Nivalenol as a possible risk in IgA nephropathy

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Introduction

The pathogenesis of IgA nephropathy (IgAN), the most common chronic glomerulonephritis in Japan, has never been elucidated. IgAN is characterized by its features: IgA is predominantly deposited in glomeruli with various grades of mesangial proliferation in every patient, and serum IgA levels are elevated in nearly half of IgAN patients1). It is also recognized that IgA specific regulatory lymphocytes are well represented in the mucosal immune system and IgA production is initiated primarily in this system. In light of these facts, we hypothesized that IgAN is triggered by some exogenous antigen(s) which induces dysregulation of the mucosal immune system. Based on this hypothesis, we have created a reproducible IgAN model in mice which is orally induced by 8-week-administration of a mycotoxin, nivalenol (NIV). It was further examined whether immunological alterations of lymphocytes isolated from Peyer's patches (PP), an important IgA inductive site or gut-associated lymphoid tissues (GALT), occurred in the NIV-induced IgAN. Moreover, we performed a long-term administration of NIV in mice and consecutively examined its IgAN-like changes. To evaluate how NIV histopathologically influences the intestinal tract, intra-intestinal injection of NIV was preliminarily carried out in mice.

In the following experiments, differences between different study groups of mice were analyzed by Student's t-test or by Mann-Whitney U test for nonparametric analyses.

The NIV model

First of all, an experimental IgAN model was established by oral administration of NIV. Six-to-week-old female C 3 H/HeN and C 3 H/HeJ mice were obtained from CLEA Japan (Tokyo, Japan). Mice were fed semi-synthetic diets containing 6 or 12 ppm NIV for 4 or 8 wk. Control mice were fed on the same semi-synthetic diets without NIV. All mice were given these diets ad lib.

After completion of the oral presentations, the levels of IgG, IgM and IgA in the sera were determined by enzyme-linked immunosorbent assay (ELISA)2-4). Sections of renal cortex were examined by immunofluorescence for glomerular immunoglobulin (Ig) and C 3 deposition, and by light and electron microscopy for histopathological changes5).

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Fig. 1  Intensity of IgA deposits in the mesangium in mice fed regular diets or those containing NIV for 4 or 8 wk. For comparison, ferritin at 10 mg in 0.2 ml was also orally given to mice by gastric intubation on the first day, followed by 1 mg/ml of drinking water for 4 wk based on the method of Genin and colleagues\(^{11}\). The intensity of immuno-fluorescence for IgA in each group was graded from 0 to 3; 0 = no deposit, 1 = weakly stained, 2 = moderately stained, 3 = strongly stained. Each column shows percentage of kidney samples belonging to each grade. Each group included nine to twelve mice.
Table 1 Serum Ig levels in C3H/HeN and C3H/HeJ mice administered NIV 12 ppm for 8 wk.

<table>
<thead>
<tr>
<th>Strain</th>
<th>NIV (ppm)</th>
<th>Serum Ig (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IgA</td>
</tr>
<tr>
<td>C3H/HeN</td>
<td>0</td>
<td>619±58</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td>12</td>
<td>25,584±6,300*</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>643±116</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>13,118±2,360*</td>
</tr>
</tbody>
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Data are expressed as means ± SEM.  
* P<0.001

Oral administration of NIV in low doses reproducibly induced marked IgA deposits in glomerular mesangium and significantly elevated serum IgA levels in mice (Fig. 1 and Table 1), findings not seen in those of controls. These changes were also confirmed in BALB/c mice. Electron microscopy revealed mesangial expansion and numerous electron dense deposits, small and large, scattered in the mesangial and paramesangial areas of glomeruli in NIV-fed mice (data not shown). The degree of these immunopathological changes analogous to human IgAN was associated with the dose and duration of NIV presentation. The findings above indicate that NIV induces some pathological changes in mice irrespective of the strain which resemble those in human IgAN, and that this mycotoxin is associated with the pathogenesis in some types of glomerulonephritis.

**Long-term administration of NIV**

IgAN is a slowly progressive glomerulonephritis in most cases, though about 25 to 50% of patients with this disease would reach end-stage renal failure 20 years after the onset. Therefore, we thought it would be meaningful to examine whether NIV administration for a longer period seriously damages glomeruli in mice after the time when the standard IgAN-like changes have been induced by 8 week-administration of this mycotoxin.

Long-term oral administration of NIV 12 ppm for 12 months significantly increased serum IgA levels as well as the intensity of glomerular IgA deposition in mice over time (Fig. 2 and 3). Histopathological examination also revealed more marked mesangial expansion in these mice (data not shown). These results indicate that persistent NIV administration is important to induce and maintain the IgAN-like pathological changes. However, neither diffuse global sclerosis of glomeruli nor interstitial fibrosis suggesting serious renal injury were observed even after 12 months of NIV administration. In addition, renal dysfunction was not induced by long-term NIV administration (data not shown).

**Immunological abnormalities in GALT of the NIV model**

Next, our emphasis was focused on the effect of orally administered NIV on Peyer's patch lymphocytes (PPL) and their immunopathological contribution to the development of IgAN. ELISPOT procedures were performed to more thoroughly examine the dysregulated immunoglobulin production of PPL in the NIV model. Namely, the number of IgA, IgG and IgM-
Fig. 2 Intensity of IgA deposits in the mesangium for a long-term NIV administration. The intensity of immunofluorescence for IgA in each group was graded from 0 to 3; 0 = no deposit, 1 = weakly stained, 2 = moderately stained, 3 = strongly stained. However, there was no kidney sample graded 0 for mesangial IgA deposition in any group. The intensity of IgA deposition in age-matched controls (left) and mice given NIV 12 ppm (right) was compared. Each column shows the percentage of kidney samples belonging to each grade. Each group included 10 to 15 mice. * P < 0.01 ** P < 0.001

Fig. 3 Time course of serum IgA levels in age-matched controls and mice given NIV 12 ppm. Left columns (○) show serum IgA levels in controls and right columns (■) show those in NIV-treated mice. Results were presented as the mean ± SEM. * P < 0.05 ** P < 0.01

producing cells were quantitated utilizing ELISPOT assay. The number of Ig-producing cells was also evaluated as well in spleen lymphocytes (SPL) isolated from the NIV model mice. Consequently, significant increase of IgA-producing cells was confirmed with an ELISPOT
Fig. 4 Frequencies of IgA-producing cells in SPL and PPL from the NIV model and controls. Regular diets or those containing NIV 12 ppm were orally administered to mice for 8 wk. After the administration period, PPL and SPL were cultured in the plates, and then final results were obtained with $1 \times 10^5$ feeding cells/well for IgA producing spots detected by ELISPOT assay. Results were presented as the mean ± SEM. * P<0.05.

Fig. 5 Analysis of cytokine mRNA expressions in CD4+ T cells from Peyer’s patches (PP) and spleens (SP) by RT-PCR. Controls were obtained from mice fed regular diets. Experimental cells were obtained from mice fed diets containing NIV 12 ppm for 8 wk. Each left lane shows mRNA expression in controls. Each right lane shows mRNA expression in experimental cells. ST: ø X174 RF DNA/Hae III fragment ladder. 1: β-actin (349 bp) 2: IFN-γ (460 bp) 3: IL-2 (502 bp) 4: IL-4 (399 bp) 5: IL-5 (243 bp) 6: IL-6 (155 bp) 7: IL-10 (455 bp) 8: TGF-β (525 bp)

procedure in PPL from the model mice (Fig. 4).

For the detection of IL-2, IL-4, IL-5, IL-6, IL-10, TGF-β and IFN-γ specific mRNA in CD4+ T cells isolated from PP of the NIV model mice, a modified standard RT-PCR amplification protocol was employed. Details of this procedure followed the method previously reported by Hiroi and colleagues89. As a result, upregulation of CD4+ T cells was also confirmed in PP of the model mice by cytokine-specific RT-PCR technique, where markedly high levels of mRNA specific for IL-4, IL-5, IL-6, IL-10 and TGF-β were noted in these cells (Fig. 5). The expressions
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of IL-4, IL-5, IL-6, IL-10 and TGF-β specific mRNA were also enhanced in CD 4+ T cells isolated from the SPL of the model mice fed with NIV 12 ppm for 8 wk just as in those of the PPL, though the expressions were slightly less intense in CD 4+ T cells of the SPL.

Given all of these facts above, it is suggested that PPL are immunologically dysregulated in the NIV-induced IgAN, and that this dysregulation in the mucosal immune system may be associated with the pathogenesis of IgAN.

Intra-intestinal injection of NIV

NIV 1 mg (50 mg/kg wt) diluted in alcohol or saline phosphate buffer was preliminarily injected into the duodenum in C3H/HeN mice to examine histopathological changes in the intestinal wall directly induced by NIV. NIV-injected mice were sacrificed 24 hr later, and sections of the small intestine were examined by light microscopy and compared with those in controls. Consequently, not only necrotic damage was observed in the proximal portion of the small intestine close to the injection site (data not shown) but also the number of PAS-positive goblet cells (GC) looked decreased even in the mid-portion of the small intestine in the NIV-injected mice compared with that in controls. For example, the number of PAS-positive GC in the cross section of intestinal walls was 489 ± 271 /mm² in mice injected with diluted NIV in alcohol, significantly lower than that in controls (1,022 ± 307 /mm²). This finding was unexpectedly obtained and of particular interest though it remains to be disclosed whether a lot of GC were damaged and collapsed or intracellular mucin was simply secreted from the cells without any marked morphological change in GC because PAS staining only colored mucinous products purple red in cells.

Conclusion

It was confirmed that NIV reproducibly and strain-nonspecifically induces pathological changes in mice which resemble those in human IgAN. IgAN-like changes in mice were exacerbated over time in a period-dependent manner with NIV administration. It was further suggested that PPL are immunologically dysregulated in NIV-induced IgAN, and that dysregulation in the mucosal immune system might be associated with the pathogenesis of IgAN.

As previously reported by Pestka and his colleagues10, there seem to be some other mycotoxins which can develop serious pathological changes in mice which resemble human glomerulonephritis. This fact, however, is not well known among nephrologists. Therefore, we finally suggest a possibility to nephrologists that mycotoxins such as NIV might have some etiologic role at least in some types of glomerulonephritis.

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