Modulating effect of gelonin gp330 conjugate on Heymann's nephritis induced in rats – A pathomorphological evaluation

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Heymann's nephritis (HN) in rats induced by injecting renal proximal tubule brush border protein gp330, is an animal model replicating human autoimmune membranous glomerulonephritis[1]. Endogenous IgG gets deposited between the foot processes in the epithelial side of the glomerulus and causes complement-mediated membrane injury, leading to proteinuria and basement membrane thickening. We investigated the effect of a toxin, gelonin conjugated to gp330 and targetted against antigp330-producing cells in ameliorating immune injury and nephrotic state in rats. The groups of animals injected with purified gp330 revealed by immunofluorescence, characteristic granular deposits of IgG along the basement membrane. The rats intravenously injected with gelonin gp330 conjugate, four days after the antigenic challenge with gp330 in two doses, showed amelioration of the nephrotic state and appreciable reduction in glomerular IgG deposits against immune injury. This substantiates our earlier biochemical results and corroborates the possibility of using toxins conjugated to specific antigen in treating antibody-mediated autoimmune diseases.

IDIOPATHIC diffuse membranous glomerulonephritis is a chronic progressive disease. It is most common in adult men and usually presents with the nephrotic syndrome. The pathogenesis of this renal disorder is not known. In animals, lesions closely resembling human membranous glomerulonephritis referred to as Heymann's nephritis (HN) can be induced by immunizing against a glycoprotein antigen, gp330, present in the brush border of renal cortical proximal tubular cells. The circulating divalent autoantibodies to gp330 cross the glomerular capillary basement membrane and get deposited between the foot processes. This antibody along with complement damage the plasma membrane of the foot processes, leading to proteinuria and diffuse membrane thickening[2]. There is a strong suspicion that an autoimmune mechanism similar to that operating in Heymann's membranous glomerulonephritis in the rat is probably responsible for

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Table 1. Protocol of treatment and histological analysis

Group	No. of rats	Immunization with gp330	Treatment on day 12	Histology: Thickening of GBM	Histology: Immunofluorescence
I	8	Day 0, Day 8	None	++++ global	++++
II	8	Day 0, Day 8	40 µg gelonin	++++ global and segmental	ND
III	8	Day 0, Day 8	450 µg gp 330	++++ glogal	ND
IV	8	Day 0, Day 8	250 µg conjugate	++ focal, segmental	++
V	8	Day 0, Day 8	500 µg conjugate	+ focal, segmental	+

Glomerular basement membrane thickening graded by silver staining and immunofluorescence.

human idiopathic membranous glomerulonephritis[3]. In long-term studies of patients treated with steroids or cytotoxic drugs, nearly 40% of patients are found to develop irreversible end-stage renal disease[4]. As the disease is mediated by activated B cells against the gp330 glycoprotein resulting in specific immunoglobulin synthesis and glomerular deposition[5], it is plausible to consider that destruction of these specific clones of B cells could retard the progression of the disease, leading to amelioration. In our earlier study[6], using biochemical markers, we have demonstrated that a toxin, gelonin-conjugated to gp330, when administered 12 days after the gp330-antigen challenge, the serum antibody titre lowered and the proteinuria almost disappeared, in contrast to the animals rendered nephrotic with the antigen administration alone. In this study, we have investigated whether a decline in circulating antibody titre and disappearance of proteinuria in animals administered with toxin–antigen conjugate, also reveal pathomorphological evidence of diminution of glomerular deposition of immunoglobulins, reflecting amelioration of the pathological lesions as well.

For the induction of HN female adult rats (IISc strain), 16–18 weeks age, maintained on chow pellets and water *ad libitum* were used. The methods for purification of renal tubular brush border glycoprotein from Wistar rat renal cortex and preparation of the toxin gelonin gp330 conjugate were described in our earlier publication[6]. The rats received two foot pad injections with 100 µg purified gp330 in Freunds Complete Adjuvant, in a total volume of 400 µl on day 0 and 8. Simultaneously, an indifferent control antigen, 40 µg of ovalbumin (Sigma) was injected in the control group of animals. The animals in which active HN has been induced were divided into five groups (Table 1), each consisting of eight rats. The conjugate gelonin gp330 was dissolved in 0.9% NaCl containing 0.018 mg of invertase per 100 g body weight of the rat and administered intravenously on day 4 after the second antigenic challenge with gp330.

Blood samples were taken from all the animals at biweekly intervals from 2nd week after initiation of the experiment and the circulating antibody titre was determined by dot-blot assay.

Rats were kept in metabolic cages for 24 h and urine samples were collected on alternate weeks till the week the blood was analysed. The quantity of urinary protein was estimated by Lowry's method[7], to confirm proteinuria following glomerular pathology.

All rats, control and experimental, were sacrificed after seven months of monitoring for gp330 specific antibody titres and proteinuria, indicators of glomerular pathology. The animals were sacrificed by deep ether anaesthesia and both kidneys were dissected. They were sliced in longitudinal plane and some were fixed in Bouin's fixative for 24 h. One kidney from four animals, each from Gr I, Gr IV and Gr V were also sliced and kept frozen at –70°C. The Bouin's fixed tissues from different groups were processed for paraffin sectioning. Serial sections (6–8 mm thick) were routinely stained with haematoxylin–eosin, periodic acid Schift–ethenamine silver to stain the glomerular and tubular basement membrane. Immunostaining to localize gp330 was carried out on 10 µm thick paraffin sections, collected on poly-lysine-coated slides, by indirect peroxidase method. After deparaffinization and stabilizing in PBS (0.05 M, pH 7.4) the endogenous peroxidase was blocked by methanol/H_2O_2 and nonspecific-binding sites were quenched in 3% normal goat serum (NGS). The sections were incubated with primary antibody (polyclonal serum from rats immunized with affinity purified gp330 antigen), 1:1000 dilution in 1% NGS–PBS for 48 h at 4°C in a humid chamber. To label the bound antibody, the sections were incubated with peroxidase-labelled goat anti-rat serum (Sigma), 1:100 for 24 h at 4°C. The washing was carried out in PBST (0.05% Tween 20). The peroxidase-labelled immune reaction was visualized by DAB/H_2O_2. Control sections were simultaneously processed identically with preimmune rat serum. The sections were dehydrated in graded alcohol and mounted in DPX mountant.

Direct immunofluorescence was used to detect the endogenous IgG deposited in rat glomeruli. Cryostat sections (5 µm) of unfixed rat kidneys were stained with FITC conjugated goat–anti rat IgG. Sections were examined under a DMRB-Leica microscope under epiillumination. The density of immunoglobulin deposition along the glomeruli and the basement membrane thickening noted by silver stain were graded visually.

All the animals which received intravenous injection of conjugate remained healthy throughout the period as indicated by the lack of effect on the antibody titres, when an indifferent antigen, ovalbumin is injected into rats. Within two weeks of antigen administration, the rats were found to have circulating antibody against gp330, reaching maximal titre by day 21 and then gradually dropping to undetectable levels by 170 days. The administration of a booster dose of gp330 or the toxin gelonin alone did not affect the antibody level and remained similar to group I. On the other hand, intravenous administration of gelonin gp330 conjugate has resulted in fall in circulating antibody levels, in a dose-dependent manner (data not shown). With time in groups I, II, III, the antibody levels in the serum declined, but HN is well established as evidenced by proteinuria and the histological features in the kidneys.

The urinary protein content from 1 mg/20 h at 56 days, steadily increased to 160–190 mg/20 h by 140 days, in the groups of animals with HN (groups I, II, III). In conjugate-treated rats (groups IV and V) the proteinuria was negligible during the course of the study, similar to control animals.

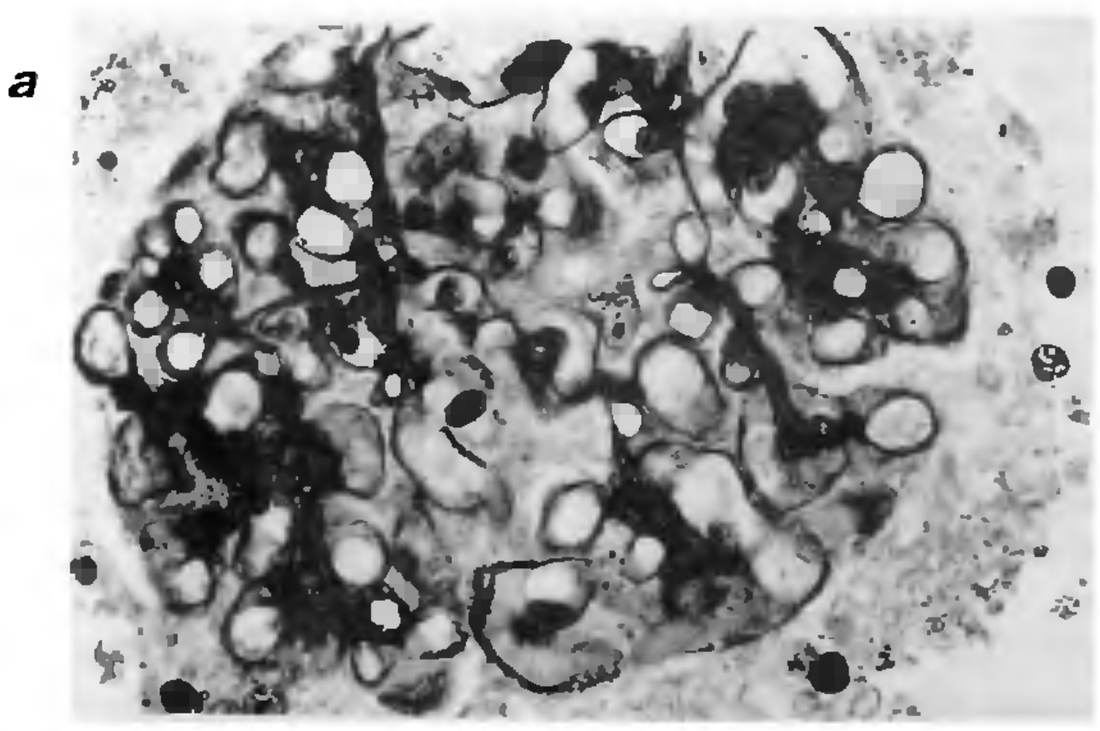

Figure 1 *a*, *b*. *a*, Heymann's nephritis induced by injecting gp330 antigen. Group I glomerulus showing widespread thickening of the capillary basement membrane, while the mesangeal cellularity is normal. Bowman's space has proteinous material. Silver mathenamine (×480); *b*, Endogenous rat IgG is deposited in a granular pattern diffusely along the glomerular basement membrane. FITC direct immunofluorescence (×360).

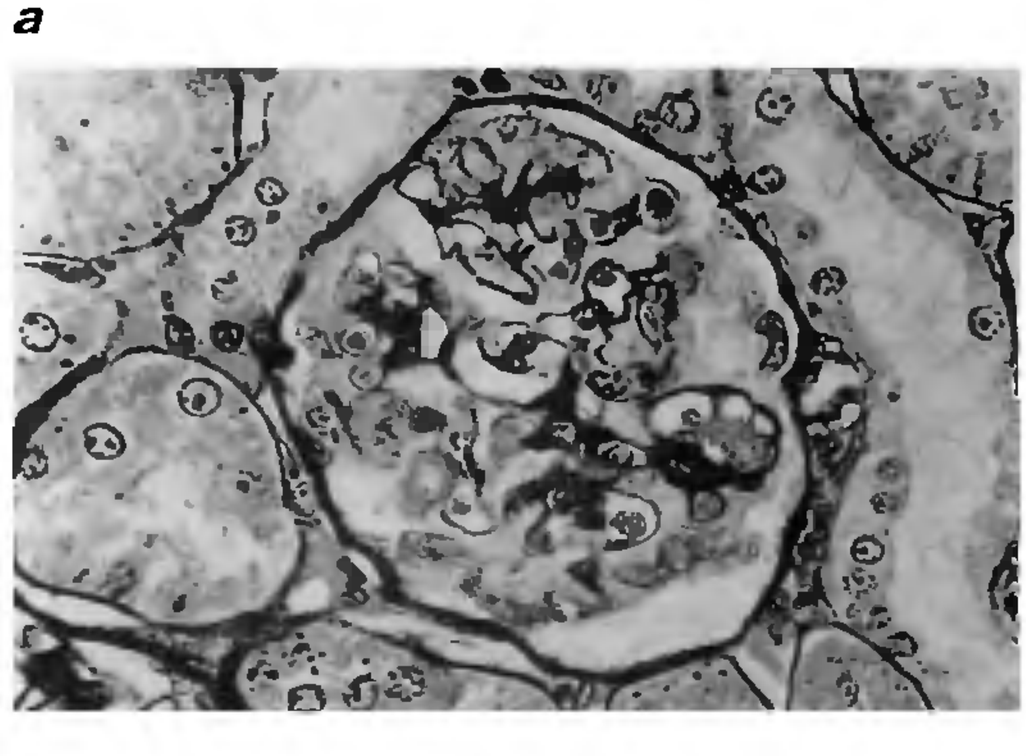

Figure 2 *a*, *b*. *a*, Gelonin gp330 conjugate injected after challenging with gp330 antigen to induce Heymann's nephritis. Glomerulus shows only segmental and sparse thickening of the capillary basement membrane (Group IV). Silver metenamine (×360); *b*, Sparse and segmental deposition of endogenous IgG along the basement membrane of some of the capillary loops (Group IV). FITC direct immunofluorescence (×360).

The glomerular pathology in HN-induced animals was mostly global and diffuse. There is a uniformly eosinophilic thickening of the capillary loops in a focal lympy manner, the mesangial cellularity being essentially normal. Silver methenamine stain has further delineated the thickening of the capillary loops both diffuse membranous and focal nodular pattern. Some of the glomeruli with late lesions had early sclerosis, revealed by mesangial thickening continuous with the capillary loops (Figure 1 *a*). The lumens of the capillaries were widely patent and there was no increase in the lobulation of the tufts. In the Bowman's space variable amount of proteinous and nuclear material was seen. The renal vessels were normal. The proximal tubules were relatively well preserved, while the distal convoluted tubules and the

collecting ducts had protein and granular casts. The degree and pattern of glomerular pathology was essentially similar in the animals belonging to groups I, II and III.

In the gelonin gp330 conjugate-injected rats, the glomerular capillary thickening and the presence of tubular casts were significantly less and focal. The basement membrane thickening, when present was segmental, involving some of the glomerular tufts only (Figure 2 *a*).

Direct immunoperoxidase staining, using serum from the gp330 challenged HN animals as the source of antibody, revealed dense labelling of the proximal tubular brush border in the renal cortex (Figure 3), confirming the specific nature of the immune response. Some of the glomerular capillaries also revealed focal staining marking the immune deposits along the basement membrane. However, the direct immunofluorescence strikingly revealed diffuse granular deposits of endogenous IgG along the glomerular basement membrane in animals belonging to groups I, II and III, corresponding to the thickening revealed by silver staining (Figure 1 *b*). In contrast, the subepithelial deposits were sparse and segmental (Figure 2 *b*) in gelonin gp330-injected animals. The degree of IgG deposition and the number of glomeruli affected were much less in group V than in group IV. In each of the conjugate-treated animals, groups of relatively normal glomeruli indistinguishable from the control animals were found intermingled with affected ones, highlighting the variable and focal nature of the pathological involvement of the kidney and resolution of the immune-mediated injury.

All toxins isolated so far from plants have been found to possess *N*-glycosidase activity. They depurinate the major rRNA, thus damaging ribosomes and arresting protein synthesis. Thus, they have been commonly termed as ribosome-inactivating proteins (RIP)[8]. This property of toxins has made them invaluable tools in cancer and immunotherapy[9,10]. This has been achieved by linking them to carrier molecules that would specifically direct them to target cells[11]. The plant toxin, gelonin, a single chain or Type I RIP has been found to be nontoxic to non-targetted intact cells[12], and, in addition, gelonin conjugates have been found to be much more effective than the ricin A chain ones[13]. Hence, it has been progressively replacing the well-known plant toxin, ricin, which belongs to the Type II RIPs[14,15]

Our previous studies with gelonin gp330 conjugate have shown that administration of the complex after induction of HN in rats caused a reduction in circulating antibody titre and the almost negligible levels of proteinuria compared to diseased animals that had not received any treatment[6].

In this study we were keen on seeing whether the diminishment of antibody titre and absence of proteinuria could be correlated with a decrease in deposition of immune complexes in the glomeruli. Following a similar protocol as our previous studies we have been able to show that there is a significant amelioration in the pathological lesions in the kidney as well. In the gelonin gp330 conjugate-treated animals, the endogenous IgG deposition along the glomerular basement membrane was focal and segmental, in addition to many of the glomeruli being relatively normal.

There have been reports of the use of various drugs in the treatment of HN. Of particular interest are the use of cyclosporin A (ref. 16) and FK506 (ref. 17). Nonetheless, both these drugs are relatively nonspecific in nature working through an immunomodulatory role by suppressing T cell activity. Toxin conjugates are extremely specific to the target cells in their mode of action and our work has clearly shown the potential in developing such conjugates for the treatment of antibody-mediated autoimmune diseases.

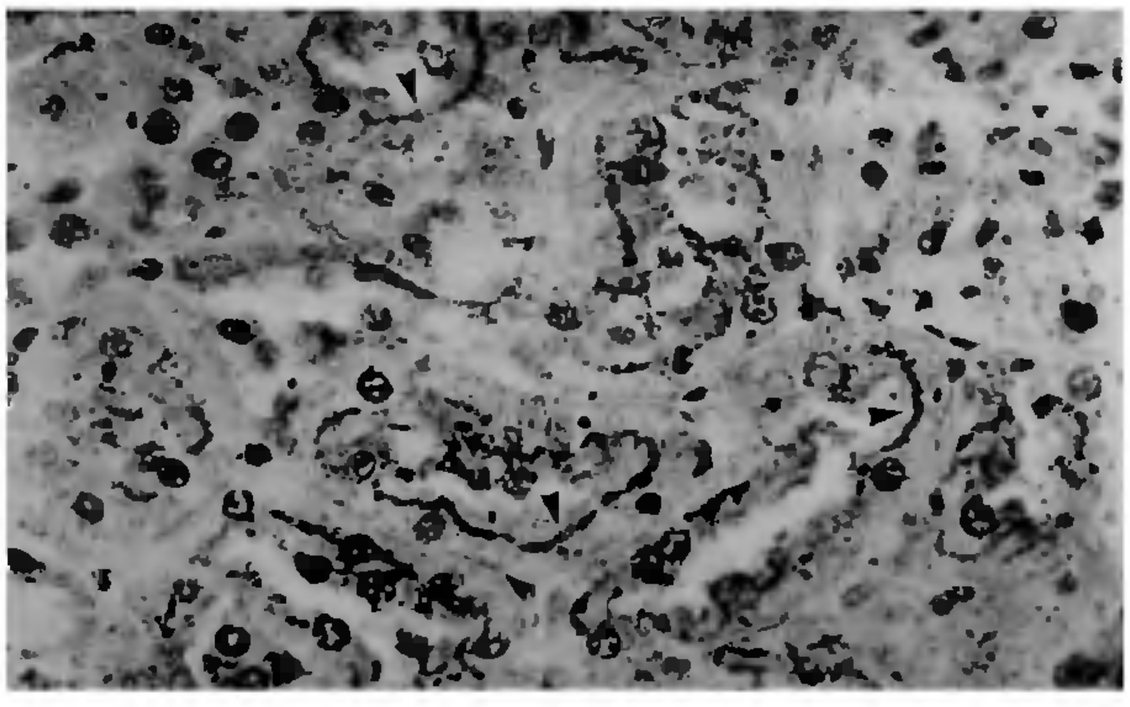

Figure 3. Indirect immunoperoxidase staining of paraffin section of normal rat kidney using serum of HN rats induced by gp330. The brush border of the proximal convoluted tubules of the renal cortex is strongly labelled (×300).

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