Functional Relevance of Activated β1 Integrins in Mercury-Induced Nephritis

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Abstract. Cell adhesion through different adhesion molecules is a crucial event in the inflammatory response. Integrins can only bind and mediate cellular adhesion after their activation by different specific stimuli. The state of β1 integrin activation can be assessed by a group of monoclonal antibodies (HUTS) that selectively recognize β1 integrins in their active form. A similar activated epitope in the rat was defined using the anti-human monoclonal antibody HUTS-21, which recognizes an activation-dependent epitope on the β1 chain. It was found that the divalent cations Mn²⁺ and Hg²⁺ were able to induce in vitro the activation of β1 integrins on rat lymphocytes. The Hg²⁺ cation induces an autoimmune disease in the Brown Norway rat characterized by synthesis and glomerular deposits of anti-glomerular basement membrane antibodies, proteinuria, and interstitial nephritis. Using the mercury model of nephritis, it was found that the expression of HUTS-21 epitope is induced in vivo in rat lymphocytes, and its appearance is correlated with the other parameters at the onset of the disease. In addition, the administration of HUTS-21 monoclonal antibody to HgCl₂-treated rats offered evidence of its protective effects (1) against infiltration of renal interstitium by leukocytes, and (2) in the reduction of anti-glomerular basement membrane synthesis and glomerular deposition. Nevertheless, urinary protein values remained unaffected. These results demonstrate a key role of β1-activated integrins in both leukocyte cell-cell interactions and leukocyte infiltration pathway mechanism, and also indicate that leukocyte migration may have less importance in the development of this disease than previously thought.

The leukocyte adhesion to endothelium and the subsequent extravasation through the vascular wall into tissues are crucial steps in the genesis of an inflammatory response, which are regulated by several proinflammatory factors (1–5). The integrins, an important family of the adhesion molecules, are central to many of these adhesion-dependent events. The β1 or very late activation antigen (VLA) are a subgroup within the integrin family comprising at least 10 members, each with a distinct α subunit noncovalently associated with the common β1 subunit (6–8). The cellular adhesion through integrins can rapidly be regulated by reversible modulation of receptor function. One mechanism implicated in the upregulation of integrin-mediated adhesion consists in the induction of transitions to a high affinity state in a small fraction of integrin receptors, probably as a consequence of conformational changes of these molecules (9). These changes can be modulated by different factors such as the concentration of divalent cations, physiologic ligands, or monoclonal antibodies (mAb), among others (7,9–11).

Other investigators have reported the existence of a specific and regulatory region in the common β1 subunit (CD29) of VLA integrins, whose expression is regulated upon cell activation, and it correlates with the ligand binding activity of these heterodimeric glycoproteins (11–16). The state of integrin activation can be assessed by a group of monoclonal antibodies (HUTS) that selectively recognize integrins in their active form. In addition, it has been shown that HUTS-21 mAb recognizes an epitope whose expression is induced upon ligand binding to the β1 integrins on T lymphoblasts (11,17).

In the present study, we have found that the human HUTS-21 mAb also recognizes β1 integrins on rat lymphocytes activated with divalent cations such as Mn²⁺ and Hg²⁺. It is well known that Hg²⁺ induces an autoimmune disease in the Brown Norway (BN) rat mediated by T-dependent polyclonal B cell activation and resulting in synthesis of autoantibodies (mainly, anti-glomerular basement membrane [GBM] antibodies) with glomerular linear deposits of IgG, proteinuria, and interstitial monoclonal cell infiltrates (18).

Considering the recently described expression of activated β1 integrins in a small proportion of lymphoid cells at sites of inflammation in human autoimmune chronic inflammatory diseases (19), we have examined the expression and possible role of activated β1 integrins in the mercury-induced model of nephritis. Our results demonstrate the capacity of HUTS-21 mAb to recognize in vivo an activation-dependent β1 epitope...
as well as its positive/negative effects on this autoimmune experimental disease.

Materials and Methods

Animals

BN rats, weighing 150 to 180 g, were obtained from IFFA-Credo (Paris, France) and from our own breeding colony and maintained under standard conditions, with free access to food and water.

Monoclonal Antibodies

The mouse anti-human HUTS-21 mAb, which reacts with an activation-dependent epitope on the VLA-β1 chain (CD29), has already been described (11,17). The mouse anti-human HP2/1 mAb is directed toward the α4 integrin (20) and cross-reacts with the rat α4 integrin (21,22). The mouse anti-rat OX1 mAb, specific for the pan-leukocyte CD45 antigen (23), was purchased from Serotec (Oxford, United Kingdom).

Flow Cytometry Assays

To assay the effects of MnCl₂ and HgCl₂ on the activated β1 subunit expression, whole-blood samples from normal control rats were incubated with a saturating concentration of HUTS-21 mAb for 15 min at 37°C in Hepes/NaCl buffer (20 mM Hepes, 150 mM NaCl, 2 mg/ml d-glucose, pH 7.4) containing 0.9 mM Mn²⁺ or 7.5 μM HgCl₂. After washing, cells were incubated with FITC-conjugated goat-anti-mouse IgG (Becton Dickinson, Mountain View, CA) secondary antibody for 30 min at 4°C in the dark. The erythrocytes were lysed, leukocytes were fixed, and cellular membranes were stabilized in fluorescence-activated cell sorter (FACS) lysing solution (Becton Dickinson). OX1 mAb and only the direct application of the secondary antibody were used as positive and negative controls, respectively. The samples were analyzed using a FACScan cytometer (Becton Dickinson).

To investigate the effects of HgCl₂ in the in vivo expression of activated β1 integrins, a group of rats (n = 5) was injected subcutaneously with HgCl₂ three times a week for 2 wk with 100 μg of HgCl₂ per 100 g body wt (18). To establish the kinetics of the expression of HUTS-21 epitope throughout the course of the disease, animals were sequentially bled on different days of the experiment by tail artery puncture. Then, whole blood samples were incubated with saturating concentration of HUTS-21 mAb for 15 min at 37°C, washed, and incubated with FITC-conjugated goat-anti-mouse IgG (Becton Dickinson) secondary antibody for 30 min at 4°C in the dark. Then, samples were processed using the same procedure as above and further analyzed by flow cytometry.

Experimental Procedure

Four groups of rats were used in this study. Groups I to III were injected with 100 μg of HgCl₂ per 100 g body wt, following the same procedure as described above to induce the disease. Group I (n = 12) did not receive any additional treatment. Rats from group II (n = 12) and group III (n = 12) also received an intraperitoneal injection (0.5 mg) of HUTS-21 or OX1 mAb on days 0, 8, and 13, respectively. Group III was used as control of HUTS-21 mAb administration. Group IV (n = 12) served as a normal control in which rats received distilled water adjusted to pH 3.8 (the same pH of the HgCl₂ solution used), following the same procedure described above for the mercury administration. The animals were sequentially bled by tail artery puncture and sacrificed on different days of the experiment.

Urinary Protein Excretion

Animals were maintained in metabolic cages for 24 h to collect urine samples and had free access to food and water. Urine samples were taken at regular intervals starting on day 0. The amount of protein was measured in triplicate by using a Bio-Rad assay (Bio-Rad, Richmond, CA), according to the manufacturer’s protocol. The optical density from each sample was measured in a Titertek Multiskan Plus spectrophotometer (Flow, Irvine, Scotland, United Kingdom) at 595 nm.

Anti-GBM Antibody Assays

Rat GBM was isolated using essentially the same procedure described by Bowman et al. (24). Briefly, glomeruli were obtained from normal BN rats by differential sieving and centrifugation of minced kidney cortices. The glomerular suspension was sonicated, washed, and lyophilized. The GBM was digested with type I collagenase (Sigma Chemical Co., St. Louis, MO) at 0.7% wt/wt for 1 h at 37°C. Anti-GBM antibodies were measured by enzyme-linked immunosorbent assay (ELISA) as described previously (24). Briefly, 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated with rat GBM (100 μl/well of 10 μg/ml GBM in 0.1 sodium carbonate buffer, pH 9.6) by overnight incubation at 4°C and then washed with PBS containing 0.05% Tween 20 (Sigma). Wells were filled with 100 μl of sera (1:100 diluted) to be tested. Plates were incubated for 1 h at 37°C. After incubation with peroxidase-labeled rabbit anti-rat IgG antibody (Serotec), peroxidase activity was revealed and absorbance was measured at 492 nm by using a Titertek Multiskan Plus (Flow).

Samples of a serum pool from untreated BN rats and from BN rats that were treated with HgCl₂ and bled on day 13 of the disease served as negative and positive controls, respectively. Results were expressed as the percentage of binding obtained with samples from positive control serum.

Kidney Tissue Processing

On day 15 of the experiment (when the interstitial cell infiltration was still highly present) (22), rats from each group were sacrificed. Kidneys were harvested and further processed for histologic, immunohistochemical, and electron microscopy studies. For light microscopy, 2-μm paraffin-embedded kidney sections were stained with hematoxylin and eosin and periodic acid-Schiff. For immunohistochemistry studies, pieces of renal tissue were snap-frozen in isopentane precooled in liquid nitrogen, and stored at −70°C until used. Direct immunofluorescence studies were performed on ethanol/ethanol-fixed serial cryostat sections by using FITC-conjugated rabbit anti-rat IgG (Serotec), as reported previously (25). To characterize OX1 and HUTS-21-positive cells in the renal interstitium, frozen kidney sections were stained with those mAb and revealed with an indirect immunoperoxidase method (26). Enumeration of interstitial infiltrating cells bearing OX1⁺ cell surface markers was determined by counting, in two kidney tissue sections per each rat, the total number of positive-labeled cells examined in 10 randomly chosen areas of interstitial infiltrates. The ratio of infiltrating cells expressing activated β1 subunit (HUTS-21⁺ cells) to the total number of OX1⁺ cells was obtained using a conventional light microscopy objective (×60), as described (27). For ultrastructural studies, the renal tissue was fixed immediately in 2.5% glutaraldehyde in 1.1 M cacodylate buffer for 2 h, followed by post-fixation in osmium tetroxide. Then, tissue was dehydrated in increasing concentrations of ethanol and embedded in Epon 812. Thin sections for ultrastructural examination were stained with lead citrate and examined at 75 kV with a Jeol 100CX electron microscope.
Statistical Analyses

The results are given as mean ± SD values obtained from the levels of proteinuria and ELISA results. Data were analyzed using two-way ANOVA, and when the statistical difference was significant, data were also analyzed using the t test with the Bonferroni correction.

Results

Recognition of Activated β1 Integrins on Rat Lymphocytes by HUTS-21 mAb

To assess the cross-reactivity of the mouse anti-human activated β1 integrin (HUTS-21) mAb with the same subunit in the BN rat, rat lymphocytes obtained from whole blood were incubated with saturating concentrations of HUTS-21 mAb in the presence of Mn²⁺ and Hg²⁺ in the medium. Samples were analyzed by flow cytometry. As shown in Figure 1, the presence of either Mn²⁺ and Hg²⁺ cations induced the expression of the epitope recognized by HUTS-21 mAb.

Induced Expression of HUTS-21 Activated Epitope on Rat Lymphocytes through the Course of the HgCl₂-Induced Nephritis

To establish the kinetics of HUTS-21 expression in vivo, whole blood samples of HgCl₂-treated rats (n = 5), obtained at different days of the disease, were incubated with a saturating concentration of HUTS-21 mAb and analyzed by flow cytometry. Figure 2 shows the time course of the expression of the activated β1 subunit epitope on rat lymphocytes. The maximal

Figure 1. Recognition of activated β1 integrins on rat lymphocytes by HUTS-21 monoclonal antibody (mAb) in the presence of MnCl₂ and HgCl₂ in the medium. Cells were incubated with a saturating concentration of the anti-activated β1 integrins HUTS-21 mAb in medium with or without Mn²⁺ and Hg²⁺. Lymphocytes were then washed and incubated with FITC-conjugated goat-anti mouse IgG. OX1 mAb and the direct application of only the secondary antibody were used as positive and negative controls, respectively. All samples were analyzed by flow cytometry.
expression of HUTS-21 epitope was obtained between days 13 and 15, with the expression declining after day 23 to reach the background levels at day 30 of the disease.

**Effect of HUTS-21 mAb Treatment on the Proteinuria of HgCl₂-Injected BN Rats**

As shown in Figure 3, HgCl₂-treated rats (group I) developed proteinuria in two different phases: a first short phase, which started immediately after the first injection of HgCl₂, followed by a second phase starting on day 11 and declining after day 16 of the disease. At the third week, all of the animals reached the background protein levels. The treatment with anti-activated β1 epitope HUTS-21 mAb (group II) did not modify the time course of the urinary protein excretion levels. Electron microscopy examination of the kidney of rats from groups I and II showed similar striking abnormalities of glomerular epithelial cells with edema and extensive effacement of the podocyte foot processes. No other ultrastructural glomerular alterations were observed (Figure 4, A through C).

**Effect of HUTS-21 mAb Treatment on Anti-GBM Antibody Production and Glomeruli Deposition**

As shown in Figure 5, the sera from rats injected with HgCl₂ (group I) presented circulating antibody anti-GBM as detected by ELISA. The maximal concentration of anti-GBM antibody was detected on day 13 of the disease, decreasing thereafter as also occurred with the proteinuria. By direct immunofluorescence methods, rats treated only with mercury showed a linear IgG glomerular deposition along the GBM on examination at day 15 of the disease, as demonstrated in Figure 6A. Rats that
in addition to mercury administration were treated with anti-
activated β1 epitope HUTS-21 mAb (group II) exhibited a
significant reduction in the anti-GBM antibody serum levels
(40%) (Figure 5). This drop in circulating anti-GBM antibody
was maintained throughout the course of the disease. Also, a
reduction in the intensity of IgG linear deposits on the GBM
was found in this group of rats (Figure 6B).

To saturate the target protein, the following alternative ther-
éapeutic protocol was performed: Mercury-treated rats received
daily an intraperitoneal injection (0.5 mg) of HUTS-21 mAb
(n = 6) or OX1 mAb (n = 6), starting on day 0 and finishing
on day 14 of the experiment. Despite having increased the total
dose of HUTS-21 mAb from 1.5 to 7.5 mg, the levels of
circulating anti-GBM antibody and the intensity of linear IgG
glomerular deposits were not further decreased, and proteinuria
remained unaltered (data not shown). These results suggest that
the doses of HUTS-21 mAb used in both protocols were
saturating.

Expression of HUTS-21 β1 Activation Epitope in
Kidney Tissue and Effects of Anti-HUTS-21 mAb
Administration on Interstitial Cell Infiltrates

Renal tissue sections from rats treated with HgCl₂ alone
(group I) and rats treated with HgCl₂ + HUTS-21 mAb (group
II) were examined by light microscopy on day 15 of the
disease. Renal tissue sections from normal rats served as con-
trol (Figure 7A).

Pronounced patchy areas of interstitial mononuclear cell
infiltrates were located preferentially in the perivascular re-
gions of the renal interstitium in HgCl₂-treated rats (Figure
7B). To characterize the total number of lymphohemopoietic
infiltrating cells and to assess the HUTS-21 expression in the
interstitial cell infiltrates, frozen kidney tissue sections were
stained with anti-CD45 (OX1) and anti-activated β1 subunit
epitope (HUTS-21) mAb. The number of OX1⁺ inflammatory
interstitial cells found in HgCl₂-treated rats was 36 ± 7 cells/
high-power field, and the proportion of HUTS-21⁺ cells that
formed part of such infiltrates was >95% (Figure 7, B and C).

The effect of HUTS-21 mAb treatment on the intensity of
interstitial cell infiltrate was also examined. Figure 7D showed
that the number of OX1⁺-infiltrated cells in the renal tissue
from rats treated with HUTS-21 (OX1⁺ cells/high-power field)
was considerably reduced (4.5 ± 0.9) compared with the
OX1⁺ cells from HgCl₂-treated rats alone (36 ± 7).

Discussion

Cell communication through different adhesion molecules is
an essential event in the inflammatory response (1–5). Cellular
adhesive properties are regulated through the selective expres-
sion of the integrin repertoire, as well as by the modulation of
the binding properties of these receptors (9). The β1 subfamily
of integrins function mainly as cellular receptors for extracel-
lar matrix proteins, and they are also implicated in cell-cell
interactions (7,28). The expression of β1 integrin activation
epitopes at sites of tissue injury in inflammatory processes has
also been described, suggesting a key role of β1 integrin
activation in the development of inflammatory processes (19).
The state of integrin activation can be assessed by a group of
monoclonal antibodies (HUTS) that selectively recognize
β1 integrins in their active form. These epitopes are expressed
on activated β1 integrins by different stimuli, and their expression
correlates with the binding activity of β1 integrins to immo-
ibilized ligands (11,16,17).

In this study, we have analyzed the expression of the β1-
activated epitope recognized by HUTS-21 mAb in an animal
model of autoimmune nephritis, induced by HgCl₂. Our results
show the expression of a similar activated β1 epitope in rat
lymphocytes, as demonstrated by the reactivity of HUTS-21
mAb with rat lymphocytes in the presence of the divalent
cations Mn²⁺ and Hg²⁺. It is well known that Hg²⁺ cation

![Figure 3. Urine protein excretion ratio in all tested groups. Results are expressed as mean ± SD. □, HgCl₂; □, HUTS-21; □, control.](image-url)
induces an autoimmune disease in the BN rat characterized by the synthesis of autoantibodies (mainly, anti-GBM antibody) with glomerular linear deposits of IgG, severe proteinuria, and acute tubulointerstitial nephritis (18,22,24,29,30). The development of this experimental autoimmune model is a consequence of the generation of an autoreactive T cell subset inducing a polyclonal B cell activation (31,32). It has been shown previously that interaction between circulating leukocytes expressing VLA molecules and endothelial cells is a crucial event in the development of this renal disease (22,29,30). Taking into account that activated β1 integrins are expressed in a small proportion of lymphoid cells in different human autoimmune chronic inflammatory diseases (19), it was of interest to know the possible expression of leukocyte-activated β1 integrins in the mercury model of nephritis. Our data demonstrate the induced expression by lymphocytes in this in vivo model of the activation-dependent epitope of the β1 (VLA) integrins, which paralleled the other parameters in the onset of the disease. In this regard, we have found that the expression of the HUTS-21 epitope on rat peripheral blood lymphocytes was maximal on days 13 to 15 of the disease, as occurred with the levels of circulating autoantibodies, proteinuria, and intensity of interstitial nephritis (22,29,30). Our findings suggest a role of β1-activated receptors in the development of this experimental autoimmune disease, and thus we have investigated whether HUTS-21 mAb was able to exert any blocking effect when it was used as a therapeutic agent. The administration of HUTS-21 mAb to HgCl2-treated rats abrogated the renal interstitial mononuclear cell infiltrates and reduced the levels of circulating anti-GBM antibodies and glomerular deposition. Nevertheless, urinary protein values were unaffected.

It is feasible that the blocking effects observed after HUTS-21 mAb administration on leukocyte extravasation and migration into the renal interstitium were due to conformational changes in the integrin molecules, inducing an increase of the ligand binding affinity. These in vivo results concur with previous in vitro studies showing that the engagement of β1 integrins by HUTS-21 causes functional inhibition of β1 integrin-dependent dynamic processes, such as cell migration on extracellular matrix proteins or endothelial cell ligands (17). It has been reported previously (33–35) that the cell migration requires a gradient of adhesive strength from the front to the rear of the migrating cell that determines cell movement and that the cell migration could be inhibited by changes in this delicate balance of adhesion/release (35–38). It is therefore conceivable that HUTS-21 mAb treatment could affect this adhesion/release balance. On the other hand, engagement of β1 epitope HUTS-21 inhibits migration of T lymphoblasts on vascular cell adhesion molecule-1 and fibronectin substrates, but has no effect over intercellular adhesion molecule-1 migration (17). Our results demonstrate that HUTS-21 mAb administration was able to abrogate the renal interstitial cell infiltrates, thus strongly supporting that leukocyte migration from blood to renal interstitium is mediated by the VLA/vascular cell adhesion molecule-1 adhesion pathway in this particular disease model, in accordance with previous studies (22,29,30). Moreover, our findings indicate that the expression of active conformations of β1 integrins is involved in the

Figure 4. Ultrastructural micrographs from rats injected with HgCl2 alone (A) and rats also treated with HUTS-21 mAb (B), showing both edema of the epithelial cell cytoplasm with prominent foot processes obliteration. Normal control rats show a well-preserved morphologic appearance of the foot-processes (C). Magnification: ×5200 in A and B; ×5600 in C.
leukocyte recruitment into the renal interstitium and also in the development of this inflammatory disease.

In addition to the protective effect of HUTS-21 mAb treatment in the development of renal cell infiltrates, we found a reduction in the levels of circulating anti-GBM antibodies, as well as in the intensity of glomerular IgG deposition. This last observation suggests a role for activated β1 integrins in T cell–B cell interactions implicated in the synthesis of the autoantibodies in this experimental model. Nevertheless, the incomplete reduction of anti-GBM antibody production after HUTS-21 mAb administration suggests that other mechanisms are at work in the autoantibody synthesis. This issue is supported by our previous observation, in which anti-α4 chain HP2/1 mAb treatment abolished anti-GBM antibody synthesis (22). Of interest, the persistence of high urinary protein excretion levels after in vivo administration of HUTS-21 mAb to HgCl2-treated rats suggests that the reduction in the levels of circulating anti-GBM antibody and glomerular IgG linear deposits are not enough to prevent the development of proteinuria. In this regard, it has been previously demonstrated that the induction of injury with heterologous anti-GBM antibody can be related to micrograms of antibody bound per gram of kidney in terms of molecules per glomerulus. Quantitative studies showed that 75 μg of antibody bound per gram of kidney, or approximately one molecule for 26 μm² of glomerular filtering surface, is necessary to induce proteinuria in the rat. Most probably and depending also on steric factors, at least half of the filtering surface would be covered by the antibody (39). By electron microscopy, we have found similar ultrastructural glomerular alterations in both HgCl2-treated rats with persistent proteinuria and in those rats that also received HUTS-21 mAb treatment, independently of the intensity of anti-GBM antibody deposition. Once proteinuria develops, it may exert a pathologic effect by itself as enhanced uptake of plasma proteins by glomerular epithelial cells does occur in proteinuric states and may eventually lead to the production of mediators with an injurious effect on the cellular components of the filtration barrier (40). On the other hand, the complete abro-

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**Figure 5.** Levels of circulating anti-glomerular basement membrane autoantibodies in HgCl2-injected rats (■), in rats treated with HUTS-21 and OX1 mAb (□), and in the normal control group (▲). Values are expressed as mean ± SD. *P ≤ 0.01, difference with the normal control group. **P ≤ 0.5, difference with the HgCl2-treated group.

**Figure 6.** Immunofluorescence staining. Positive linear pattern deposition of rat IgG in rats treated with HgCl2 alone (A) and in rats that were also injected with HUTS-21 mAb (B). Panel B shows a reduction in the intensity of the deposits.
gation of interstitial cell infiltrates in the presence of proteinuria, found after in vivo administration of HUTS-21 mAb to HgCl₂-treated rats, indicates that the leukocyte migration may have less importance in the development of renal tissue damage than previously thought. These findings are consistent with those reported by our group, in which anti-α4 mAb HP2/4 treatment was able to abrogate the interstitial leukocyte infiltration without affecting the proteinuria levels (30). They are also in agreement with those described in allergic inflammatory reactions, in which the administration of anti-α4 mAb HP1/2 suppressed the leukocyte accumulation but had no effect on stimulated edema formation (41). Nevertheless, there are conflicting data in the literature regarding the protective effect of integrin blockade on renal function but not on inflammation. A similar apparent discrepancy on the functional effects of the leukocyte adhesion molecules has been reported in allergic airway inflammatory disorders (42–44). Although it is well established that leukocyte adhesion molecule blockade is tissue-protective in ischemic reperfusion injury in muscle or heart, the interpretation of responses of the kidney is still a matter of controversy. CD11/CD18 and intercellular adhesion molecule-1 blockade are usually protective in experimental renal ischemia-reperfusion injury. In contrast, induction of systemic neutropenia and selectin function blockade do not have a protective effect, suggesting a neutrophil-independent mechanism for renal protection (45). Leukocyte adhesion molecules might mediate renal ischemia-reperfusion injury by mechanisms other than simply leukocyte migration, such as signal transduction and cell transport (46).

In conclusion, we have reported a key role of the β1-activated integrins in the development and progression of this renal inflammatory disease, in both leukocyte cell-cell interactions and leukocyte infiltration pathway mechanisms. Our results confirm the important role played by VLA integrins in this model and provide further evidence that cellular activation and synthesis of anti-GBM antibody can be implicated in the renal dysfunction observed in rats with HgCl₂-induced disease.

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