Immunological Abnormalities in Family Members of Patients with IgA Nephropathy

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We examined several immunological parameters such as the number of T cells with CD4 antigen and the receptor for the Fc portion of IgA (Tα cells), in vitro immunoglobulin production by peripheral blood lymphocytes with or without pokeweed mitogen and serum levels of immune complexes in 19 healthy family members of patients with IgA nephropathy (IgAN). It was shown that the levels of serum IgA, T4 cells, CD4/CD8 ratio of T cell subsets, Tα cells, IgA circulating immune-complexes and spontaneous synthesis of immunoglobulins were significantly increased in family members of patients with IgAN. No family members had proteinuria or hematuria. It was concluded that immunological abnormalities, including abnormalities of T cells and B cells, were found not only in patients with IgAN but also in their healthy family members. It was suggested that some factors in addition to these immunological abnormalities may be involved in the development of IgAN.

Key words: Tα cells, Family study, Cellular immunity

IgA nephropathy (IgAN) is the most common primary glomerulonephritis in Japan as well as in several other countries (1, 2). A marked geographical heterogeneity has been observed in the incidence of this disease (2). Cases of familial IgAN (3), an occasional association of IgAN with certain HLA antigens (4, 5) and polymorphism in the immunoglobulin heavy chain switch region (6) have been reported. These results suggested that some genetic factors might be involved in the development of IgAN.

IgAN is considered to be an immune-mediated disorder (7) and many immunological abnormalities have been observed, including elevated serum IgA levels (8), enhanced production of IgA by peripheral blood lymphocytes (9, 10), increases in IgA-bearing lymphocytes (11), elevated CD4/CD8 ratio in T cell subsets (12) and various types of autoantibodies of the IgA class in the sera (13, 14). Some of these immunological abnormalities have also been observed in healthy relatives of patients with IgAN. IgA-bearing lymphocytes were increased in some of the family members of patients with IgAN (15). B cells of healthy relatives of patients with IgAN showed an enhanced production of immunoglobulin (10, 16, 17).

IgA synthesis is thought to be highly T cell dependent. IgA-specific switching T cells were found in murine Peyer's patches (18). It was shown that T cells with the receptor for the Fc portion of IgA (Tα cell) had IgA-specific helper activity (19, 20) and that these cells might play some role in IgA-specific switching (21).

In the present study, T cell subsets including Tα cells, and in vitro immunoglobulin production in healthy relatives of patients with IgAN were investigated to elucidate immunological abnormalities in family members of patients with IgAN.

MATERIALS AND METHODS

Subjects

Nineteen first-degree relatives (11 parents and...
eight siblings) in eight families of patients with IgAN were studied. Fifteen healthy adults served as controls. The diagnosis of IgAN was confirmed by open renal biopsy, followed by light microscopic and immunofluorescence studies. Systemic diseases such as lupus erythematosus, rheumatoid arthritis, diabetes mellitus and liver diseases were excluded. None of the patients took any medications prior to this study. All patients had proteinuria and/or microscopic hematuria, but none of them had elevated levels of serum creatinine or blood urea nitrogen. None of the family members or healthy controls had proteinuria, microhematuria or any other abnormalities in urinary sediments. Blood samples were collected early in the morning before breakfast.

**Purification of human IgA myeloma protein**

Human IgA protein was purified from IgA1 (lambda) myeloma serum by jacalin affinity column chromatography (22). Briefly, the IgA-rich fraction was precipitated by 50% ammonium sulfate saturation, and was applied to a jacalin column (Sepharose 4B coupled with protein extracted from the seeds of the jack fruit). After washing with borate-buffered saline, IgA was eluted with 0.8 M galactose solution. Purified IgA showed a single precipitating line against anti-whole human serum as examined by immunoelectrophoresis; it was not contaminated with IgG or IgM as proven by enzyme-linked immunosorbent assay (ELISA). Polyacrylamide gradient gel electrophoresis (PAA2/16, Pharmacia, Piscataway, NJ) showed that this fraction contained polymeric IgA1. This purified human IgA myeloma protein was conjugated with FITC using the method of Kawamura (23). The F/P ratio of this FITC-labeled IgA was 1.8.

**T cell subsets**

Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Paque (Pharmacia LKB Biotechnology Inc.) density gradient centrifugation after collection of heparinized blood. Monocytes and macrophages were removed by an adherent procedure. Phycoerythrin-labeled anti-CD3, CD4 and CD8 (OKT3, OKT4 and OKT8, Ortho Diagnostic Systems Inc., Raritan, NJ) were added to one million mononuclear cells and incubated for 30 min on ice. After washing three times with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), the receptors for the Fc portion of IgA (Fc α R) were stained by FITC-labeled human IgA for 30 min on ice. To examine the specific binding of IgA to Fc receptor of IgA on T cells, human IgG, IgM or IgA was added to FITC-labeled IgA. Two-color fluorescent flow cytometry (FACStar, Becton Dickinson, Mountain View, CA) was employed to enumerate Tα cells which had CD3, CD4 or CD8 antigen and Fc α R. A pure lymphocyte population was obtained by two-dimensional gating (forward light scattering vs. side light scattering). Ten thousand lymphocytes were enumerated each time to study T cell subsets.

**Bα cells**

When measuring Tα3 cells (Tα cells with a CD3 surface marker), the number of non-T cells with receptors for the Fc portion of IgA was enumerated; these cells were defined as Bα cells. The subset of non-T3 cells included less than 0.5% monocytes, as confirmed by flow cytometry.

**Cell culture conditions**

After separating mononuclear cells from peripheral blood, washed cells were cultured at a concentration of 1 x 10⁶ cells/ml with or without the addition of 10 μg/ml of pokeweed mitogen (PWM, Sigma, St. Louis, MO) in RPMI 1640 containing 100 U/ml of penicillin and 100 μg/ml of streptomycin supplemented with 10% heat-inactivated fetal calf serum (GIBCO, Grand Island, NY) at 37°C in 5% CO₂ in air at 100% humidity. The culture supernatants were collected on day 7 and stored at -20°C until assay.

**Enzyme-linked immunosorbent assay**

The concentrations of IgG, IgA and IgM in the supernatant of lymphocyte cultures were measured by ELISA as follows. A 96-well polystyrene microplate (Linbro/Titertek, Flow Laboratories, Netherlands) was coated with F(ab')2 fragments of goat anti-human IgG, IgA or IgM antisera (Cappel Laboratories, Malvern, PA), and then blocked with 1% BSA in PBS. Appropriately diluted samples were added to the wells, incubated at 4°C overnight, and then washed three times with 0.1% Tween in PBS. After incubation with alkaline phosphatase-conjugated affinity-purified goat anti-human IgG, IgA and IgM antisera (Sigma), the wells were washed and p-nitrophenyl phosphate disodium solution (Sigma) was added. The optical absorption was measured at 405 nm.
measured by a microplate photometer (Model MTP-22, Corona Electric, Japan) at a wave length of 405 nm. Log linear curves were obtained from standard sera and were used for quantitation of immunoglobulins.

**Concentration of serum immunoglobulins**

The levels of serum IgG, IgA and IgM were measured by laser nephelometry (Behring Institute, West Germany).

**Levels of circulating immune-complexes**

The levels of IgG and IgA immune-complexes were measured by solid phase anti-C3 Facb ELISA methods (Teijin Lab, Tokyo, Japan) (24).

**Statistical analysis**

The Mann-Whitney U test and the Spearman-Kendall test were employed for statistical analysis.

**RESULTS**

**Levels of serum IgA**

As shown in Fig. 1, the serum levels of IgA were significantly increased not only in patients with IgAN but also in their healthy relatives (IgAN patients: 429±138 mg/dl; family members: 332±111 mg/dl; controls: 269±82 mg/dl, p<0.01). There were no differences in the levels of serum IgG and IgM among these three groups.

**Ta cells**

Figure 2 shows the amount of Ta 3 cells. No significant differences in the number of Ta 3 cells were observed among IgAN patients, their family members and controls (patients: 5.8±2.6%; family...
The number of Tα4 cells was significantly increased in patients with IgAN (5.5 ± 3.0%; controls: 2.3 ± 1.0%, p < 0.005). Their relatives also had significantly elevated levels of Tα4 cells (3.9 ± 2.1%, p < 0.01). Patients with IgAN had higher levels of Tα8 cells than their family members (p < 0.05). The number of Tα8 cells was significantly higher in patients with IgAN (6.0 ± 2.5%; controls: 3.9 ± 1.2%, p < 0.01). Some of the family members had more Tα8 cells in their peripheral blood than controls although statistical analysis showed no significant differences (family members: 4.3 ± 2.1%). No correlations were observed between increased Tα cells and histological severity, the level of proteinuria or other clinical data in patients with IgAN. The binding of IgA to Fc receptor of IgA on T cells was not inhibited by IgG or IgM; it was only inhibited by IgA. Therefore, IgA binding to T cells was considered as an immunoglobulin class-specific phenomenon.

**Bα cells**

The percentages of Bα cells in patients with IgAN, family members and controls were 5.9 ± 3.4%, 6.4 ± 4.3% and 4.3 ± 3.1% (Fig. 3). Three out of eight patients and eight of 19 family members showed an increase in Bα cells compared to the control group although there was no statistical significance.

**CD4- and CD8-positive T cells and CD4/CD8 ratios**

CD4-positive T cells was increased in the peripheral blood from patients compared with controls (patients: 45.0 ± 5.8%; controls: 39.3 ± 5.1%, p < 0.05). A significant increase in CD4 cells in family members was also observed (family members: 48.7 ± 8.4%, p < 0.005). There were no significant differences in the amount of CD8-positive T cells among these three groups. The ratio of CD4/CD8 was significantly increased in family members com-
pared with controls (family members: 1.86 ± 0.59; controls: 1.31 ± 0.17, p<0.005). Only one patient and about one half of the family members had a CD4/CD8 ratio of more than 1.65 (mean ± 2SD) compared with the control group.

**Circulating immune-complexes (CIC)**

As shown in Fig. 4, the levels of IgA class CIC were significantly increased in the patients with IgAN and their family members compared with controls (patients: 4.8 ± 2.8 µg/ml; family members: 2.8 ± 1.2 µg/ml; controls: 2.1 ± 0.9 µg/ml, p<0.05). There was no significant difference in the levels of IgA-CIC between the patients and their family members. No significant increase in IgG-CIC was observed among these three groups (patients: 3.6 ± 2.3 µg/ml; family members: 2.5 ± 0.8 µg/ml; controls: 2.2 ± 0.7 µg/ml).

**In vitro immunoglobulin production by peripheral blood mononuclear cells (PBMC)**

Significant increases were observed in spontaneous IgA synthesis by PBMC from patients with IgAN (p<0.05) and their family members (p<0.005) compared with the controls (Fig. 5). The degree of the increases of IgA production did not differ in patients and family members. Marked increases in IgA synthesis (p<0.05) were observed in PWM-stimulated PBMC cultures only from family members (Fig. 6).

Results of IgG, IgA and IgM production with and without PWM are summarized in Table 1. Spontaneous IgG, IgA and IgM synthesis was increased in patients and their family members compared with the controls (p<0.05). In PWM-stimulated cultures, significant increases in IgG, IgA and IgM production were observed only in the family members (p<0.05). Immunoglobulin production with PWM in the culture supernatant was not increased in patients with IgAN when compared to the controls. The increased IgA synthesis with PWM was observed in ten of eleven controls, but it was not a statistically increase.

The altered IgA synthesis due to the addition of PWM is shown in Table 2. IgA synthesis in cultures with PWM decreased in all patients compared to those without PWM. Only two persons in the control group showed a decrease in IgA synthesis in the

![Fig. 5. In vitro IgA production by peripheral lymphocytes without pokeweed mitogen (PWM). Patients (p<0.05) and their relatives (p<0.005) produced much more IgA in PBMC culture than controls. There was no difference between patients and their relatives.](image)

![Fig. 6. In vitro IgA production with the addition of pokeweed mitogen (PWM). Lymphocytes from family members showed enhanced IgA production with PWM compared those from patients and controls (p<0.05). No significant increase in IgA production was observed in the patients.](image)
Table 1. Immunoglobulin production by PBMC.

<table>
<thead>
<tr>
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<th>PWM(−)</th>
<th>PWM(+)</th>
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<tbody>
<tr>
<td></td>
<td>IgG</td>
<td>IgA</td>
</tr>
<tr>
<td></td>
<td>1749 ± 1365</td>
<td>1534 ± 1091</td>
</tr>
<tr>
<td>Patient</td>
<td>Family</td>
<td>Control</td>
</tr>
<tr>
<td>(n = 8)</td>
<td>1331 ± 923</td>
<td>383 ± 353</td>
</tr>
<tr>
<td></td>
<td>1709 ± 1093</td>
<td>689 ± 964</td>
</tr>
<tr>
<td></td>
<td>1489 ± 1387</td>
<td>330 ± 335</td>
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<tr>
<td></td>
<td>4214 ± 3232</td>
<td>1767 ± 1108</td>
</tr>
<tr>
<td></td>
<td>2892 ± 3443</td>
<td>758 ± 668</td>
</tr>
<tr>
<td></td>
<td>3231 ± 2228</td>
<td>4567 ± 3672</td>
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* p < 0.05

Without pokeweed mitogen (PWM), patients and family members showed a significantly enhanced production of IgG, IgA and IgM as compared to controls (p < 0.05). When cultured with PWM, only the family members produced a significant amount of IgG, IgA and IgM in comparison with patients and controls (p < 0.05). No increase in immunoglobulin production was observed in the patient.

Table 2. Altered IgA synthesis with PWM compared to that without PWM.

<table>
<thead>
<tr>
<th></th>
<th>Increase</th>
<th>Decrease</th>
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<tbody>
<tr>
<td>Patient (n = 8)</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Family (n = 19)</td>
<td>11*</td>
<td>8</td>
</tr>
<tr>
<td>Control (n = 11)</td>
<td>9</td>
<td>2</td>
</tr>
</tbody>
</table>

*p < 0.05

All patients showed decreased IgA synthesis when cultured with pokeweed mitogen (PWM) compared to that without PWM. An increase in IgA production with PWM was found in two of the control group and in 11 of the family members. About one half of the family members manifested an increase in IgA synthesis, while others showed a decrease in IgA synthesis.

cultures with PWM compared to those without PWM. Eight of 19 family members showed a decrease in IgA synthesis in cultures with PWM. However, other family members showed an increase in IgA synthesis in PBMC cultures with PWM.

DISCUSSION

It has been reported that an elevated serum level of IgA was observed in approximately 50% of patients (8). One-third of the family members of patients with IgAN had higher serum levels of IgA than those of controls (16). In this study, we showed that healthy relatives also had significantly elevated serum IgA levels. Although the elevated serum IgA in patients with IgAN might be due to the hyperproduction of IgA, an impaired clearance of IgA remains a possible cause. IgA synthesis is known to be highly T cell dependent. Activated T cells which have IL-2 (interleukin-2) receptors are reported to be increased in patients with IgAN (25). The helper activity of T cells in patients was found to be parallel with the elevated CD4/CD8 ratio (12). In the current study, CD4-positive T cells were increased in both patients and family members, and the CD4/CD8 ratio was also elevated in family members. It is interesting to note that all patients had at least one relative with an elevated CD4/CD8 ratio (greater than the mean of the controls) in this study. An increased CD4/CD8 ratio might be due to hyperactivity of CD4-positive T cells because the numbers of CD8-positive T cells did not differ among these three groups. T cells with receptors for the Fc portion of IgA (Tα cells) were considered as prospective IgA-specific helper T cells (19, 20). Furthermore, the majority of Tα cells have CD4 antigen on their surface (Tα 4 cells) (26) and these...
cells have been shown to switch from IgM-bearing B cells to IgA-bearing B cells in vitro (21). Tα4 cells have been reported to be significantly increased in patients (27) and the number of Tα4 cells is reported to correlate with the amount of spontaneous IgA synthesis (28). The number of Tα4 cells was also increased in family members in the present study without any significant increase in the number of Tα3 or Tα8 cells. It is suggested that the increase in Tα4 cells might play an important role in the elevated serum IgA in both patients and their family members.

There have been several studies on in vitro immunoglobulin production by lymphocytes from patients with or without the addition of mitogens. Several authors have reported that in vitro IgA synthesis is increased with or without the presence of mitogens (9, 10), but others have indicated no increase (29). In this study, not only the spontaneous production of IgA, but also that of IgG and IgM was significantly increased in both patients and their family members. Enhanced T cell helper activity might have effects on not only IgA but also on IgG and IgM via a non-specific mechanism. However, only family members showed enhanced immunoglobulin production in PWM-stimulated cultures compared with patients and controls. There was no difference in immunoglobulin synthesis by PBMC with PWM between patients and the control group. When IgA synthesis by PBMC with PWM was compared to that without PWM, IgA production with PWM was decreased in all patients. This decrease of IgA synthesis with PWM was observed in two of eleven controls and eight of nineteen family members. The results of the present study suggested that about one half of the healthy relatives had almost the same T cell function as the patients.

Impaired immunoglobulin synthesis by peripheral blood lymphocytes in systemic lupus erythematosus (SLE) is well known (30). One possible explanation for this depressed PWM-stimulated immunoglobulin response is that lymphocytes from patients with IgAN were already activated in vivo and that these preactivated cells did not respond to a second stimulation such as PWM, as observed in SLE. It might also be speculated that helper T cell activity is inhibited in in vitro cultures with PWM because CD4 T cells are known to become refractory to IL-2 growth promotion earlier than CD8-positive T cells (31). This enhanced helper activity of T cells in peripheral blood might be associated with a hyperresponse to exogenous antigens in patients and family members with IgAN. It has been shown that the administration of influenza vaccines to patients with IgAN induces not only an increase in serum levels of IgA-class anti-influenza antibodies but also a simultaneous increase in IgA-class rheumatoid factor (32); this phenomenon was also observed in some healthy relatives of patients with IgAN (33).

The level of IgA-class CIC was shown to be elevated in patients and their relatives. Although the physiochemical properties of these immune complexes were not examined in this study, a mechanism for the enhancement of IgA-CIC might be present in family members of patients with IgAN, probably due to the hyperactivation of lymphocytes.

B cells with receptors for the Fc portion of IgA (Bα cells) have been observed in human peripheral blood (34) and are reported to be increased in the peripheral blood of patients with IgAN (35). Although the role of these cells remains obscure, they are reported to be activated B cells (36). Furthermore, activated B cells with the Fc receptor for IgE, known as CD23, were also increased in patients with IgAN (unpublished data). In this study, more Bα cells were seen in some family members of IgAN patients than in the controls. It is postulated that some of the healthy family members of patients with IgAN might have activated B cells in their peripheral blood.

These observations suggest that several immunological abnormalities might exist even in healthy family members who have no clinical signs of glomerulonephritis. Some unknown factors in the development of IgAN might exist in addition to the dysregulation of IgA synthesis.

In conclusion, healthy relatives of patients with IgAN were “high responders”, especially in IgA synthesis. Several immunological abnormalities, including an increase in the concentration of serum IgA, elevated IgA-CIC and enhanced in vitro IgA synthesis with and without PWM were observed in the healthy relatives of patients with IgAN. These phenomena might be associated with an increase in
T α 4 cells observed not only in the patients but also in the healthy family members. Factors other than the high IgA response might also be involved in the development of IgAN.

ACKNOWLEDGEMENT: The author is grateful to Dr. Masayuki Endoh and Dr. Hideto Sakai for their very helpful technical advice and discussions.

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