In vivo treatment of Heymann's Nephritis using a cytotoxic protein–toxin conjugate

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Abstract In this study we investigated the possibility of treating Heymann's Nephritis (HN) by destroying antibody producing cells by targetting a toxin, gelonin – conjugated to gp330, the renal brush border antigen. HN was induced in rats by immunizing them with purified gp330. The gelonin–gp330 conjugate was administered 12 days after the antigenic challenge. Serum was screened for circulating antibodies. Proteinurea was estimated. The gp330–gelonin conjugate-treated animals had a circulating antibody titre in the serum much lower than that of diseased (untreated) animals. Proteinurea seen in diseased animals was not observed in treated animals. This work suggests the possibility of using a toxin–antigen conjugate for immunomodulating antibody mediated autoimmune renal disease.

Key words: Heymann's Nephritis; Gelonin–gp330 conjugate; Cytotoxicity

1. Introduction

Heymann's Nephritis is an autoimmune disease, that can be experimentally induced in rats. It has been used extensively as a model to study membranous glomerulonephropathy in humans. This disease was first induced by Heymann in 1959 [1], by immunizing rats with kidney cortex homogenate in Freunds Complete Adjuvant. The disease is initiated by circulating antibodies [2,3], which deposit on the epithelial aspect of the glomerular capillary wall resulting in heavy proteinuria [4]. It has been shown that the antigen is a glycoprotein [5] located in the membrane of the proximal tubule brush border of the rat kidney [6], sharing a high homology with the low density lipoprotein receptor (LDLR) [7] and is called gp330.

As circulating antibodies seem to be the mediators of HN, causing glomerular pathology and proteinuria, activation of specific B cells appears to be the pivotal event in the evolution and pathogenesis of this disease [8]. Hence, we felt that destruction of B-cells expressing anti-gp330 on their surface could prevent the subsequent steps in the pathway leading to severe kidney damage. Immunosuppressants have been used in the prevention of immune complex deposition in glomerulonephritis induced in rats [9]. As they are nonspecific and have a generalised action on lymphocytes, it would be more efficacious to use specific drug targetting directed to gp330 primed cells. This can be achieved by directing a toxin, like gelonin, a plant ribosome inactivating protein [10] to these cells. The toxin was conjugated to gp330 with the help of a heterobifunctional crosslinking agent *N*-succinimidyl-3-(2-pyridyldithio) propionate (SPDP). In this report we show, using biochemical techniques, how a toxin conjugate is useful in ameliorating the damaging effects of this autoimmune disease of the kidney.

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Abbreviations: Heymann's Nephritis (HN); Low density lipoprotein receptor (LDLR); *N*-Succinimidyl-3-(2-pyridyldithio)propionate (SPDP).

2. Materials and methods

2.1. Animals

Two strains of rats were obtained from the Central Animal Facility (CAF), Indian Institute of Science, Bangalore. These were namely Wistar and IISc strains. The latter are inbred Wistar rats maintained at the CAF and found by us to be more susceptible to autoimmune disease. The animals used were approximately all of the same age (16–18 weeks) and female, and were fed ad libitum with chow pellets and water during the entire course of the experiment.

2.2. Preparation of gp330

Crude cortex preparation: The antigen gp330 was prepared according to the combined methods of Malathi et al. [11] and Kerjaschki and Farquhar [7]. Sephacryl S-300 and Sepharose 4B were purchased from Pharmacia Biotech, Sweden. Lentil lectin was prepared according to the method of Hayman and Crumpton [12] and coupled to Sepharose 4B as described by March et al. [13]. The sample was tested for purity by SDS-PAGE as described by Kerjaschki and Farquhar [7].

2.3. Preparation of Gelonin

Gelonium multiforum seeds were purchased from United Chemical and Allied products, Calcutta. Gelonin was extracted from these seeds as described by Stirpe et al. [14]. Its purity and activity were tested by SDS-PAGE and the extent to which it inhibited protein synthesis in a rabbit reticulocyte lysate.

2.4. Preparation of gelonin–gp330 conjugate

The gelonin–gp330 conjugate was prepared based on the method of Pain and Surolia [15] using the heterobifunctional crosslinking agent SPDP. The purity and efficacy of the product was tested for by SDS-PAGE and inhibition of the protein synthesis as for gelonin.

2.5. Preparation of anti-gelonin-Sepharose affinity column

Antibodies against gelonin were raised in rabbits. The IgG fraction was purified using Protein G-Sepharose. The purified IgG was coupled to Sepharose 4B according to the method of March et al. [13].

2.6. In vitro inhibition of protein synthesis

Rabbit reticulocyte lysate was prepared as described by Jagus [16]. The efficiency of the lysate was tested using [^{35}S]methionine in the cocktail, which also contained DTT. The ability of gelonin and gelonin–gp330 to inhibit protein translation was then carried out using this system.

2.7. Induction of active HN

Adult IISc strain rats were administered two foot pad injections with 100 μg purified gp330 in Freunds Complete Adjuvant in a total volume of 400 μl on day 0 and day 8. Simultaneously a control antigen, namely 40 μg of ovalbumin (Sigma) was injected.

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2.8. Administration of gelonin–gp330 conjugate, or gp330 or gelonin

The animals in which active HN had been induced were divided into five groups. Each group consisted of eight rats. The first group (G-I) was given a placebo namely phosphate-buffered saline (PBS). The second group (G-II) was given gelonin (40 μg) and the third group (G-III) was administered gp330 (450 μg) in a similar schedule as for the gelonin-gp330 conjugate. The fourth group was further subdivided into 2 groups (G-IV and G-V, respectively) and intravenously injected with two different doses of the conjugate, one set was given 250 μg while the other was given 500 μg. The conjugate, gelonin and gp330 were dissolved in 0.9% NaCl containing 0.018 mg of invertase per 100 g body weight of the rat and administered on the 4th day after the second immunization.

2.9. Detection of anti-gp330 antibodies in serum

Blood samples were taken from all animals at bi-weekly intervals starting from the 2nd week after initiating the experiment (day 0). The circulating anti-gp330 antibodies present in the serum were detected using the dot-blot assay. The circulating antibody titre was determined as described by Matsukawa et al. [17]. Briefly, serum samples were serially diluted in 0.02 M PBS pH 7.2, according to the general formula $1:4^N \times 1000$ where N varied from 0–4. The titre was determined as the highest dilution of the serum giving positive dot blot assay. Antibodies against ovalbumin were also tested for in the same manner as for anti-gp330 antibodies.

2.10 In vitro production of anti-gp330 antibodies by lymph node cells

The production of anti-gp330 antibodies by brachial and axillary lymph node cells taken from the front legs was assessed as described by De Heer et al. [8].

2.11 Urine analysis

Rats were kept in metabolic cages for 24 h and urine samples were collected. This was done every alternate week to the week blood was collected. The quantity of protein in the urine was estimated by Lowry's method [18], after dialysing out urea, and other small molecular weight components. Proteinuria was graded according to the amount excreted.

3. Results

3.1. Efficiency of gelonin and gelonin–gp330 conjugate

Gelonin is a single chain ribosome inactivating protein. It was conjugated to gp330 using SPDP. Free gelonin was separated from gelonin–gp330 by gel filtration through a Sephadex G-100 column. More than 90% of the conjugate was retained on an anti-gelonin-Sepharose affinity matrix, indicating the absence of any free gp330. To assess the stoichiometry of gp330: gelonin in the conjugate it was rechromatographed, on the same gel filtration column, in the presence and absence of DTT (Fig. 1). Under reducing conditions two protein peaks comprising of gp330 and gelonin were obtained. Based on the protein content of each peak, the molar ratio of gp330 and gelonin calculated to be 1:1.

The efficacy of gelonin and gelonin–gp330 conjugate were tested in vitro using rabbit reticulocyte lysate, containing DTT in the cocktail mixture. The concentration of gelonin required to achieve 50% inhibition was 13.8 ng/ml and for 80% inhibition was 35 ng/ml (1.13 nM) which is similar to that reported by Stirpe et al. [14]. The conjugate also inhibited 80% protein synthesis at a similar concentration (1.01 nM) (Fig. 2). Thus, these results reinforce the point that (a) conjugation of gelonin by SPDP did not destroy its activity in vitro, in fact we also found that if the molar ratio of gelonin:SPDP is kept to 1 in the reaction mixture the activity of the toxin is not compromised at all; (b) gelonin is conjugated to gp330 with a stoichiometry of 1:1.

Table 1
In vitro production of anti-gp330 antibodies by lymph node cells of treated and untreated rats. N is normal animal. G-I to G-V are groups I to V as mentioned in materials and methods

No. of cells/well	Antibody produced in ng					
	N	G-I	G-II	G-III	G-IV	G-V
1×10^7	2.1	8.2	7.9	7.6	3.6	2.8
2.5×10^7	3.2	18	25	22	5.7	3.7
5.0×10^7	2.7	43	40	35.8	7.2	5.2
10.0×10^7	3.6	96	92	98	9.1	6.8

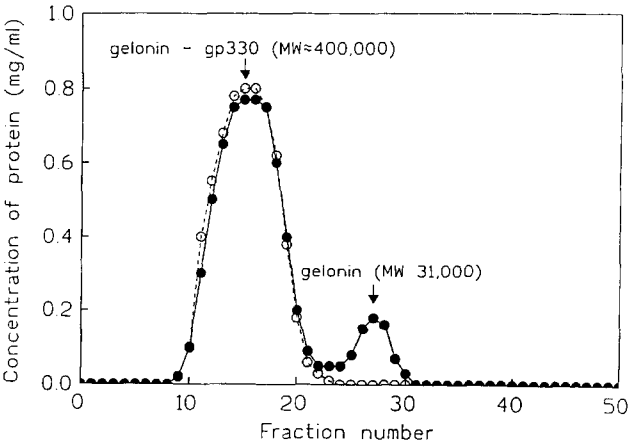

Fig. 1. Gel filtration of gelonin–gp330 conjugate on a Sephadex G-100 column in the presence (●) and absence of DTT (○). Under reducing conditions two peaks are obtained, one which appears in the void volume and corresponds to gp330 while the other is in the inner volume and elutes at the same position as gelonin.

3.2. Detection of anti-gp330 antibodies in serum

The experimental animals in which HN had been induced can be essentially classified into two groups: (a) untreated animals, rats which received PBS, or gelonin or gp330 only as controls; (b) treated animals, rats which were intravenously administered with the gelonin–gp330 conjugate.

Within two weeks of sensitising the rats with gp330, the animals were found to have circulating antibody in the serum, reaching a maximum titre by the 21st day. Subsequently, the antibody titre tempered gradually, to relatively undetectable levels by 168 days (Fig. 3). Further, administration of gelonin alone or a third dose of gp330 on 12th day (Group II and Group III respectively, Fig. 3) did not alter the serum antibody levels and remained same as in Group I, with disease. On the other hand intravenous administration of gp330–gelonin conjugate has resulted in significant fall in circulating antibody titres, more so in Group V, which received a higher concentration (500 μg) of the conjugate. This indicates that booster with gp330 or the administration of gelonin alone does not ameliorate the condition, nor the antibody response. With time in groups I, II and III, the antibody levels in the serum declined, but by then Heymann's Nephritis was well established as evidenced by proteinuria.

3.3. Production of anti-gp330 antibodies by lymph nodes

To test our hypothesis that gp330–gelonin was indeed killing antibody producing cells we assessed the in vitro production of antibodies by cells in the lymph nodes.

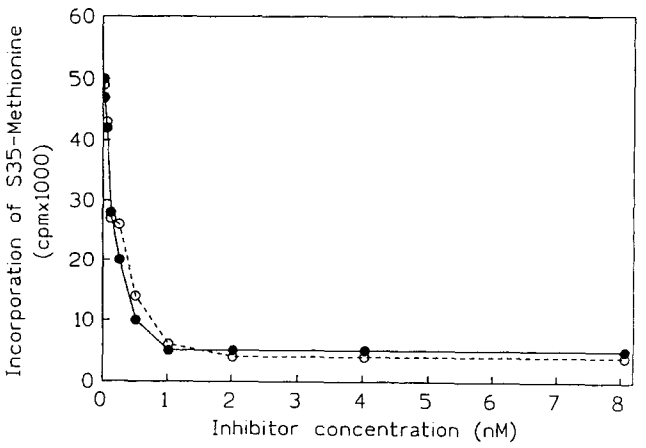

Fig. 2. In vitro inhibition of protein synthesis by gelonin (●) and gelonin-gp330 conjugate (○). IC_{50} of gelonin and gelonin–gp330 are 0.45 nM and 0.40 nM, respectively.

We observed that antibody production by lymph node cells from treated animals was negligible as compared with cells isolated from animals which had not received any conjugate. As in the case of circulating antibodies, the titre was high for groups I, II and III while it was just above threshold level for groups IV and V (Table 1). When an unrelated control antigen, ovalbumin, was coated in the wells instead of gp330 there was no production of antibody against ovalbumin by the lymph node cells isolated from the diseased animals (results not shown).

3.4. Urine analysis

Urine samples collected from the two classes of rats, treated and untreated, were graded according to the degree of proteinuria.

In our preliminary experiments, we had collected urine from the second week after induction of HN, and found that proteinuria was manifested only after the 8th week. Hence, urine was collected for a period of 20 h every 14 days from the 56th day to the 168th day. Urinary protein content in untreated animals initially (at 56th day) was approximately 1 mg/20 h, with time there was a steady increase in the protein excreted, till it reached about 190 mg/20 h, and remained at this point even at the 126th day then, at the 140th day it was found to have diminished to about 160 mg/20 h and remained at this level till animals were sacrificed after the 168th day (Fig. 4).

The conjugate treated rats (Group IV and V) had negligible levels of proteinuria during the course of the study, similar to control rats (Fig. 4).

4. Discussion

Toxins conjugated to proteins have been used widely in selectively killing target cells [19]. Most plant toxins act by inhibiting protein synthesis machinery. The most commonly used plant toxin is probably ricin. However, due to the presence of its B chain, it could bind to non-target cells as well. Removal of the B-chain seems to lower toxicity of the molecule by a thousand fold [20]. This is overcome by using single chain polypeptide toxins like gelonin, which is known to be non toxic to intact cells [10].

Immunotoxins have been used in immuno and cancer therapy, and in some cases clinical trials have also begun (reviewed in [19,21,22]). The in vitro and ex vivo use of toxin–antigen conjugates for the therapy of myasthenia gravis, where serum antibodies are formed against the acetylcholine receptor, have also been reported [23,24]. In the present study, we have shown that the administration of gp330–gelonin conjugate into rats with Heymann's Nephritis, could alter the course of the disease, offering a protective effect. This is probably brought about by selective killing of the specific gp330 antibody producing cells in the circulation, as seen by the reduction in antibody production by cells of the lymph node in vitro. This leads to a reduction in circulating antibody levels, reducing the load on the glomerular filtration of these immunoglobulins and indirectly abrogating proteinuria.

The fact that gp330–gelonin conjugate is antigen specific is indicated by the unaltered immune response against an unrelated control antigen, ovalbumin. The in vivo efficacy of the conjugate in alleviating the clinical and pathological symptoms of HN has been documented by a variety of biochemical markers, which include marked reduction in circulating antibodies as compared to untreated animals and the absence of proteinuria. Equivalent amounts of either gelonin (40 μg) or gp330 (450 μg) alone displayed no therapeutic efficacy in abrogating HN. This excludes any non-specific immunosuppressive effect of either gelonin or gp330 moiety on HN.

The results reported here establish two points: (a) the toxin–protein conjugates are effective as therapeutic agents in autoimmune diseases, where the cells, implicated in the disease (in this case B cells), can be selectively destroyed; (b) membranous glomerulonephritis is initiated by circulating antibodies, as proteinuria can be prevented by the destruction of B cells producing antibodies against gp330, thereby corroborating the evidence of Van Damme et al. and Couser et al. [2,3]. Most immunotoxins are composed of antibody or anti-idiotypic antibody linked to a toxin molecule. This approach is valuable when dealing with autoimmune diseases caused by an antigen with a single epitope or those antigens which display an antigenic region confined to a single site, as is observed for the main immunogenic region in acetylcholine receptor [25]. However, for antigens such as gp330, which evokes antibodies against multiple epitopes [26], it would be practical to use the whole

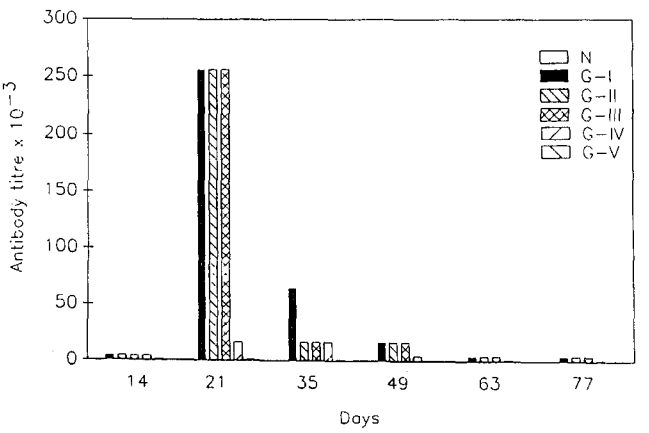

Fig. 3. Circulating anti–gp330 antibodies. The N value titre (as explained in materials and methods) is plotted against the day serum was collected from the orbital vein of the rat.

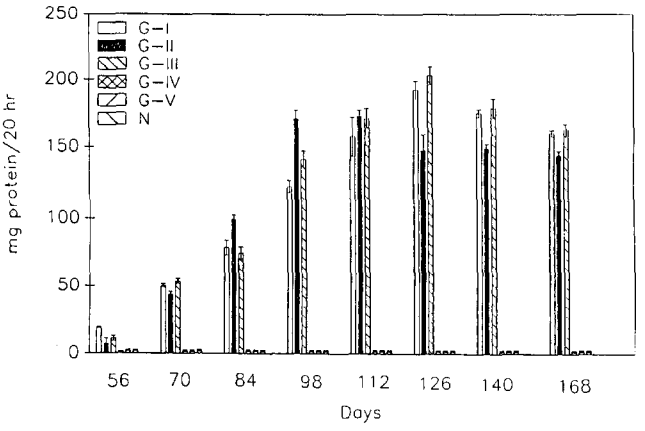

Fig. 4. Sequential urinary protein secretion in the different groups of animals (*I–V*). The values are a mean of the 8 rats in each group.

molecule rather than any particular anti-idiotypic antibody. This would ensure the destruction of B-cells producing antibodies directed against all the epitopes of the antigen.

The fact that the conjugate has been effective in significantly diminishing the amount of circulating antibodies and preventing proteinuria, shows that it can be considered as a therapeutic modality in autoimmune nephritis.

Immunonosuppressants like FK506 [20] and cyclosporin A [13], also have been used in the therapy of immunemediated diseases. However, their nonspecific and generalised action on the immune system, usually necessitates antibiotic therapy to ward of infections as occurring as a secondary phenomenon. Hence, our approach seems to be more specifically targeted to cells implicated in the disease, and could have a wider application in autoimmune therapy.

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