell epitope elicited an Ab to the peptide only but not to native GBM (11, 25). Several mAbs to different chains of the collagen NC were generated using synthetic peptides as Ags for investigation of the differential expression of the collagens (24). However, the Abs reacted only with unmasked epitopes of collagens by high concentrations of urea at very low pH, which presumably denatured the proteins (24). Several unsuccessful attempts were made to map linear B cell epitopes by synthetic peptides covering Goodpasture Ag or other collagen chains (26, 27). The single exception was a mAb to Col4 $\alpha$ 4NC1, which bound to native GBM as well as a peptide (9). Based on all those studies, it is reasonable to conclude that the native GBM may lack linear B cell epitopes, probably due to its complicated quaternary structure composed of the multiple protein complexes (28). Our study supports this hypothesis. Second, our study suggests that anti-GBM Ab in pCol<sub>28-40</sub>-immunized rats may react with more than one epitope/protein. The diverse specificity of Goodpasture Ab has been well documented in clinical observations (12–18). Eluted anti-GBM Ab showed a broader staining pattern in immunofluorescence than SR13, a mAb to Col4 $\alpha$ 4NC, suggesting that the eluted Ab may bind to some components of GBM other than collagen chains. This was supported by our immunoprecipitation study, in which several proteins were shown to be precipitated by the eluted Ab. Precipitation of multiple proteins in our experiment may not necessarily mean that all those proteins are recognized by the Ab, because some of them may be coprecipitated as a protein complex (28). Nevertheless, a group of proteins with molecular mass higher than NC dimers should be independently precipitated proteins (Fig. 8*B*). Fourth, our experiments showed that the eluted Ab from the diseased rat kidneys reacted strongly with human GBM as well. Thus, our model may be used to elucidate the mechanism for highly diverse anti-GBM Ab response in human anti-GBM disease.

#### *How does a single nephritogenic T cell epitope elicit production of IgG-type Ab to diverse native GBM Ags?*

It has been reported that a self T cell epitope can initiate Ab response in several autoimmune disease models and a transgenic mouse model with the HBV e Ag (34–38). For example, a T cell epitope of autoantigen ZP3 induced Ab response to native ZP (34, 38). Based on several observations in our recent study, we hypothesize the mechanism for production of anti-GBM Ab as follows: 1)

self-reactive T cells to pCol<sub>28–40</sub> are activated; 2) the activated T cells migrate into glomeruli to initiate glomerular injury, but some T cells home to the draining lymph nodes of kidney; 3) the released glomerular Ags, including collagen proteins, are transported to the draining lymph nodes by a certain type of APC; 4) autoreactive B cells are ultimately activated by pCol<sub>28–40</sub>-specific T cells and the glomerular Ags in the draining lymph nodes. Our recent unpublished data support this hypothesis. We identified a CD11b/c<sup>+</sup>CD8<sup>+</sup> cell population present only in damaged glomeruli of immunized rats, which displayed a similar shape to that of dendritic cells upon culture. A population of CD8<sup>+</sup> cells in damaged glomeruli has been described by several studies. However, these studies did not determine whether the cells also expressed CD11b/c (39, 40). These dendritic cell-like cells are a promising candidate for the APC that transport the released GBM Ags. Another interesting observation was the elevated production of IgG in the kidney draining lymph nodes of rats immunized with pCol<sub>28–40</sub>. The produced IgG was not the pCol<sub>28–40</sub> Ab. We believe that the IgG may be anti-GBM Ab. These preliminary observations suggest that the draining lymph nodes of the kidney may be the site of anti-GBM Ab production. The production of anti-GBM Ab, which was confined to the local draining lymph nodes, may explain why we were never able to detect anti-GBM Ab in circulation. The identification of the location of autoreactive B cell activation will facilitate our studies to understand the mechanism for T cell-initiated autoantibody response.

Because a pure T cell epitope is sufficient to trigger activation of autoreactive B cells and autoantibody production, it is interesting to ask why transfer of the Col4 $\alpha$ 3NC1-specific CD4<sup>+</sup> T cells failed to induce anti-GBM Ab production. Based on our previous observations and other studies on T cell trafficking, it may be due to trafficking pattern of the T cells, which have been activated in vitro (41). The activated Ag-specific T cells are not able to home into the local lymph nodes and, thus, could not provide the help for B cells.

#### *Autoantibody response to diverse GBM Ags may be a consequence of T cell-mediated GN*

The discovery of anti-GBM Ab is a milestone for autoimmune disease studies. Clinically, the production of anti-GBM Ab is the most important hallmark for human anti-GBM diseases. However, our previous studies have clearly demonstrated that a T cell mechanism alone is sufficient to induce anti-GBM GN (31, 32). Some may argue that a T cell-mediated GN should not be considered an anti-GBM disease, because it may lack anti-GBM Ab, and thus this form of GN might be clinically irrelevant. However, the present study is significant in that it demonstrated that T cell-mediated mechanism induces not only severe GN but also anti-GBM Ab.

The present study also argues that the anti-GBM Ab, which has been thought to be the cause of anti-GBM GN, may be merely the consequence of T cell-mediated glomerular injury. Our study suggests the need to re-examine the cause-effect relationship between anti-GBM Ab and human anti-GBM disease. Heterogenic anti-GBM Abs have been shown to induce GN in several animal models (42–45). However, at least in this model, anti-GBM Ab may not play a crucial role in the pathogenesis, as evidenced by lack of differences in disease severities between the experimental rats with or without anti-GBM Ab. Although anti-GBM Ab is the most important diagnostic index for human anti-GBM GN, there was no significant correlation between severity of GN and anti-GBM Ab titer in GN patients (46, 47).

Many elegant studies revealed highly diverse specificities of anti-GBM Ab in human anti-GBM disease (12–18). In addition to

Col4 $\alpha$ 3NC1, human anti-GBM Abs reacted with other collagen chains or even other glomerular Ags. Interestingly, anti-GBM Abs were not limited to anti-GBM disease, but also were present in some other types of GN (48). Detection of anti-neutrophil cytoplasmic autoantibody in anti-GBM disease and several types of GN has been well documented (17, 18). What mechanism leads to an Ab response to such diverse autoantigens? The present study indicates that such diversified anti-GBM Abs could be simply elicited by a single nephritogenic T cell epitope, or even a microbial peptides that mimic the T cell epitope.

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