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INHIBITORY OLIGODEOXYNUCLEOTIDE IMPROVES GLOMERULONEPHRITIS AND PROLONGS SURVIVAL IN MRL-lpr/lpr MICE

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Abstract: Inhibitory oligodeoxynucleotides (ODNs), which are capable of blocking CpG-induced inflammation, have been anticipated to be beneficial therapeutic agents for autoimmune diseases. In this study, we show that GpC ODN, which inverted the cytosine guanine sequence of CpG motif to guanine cytosine sequence, is an inhibitory ODN. The inhibitory effects of GpC ODN on CpG ODN-induced immune activation were confirmed by cytokine assay using splenocytes from lupus-prone MRL-lpr/lpr mice. *In vivo,* injecting MRL-lpr/lpr mice with GpC ODN did not reduce the deposition of IgG and C3 in the glomeruli, the serum level of IL-12, the serum level of rheumatoid factors and anti-ds DNA antibody, or alter the composition of IgG isotypes of anti-ds DNA antibody. However, the mice in the GpC group showed less proteinuria, significantly lower blood urea nitrogen levels (BUN) and significantly prolonged survival. Our results suggest that inhibitory ODNs, such as GpC ODN, have the potential to become a treatment for autoimmune diseases, like lupus nephritis.

Key words: MRL-lpr/lpr mouse, Systemic lupus erythematosus, Glomerulonephritis, Inhibitory oligodeoxynucleotides

INTRODUCTION

Bacterial DNA contains pathogen-associated molecular patterns that are recognized by Toll-like receptor 9 (TLR9) leading to an innate immune response. This response requires unmethylated cytosine-phosphate-guanosine sequences, so called

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CpG motifs[1]. Immune cells such as B cells, macrophages and plasmocytoid dendritic cells express TLR9, although these cell populations differ between species. Unmethylated CpG is internalized into endosomal and lysosomal vesicles and interacts with TLR9 initiating intracytoplasmic activation signals[2-4]. Activation of these cells leads to activation of other immune cells, such as T cells, to create a Th1-biased immune milieu.

While unmethylated CpG-induced immune responses can protect the host from infectious pathogens, exposure to stimulatory motifs can have harmful consequences. Marshak-Rothstein and colleagues reported that the innate immune pathway involving TLR9 can activate autoreactive B cells independently of T cells; this finding relates the innate and adaptive immune systems to autoimmune disease development[5]. Importantly, most CpG motifs of vertebrates are favorably methylated so that they do not trigger innate immune responses.

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by the production of pathogenic autoantibodies. Recent studies have shown that SLE patients have elevated levels of circulating plasma DNA containing immunostimulatory CpG motifs because of increased cell apoptosis and insufficient clearance of apoptotic cells[6,7]. It has also been reported that SLE patients have decreased levels of DNA methylation[8]. These findings suggest that circulating plasma DNA containing immunostimulatory CpG motifs may stimulate immune cells to produce pathogenic autoantibodies and cytokines.

Some immunomodulatory ODNs that are capable of blocking CpG-induced inflammation have been referred to as inhibitory, suppressive or neutralizing ODNs[9-14]. ODNs rich in poly G sequences, methylated CpG[15] and the single base switch from CpG to GpG[16] reportedly work as inhibitory ODNs. Ho *et al.* reported that GpG ODN successfully reduced the incidence and severity of experimental autoimmune encephalomyelitis by inhibiting the activation of Th1 cells[16]. Li *et al.* reported that poly G-containing ODNs attenuated collagen-induced arthritis[17]. These reports led us to hypothesize that inhibitory ODNs can attenuate the severity of autoimmune glomerulonephritis if intrinsic unmethylated CpG is one of the pathogenesis factors.

GpC ODN has been used as an inactive ODN that has no immunomodulatory effect[9,18]. However, in the present study, we show that GpC ODN works as an inhibitory ODN. To test our hypothesis that inhibitory ODN can attenuate the severity of glomerulonephritis, we administered GpC ODN to MRL-lpr/lpr mice, a murine model of SLE, and measured serologic and clinical disease activity.

MATERIALS AND METHODS

Mice

Female MRL-lpr/lpr mice were obtained at 8 to 9 weeks of age from SLC Japan (Shizuoka, Japan). Mice were maintained under specific pathogen-free conditions

in the animal facility at the Fukushima Medical University School of Medicine. Procedures involving animals and their care were conducted in conformity with national and international laws and policies.

Oligodeoxynucleotides

Phosphorothioate stabilized ODNs were purchased from BEX Inc. (Tokyo, Japan). ODN sequences were as follows: 1668 CpG ODN, TCCATGACGTTCCTGATGCT; and 1720 GpC ODN, TCCATGAGCTTCCTGATGCT.

In vitro cytokine analysis

Whole splenocytes (2×10^6) from MRL-lpr/lpr mice were cultured in RPMI 1640 and 10% FCS with the indicated ODN at the indicated microgram per milliliter concentrations for 48 h. The supernatants were collected and tested by sandwich ELISA. Briefly, polystyrene microtiter plates (EIA/RIA Plate; Corning Incorporated, NY, USA) were coated with capture antibody in carbonate coating buffer (35 mM $NaHCO_3$, 15 mM Na_2CO_3, pH 9.6) at 4°C overnight. For the IL-6 and IL-12 ELISA, rat anti-mouse IL-6 capture antibody and rat anti-mouse IL-12 capture antibody (BD Biosciences, CA, USA) were diluted to 5 μg/ml. Seven serial three-fold dilutions of recombinant mouse IL-6 and IL-12 (BD Biosciences) starting at 30 ng/ml were used as standards. Plates were blocked with 1% BSA in PBS (PBS-B) for 1 hr prior to adding standards and samples and incubating at 4°C overnight. After washing, biotinylated rat anti-mouse IL-6 and IL-12 monoclonal antibody (1: 1,000 in PBS-B) (BD Biosciences) were added and incubated for 1 hr. Streptavidin-HRP conjugates (Zymed Laboratories Inc., CA, USA) were added followed by equally mixed 3, 3′, 5, 5′ tetramethylbenzidine peroxidase substrate and peroxidase solution B (KPL, MD, USA). Optical density of the reaction product was measured at 450 nm using a 650 nm reference on the Benchmark Plus microplate spectrophotometer (Bio-Rad, CA, USA).

Administration of ODN

Mice were divided into two groups; each group consisted of 20 mice. All mice were injected intraperitoneally with 50 μl of saline or 50 μg of GpC ODN in 200 μl saline three times at 2-wk intervals. The initial injection was given when the mice were 10-wks old. Three mice from each group were sacrificed by cervical dislocation at 23 wks to evaluate glomerular disease. The remaining mice were observed for survival study.

Measurement of anti-dsDNA antibody

Total anti-dsDNA IgG antibody (Ab) and IgG isotype levels were measured by ELISA as previously described[19]. Briefly, ELISA plates were coated with 5 μg/ml double-stranded calf thymus DNA diluted in carbonate coating buffer at 37°C overnight. The plates were blocked with PBS-B for 60 min. After washing with

PBS containing 0.1% Tween 20 (PBS-T), sera were added in serial dilutions (starting at a 1/100 dilution) to each well and incubated for 60 min at room temperature. After washing with PBS-T, peroxidase-conjugated goat anti-mouse IgG (γ-chain specific; Sigma-Aldrich, MO, USA), IgG1 or IgG2a (γ_1, γ_{2a}-chain specific respectively; Southern Biotechnology Associates, AL, USA) was added at the dilution of 1/4,000 in 1% BSA PBS-T and incubated for 1 hr. After washing, the assay was developed by adding 3, 3′, 5, 5′-tetramethylbenzidine (TMB)+substrate-chromogen (Dako, CA, USA), and the OD 405 was determined on a Benchmark Plus microplate spectrophotometer (Bio-Rad, CA, USA).

To calculate the IgG2a/IgG1 ratio, preselected high-titer serum was assigned as the internal standard. The titer of each sample was calculated by comparing it with the standard serum, which was arbitrarily defined as 100 ELISA units.

Measurement of rheumatoid factor (RF)

RF IgG and RF IgM reactivity were measured as previously described[19]. Briefly, ELISA plates were coated with 2 μg/ml rabbit IgG (Sigma-Aldrich) diluted in carbonate coating buffer overnight at 4°C. After blocking and washing, serial dilutions of sample sera (starting at a 1/100 dilution) in PBS-B were added to the plates and incubated for 60 min at room temperature. After washing, peroxidase-conjugated goat anti-mouse IgG (γ-chain specific) or goat anti-mouse IgM (μ-chain specific) (Sigma-Aldrich) was added to the plates at a dilution of 1/1,000 or 1/250, respectively, in 1% BSA PBS-T. After washing, the assay was developed as described above for anti-DNA ELISA.

Serum cytokine analysis

The serum IL-12 level was measured as described above for *in vitro* cytokine analysis.

Measurement of blood urea nitrogen (BUN)

BUN was measured by the urease/indophenol method[20,21]. The urease reagent was prepared by adding 50 mg of urease from Jack Bean (WAKO, Japan) to 200 mg of disodium ethylenediaminetetraacetate (EDTA-2Na). This solution was diluted to 500 ml in distilled water and adjusted to pH 7.0 with NaOH. The phenol color reagent was prepared by adding 5 g of phenol to 25 mg of $Na_2Fe(CN)_5NO\text{-}2H_2O$. This solution was diluted to 500 ml in distilled water. To prepare the alkaline hypochlorite solution, 2.5 g of NaOH was dissolved in distilled water. Then, 3 ml of 10% NaOCl was added and diluted to 500 ml in distilled water. To measure the BUN, 4 μl of each serum sample and standard urea solutions were incubated with 200 μl of urease reagent at 37°C for 15 min. Then, 400 μl of phenol color reagent was added, followed by generous vortexing. After adding 400 μl of alkaline hypochlorite solution, sample tubes were incubated at 37°C for 20 min. The OD 560 was measured, and the BUN levels were calculated using standard curve.

Urine studies

Mice were placed in metabolic cages (1 or 2 mice in each cage) for 24-h urine collection. To prevent bacterial growth, antibiotics (penicillin and streptomycin) were added to collection tubes. Urine protein excretion was measured with the Bio-Rad protein assay kit (Bio-Rad) according to the manufacturer's instructions. Results were reported as milligrams of protein per mouse per day.

Pathology

Three mice from each group were sacrificed, and their kidneys were removed. The right kidney was placed in 10% buffered formalin followed by paraffin embedding, cutting, and hematoxylin and eosin (H & E) staining. Twenty glomeruli from each H & E stained kidney section were photographed. The number of glomerular cells and the glomerular diameter of each glomerulus were recorded. The left kidney was frozen and placed in OCT medium for immunofluorescence staining. Four micron sections were cut and then stained with FITC-conjugated anti-mouse IgG or C3. The fluorescence intensity of individual samples was graded on a scale from 0 to 4+.

Statistical analysis

The Kruskal-Wallis test was used to determine statistical values for the cytokine assays. The Mann-Whitney U test was used for proteinuria, anti-dsDNA Ab and RF evaluation. Student's t-test was used for BUN evaluation. Survival statistics were analyzed using the log-rank test. P-values less than 0.05 were considered statistically significant.

RESULTS

In vitro cytokine analysis

Unmethylated CpG motifs stimulate immune cells to proliferate and secrete cytokines (IL-6, IL-10, and IL-12) and up-regulate the expression of MHC and costimulatory molecules[22-24]. We investigated the effect of GpC ODN, which has been used as a non-immunomodulatory ODN, on CpG ODN-induced cytokine production. GpC ODN did not induce IL-6 and IL-12 production from splenocytes (Fig. 1A). But, GpC ODN reduced CpG ODN-induced production of IL-6 and IL-12 in a dose-dependent manner (Fig. 1B).

Measurement of serum autoantibody levels

MRL-lpr/lpr mice develop hypergammaglobulinemia and produce autoantibodies including anti-dsDNA Ab and RF[19,25,26]. To determine whether the administration of ODN modifies the production of autoantibodies, serum levels of anti-dsDNA Ab and RF were measured by ELISA. The serum levels of both anti-

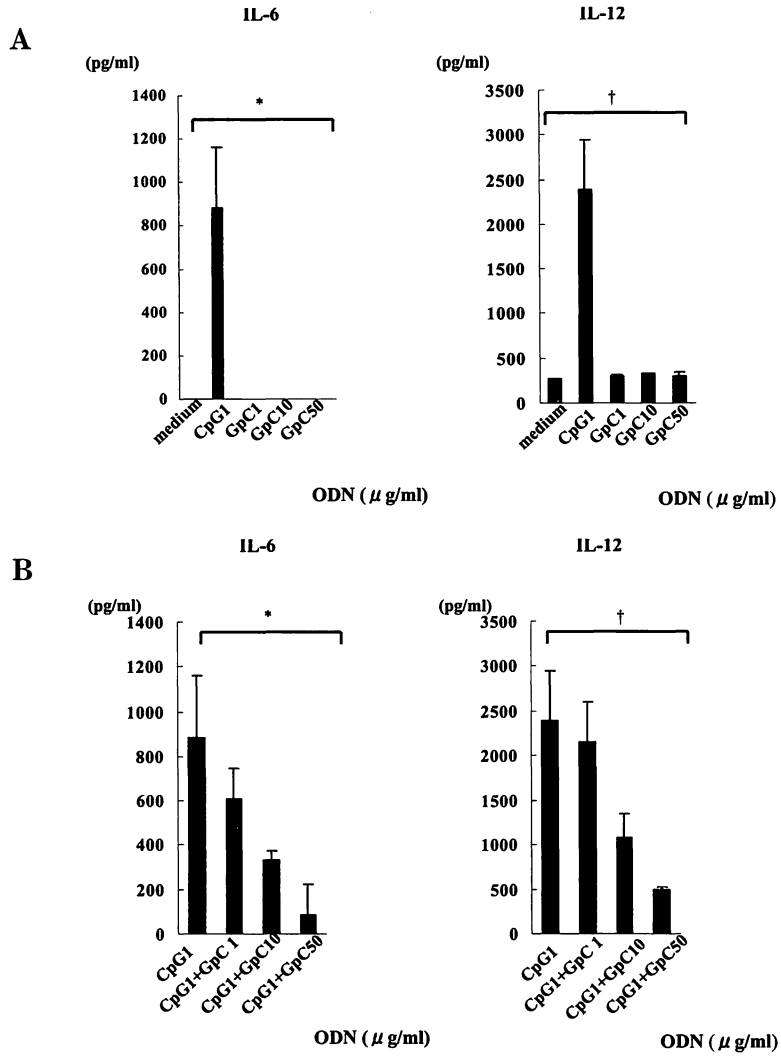

Fig. 1. Cytokine analysis. A, Whole splenocytes from MRL-lpr/lpr mice were cultured with phosphorothioate backbone CpG and/or GpC ODNs. GpC ODN does not induce cytokine production in splenocytes. IL-6 and IL-12 in culture supernatants of splenocytes from MRL lpr/lpr mice were measured by ELISA. Data represents the mean±SD of three independent experiments. Values are analyzed by the Kruskal-Wallis test. (*, $p=0.0080$; †, $p=0.033$) B, CpG ODN-induced cytokine production was blocked by GpC ODN in a dose-dependent manner. IL-6 and IL-12 in culture supernatants of splenocytes from MRL lpr/lpr mice were measured by ELISA. Data represents the mean±SD of three independent experiments. Values are analyzed by the Kruskal-Wallis test. (*, $p=0.021$; †, $p=0.024$)

dsDNA Ab and RF showed no statistically significant differences between the two groups (Fig. 2A, 3). The anti-dsDNA Ab isotypes were also measured. No significant differences in IgG2a/IgG1 ratios were found (Fig. 2B).

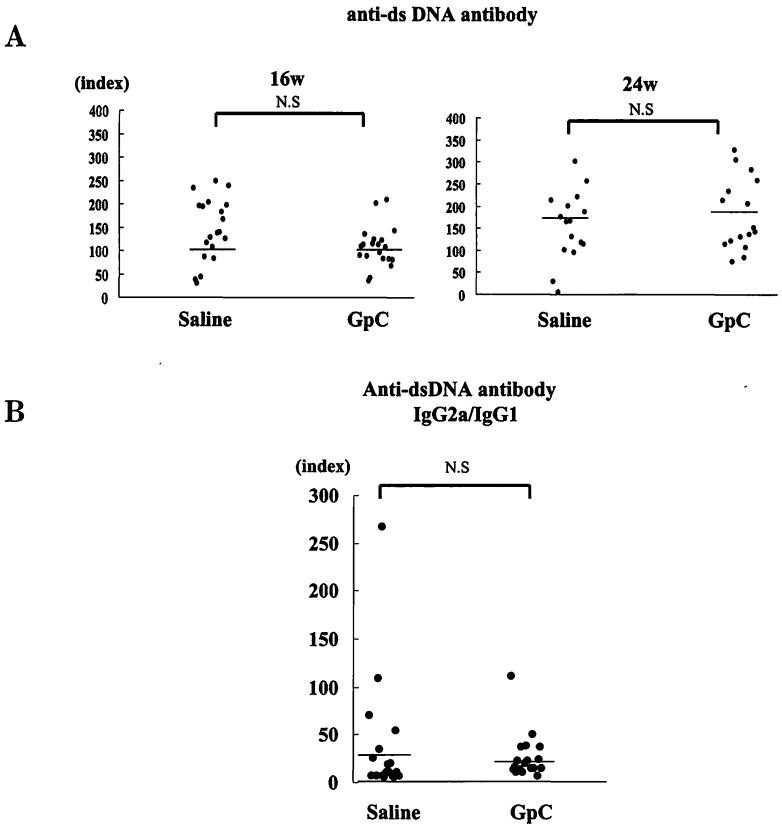

Fig. 2. Measurement of anti-dsDNA Ab. A, Levels of anti-dsDNA Ab in mice sera were measured by ELISA. Data presented are the index values at 16 and 24-wks old. The index values of ELISA were defined as (OD_{405} of serum sample $-$ OD_{405} of negative control)/(OD_{405} of positive control $-$ OD_{405} of negative control) $\times$ 100. Statistical analysis showed no significant differences. B, The level of each anti-dsDNA IgG isotype was measured by ELISA. The titer was calculated as described in materials and methods. GpC ODN had no effect on class switching of anti-dsDNA IgG isotype. Data presented are IgG2a/IgG1 ratios at 22-wks old.

Serum cytokine analysis

In addition to autoantibodies, cytokines are also pivotal for the modification of the progression of autoimmune glomerulonephritis. Th1 cytokines, such as IL-12, IL-18 and IFNγ, play an important role in exacerbating autoimmune nephritis in MRL-lpr/lpr mice[27-32]. Serum IL-12 levels in the GpC group of mice tended to be reduced compared with those of the saline-injected control mice; however, the reduction was not statistically significant (Fig. 4).

BUN and urinary protein excretion

Glomerulonephritis usually leads to renal failure in MRL-lpr/lpr mice, and it is

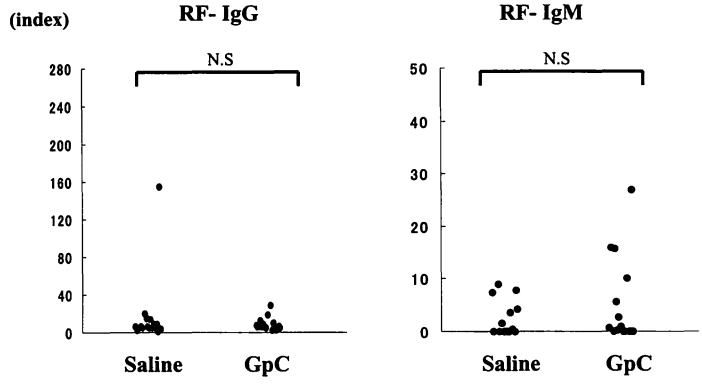

Fig. 3. Levels of RF-IgG and IgM in mice sera were measured by ELISA. Data presented are the index values at 24-wks old. The index values of ELISA were defined as in anti-dsDNA Ab measurement. Statistical analysis showed no significant differences.

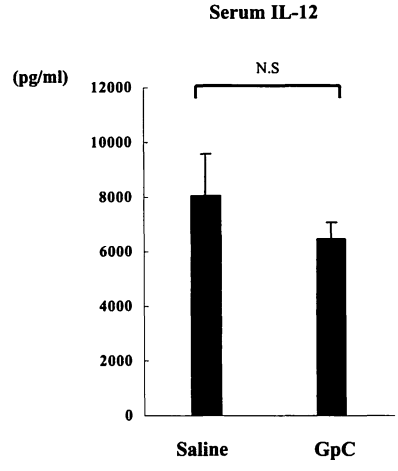

Fig. 4. Serum IL-12 levels at 23 to 24 wks were measured in 19 mice from each group. The bar represents the mean±SE. Although not statistically significant, there was a tendency for increased IL-12 in the saline-injected control group.

a major cause of mortality in this strain. To investigate the effect of ODN on renal disease, BUN levels were measured, and serial urine samples were assessed for 24-h protein excretion. Some mice in the saline-injected control group showed abnormally increased BUN levels, which indicate the loss of renal function. The GpC group showed consistently low BUN levels. This result was statistically significant at 22 wks (Fig. 5, $p=0.0406$). In the urine study, 62.5% and 87.5% of urine samples in the saline-injected control group were found to exhibit proteinuria (>2 mg/mouse/day) at 22 wks and 24 wks, respectively. On the other hand, the onset of proteinuria was delayed in the GpC group. Only 25% of samples in the GpC group were found to have proteinuria at both 22 wks and 24 wks. There was a statistically significant difference ($p=0.036$) in this result at the age of 24 wks (Table 1).

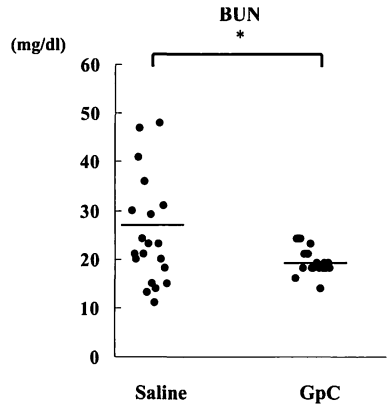

Fig. 5. The BUN at 20, 22 and 24 wks was measured in 19 to 20 mice from each group. The saline-injected control group had a higher average BUN at all time points. The data from mice that were 22-wks old are statistically significant (*, $p=0.041$).

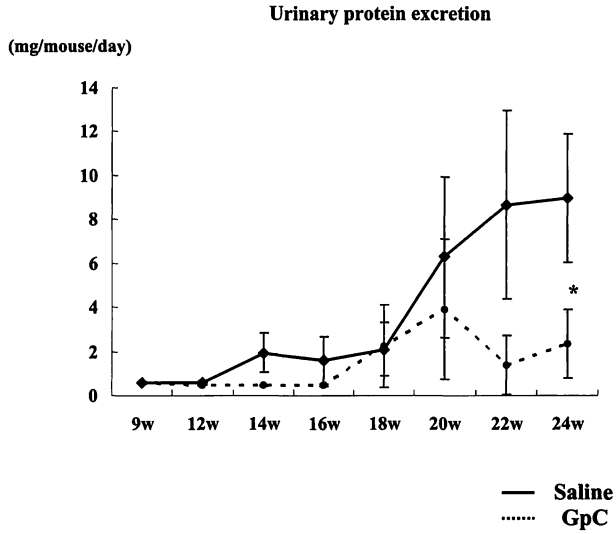

Fig. 6. The results from urinary protein excretion assays are demonstrated. Data represents the mean 24-h protein excretion±SE of 8 metabolic cages/group. The GpC group (dotted line) showed significantly reduced protein excretion compared with the saline-injected control group (solid line) at 24-wks old (*, $p=0.0157$).

The mean 24-h protein excretion was also analyzed. GpC ODN reduced the mean amount of urinary protein; this result reached statistical significance ($p=0.0157$) at 24 wks (Fig. 6).

Renal pathology and immunofluorescence analysis

Three mice from each group were randomly selected and sacrificed at 23 wks. The kidneys were removed for histological evaluation of glomerulonephritis.

Table 1. The percentage of proteinuria

	Proteinuria (%) (n=8-10)						
Week	9	16	18	20	22	24	26
Saline	0	0	25	50	62.5	87.5	62.5
GpC	0	0	12.5	12.5	25	25*	25

Urine protein excretion of > 2 mg/mouse/day is considered to be proteinuria. The difference between the saline injected group and the GpC ODN injected group was significant at the age of 24-wk-old.
*Result showing the statistical significance of $p=0.036$

Table 2. Histological Evaluation

	Saline	GpC ODN
Cell counts (cells/glomerulus)	56.7±17.2	46.8±8.4*
Diameter of glomerulus (μm)	100.5±16.5	87.5±11.8*

Three mice form each group were sacrificed at 24-wk-old. Values are means±SD from 60 glomeruli per group.
*Result showing $p<0.01$ of statistical significance.

Prominent cell infiltration and glomerular proliferation were observed in kidneys from both groups. However, the average number of cells in the glomeruli was significantly decreased in the GpC group compared with that of the saline-injected control group. Furthermore, the mean glomerular diameter was significantly smaller (a reflection of cell infiltration) in the GpC group (Table 2, Fig. 7A). On the other hand, immunofluorescence staining analysis for C3 and IgG deposition showed no significant differences between the groups (Fig. 7B).

Survival

Mice in the GpC group had significantly decreased mortality compared with mice in the saline-injected control group (GpC, 12% mortality; saline, 53% at 30 wks) (Fig. 8). The difference in survival between the GpC group and the saline-injected control group was statistically significant ($p=0.0177$).

DISCUSSION

1720 GpC ODN, which we used in this study, has the same sequence as immunostimulatory 1668 phosphorothioate CpG ODN except that CpG was replaced with guanosine-phosphate-cytosine, GpC, dinucleotide. This synthesized GpC ODN has no immunostimulatory effect and is often used as a control ODN. Lately, some non-CpG ODNs, including phosphodiester GpC ODN, have been reported to inhibit the stimulatory effect of CpG ODN[33]. In the present study, we investigated the inhibitory effect of phosphorothioate GpC ODN, and we examined whether this ODN

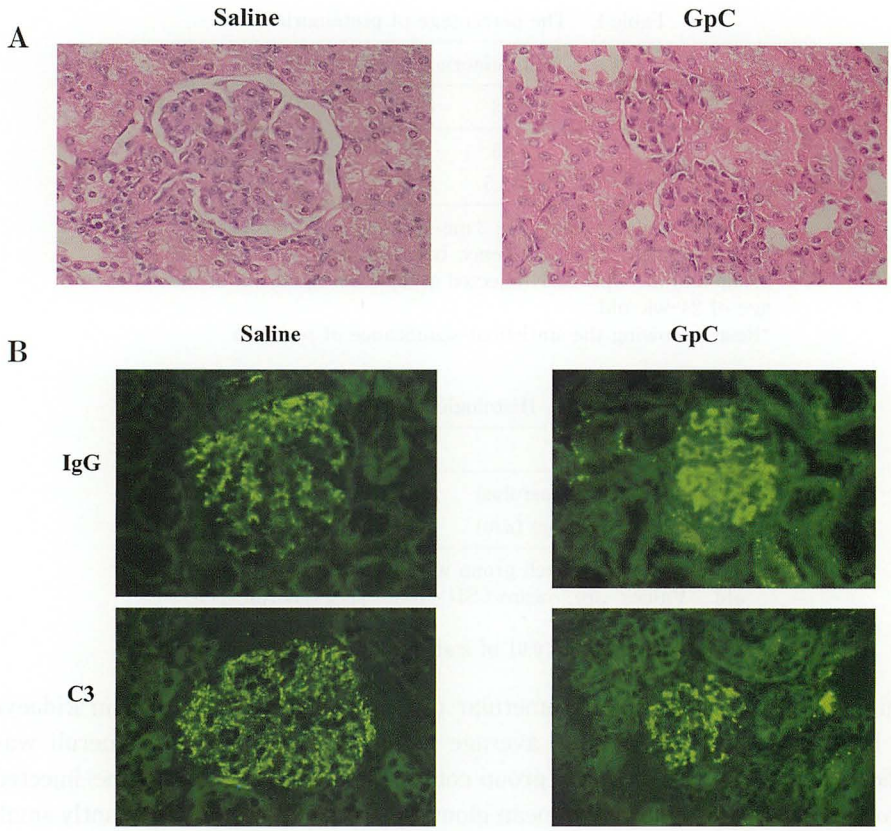

Fig. 7. Pathological evaluation. A, Effects of GpC ODN injections were evaluated on H & E staining. Severe cell infiltration and enlarged glomeruli were observed in the kidneys of saline-injected mice. The data is summarized in Table 2. B, Immunofluorescence staining analysis of IgG and C3 deposition in the kidneys of 24-wk old MRL-lpr/lpr mice. Neither the fluorescence intensity nor the distribution of deposition was significantly different between the saline-injected mice and the GpC ODN-injected mice.

ameliorates the progression of autoimmune glomerulonephritis in lupus-prone MRL lpr/lpr mice.

In vitro, GpC ODN showed an inhibitory effect on IL-6 and IL-12 production from splenocytes stimulated by CpG. *In vivo*, GpC-injected mice had reduced proteinuria, low BUN levels and prolonged survival. Although serum levels of autoantibodies and isotypes were identical between the two groups, IL-12 showed a tendency to be reduced in GpC-injected mice compared with saline-injected control mice. Histopathologically, glomeruli from the GpC group had less cell proliferation, less infiltration, and a smaller diameter. There was no significant difference in the intensity of IgG and C3 deposition between the two groups. We speculate that cytokine milieu modulation by inhibitory GpC ODN is relevant to delaying the progression of renal disease in MRL-lpr/lpr mice.

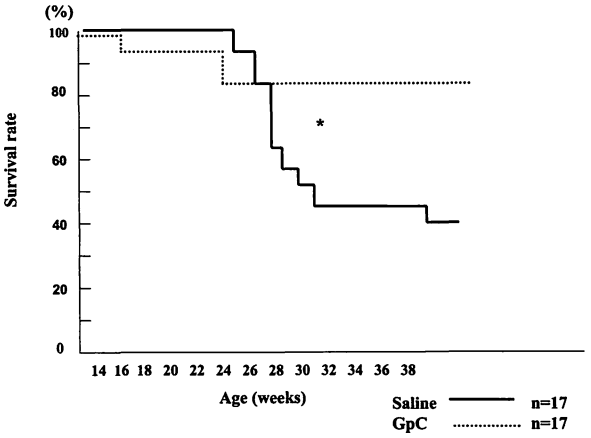

Fig. 8. Data presented are the percentage of surviving mice plotted against their age in weeks. The survival of 17 mice in each group injected with saline (solid line) and GpC (dotted line) are indicated. Survival in the GpC group was significantly greater than in the saline group (*, $p=0.0157$).

Internalized CpG ODN co-localizes with TLR9 in lysosomal compartments and initiates cell signaling transduction. This leads to the activation of downstream kinases, such as IκB kinase, resulting in translocation of NF-κB into the nucleus to induce proinflammatory cytokines such as IL-6 and TNF. Competition of ODN cell surface binding and uptake has been reported to explain the inhibitory effect[34]. But, there is opposing data showing that neither binding nor uptake is prevented by inhibitory ODN[9]. Moreover, delayed addition of inhibitory ODN can still block the effects of stimulatory ODN, suggesting the existence of an unknown intracellular active signaling pathway used by inhibitory ODN[35].

MRL-lpr/lpr mice develop autoimmune glomerulonephritis. Renal failure is the most common cause of death in this strain. Th1-type cytokines, such as IL-12, IL-18 and IFN-γ, are known to play important roles in the progression of glomerulonephritis. Administration of synthesized CpG ODN exacerbates autoimmune glomerulonephritis[36,37], suggesting innate hypomethylated CpG DNA and/or CpG DNA from microorganisms to be the factors that aggravate disease activity. CpG ODN increases the serum IgG DNA autoantibodies, especially IgG2a, which are correlated to disease aggravation. But, our result showed a significant improvement in renal disease without a change in the level or isotype of autoantibodies. The inhibitory effect on cytokine production and the tendency for reduced serum IL-12 levels may explain that changes in the cytokine milieu are possibly responsible for the less severe glomerulonephritis of GpC-injected mice. In mice with glomerulonephritis, administered ODNs are detected in the glomeruli with a capillary and mesangial pattern[36,38,39], indicating that infiltrating immune cells can respond to these ODNs. A recent study reported that administration of polyinosinic-cytidylic acid, an analogue of viral dsRNA, aggravated glomerulonephritis in MRL-lpr/lpr

mice. In that study, serum DNA autoantibody levels were unaltered, but IFN-α, IL-12p70 and IL-6 levels were increased. The authors concluded that the process of aggravation of renal injury was facilitated through activation of TLR3-expressing antigen-presenting cells and mesangial cells[40]. TLR9 is not expressed on mesangial cells; but, immune cells infiltrating into inflammatory sites may have taken up GpC ODN to reduce pathological cytokine production.

Another previous study, which used almost the same GpC ODN and the same mouse strain, reported that GpC ODN showed no effect on improving glomerulonephritis, although the mice received a greater amount of GpC ODN than in our experiment[36]. This discrepancy may be explained by the difference in the time of evaluation. In the previous study, mice were sacrificed at 18 wks and no effects of GpC were found. Our results also showed no significant effect at the same time point. We found a significant effect of GpC ODN on reduced urine protein excretion in mice at 24 wks.

There are two recently reported studies showing that the inhibitory ODN successfully delayed the onset of glomerulonephritis in MRL-lpr/lpr and NZB$\times$NZW mice[39,41]. Significantly reduced levels of serum autoantibody, which are inconsistent with our data, may have been caused by the administration of high-dose inhibitory ODN, the difference in the inhibitory ODN sequence, or the difference in the mouse strain. It should be considered that GpC ODN can act on not only immune cells, but also some other cell types, such as stromal cells, to modulate the course of glomerulonephritis through unknown mechanisms. Further experiments are required to elucidate the precise mechanisms responsible for our observation.

In summary, inhibitory oligodeoxynucleotide improved glomerulonephritis and prolonged survival in MRL-lpr/lpr mice. Further studies of GpC ODN may provide a feasible therapeutical strategy for the treatment of autoimmune diseases such as lupus nephritis.

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