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J Clin Invest. 1992;**89**(2):517-523. <https://doi.org/10.1172/JCI115615>.

Research Article

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Tubular Antigen-binding Proteins Repress Transcription of Type IV Collagen in the Autoimmune Target Epithelium of Experimental Interstitial Nephritis

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Abstract

We have been studying immune interactions with somatic cells using a tubular antigen-binding protein (ThF) secreted by helper T lymphocytes harvested from mice that have an autoimmune form of interstitial nephritis called anti-tubular basement membrane disease. This ThF, although characterized originally because of its ability to induce effector T cells, additionally recognizes the nephritogenic 3M-1 antigen expressed by its target renal tubular epithelium. We believe these proteins, in general, may modulate directly some homeostatic functions in organ-derived cells, and now report that our ThF represses specifically the cellular transcription and secretion of basement membrane type IV collagen in tubular epithelium. These *in vitro* findings of reduced levels of mRNA encoding type IV collagen correlate well with *in situ* hybridization studies performed on kidneys expressing early autoimmune lesions, and predict a progressive drop in the expression of type IV collagen in the interstitium. Such a novel and unexpected repression of transcription of type IV collagen might easily impart or facilitate permanent change in the infrastructure of kidney architecture during autoimmune injury and, perhaps, contributes to the process of tubular atrophy attendant to prolonged renal inflammation. (*J. Clin. Invest.* 1992; 89:517-523.) Key words: renal epithelium • basement membranes • T cells • autoimmune tubular atrophy

Introduction

Approximately 10 years ago we observed that crude culture supernatants from immune lymphocytes in autoimmune interstitial nephritis could alter the biology of kidney cells *in vitro* (1, 2). These early experiments suggested the possibility of interactions between immune and somatic cells. Since that time we have been able to both characterize the relevant nephritogenic 3M-1 target antigen (3, 4) as well as establish 3M-1-specific CD4⁺ helper T cell clones (5). These helper T cells are present within inflammatory tubulointerstitial infiltrates (5), secrete a 3M-1-binding protein (ThF)¹ of ~ 78,000 *M_r* (6), and

recognize 3M-1 on the surface of cultured proximal tubular epithelium (MCT cells; 7, 8) from the kidney. In the presence of 3M-1 antigen, precursor cells, and IL-2, ThF can induce CD8⁺ effector T cells that produce nephritogenic injury (6, 9).

Cortical epithelium similar to our MCT cells rests normally *in situ* on a traditional basement membrane that, for the most part, is composed of type IV collagen (10-12). These cells and their basement membrane form elongated tubules which are held together by a scaffolding of types I and III interstitial fibrillar collagens (10). MCT cells in culture secrete measurable amounts of basement membrane type IV collagen, as well as lesser amounts of interstitial types I and III collagens (7). Since much of the cortical tubular architecture of the kidney undergoes destructive remodelling during autoimmune interstitial inflammation (13, 14), and since our ThF recognizes an autoantigen distributed only along cortical tubules (3, 4), we attempted in this report to determine what biologic effect ThF might have on the transcription and secretion of types I and IV collagens by MCT epithelium in culture.

Methods

Cell culture and preparation of antigen-binding protein. MCT cells from the kidneys of SJL mice were passaged in serum-free media supplemented with transferrin (5 µg/ml), insulin (5 µg/ml) at 37°C in 5% CO₂ every 48-72 h (7). ThF was prepared from the cultures of CD4⁺ M-30 (3M-1-specific) or M-12 (PPD-specific) helper T cell clones by immunoaffinity chromatography using a reverse-elution column (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) containing solubilized tubular antigen (1 mg/ml) linked to Sepharose 4B (6, 9). Purifications were verified by the iodination of aliquots of each preparation of M30 ThF followed by resolution on 8% SDS-PAGE gels and autoradiography (4, 7).

Collagen radioimmunoassays. Serum-free cultures of 10⁴ near-confluent MCT cells (7), in flat-bottomed microtiter plates, were grown in serum-free media supplemented with transferrin (5 µg/ml), insulin (5 µg/ml), ascorbic acid (50 µg/ml), and β-aminopropionitrile (50 µg/ml), containing 50 µg/ml of ultrapure BSA, and various concentrations of ThF. M12-ThF and M30-ThF were secreted originally into a known volume of T cell culture media in cultures starting with 5 × 10⁵ CD4⁺ T cells/ml. These ThFs, after affinity purification, were restored to that original volume, and this volume was considered to contain or be equivalent to 100% of the original concentration. If the ThF were added to MCT cultures, for example, at 50% concentration, it would mean it had been added at 50% of its original secreted concentration. The BSA concentration, far in excess of the ThF, served to stabilize protein content. After 72 h of culture, the supernatants were harvested and assayed for types I or IV procollagens by radioimmunoassay, and cell growth was assessed by [³H]TdR incorporation (7). Absolute amounts of measured collagen were normalized for cell growth detected by simultaneous thymidine incorporation. Replicate measurements were within 7% SEM.

RNA analyses. Total RNA from near-confluent MCT cells (20 µg) was electrophoresed through a 1.2% agarose-formaldehyde gel and transferred to Zetabind (Cuno, Meriden, CT) for Northern hybridizations (4, 15). Hybridizations were performed at 62°C in 7% SDS, 0.5 M

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Received for publication 26 March 1991 and in revised form 14 August 1991.

1. Abbreviations used in this paper: EGF, epidermal growth factor; MCT, proximal tubular epithelium; RTA, renal tubular antigen; ThF, tubular antigen-binding protein; TGFβ, transforming growth factor β.

J. Clin. Invest.

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0021-9738/92/02/0517/07 \$2.00

Volume 89, February 1992, 517-523

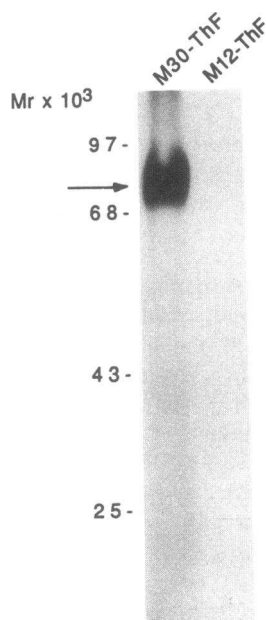


Figure 1. Visualization of ThF eluted from a soluble renal tubular antigen affinity column. Protein lysates harvested from M-30 T cells were passaged over a tubular antigen affinity column. Part of the eluate was then iodinated and electrophoresed on an 8% SDS-PAGE gel under reducing conditions to produce a band at $\sim 78,000 M_r$ (arrow; 6). No such band was observed with protein lysates prepared from M12 cells reactive with purified protein derivative.

sodium hydrogen phosphate, 1 mM EDTA, and 50 $\mu\text{g}/\text{ml}$ polyadenylic acid with 50 $\mu\text{g}/\text{ml}$ salmon sperm. Filters were washed at 65°C in 0.1% standard saline citrate (SSC) with 0.5% SDS, and exposed to Kodak XAR film at -70°C . Dot-blot hybridizations were performed in duplicate with cytoplasmic RNA harvested from 10^6 MCT cells cultured with ThF using 0.1% trypsin in 1 mM EDTA (15). The cells were washed in PBS, and lysed with 0.05% NP-40 in Tris-EDTA buffer.

Cytoplasmic RNA equivalent to 2×10^5 cells was denatured in $12 \times \text{SSC}/15\%$ formalin at 65°C and spotted onto Nytran (Gibco Laboratories, Grand Island, NY) by using a Hybridot manifold (Bethesda Research Laboratories, Gaithersburg, MD). The Northern and dot-blot filters were probed with ^{32}P -labeled cDNAs ($\text{SA} = 0.5\text{--}1.0 \times 10^9 \text{ cpm}/\mu\text{g}$) representing either a 0.85-kb XhoI fragment of murine type $\alpha 2(\text{I})$ collagen from pAZ1002 [16], a 0.66-kb PstI fragment of murine type $\alpha 1(\text{IV})$ collagen from pc15 [17], or a 1.2 kb-PstI fragment of murine beta-actin from pAL41 [18]) in hybridization fluid comprised of 50% formamide, $2 \times$ Denhardt's solution, 150 $\mu\text{g}/\text{ml}$ ssDNA, 0.1% SDS, and $5 \times \text{SSC}$ incubated at 42°C for 16 h. The filters were washed 15 min twice in $6 \times \text{SSC}$, 0.5% SDS at 22°C , and once in $0.1 \times \text{SSC}$, 0.5% SDS at 65°C for 1 h, and then autoradiographed with intensifying screens at -70°C . Hybridization signals were quantitated using a densitometer of integration of signals by triangulation. Exposures were within the linear range of the film.

Transcriptional run-off assays. The in vitro transcription assay was performed on 10^7 nuclei harvested from near-confluent monolayer cultures of MCT cells washed in PBS, released with trypsin, pelleted, and resuspended in 1 ml of 0.5% NP-40, 10 mM Tris (pH 7.0), 10 mM NaCl, 3 mM MgCl_2 for 5 min on ice (15). The samples were spun again and washed in 2 ml of NP-40 buffer. Nuclear pellets were suspended in 100 μl of 50 mM Tris (pH 8.3), 40% glycerol, 5 mM MgCl_2 , and 0.1 mM EDTA and kept frozen. For labelling, nuclei were thawed and mixed with 100 μl of 10 mM Tris (pH 8.0), 5 mM MgCl_2 , 300 mM KCl, 0.5 mM each of ATP, GTP, CTP, and 100 μl of $[\text{P}^{32}]\text{UTP}$ ($> 600 \text{ Ci}/\text{mmol}$) for 30 min at 26°C . Reactions were terminated on ice. An aliquot of 10 μl of 10 mg/ml tRNA was added, and nuclei were digested with 0.5 mg/ml DNase I. Nuclear proteins were digested with 5 mg/ml of proteinase K, followed by chloroform:phenol extraction and ethanol precipitation. Hybridization filters were prepared by linearizing and

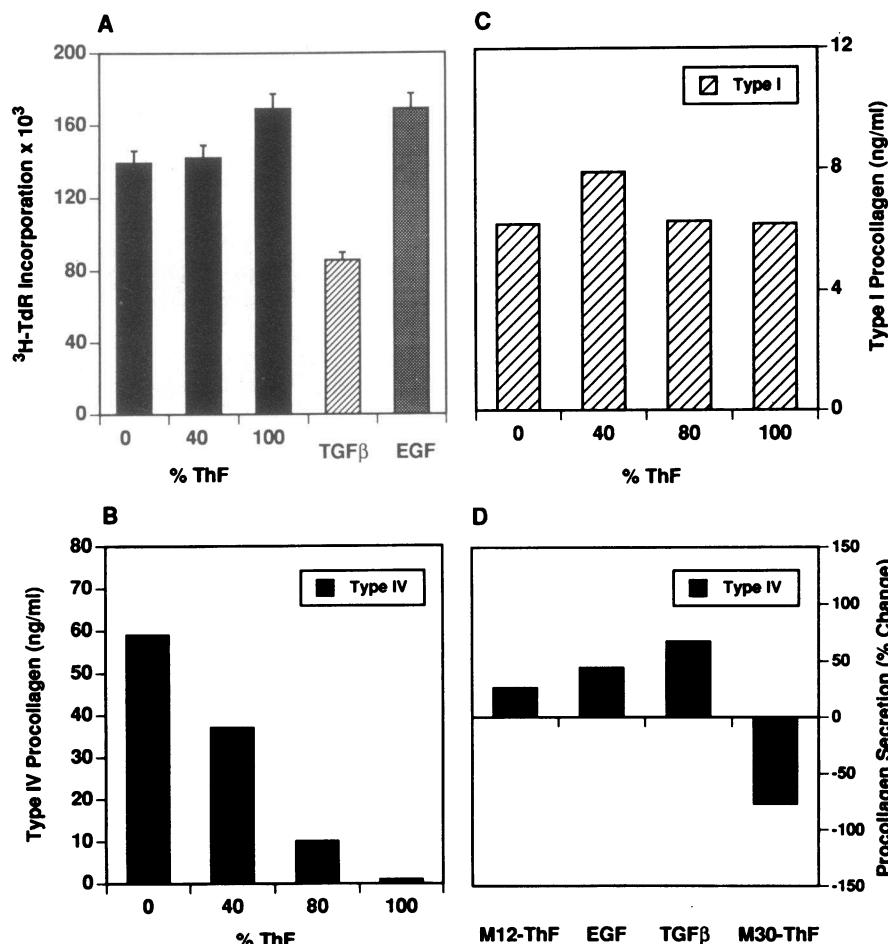


Figure 2. The effects of tubular antigen-binding proteins on collagen secretion by MCT epithelium (15). Experiments were performed multiple times with SEM for each assay varying only by 5–7%. (A) The addition of increasing amounts of M30-ThF to cultures of 3M-1-secreting MCT epithelium slightly increased the incorporation of $[\text{P}^{32}]\text{H}$ -TdR at 72 h. TGF β at 1 ng/ml reduced MCT growth while EGF at 10 ng/ml increased it slightly; (B) the addition of increasing amounts of M30-ThF to cultures of 3M-1-secreting MCT epithelium progressively inhibited the secretion of type IV collagen as measured by radioimmunoassay; (C) the same ThF had little to no effect on type I secretion, except to increase secretion slightly at mid-level doses. (D) M30-ThF inhibits the secretion of type IV collagen at 100% equivalent concentration, while ThF harvested from purified protein derivative-reactive M12 T cells (M12-ThF; also at 100% equivalent concentration), TGF β (1 ng/ml), and EGF (10 ng/ml) produced increases in collagen secretion.

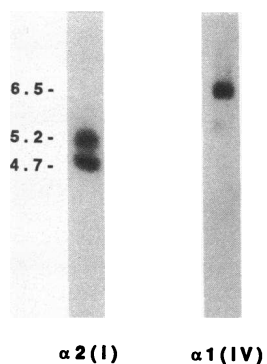


Figure 3. Northern hybridization of MCT RNA with ^{32}P -labelled probes encoding for $\alpha 1(\text{IV})$ and $\alpha 2(\text{I})$ genes. Hybridizations revealed the presence of a 6.5-kb transcript for $\alpha 1(\text{IV})$, and 4.8 plus 5.8-kb transcript for $\alpha 2(\text{I})$ in MCT cells (19).

denaturing various test plasmids, spotting 2- μg equivalents of insert DNA onto nitrocellulose, and prehybridizing in 40% formamide, 4 \times SSC, 70 mM Tris (pH 7.5), 1 \times Denhardt's solution, 0.4% SDS, 5 mM EDTA, 150 $\mu\text{g}/\text{ml}$ ssDNA containing 60 mg/ml each of poly(A) and poly(C) for 72 h at 42°C. Fresh hybridization fluid was added with 2–3 $\times 10^5$ cpm ^{32}P -labelled RNA, and hybridization was continued for another 72 h at 42°C. Blots were dried and autoradiographed with hybridization signals quantified as above. Exposures were within the linear range of the film.

In situ hybridizations. SJL mice were immunized with renal tubular antigen (RTA)/CFA to produce anti-tubular basement membrane disease (13). Control mice were immunized with CFA alone. Kidney sections of 5 μm were cut from paraffin blocks after fixation in ethanol formaldehyde, and then applied to chromalum-treated slides (15). Sections were deparaffinized in 100% xylene, incubated in 1% Triton X-100 for 10 min at 22°C, and permeabilized in 10 mg/ml of proteinase K in 20 mM Tris, 2 mM CaCl_2 (pH 7.4) for 20 min at 37°C. Hybridization fluid containing 50% formamide, 5 \times Denhardt's solution, 5 \times SSC, 10% dextran sulfate, 500 $\mu\text{g}/\text{ml}$ ssDNA, 0.1% Triton X-100, and 0.5 $\mu\text{g}/\text{ml}$ of ^{35}S -labelled cDNA probe ($0.5\text{--}1.0 \times 10^8$ cpm/ μg) was heated to 100°C for 2 min, iced, and admixed with DTT to final concentration of 10 mM. An aliquot of 40 μl of fluid was applied to each slide, enclosed with a glass cover slip, and hybridized at 40°C for 4 to 18 h. Sections were then washed 30 min with 2 \times SSC at 22°C, 30 min each with 0.1 \times SSC and 0.1 \times SSC at 65°C, and 15 min 0.1 \times SSC at 37°C. Slides were dipped in Kodak NTB-2, exposed for 2–5 d at room temperature, and the reaction product was developed with D19 and Kodak fixer. Slides were then counterstained with hematoxylin and examined under the microscope. Silver grains were counted in tubular cells expressing visible brush border from 10 random high power fields ($\times 800$) on slides hybridized with ^{35}S -labelled types I and IV collagen probes. Grain counts from slides hybridized with pBR322 were subtracted as background, and the corrected counts were then expressed as a mean \pm SEM. All controls were run simultaneously.

Immunofluorescence. Portions of renal tissue were sectioned, placed on a cellulose sponge, and snap frozen in isopentane prechilled in liquid nitrogen and stored at -70°C . Cryostat sections, 4 μm in thickness, were stained with conjugated antisera and evaluated in a Zeiss Universal microscope as described previously (3, 7, 13, 15). Optimal concentrations of FITC-conjugated affinity-purified mouse anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was used for indirect immunofluorescence to detect kidney tissue binding by primary monospecific rabbit antibodies to M30-ThF (6), and to types I and IV collagen (7). Normal rabbit sera were used as a control.

Results

In preparation for our initial experiments we purified antigen-binding ThF from a CD4 $^+$ helper T cell clone (M30) reactive with the 3M-1 renal tubular antigen expressed by cortical tubu-

lar epithelium using a tubular antigen affinity column (6). This ThF resolved at $\sim 78,000 M_r$ under reducing conditions on SDS-PAGE (Fig. 1), and was of predicted purity (6).

We next observed in Fig. 2A that the addition of increasing concentrations of affinity-purified M30-ThF to cultures of MCT epithelium only increased slightly their incorporation of [^3H]TdR, whereas the recombinant cytokine transforming growth factor β (TGF β) predictably decreased the growth of MCT cells. The addition of M30-ThF also attenuated progressively their secretion of type IV collagen as measured at 72 h (Fig. 2B). In contrast, ThF did not alter greatly the secretion of type I collagen, except to raise it slightly at a mid-dose level (Fig. 2C). To determine if the inhibition of secretion of type IV

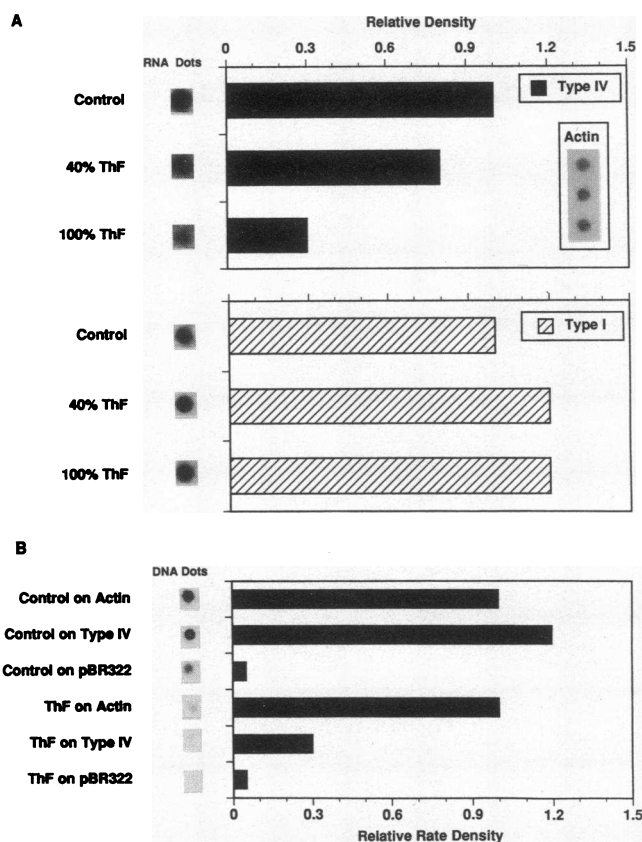


Figure 4. The effects of ThF on the transcriptional activity of collagen in MCT epithelium. (A) Cytoplasmic dot-blot hybridizations of MCT cell RNA with cDNA encoding types I and IV collagen following coculture with ThF. 10^6 MCT cells were cultured with ThF (40% and 100% equivalent concentrations) for 24 h, after which 2×10^5 cell equivalents of cytoplasmic RNA were spotted onto Nytran and hybridized with ^{32}P -labelled cDNA probes for types I and IV collagen (15). The results show a reduction in hybridizing transcripts for type IV collagen with a minimal increase in type I collagen between treated groups. A ^{32}P -labelled cDNA probe encoding actin did not reveal differences in RNA loading (inset box in type IV bar graph); (B) Nuclear run-off transcription assay for type IV collagen. 10^7 MCT cells were cultured in serum free media with or without ThF at 100% equivalence for 48 h. The transcription of their isolated nuclei was completed in the presence of [^{32}P]UTP and hybridized to DNA specific for type IV collagen, actin, and pBR322 (15). The results indicate there is less transcription of type IV collagen in MCT cells treated with ThF when compared to controls.

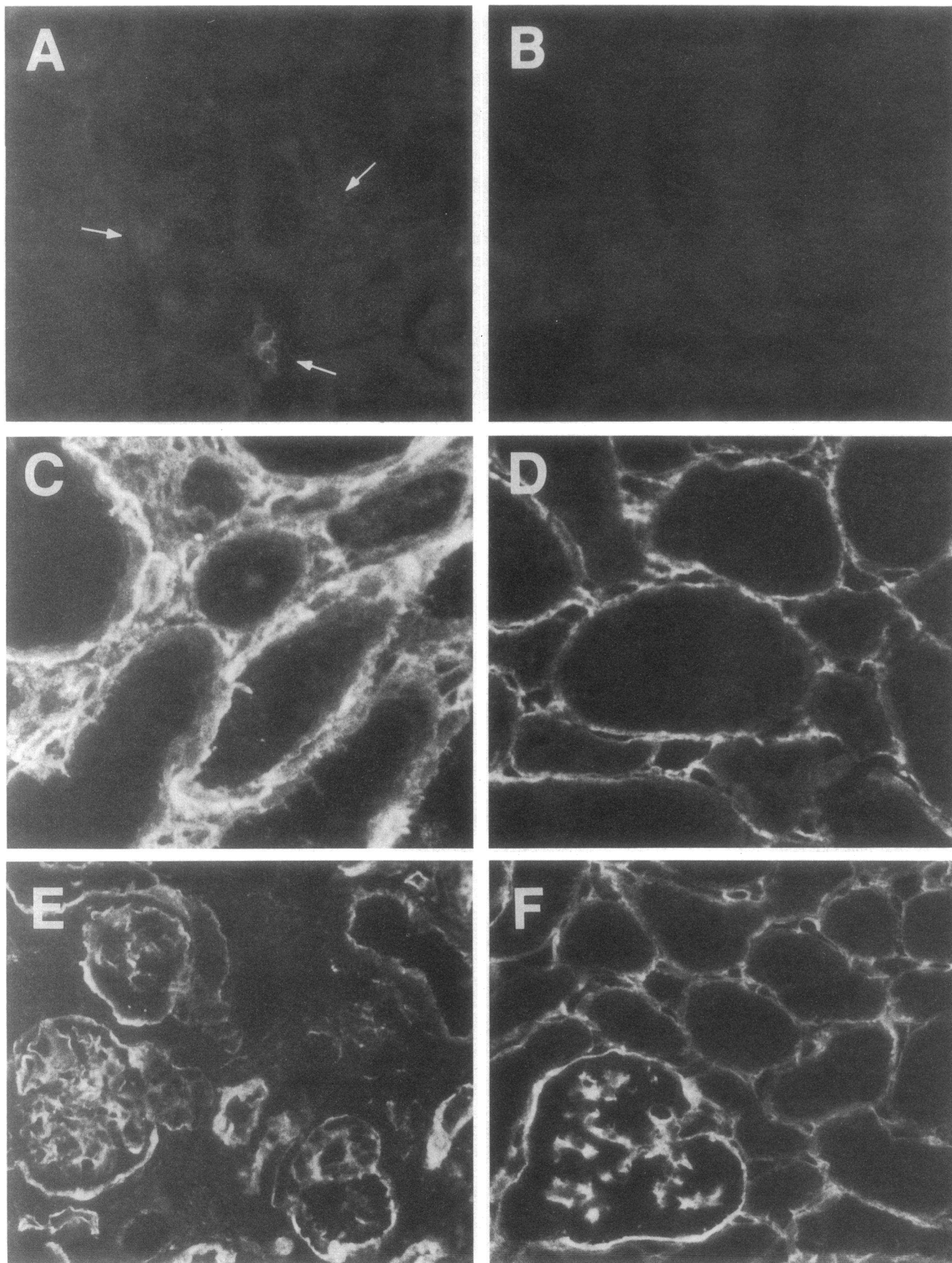


Figure 5. Immunofluorescent staining of kidney tissue expressing anti-tubular basement membrane disease and interstitial nephritis. (A) 5-wk early nephritic kidneys stained with monospecific rabbit anti-ThF antibody plus FITC-mouse anti-rabbit IgG (6) identifies occasional mononuclear cells (arrows) located in the interstitium between tubules ($\times 160$); (B) FITC-mouse anti-rabbit IgG plus control rabbit antibody does not

collagen by M30-ThF had immunologic specificity, we next examined the effects of other ThFs from CD4⁺ helper cells (M12) reactive to purified protein derivative (5, 9) as well as the impact of the recombinant cytokines TGF β and epidermal growth factor (EGF). In Fig. 2 *D*, M12-ThF, TGF β , and EGF all produced slight to substantial increases in the secretion of type IV collagen when cocultured in the presence of MCT cells, but did not yield the inhibitory effects observed with 3M-1-binding ThF obtained from M30 cells.

To examine the level at which ThF might have exerted its influence on the secretion of epithelial collagen, we compared the relative abundance of mRNA transcripts in MCT cells stimulated with 3M-1-binding ThF. First, in Fig. 3, we established that our labelled cDNA probes hybridized to appropriately sized mRNA harvested from MCT cells (α 1[IV] mRNA: \sim 6.5 kb, and α 2[I] mRNA: \sim 4.8 and 5.8 kb; 19). We next determined the relative change in α 1(IV) and α 2(I) mRNA collagen transcripts in MCT cells using RNA dot-blot hybridizations (15). Our findings in Fig. 4 *A* demonstrate that ThF raised slightly the transcript levels for type I collagen, whereas transcript levels for type IV collagen fell substantially. To determine whether this latter effect of ThF on α 1(IV) transcripts was due to a change in the rate of transcription, we performed in vitro nuclear run-off experiments on nuclei isolated from MCT cells cocultured with ThF. In Fig. 4 *B*, while ThF did not produce much change in the rate of transcription of actin, there was a substantial reduction in rate of transcription for type IV collagen.

To further extend our in vitro findings regarding changes in copy numbers of collagen transcripts, we next performed immunohistochemistry and in situ hybridizations on kidney sections obtained from mice immunized to produce anti-tubular basement membrane disease and interstitial nephritis (15). 3M-1-specific helper T cells producing ThF are present in such lesions (5, 6). Immunofluorescent examination of kidneys harvested 5 wk after immunization with RTA/CFA, at an early stage just before major damage begins to destroy the interstitial architecture (13), revealed in Fig. 5, *A* and *B*, the presence of ThF-expressing mononuclear cells in the tubulointerstitium of nephritic mice. ThF-expressing mononuclear cells were not found in control kidneys immunized with CFA alone. In situ hybridizations, depicted in Fig. 6 (*A*–*D*), were also performed by overlaying 5-wk nephritic kidney sections with ³⁵S-labelled cDNA encoding types I or IV collagen. Our findings indicate that the numbers of type IV collagen transcripts in cortical tubular cells were reduced to 10.2 ± 2.2 grains/high power field (hpf) (Fig. 6 *D*) in tubules involved in interstitial infiltrates when compared with control kidneys expressing 48.0 ± 3.8 grains/hpf (Fig. 6 *B*); $P < 0.01$. The transcript reaction product for type I collagen was increased from 3.5 ± 0.9 grains/hpf (Fig. 6 *A*) in controls to 15.5 ± 1.1 grains/hpf in kidneys from mice with early disease (Fig. 6 *C*); $P < 0.01$. These changes in mRNA were confirmed in more advanced lesions by immunofluores-

cent staining. Kidneys harvested 12 weeks after immunization demonstrated an increased staining for collagen type I in the interstitium and perivascular areas (Fig. 5, *C* and *D*) and a decreased staining for collagen type IV as evidenced by a reduced fraction of cortical tubules that were ringed by basement membrane (Fig. 5, *E* and *F*).

Discussion

The antigen-binding ThF of anti-tubular basement membrane disease is secreted by CD4⁺ helper T cells (6), and these helper cells can be harvested from interstitial lesions (5). In the present experiments we were able to detect additionally the presence of this ThF in mononuclear cells scattered within the tubulointerstitial space. Although we only tested the ThF effect on proximal tubular cells in vitro, the 3M-1 target antigen is found all along the cortical nephron of mice (13), and these ThF proteins might likely interact with any 3M-1 antigen-secreting tubular segment.

Our experiments indicate that the in vitro transcription and secretion of type IV collagen in target epithelium can be repressed specifically by antigen-binding ThF. The effect of ThF on the secretion of type IV collagen was dose dependent, and the actions of EGF and TGF β further demonstrate that type IV secretion can be modulated bi-directionally. Not a great deal of information exists currently regarding the effects of TGF β and EGF on the secretion of type IV collagen in organ-derived epithelial cells. Under our culture conditions they appear to stimulate the secretion of type IV, a finding which might be predicted from work with TGF β on type I collagen in fibroblasts (20), or with EGF on type IV secretion in myoepithelium (21). The unique effects of antigen-binding ThF that inhibit the secretion of type IV basement membrane collagen in its target epithelium was also not anticipated from previous work (1, 2). These novel findings nevertheless reinforce the notion that cytokine-like proteins may influence differentially the secretion of distinct phenotypes of collagen originating from the same cell.

We believe the mechanism of the effect of ThF on type IV secretion is likely related to a reduction in the rate of transcription of type IV in affected epithelium. The 20-fold decrease in the secretion of type IV collagen in MCT cells, however, is significantly greater than the observed 3-fold reduction in levels of α 1(IV) mRNA, suggesting that the repression of transcription of type IV collagen in MCT epithelium by ThF may represent only one of several potential mechanisms for the control of expression of collagen genes by paracrine regulatory proteins (22). The fall in type IV transcripts in MCT cells following the addition of ThF in vitro paralleled the reduction in type IV reaction product observed with the in situ hybridization of early nephritic kidneys. The increase in transcripts encoding type I collagen in vivo, however, is not what we observed in vitro with ThF. Since a variety of mononuclear cells are present in interstitial lesions (23), it is likely that other as yet

stain any cortical structures ($\times 160$); (*C*) nephritic kidney sections harvested at 12 weeks after immunization with RTA/CFA (13) were stained with monospecific rabbit anti-type I collagen antibody (7) showing a marked increase in the deposition of type I collagen within the interstitial space and near thickened tubular basement membranes ($\times 400$); (*D*) control kidney sections from mice immunized with CFA 12 weeks before harvest and stained with rabbit anti-type I collagen antibody ($\times 400$); (*E*) nephritic kidney sections were harvested 12 weeks after immunization with RTA/CFA (13) and stained with monospecific rabbit anti-type IV collagen antibody (7) demonstrating a marked decrease in the numbers of cortical tubules resting on basement membrane containing type IV collagen ($\times 250$); (*F*) control kidney sections from mice immunized with CFA 12 weeks before harvest and stained with rabbit anti-type IV collagen antibody to highlight normal tubules ($\times 250$).

Type I Procollagen

Type IV Procollagen

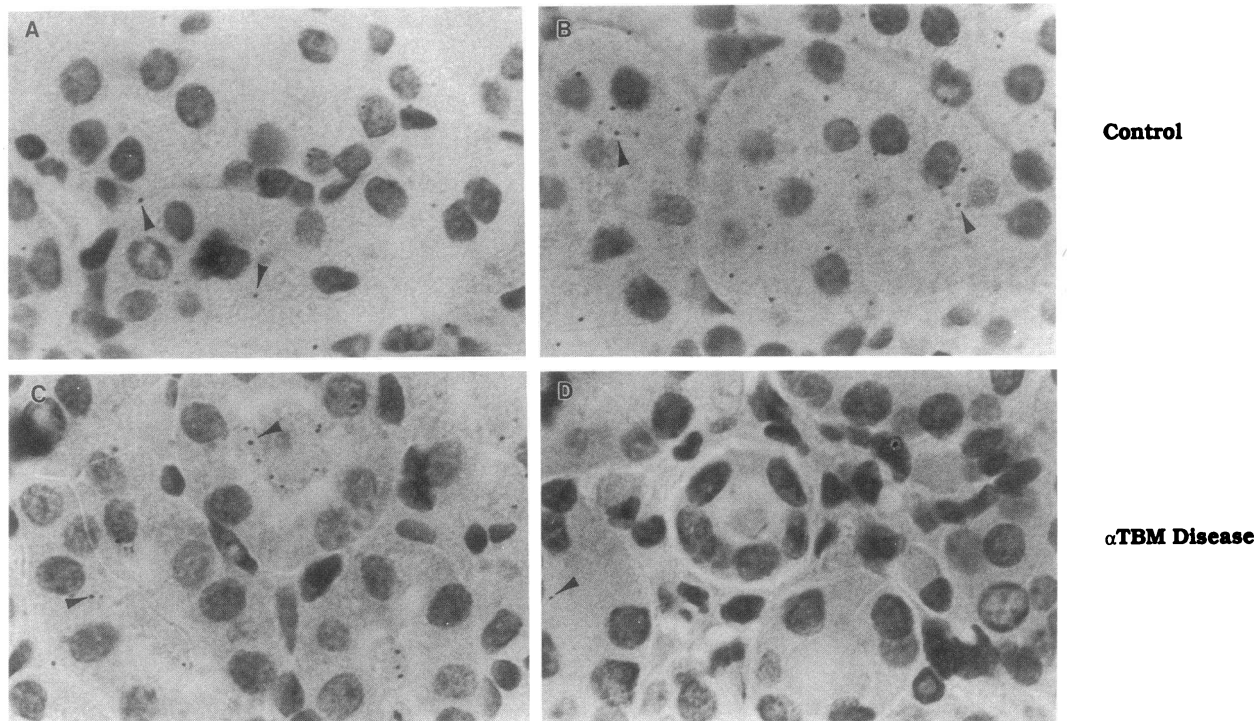


Figure 6. In situ hybridization of kidney sections using ^{35}S -labelled cDNA encoding types I and IV collagen (15). (A) Control kidneys from SJL mice immunized with CFA and hybridized with cDNA probes for type I collagen. (B) Control kidneys harvested from SJL mice immunized with CFA and hybridized with cDNA probes for type IV collagen. (C) Kidneys harvested five weeks after the immunization of SJL mice with RTA/CFA to produce anti-tubular basement membrane disease (5) were hybridized with cDNA probes for type I collagen. (D) Kidneys harvested five weeks after the immunization of SJL mice with RTA/CFA to produce anti-tubular basement membrane disease were hybridized with cDNA probes for type IV collagen. These in situ results demonstrate a decrease in reaction product for type IV collagen and an increase in reaction product for type I collagen among cortical tubules involved with early interstitial infiltrates.

unidentified cytokines expressed concomitantly in this interstitial microenvironment may both increase type I secretion and/or produce the same effects on type IV as ThF.

Cortical tubular epithelium and its associated basement membrane structures begin to wither with extended renal inflammation (24, 25). Our demonstration that disease-relevant antigen-binding T cell proteins can modulate selectively the transcription and secretion of collagens in epithelium expressing appropriate target antigens provides additional new information regarding the process of parenchymal cell modification by autoimmune signalling events (15). A pathophysiologic bridge between renal tubular atrophy and the specific action of an antigen-binding protein has not been proposed before. The 3M-1 antigen is a complex structure expressed as multiple isoforms (4). Its framework domain contains nephritogenic epitopes and has some structural similarity to intermediate filament-associated proteins. How ThF interactions with the 3M-1 ligand might influence the biology of type IV collagen is currently under further investigation. The demonstration, nevertheless, of a direct effect of immune-somatic cell interactions on local organ remodelling lends support to the notion that some immune events may mediate progressive tubular atrophy. In our example the argument favors an architectural regression of otherwise mature renal parenchymal structures.

Acknowledgments

We thank Dr. Nicholas Kefalides, Director, Connective Tissue Research Institute, University of Pennsylvania, and Dr. Thomas Ka-

desch, Howard Hughes Medical Institute, University of Pennsylvania, for helpful discussions of this manuscript.

This work was supported in part by grants AR-20553, DK-07006, DK-30280, DK-33501, DK-42155, and DK-41110 from the National Institutes of Health. E. G. Neilson is the recipient of an Established Investigator Award (85-108) from the American Heart Association and its Pennsylvania Affiliate. C. J. Kelly is the recipient of a Markey Scholar Award (86-019) from the Lucille P. Markey Charitable Trust. T. P. Haverty was supported by the Measey Foundation.

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