Mesangial Cell–Binding Anti-DNA Antibodies in Patients with Systemic Lupus Erythematosus

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Abstract. The mechanisms by which anti-DNA antibodies contribute to the pathogenesis of lupus nephritis (LN) remain to be elucidated. This study investigates the binding of polyclonal anti-DNA immunoglobulins from patients with systemic lupus erythematosus (SLE) to human mesangial cells (HMC) in vitro. Testing of cross-sectional serum samples from 280 LN patients (108 during active disease; 172 during remission), 35 SLE patients without renal involvement, 72 patients with non-lupus primary glomerular diseases, and 37 healthy subjects with a cellular enzyme-linked immunosorbent assay showed significant IgG mesangial cell-binding activity in patients with SLE, particularly those with active LN (P < 0.0001). Significant HMC-binding activity was demonstrated in 83.9%, 42.8%, and 47.1% of patients with active LN, inactive LN, and non-renal SLE, respectively. This was predominantly attributed to binding by anti-DNA antibodies, and immune complex binding accounted for 4.6%, 3.5%, and 2.8% of seropositive samples in the respective groups. Longitudinal studies in 27 LN patients demonstrated correlation between serial levels of anti-DNA antibodies, serum HMC-binding activity, and disease activity in 18 patients (66.7%). Affinity-purified polyclonal IgG anti-DNA antibodies from sera with HMC-binding activity showed significant binding to cultured HMC, and to a lesser extent glomerular and proximal tubular epithelial cells and human umbilical vein endothelial cells, but not tumor cell lines, peritonal mesothelial cells, bronchial epithelial cells, or fibroblasts. The binding of anti-DNA antibodies to HMC was increased 1.47-fold (P = 0.0059) after the removal of Ig-associate DNA by DNase treatment, but it was unaffected by DNase treatment of HMC membrane. Controlled trypsinization of membrane proteins in HMC resulted in a 1.26-fold (P = 0.0025) increase in their binding by anti-DNA antibodies. In conclusion, subsets of anti-DNA antibodies from patients with SLE are capable of binding to HMC. The association of such binding with renal involvement and disease activity and its modulation by DNA concentration suggest that Ig binding to HMC can be a potential marker for disease activity in selected patients and that the binding of anti-DNA antibodies to HMC may be a pathogenetic mechanism in LN.

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by the presence of autoantibodies against nuclear antigens (1). Lupus nephritis (LN) is a frequent and severe organ manifestation in which mesangial proliferation and deposition of immunoglobulins and complement components within the mesangium are prominent features (2). Anti-DNA antibodies have been demonstrated in renal and glomerular eluates obtained from SLE patients and lupus mice (3–5). The correlation between circulating anti-DNA antibodies and disease activity presents further evidence that these antibodies participate in pathogenetic mechanisms. Furthermore, glomerulonephritis could be induced in non-autoimmune mice upon inoculation with the transgene that encodes the secreted form of an IgG anti-DNA antibody, indicating that anti-DNA antibodies are instrumental in the initiation of LN (6).

However, the role of anti-DNA antibodies in the pathogenetic mechanisms of LN has not been fully elucidated (7,8), and the heterogeneity of these antibodies implies variable pathogenetic involvement. Characteristics of anti-DNA antibodies that have been associated with pathogenicity include an IgG isotype, cationic charge, high affinity for binding to double-stranded DNA, and cross-reactivity with different glomerular components, such as laminin, collagen, and heparan sulfate proteoglycans (8–14). Glomerular binding by autoantibodies has been associated with pathogenicity and disease activity (15–17). It remains unclear how these antibodies interact with resident cells or components in the glomerulus.

The strategic location of mesangial cells in the glomerulus, juxtaposed to adjacent capillary loops, facilitates their interaction with immune deposits, complement components, and inflammatory mediators (18). Mesangial cell proliferation and the deposition of anti-DNA antibodies in the mesangium are characteristic features in LN (2). It is thus pertinent to examine the mechanisms by which anti-DNA antibodies interact with mesangial cells and the pathogenetic consequences of such interactions. We have investigated the binding of immuno-
globulins from patients with SLE to human mesangial cell (HMC) in vitro. Our results showed that anti-DNA antibodies accounted for the serum HMC-binding activity. The latter also correlated with clinical activity in selected patients. The binding of anti-DNA antibodies to HMC could be modulated by Ig-associated DNA and was mediated in part by membrane proteins.

Materials and Methods

All chemicals were purchased from Sigma (China South, Hong Kong) unless otherwise stated and were of the highest purity. Tissue culture flasks were purchased from Falcon (Becton Dickinson, Hong Kong), and culture media from Life Technologies (Hong Kong).

Serum Samples from Patients with LN

For the cross-sectional study to determine the prevalence of HMC-binding immunoglobulins, 280 sera from 280 patients (222 women, 58 men; mean age, 45.7 ± 14.9 yr) with a history of biopsy-proven diffuse or severe focal proliferative LN and who satisfied four or more of the American Rheumatism Association criteria for SLE (19) were included. These serum samples were classified as either “active” or “inactive” on the basis of both clinical and serologic assessment. Active disease was associated with an SLE Disease Activity Index (SLEDAI) ≥10, while remission samples were obtained when the SLEDAI was ≤4 (20). Serum samples from SLE patients without renal involvement (n = 35), patients with non-lupus primary glomerular diseases (n = 72); thin membrane disease, 7; membrano-proliferative glomerulonephritis, 16; membranous nephropathy, 16; focal segmental glomerulosclerosis, 15; minimal change disease, 18), and healthy subjects (n = 37) were included for comparison. In addition, multiple serial serum samples were obtained from 27 patients with diffuse proliferative LN (19 women, 8 men; mean age, 35.1 ± 11.5 yr) over 4.5 ± 1.2 yr to study the association between Ig binding to HMC, the level of anti-DNA antibodies, and clinical disease activity.

Cell Culture

HMC were cultured from nephrectomized kidneys according to established methods (21). Briefly, cells derived from collagenase-treated glomeruli were maintained in RPMI 1640 medium supplemented with 20% (vol/vol) fetal calf serum (FCS), glutamine (2 mM), transferrin (5 μg/ml), insulin (5 μg/ml), sodium selenite (5 ng/ml), penicillin (100 IU/ml), and streptomycin (100 μg/ml). HMC were characterized by their stellate morphology, ability to form hillocks, and immunohistochemical staining (positive for vimentin; negative for cytokeratin and von Willebrand Factor). HMC were passaged at a split ratio of 1:3, and growth-arrested cells of the 4th through 7th passage were used in experiments. These cells showed no significant variation in viability, proliferation rate, cellular protein concentration (10^6 cells), and the expression of smooth muscle actin, vimentin, or extracellular matrix synthesis (data not shown).

Glomerular epithelial cells (GEC) were isolated from the supernatant of collagenase-treated glomeruli (22). Human peritoneal mesothelial cells (HPMC) were isolated from normal omental specimens according to established protocol (23). Proximal tubular epithelial cells (PTEC), human umbilical vein endothelial cells (HUVEC), human pleural mesothelial cells (McT-5A), and human bronchial epithelial cells (NHBE) were purchased from Clonetics (Advanced Tech and Ind Comp Ltd, Hong Kong); CHO cells and 3T3 Swiss fibroblasts were kind gifts from Dr. Nancie Chen (University of Hong Kong, Hong Kong).

Determination of Cellular Protein Concentration

Confluent growth-arrested cells (HMC, GEC, PTEC, HUVEC, HPMC, McT-5A, NHBE, CHO cells, and 3T3 Swiss fibroblasts) cultured in triplicate in 96-well plates were lysed with 4 M urea buffer, 20 mM sodium acetate, pH 6.0, containing 1% (vol/vol) Triton X-100 (50 μl). The protein concentration in each sample was determined using a modified Lowry assay according to the manufacturer’s instructions (Bio-Rad, Hong Kong).

Removal of Immune Complexes (IC) from Serum Samples

Two approaches were used to remove IC in serum samples from patients with SLE.

Precipitation of IC with polyethylene glycol (PEG). IC in sera (0.5 ml) were precipitated with PEG (3.4% [wt/vol] final concentration) for 30 min at 4°C. Samples were centrifuged at 15,000 g for 30 min to separate the IC-free sera from the precipitate.

Removal of IC by Protein G-Sepharose. Sera (5 μl) diluted in phosphate-buffered saline (PBS; 80 μl) were incubated with protein G-Sepharose beads (10 μl, Amersham Pharmacia Biotech, Hong Kong) for 15 min at room temperature with constant agitation before centrifugation to remove the protein G-Sepharose–bound IC (24).

HMC-binding activity was compared between serum samples with or without the removal of IC by the above procedures.

Cellular Enzyme-Linked Immunosorbant Assay (ELISA) to Measure HMC Binding by Either Serum Ig or Polyclonal Anti-DNA Antibodies

HMC were seeded into 96-well tissue culture plates at 20,000 cells/well in RPMI 1640 medium containing 20% FCS. 90% confluent cells were depleted of FCS for 72 h before being used in experiments. Measurement of lactate dehydrogenase (LDH) release confirmed maintenance of cell viability (5.12 ± 2.14% versus 6.01 ± 3.32% cytotoxicity before and after serum starvation, respectively; P = 0.147; n = 6). HMC were fixed with 1% (wt/vol) paraformaldehyde in PBS (pH 7.5) for 5 min at room temperature. The cells were washed thrice with PBS between incubations, and all incubations were for 1 h at 37°C unless otherwise stated. HMC were blocked with 3% (wt/vol) bovine serum albumin (BSA) followed by normal human IgG (100 μg/ml) to block Fc receptor–mediated binding. HMC were subsequently incubated with 100 μl of test sera (dilution 1:100) or anti-DNA preparations (final IgG concentration, 10 μg/ml). In some experiments, increasing concentrations of DNA (0 to 10 μg/ml) were added to anti-DNA antibodies for 2 h at 37°C before incubation with HMC to assess competition for the binding antigen on the cell surface. HMC were then incubated with goat anti-human IgG/F(ab) conjugated with alkaline phosphatase (5 μg/ml; Biosource Int, Hong Kong). Goat anti-human IgA or IgM F(ab) was used as the second antibody in experiments to assess binding by IgA or IgM immunoglobulins. Cross-reactivity of anti-human IgG, IgA, and IgM antibodies toward other isoforms were 8.2 ± 3.5%, 5.2 ± 3.6%, and 6.1 ± 3.1%, respectively. Ig binding to HMC was detected by incubation with para-nitrophenol phosphate for 45 min at room temperature, and optical density (OD) was measured at wavelength A405/420 on a Titertek Multiscan MCC/340 spectrophotometer (Bio-Rad, Hong Kong) when the positive control showed an OD of 1.5. Pooled serum or polyclonal anti-DNA antibodies from a patient who demonstrated persistent high HMC-binding activity due to anti-DNA antibodies were used as positive controls in respective experiments. Intra-assay and inter-assay coefficients of variance were 4.5 ± 0.7% and 5.3 ± 0.6%, respectively. A standard curve was obtained by plotting mean
OD against mean IgG bound to HMC (the latter determined by the
difference in IgG concentration of the tested sample before and after
incubation with HMC) from 20 LN patients who showed variable
degrees of HMC-binding activity. This standard curve (correlation
coefficient = 0.97) was subsequently used to determine the amount of
IgG binding to HMC in control and tested samples. The amount of
IgG bound to HMC was expressed as μg of bound IgG/μg of cell
protein. Seropositivity for HMC-binding of any tested sample was
denoted by results that exceeded mean + 3 SD of the corresponding
specimens from healthy subjects.

Cellular ELISA with GEC, PTEC, HUVEC, HPMC, MeT-5A,
NHBE, CHO cells, and 3T3 Swiss fibroblasts as substrate were also
performed to examine the cellular specificity of Ig binding.

Isolation of Polyclonal Anti-DNA Antibodies from
SLE Sera

This was achieved through sequential affinity chromatography
(25). Aliquots (0.5 ml) of serum samples were loaded onto native
DNA-cellulose column (Amersham Pharmacia Biotech) equilibrated
with 25 mM Tris buffer (pH 8.0) at a flow rate of 0.5 ml/min.
Non–DNA-binding fractions were flushed with the above buffer, and
anti-DNA antibodies were eluted with a linear NaCl gradient. The
absorbance at A_{280} was measured continuously throughout the purifi-
cation process. The columns were subsequently washed with 20 mM
Tris-HCl (pH 7.4) containing 2 M NaCl, 1 mM ethylenediaminetet-
raetic acid (EDTA), and 1 mM β-mercaptoethanol before further
elution. This did not yield additional immunoglobulins. Control ex-
periments were performed by passage of serum samples through a
column of microgranular cellulose in the absence of immobilized
DNA. Anti-DNA antibodies of the IgG class were isolated by protein
A Sepharose affinity chromatography (25,26). Isolated anti-DNA and
non–DNA-binding samples were desalted and concentrated 20-fold
using Ultrafree-4 centrifugal filter units (Millipore Asia, Hong Kong)
before determination of IgG concentration, anti-DNA activity, or use
in experiments. The purity of eluted IgG was confirmed by 10%
sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Flow Cytometry to Examine Ig Binding to HMC

Confluent HMC were treated with 0.02% (wt/vol) trypsin with
0.02% (wt/vol) EDTA for 5 min at 37°C and then neutralized with
trypsin inhibitor (1 mg/ml). HMC were then cultured in suspension
for 4 h, pelleted by centrifugation at 1500 g for 5 min, washed thrice
with PBS, and incubated with serum samples, anti-DNA antibodies, or
control IgG (10 μg/ml) for 30 min at 4°C in Kreh-Ringer bicarbonate
buffer containing 1% BSA. Unbound immunoglobulins were washed
with the above buffer. HMC were then incubated with goat anti-
human IgG-F(ab) conjugated with FITC (final concentration, 5 μg/ml)
at 4°C for 1 h, washed thrice with PBS, and resuspended in 0.5 ml of
PBS containing 0.5% (vol/vol) formaldehyde. Ig binding was ana-
lyzed by flow cytometry (Coulter Epics XL Flow Cytometer, Coulter,
FL), with 10,000 cells counted for each sample. Analysis was per-
formed with XL System II software.

Measurement of Anti-DNA Activity in Serum and
Isolated Anti-DNA Fractions

This was measured using a commercial ELISA (Microplate auto-
immune anti-DNA quantitative ELISA, Bio-Rad, Hong Kong). Sam-
ples giving a value >60 IU/ml were considered positive. The lower
and upper limits of detection for this assay were 20 IU/ml and 1000
IU/ml, respectively.

Measurement of IgG, IgA, or IgM Concentration
in Anti-DNA Antibody Preparations

IgG, IgA, and IgM concentration in anti-DNA antibody prepara-
tions were measured by ELISA. Goat anti-human IgG, IgA, or IgM
(10 μg/ml, 200 μl; Biosource International, Hong Kong) in 0.05 M
carbonate buffer (pH 9.5) were coated onto 96-well microtiter plates
(Immulon 2; Dynex Technologies, Hong Kong) at 4°C overnight.
After washing thrice with PBS/0.1% (vol/vol) Tween-20 (PBS/T),
the plates were blocked with 3% (wt/vol) BSA in PBS for 1 h at 37°C.
After washing with PBS, samples (starting dilution, 1:1000) or stand-
ards (range, 0 to 500 ng/ml; ICN Biomedicals, Eschwege, Germany)
were added in duplicate in serial dilutions and incubated for 1 h at
37°C. After washing in PBS, the relevant alkaline phosphatase–
conjugated goat anti-human Ig (5 μg/ml; Biosource, Hong Kong) was
added and incubated for 1 h at 37°C. Bound immunoglobulins were
detected by the addition of para-nitrophenol phosphate, and the ab-
sorbance was read at A_{405nm}. Prior experiments have demonstrated
<5% isofrom cross-reactivity of the antibodies. Intra-assay and inter-
assay coefficients of variance were 3.5 ± 0.3% and 4.9 ± 0.4%,
respectively, for IgG, 2.1 ± 1.3% and 6.2 ± 2.4%, respectively, for
IgA, and 4.3 ± 2.7% and 7.1 ± 0.2%, respectively, for IgM.

Digestion of DNA with DNase I

Removal of DNA from Anti-DNA Antibodies. Sera or anti-
DNA antibodies (final IgG concentration, 10 μg/ml) were incubated
with DNase I (40 μg/ml) and MgCl₂ (10 mM) for 1 h at 37°C, and
the reaction was stopped by the addition of EDTA (15 mM) (27).

Removal of DNA from the Surface of HMC. DNA was re-
moved from HMC surface after fixation with 1% (wt/vol) parafo-
maldehyde. After washing with PBS, the cells were incubated with 25
μg/ml DNase I in PBS containing 10 mM MgCl₂ for 1 h at 37°C,
after which the reaction was stopped by the addition of EDTA (15 mM).
Prior experiments had confirmed that DNase I at 25 μg/ml removed
cell surface DNA maximally (determined by agarose gel electrophore-
sis and ethidium bromide staining of isolated plasma membrane from
HMC before and after DNase treatment) without causing cell detach-
ment or cytotoxicity (assessed in parallel experiments by phase con-
trast microscopy and LDH release respectively).

Removal of Transmembrane Proteins from HMC by
Controlled Trypsin Digestion

Fixed HMC were treated with 10 μg/ml trypsin (tissue culture
grade) in PBS for 10 min at 4°C before neutralization with trypsin
inhibitor (1 mg/ml) in PBS. The cells were washed thrice with PBS.
This concentration of trypsin resulted in maximal release of [35S]-
methionine-labeled transmembrane proteins without affecting cell
attachment (data not shown).

Immunohistochemical Staining

HMC were seeded onto glass coverslips at a density of 10,000
cells/cm² and cultured for 48 h before fixing with 3.5% (wt/vol)
parafomaldehyde in PBS for 10 min at room temperature. Half of the
slides were incubated with trypsin (10 μg/ml) for 10 min at 4°C
before staining. Cells were then incubated with anti-DNA antibodies
or normal human IgG controls (final IgG concentration, 10 μg/ml) for
1 h at 37°C, washed thrice with PBS, and incubated with second
antibody conjugated with FITC for 1 h at 37°C in a darkened humid-
ified chamber. Cells were washed in PBS and mounted in fluores-
cence mounting medium (DAKO, Gene Company Ltd, Hong Kong),
and epifluorescence was monitored using a Zeiss Axiowert 135 mi-
croscope (Carl Zeiss, Gold Pacific Ltd, Hong Kong). Photographs
were taken on Kodak Max 400 film (Eastman Kodak, Rochester, NY). The localization of antibody staining was further confirmed using confocal microscopy (Aviovert 135M and Microsystems LSM, Carl Zeiss).

**Statistical Analyses**

All experiments were repeated at least three times using HMC from three separate donors. Results are expressed as mean ± SD. Statistical analyses were performed using InStat GraphPad software (GraphPad, San Diego, CA). Comparison between active and inactive samples was performed by the Mann-Whitney U test. Anti-DNA antibody binding to HMC was compared with that of non–anti-DNA Ig fractions from the same patient by the Wilcoxon signed-ranks test. Correlation between HMC-binding IgG and the level of anti-DNA IgG antibodies was examined with Spearman’s method. Two-tailed \( P < 0.05 \) was considered statistically significant.

**Results**

**HMC-Binding Immunoglobulins in Patients with SLE**

Preliminary studies using 20 serum samples with positive HMC-binding activity showed similar HMC-binding results for paraformaldehyde-fixed and live HMC (4.2 ± 1.2 \( \mu \)g of bound IgG/\( \mu \)g of cell protein and 4.0 ± 1.5 \( \mu \)g of bound IgG/\( \mu \)g of cell protein respectively; \( P = 0.1703 \)). Fixed cells were used in subsequent cellular ELISA.

Cellular ELISA with HMC, GEC, PTEC, HUVEC, HPMC, MeT-5A, NHBE, CHO cells, and 3T3 Swiss fibroblasts as substrate to examine the cellular specificity of Ig binding, demonstrated IgG binding in serum samples from patients with SLE to HMC, glomerular and tubular epithelial cells, and HUVEC, but insignificant binding to the other cell types relative to normal IgG (Figure 1). We have previously investigated the binding of anti-DNA antibodies to HUVEC (25). HMC binding by anti-DNA antibodies were investigated in this study.

IC may be present in SLE serum and may contribute to HMC-binding activity as measured by the cellular ELISA; we therefore
examined the differences in HMC-binding activity before and after the removal of IC in all samples. Indeed, a small proportion of active LN (4.6%; 5 of 108), inactive LN (3.5%; 6 of 172), and non-renal SLE (2.8%; 1 of 35) sera demonstrated significant HMC-binding activity attributed to circulating IC. The quantity of DNA–anti-DNA IC amounted to 3.2 ± 1.3 μg of DNA/ml, 2.5 ± 1.6 μg of DNA/ml and 2.2 μg of DNA/ml in these samples, respectively. These samples were excluded from further studies that aimed to examine IgG and anti-DNA antibody binding to HMC. Significant IgG binding to HMC was observed in sera from SLE patients with or without LN and to a lesser extent in patients with non-lupus glomerular diseases compared with healthy controls (Figure 2A). Testing of serum samples from patients with LN showed that the HMC-binding Ig was restricted to the IgG isotype (Figure 2B). The prevalence rates for HMC-binding activity in active LN, inactive LN, SLE without renal involvement, and non-lupus glomerular disease were 83.9%, 42.8%, 47.1%, and 23.6%, respectively. Ig binding of sera from nonrenal SLE patients was similar to that of inactive LN samples (P = 0.348).

Active disease in patients with LN was associated with increased IgG binding to HMC, higher levels of anti-DNA antibodies, and increased total IgG (Table 1). Fluorescence Activated Cell Sorter (FACS, Becton Dickinson) analysis confirmed the binding of IgG from lupus sera to HMC, especially during active disease (Figure 3). Furthermore, the degree of IgG binding to HMC correlated with the titer of anti-DNA antibodies in serum samples during active diffuse proliferative LN (Figure 4).

### The Binding of Anti-DNA Antibodies to HMC

In view of this correlation, we investigated the binding of anti-DNA antibodies from patients with LN to HMC. Polyclonal anti-DNA antibodies were isolated from 30 active and 26 remission sera. These 56 samples demonstrated significant HMC-binding activity in their whole-sera form. IgG antibodies accounted for 89.2% of the anti-DNA activity and 4.2% of total IgG in serum samples from LN patients. Recovery of anti-DNA antibodies after passage through DNA cellulose columns was 67.2 ± 5.3%. The anti-DNA activities in the eluted and unbound fractions were 263.1 ± 288.2 IU/ml and 48.8 ± 2.7 IU/ml, respectively (P < 0.0001). Elution from neat cellulose control columns did not yield anti-DNA activity. In all samples, the isolated anti-DNA antibodies demonstrated HMC-binding activity compared with control IgG. In contrast, HMC-binding activity was shown in the non-anti-DNA fraction of only 1 of the 56 samples. The mean HMC-binding activity of anti-DNA fractions was 5.4 ± 1.2 μg of bound IgG/μg of cell protein, compared with 1.2 ± 0.3 μg of bound IgG/μg of cell protein in the non–anti-DNA fractions (P < 0.0001; Figure 5A). HMC-binding by IgG from the non–anti-DNA fractions was similar to that of IgG from healthy controls (1.4 ± 0.5 μg of bound IgG/μg of cell protein; P = 0.075). Also, HMC-binding activity was restricted to anti-DNA antibodies of the IgG isotype (data not shown). HMC-binding activity was enhanced in the anti-DNA preparations compared with the corresponding whole serum in 55 of 56 samples when tested at identical IgG concentrations (5.2 ± 1.1 μg of bound IgG/μg of cell protein versus 3.9 ± 0.7 μg of bound IgG/μg of cell protein; P < 0.001; Figure 5B). When the volume of each isolated anti-DNA antibody preparation was reconstituted to the same as the original whole serum, the corresponding samples demonstrated a positive correlation in their Ig binding to HMC (Figure 5C), which gave further evidence that anti-DNA antibodies accounted for the HMC-binding activity in the original serum.

### Longitudinal Studies

The relationship among serum HMC-binding IgG, anti-DNA antibody level, and disease activity was examined longitudinally in 27 patients (19 women, 8 men; age, 35.1 ± 11.5 yr) with a history of biopsy-proven diffuse proliferative LN and who had tested positive for HMC-binding at least once during the course of follow-up. Serum samples were collected serially at intervals not exceeding 6 mo over a follow-up duration of 54.0 ± 14.4 mo. Eighteen patients (66.7%) demonstrated similar serial variations between the levels of IgG binding to HMC and the titers of anti-DNA antibodies (representative profile of one patient shown in Figure 6A), with higher levels of HMC-binding noted during active disease. 8 (29.6%) patients demonstrated positive HMC-binding activity that showed moderate temporal changes that were less marked than those of anti-DNA antibodies (Figure 6B). One patient (3.7%) showed high titers of HMC-binding IgG perennially, despite prolonged clinical remission (Figure 6C).

### Mechanisms by which Anti-DNA Antibodies Bind to HMC

Anti-DNA antibodies isolated from 12 patients were used to examine their mechanisms of binding to cultured HMC. The non–anti-DNA fractions showed insignificant binding to HMC, which was unaffected by addition or removal of DNA (data not shown). DNase treatment of all anti-DNA Ig preparations increased their binding to HMC 1.47-fold (7.5 ± 1.7 μg...
of bound IgG/µg of cell protein versus 5.1 ± 0.6 µg of bound IgG/µg of cell protein, with or without DNase treatment of antibodies respectively; \( P = 0.0059 \); Figure 7A). Anti-DNA binding to HMC was unaffected by DNase treatment of the cells alone (\( P = 0.5703 \)). DNase treatment of both HMC and anti-DNA antibodies resulted in a 1.36-fold increase in their binding (\( P = 0.0059 \)).

The Role of Cell Membrane Proteins in Mediating Anti-DNA Antibody Binding to HMC

HMC were treated with trypsin and/or DNase to examine the importance of cell membrane protein(s) and cell surface DNA in mediating the binding by anti-DNA antibodies. The latter were treated with DNase before the experiments to maximize their binding to HMC. Controlled trypsinization of HMC resulted in a 1.37-fold increase in their binding by anti-DNA antibodies (6.4 ± 0.8 µg of bound IgG/µg of cell protein versus 8.2 ± 1.1 µg of bound IgG/µg of cell protein before and after trypsinization, respectively; \( P = 0.0025 \); Figure 7B). Treatment of HMC with DNase had no additional effect on their binding by anti-DNA antibodies. Prior incubation of DNase-treated anti-DNA antibodies with exogenous DNA inhibited their subsequent binding to HMC in a dose-dependent manner (Table 2).

Immunohistochemical Studies

Immunohistochemical staining demonstrated the binding of anti-DNA antibodies to both the cell membrane and the cytoplasmic region of HMC (Figure 8B). This was confirmed by confocal microscopy (Figure 9). Insignificant Ig binding was observed within the extracellular matrix. Prior trypsinization of HMC resulted in enhanced binding by anti-DNA antibodies (Figure 8C), corroborating data from the cellular ELISA. Con-
Figure 5. (A) Comparison of HMC binding by anti-DNA antibodies and non-DNA-binding IgG fractions isolated from patients with LN. Anti-DNA antibodies accounted for the HMC-binding immunoglobulins in all but one patient, who demonstrated significant binding to HMC in the non-anti-DNA fraction. Horizontal bars represent mean values. (B) Comparison of IgG binding to HMC between whole sera and the anti-DNA preparations isolated from the corresponding samples. HMC-binding activity was enhanced in the anti-DNA preparations. (C) Correlation between HMC binding by IgG anti-DNA antibody preparations and by IgG in the original whole serum samples.

Figure 6. Relationship between HMC-binding activity (●), the level of anti-DNA antibodies (○), and clinical disease activity in serial serum samples of three representative patients with LN. In 66.7% of patients, HMC-binding correlated with anti-DNA antibody levels and disease activity (as shown in panel A). 29.6% of patients demonstrated positive HMC-binding activity that showed moderate temporal changes, which were less marked than those of anti-DNA antibodies (as shown in panel B). One patient (C) (3.7%) had persistent high HMC-binding despite remission.
trol experiments demonstrated insignificant binding of normal IgG to HMC (Figure 8A), which was unaffected by trypsin treatment.

**Discussion**

Immune-mediated glomerulonephritis affects more than half of the patients with SLE, and it is characterized by the deposition of anti-DNA antibodies and inflammatory mediators in different parts of the glomerulus. Mesangial cell proliferation and immune deposition in the mesangium are prominent histologic manifestations. Anti–mesangial cell antibodies have been demonstrated to induce mesangial proliferation in a rat model of anti-Thy 1–mediated glomerulonephritis (28). We have previously reported the binding of murine monoclonal anti-DNA antibodies to human mesangial cells (27). To date, there has been no data on the interactions between autoantibodies and mesangial cells in human LN. For the degree of Ig binding to HMC be more accurately assessed and compared between experiments, we have expressed the results of cellular ELISA as the amount of cell-bound Ig per unit of cell protein rather than by OD readings. In this study, we have demonstrated significant IgG HMC-binding activity from patients with LN, especially during active disease. Increased cellular binding during active disease could not be attributed totally to increased IgG concentrations; our finding therefore suggested variations in the properties of HMC-binding immunoglobulins and/or the mechanisms of cellular binding. We have previously demonstrated that human polyclonal anti-DNA antibodies could bind to HUVEC and that the binding correlated with disease activity (25). It is unlikely that the binding of immunoglobulins to glomerular or tubular epithelial cells was consequent to nonspecific adhesion, because similar binding was not observed with a variety of other cell types that have been included in the experiments. Instead, these observations suggest that the binding of immunoglobulins to different renal parenchymal cells could be important in pathogenesis.

We observed that the HMC-binding activity in LN sera was predominantly attributable to the binding of anti-DNA antibodies to HMC. The pathogenetic significance of anti-DNA antibodies has been implicated by their correlation with disease activity (29–31) and their deposition at sites manifesting histopathologic changes (32–35). It would be of interest to spec-
ulate whether binding of anti-DNA antibodies to HMC might also contribute to the pathogenetic mechanisms of disease. In line with previous reports, we have also found that circulating anti-DNA antibodies were predominantly of the IgG isotype (36,37). Furthermore, only anti-DNA antibodies of the IgG isotype showed significant binding to HMC. Heterogeneity among the HMC-binding anti-DNA antibody populations is suggested by the observation that the relationship between IgG binding to HMC and clinical disease activity was observed in only 67% of patients.

Our findings demonstrate the binding of anti-DNA antibodies to the HMC cell surface and intracellularly, while no staining was observed within the extracellular matrix. It has been proposed that the binding of anti-DNA antibodies to glomerular structures can occur through two mechanisms: the direct binding of these antibodies to crossreactive epitopes or indirect binding via intercalating DNA and/or chromatin material (11,13,15,38–41). It has been previously reported that indirect binding was mediated by heparan sulfate or other cell surface molecules through interactions with DNA, histones, and nucleosomes (12,13,38). On the other hand, direct crossreactivity has been associated with pathogenicity (39,40,42). In this context, we observed markedly increased binding of human polyclonal anti-DNA antibodies to HMC upon DNase treatment of the antibodies, suggesting that the predominant mechanism by which these antibodies bound to HMC was through direct crossreactivity with HMC membrane epitopes. This was confirmed by the inhibition of HMC-binding when anti-DNA antibodies had been incubated with exogenous DNA. That DNase treatment of mesangial cells did not alter their binding by anti-DNA antibodies could be explained by the already insignificant amount of DNA on the cell surface. Previous studies have demonstrated preferential localization of DNA in the glomerular basement membrane but not on the cell surface in LN (43). On the other hand, the enhancement of HMC-binding activity observed with affinity isolated poly-

![Figure 8](image1.jpg)

**Figure 8.** Immunohistochemical staining to compare the binding of normal IgG (A) or IgG anti-DNA antibodies (B) to HMC. Cell surface and cytoplasmic staining was observed with anti-DNA antibodies but not with control normal IgG. Trypsinization of cell surface proteins further increased the binding by anti-DNA antibodies (C). Magnification, ×400.

![Figure 9](image2.jpg)

**Figure 9.** Immunohistochemical and confocal analyses of anti-DNA antibody staining in HMC. HMC were sectioned at 1-μm intervals beginning from the cell surface (A). HMC binding by anti-DNA antibodies was observed on the cell membrane (depicted by arrow) and within the cytoplasmic area. Magnification, ×600.
clonal anti-DNA antibodies compared to the corresponding original whole serum suggests the presence of serum factors that could inhibit the binding of anti-DNA antibodies to HMC.

Treatment of HMC with trypsin markedly increased their binding by anti-DNA antibodies. Trypsin is a serine protease with substrate specificity toward positively charged lysine and arginine side chains. Proteases are secreted by infiltrating inflammatory cells and mesangial cells in inflammatory renal diseases (44–46). Whether the synthesis of trypsin is modulated in LN remains unknown. Mesangial cells synthesize many of the cell surface or matrix components that have been demonstrated to crossreact with anti-DNA antibodies, such as collagen type IV, fibronectin, laminin, and heparan sulfate proteoglycans (47), many of which are susceptible to trypsin digestion. Thus, the modulation of anti-DNA binding to HMC by trypsin could be of pathogenetic relevance, although the nature of HMC membrane proteins that mediate the binding by anti-DNA antibodies have yet to be identified. The marked increase in anti-DNA binding upon trypsin treatment of HMC suggests that cell membrane protein(s) might have masked the binding sites for these antibodies, or that anti-DNA antibodies have higher affinity of binding to protein fragments generated after trypsin treatment. The cell membrane molecules that mediated anti-DNA binding were not likely to be associated with DNA in their normal state, because DNase treatment of the cells did not induce further changes in antibody binding. It has been reported that hyaluronan could bind anti-DNA antibodies through its negative carboxyl groups (48). Hyaluronan is a glycosaminoglycan present on the cell surface and in the extracellular matrix (49). It is devoid of a core protein and is thus resistant to trypsin digestion. Binding of hyaluronan to its cell surface receptors can reduce its binding to other molecules (49). Trypsin digestion of hyaluronan binding proteins on the cell surface may therefore unmask or enhance the capacity of hyaluronan to bind anti-DNA antibodies. Studies are being carried out to characterize the HMC membrane molecules that mediate the binding by anti-DNA antibodies.

In conclusion, we have demonstrated in patients with LN the presence of IgG anti-DNA antibodies that were capable of binding to HMC. The relationship between HMC binding by anti-DNA antibodies and disease activity and the data implicating direct crossreactivity with HMC membrane epitopes suggest that HMC-binding anti-DNA antibodies may participate in the pathogenesis of LN. Human polyclonal anti-DNA antibodies have been reported to induce cytotoxicity and apoptosis in rat mesangial cells (50). It remains to be determined whether their binding to HMC can affect mesangial cell functions, such as the clearance of immune deposits, the production of cytokines and growth factors, or contractile properties that can affect intrarenal hemodynamics, cell proliferation, and apoptosis.

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References


