Decreased cytokine-induced IgA subclass production by CD40-ligated circulating B cells in primary IgA nephropathy

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Abstract

Background. In IgA nephropathy (IgAN), the abnormalities in the IgA immune system are apparently restricted to the IgA1 subclass in the systemic compartment, as evidenced by the antigen-specific responses to recall antigens. Since precursors of IgA producing B cells in human peripheral blood belong predominantly to the mucosal compartment, we took the opportunity to assess the capacity of circulating B cells in peripheral blood (PBMC) of 20 IgAN patients and matched controls to produce IgA, IgA1, and IgA2.

Methods. Supernatants from T cell- (immobilized anti-CD3) and B cell-specific (CD40 ligation) activated cultures were assessed for immunoglobulin isotypes by ELISA. In addition, we compared the sensitivity of T and B cells to various cytokines (IL-2, IL-10, TGF-β) in both culture systems.

Results. In contrast to significantly higher plasma IgA1 levels ($P<0.01$), no significant differences in salivary IgA1 ($P=0.73$) and IgA2 ($P=0.96$) levels or ratios ($P=0.91$) were found. In the absence of exogenous cytokines, none of the different culture systems led to significant differences in IgA or IgA subclass synthesis by PBMCs of patients and controls. However, in IgAN patients, the addition of IL-2 did not enhance the production of the IgA subclasses as was found in controls. Furthermore IL-10 led to significantly ($P<0.05$) lower IgA1 and IgA2 synthesis in patients than in controls. TGF-β induced suppression of all isotypes in patients and controls. None of the different conditions resulted in a selectively enhanced production of any one of the IgA subclasses. When both IL-10 and TGF-β were added to the cultures, IgM was the predominant immunoglobulin synthesized both in patients and controls with a significantly ($P<0.05$) lower synthesis of IgM, IgG, IgA1, and IgA2 in patients.

Conclusion. These in vitro data suggest that PBMCs from patients contain more mature and further differentiated B cells. However, there was no selective IgA or IgA1 dysregulation of circulating B cells in IgAN. These results do not confirm the widely believed paradigm that patients with IgAN are primary hyperresponders.

Introduction

Primary IgA nephropathy (IgAN) is characterized by deposits of IgA1 in the glomerular mesangium. Although the mechanism of the mesangial deposition is still unclear, it is commonly accepted that the mesangial IgA is derived from the circulation [1,2]. The most consistent observation has been that the abnormalities in the IgA immune system in IgAN are predominantly or exclusively restricted to the IgA1 subclass in the systemic compartment, as evidenced by antigen-specific responses after immunization with recall antigens [3,4]. In contrast, IgA and IgA subclass concentrations in saliva and nasal washes, secretions of the mucosal system, were found to be comparable in patients with IgAN and controls [5]. How upper respiratory tract infections can induce an overproduction of IgA1 by the bone marrow of IgAN patients remains to be elucidated.

Precursors of IgA producing cells in the peripheral blood belong predominantly to the mucosal immune system and contribute very little to the synthesis of circulating immunoglobulins [6,7]. Previous studies of spontaneous and mitogen-induced IgA production by peripheral blood lymphocytes from patients with IgAN have provided conflicting results [8]. Spontaneous or pokeweed mitogen-driven (PWM) culture systems can be biased by the presence of monocytes and T cells or by the activation of only a minor population of B cells [9]. Likewise, Epstein–Barr virus transformation induces immunoglobulin synthesis from a very small subpopulation of B lymphocytes, that is different from the subpopulation activated by PWM [9,10].
The proliferation and differentiation of IgA-producing B cells is regulated by the interaction of antigen presenting cells and T cells and by the generation of cytokines. The interaction of CD40 with its ligand is one of the most prominent molecular interactions in the development of a T-cell dependent humoral immune response. In vitro activation of human B lymphocytes through CD40 crosslinking has been extensively studied and mimicks the in vivo interaction between B cells and activated T cells. These culture conditions allow the proliferation of various B cell populations and induction of isotype switching of which the specificity is subsequently provided by cytokines [11].

Addition of IL-10 to CD40-activated B cell cultures was found to result in enhanced B lymphocyte proliferation, differentiation and the production of considerable amounts of IgM, IgG, and IgA [11]. Furthermore IL-10 upregulates the expression of CD25/Tac on CD40 activated B cells and in this context IL-2 strongly enhanced B cell proliferation [12]. In the presence of IL-10, TGF-β is an IgA-switch factor and addition of this factor to CD40-activated naive B cells cultured with IL-10 induces large amounts of IgA, while inhibiting IgM and IgG production [13].

The precise relationship between the mucosal immune system and the bone marrow response is still unknown, but the expansion of IgA1-producing cells in IgAN appears to be largely restricted to the systemic compartment. This restriction may be caused by the chemical nature of the responsible antigens, or a second explanation could be a more frequent exposure to antigens of specific mucosal sites [7]. The present study examined a third possibility, namely selective dysregulation of IgA1-producing B lymphocytes in patients with IgAN. We assessed the capacity of B cells in peripheral blood of patients and matched controls to produce IgA, IgA1, and IgA2 in T cell- (immobilized anti-CD3) and B cell-specific (CD40 ligation) activated cultures. In addition, the sensitivity to various cytokines in both culture systems was compared.

**Subjects and methods**

**Patients**

The study protocol was approved by the Ethical Committee of the Leiden University Hospital. The subjects included 20 patients (18 males and two females, mean age 36 years; range 19–59 years) with biopsy proven IgAN, defined by mesangial deposits of IgA as the dominant isotype. None of the patients had clinical or laboratory evidence of Henoch–Schönlein purpura, systemic lupus erythematous, or liver disease or received immunosuppressive therapy. Kidney function was normal or mildly impaired (creatinine clearance > 80 ml/min). None of the patients had macroscopic haematuria or proteinuria > 2 g/24 h. As controls, 20 matched healthy volunteers were recruited. Neither patients nor controls had symptoms or signs of mucosal infection in the 2 weeks preceding the study.

**Biological samples**

Sera from patients and healthy volunteers were obtained from fresh venous blood after overnight fasting. At the same time, 5 ml of whole unstimulated saliva was collected by drooling in plastic tubes. After centrifugation to remove insoluble material, the supernatant was collected. To correct for dilution of the saliva samples, the albumin concentrations were determined in each sample by rate nephelometry (Array Rate Nephelometer, Beckman, Brea, CA, USA). Sera and saliva supernatants were aliquoted and stored at −20°C until assayed.

**Cell culture**

PBMCs were isolated by centrifugation using a standard Ficoll–Hypaque (Sigma Chemical Co., St Louis, MO, USA) density gradient. After lysis of contaminating red blood cells and three washings, the cells were placed in complete culture media consisting of Iscove’s modified Dulbecco’s medium (IMDM) glutamax (Gibco BRL, Breda, The Netherlands) with 10% heat-inactivated foetal calf serum (FCS: Hyclone Laboratories Inc., Logan, UT, USA) and ITS (Insulin 5 μg/ml, transferrin 5 μg/ml and selenium 5 ng/ml final concentration; Sigma). The number of viable cells was determined by Trypan blue exclusion in a haemocytometer. The different cell populations present in PBMCs were estimated using human CD40 ligand-transfected L cells (L-CD40L) and L cells were grown in IMDM with 10% FCS and after harvesting by trypsinization non-transfected mouse fibroblast Ltk− cells were used as negative control. The L-CD40L and L cells were grown in IMDM with 10% FCS and after harvesting by trypsinization non-transfected mouse fibroblast Ltk− cells were used as negative control. The L-CD40L and L cells were grown in IMDM with 10% FCS and after harvesting by trypsinization non-transfected mouse fibroblast Ltk− cells were used as negative control. The L-CD40L and L cells were grown in IMDM with 10% FCS and after harvesting by trypsinization non-transfected mouse fibroblast Ltk− cells were used as negative control. The L-CD40L and L cells were grown in IMDM with 10% FCS and after harvesting by trypsinization non-transfected mouse fibroblast Ltk− cells were used as negative control. The L-CD40L and L cells were grown in IMDM with 10% FCS and after harvesting by trypsinization non-transfected mouse fibroblast Ltk− cells were used as negative control. The L-CD40L and L cells were grown in IMDM with 10% FCS and after harvesting by trypsinization non-transfected mouse fibroblast Ltk− cells were used as negative control. The L-CD40L and L cells were grown in IMDM with 10% FCS and after harvesting by trypsinization non-transfected mouse fibroblast Ltk− cells were used as negative control. The L-CD40L and L cells were grown in IMDM with 10% FCS and after harvesting by trypsinization non-transfected mouse fibroblast Ltk− cells were used as negative control.

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The T cell specific activation of B cell proliferation and differentiation was studied using immobilized monoclonal antibodies to CD3 as has been described previously [14]. Culture wells were precoated with OKT3 (anti-CD3, ATCC, Rockville, MD, USA; 200 μl/well at 1/1000 dilution of ascites in sterile phosphate buffered saline [PBS], pH 7.4) overnight at room temperature.

The B cell specific activation of B cell proliferation and differentiation was studied using human CD40 ligand-transfected L cells (L-CD40L) as described [15,16]. The non-transfected mouse fibroblast Ltk− cells were used as negative control. The L-CD40L and L cells were grown in IMDM with 10% FCS and after harvesting by trypsinization and irradiation (80 Gy), used in a final concentration of 5 × 10^4 irradiated cells/well.

Additional cultures in both systems were performed using single growth factors and various combinations of these factors. All growth factors were added at the beginning of the 14-day culture period. Purified human interleukin (IL)-2 (a gift from P Schrier, University Hospital Leiden, The Netherlands), recombinant IL-10 (provided by J. Banchereau, Laboratory for Immunological Research, Sherching Plough, Dardilly, France) and human TGF-β1 (R&D Systems Europe Ltd, Abingdon, UK) were used at a final concentration of 20 U/ml, 50 ng/ml and 1 ng/ml respectively. At the end of the 14-day culture period, the cultures were centrifuged, and the cell-free supernatants stored at −20°C until measurement of immunoglobulin isotypes.

**Enzyme-linked immunosorbent assay**

Culture supernatants were tested for antibodies of the various isotypes by sandwich enzyme-linked immunosorbent assay.
Decreased cytokine-induced IgA subclass synthesis in IgAN

(ELISA) [17]. Polystyrene 96-well ELISA plates (Greiner, Alphen a/d Rijn, The Netherlands) were coated, overnight at room temperature, with 100 µl/well of the capturing antibody, appropriately diluted in PBS. Primary antibodies were heavy chain specific mAb 4E8 (specific for IgA) and heavy chain specific, affinity purified goat F(ab\(^{-}\))\(_2\) fragments against human IgG or IgM (Jackson, West Grove, PA, USA). In the IgA subclass ELISA the primary antibodies were subclass specific mAb 69–11.4 (specific for IgA1) and 16–512-H5 (specific for IgA2). The quality, specificity and sensitivity in measuring IgA subclasses in different body fluids of these mAbs previously has been described in detail [17]. After three washings with PBS containing 0.05% Tween 20 (PBST), non-specific binding sites were blocked with PBST containing 1% bovine serum albumin (Sigma).

Appropriate serial dilutions of sera, saliva supernatants, or cell-free culture supernatants were added to duplicate wells and incubated for 2 h at 37 °C. Serial twofold dilutions of a normal human serum pool (NHS) with known concentrations of IgA, IgA1, IgA2, IgG, and IgM served as a standard. The standard serum yielded increasing OD values in a dose-dependent linear fashion.

Bound immunoglobulins were detected by heavy chain-specific, affinity-purified goat F(ab\(^{-}\))\(_2\) fragments against human IgA (specific for IgA, IgA1, and IgA2), IgG, or IgM coupled to biotin (Tago, Burlingame, CA, USA). Consecutive incubations followed with streptavidin conjugated to horseradish peroxidase (Zymed, Sanbio BV, Uden, The Netherlands) and enzyme substrate (2,2-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid, Sigma) containing 0.0075% H\(_2\)O\(_2\). Between each step the wells were washed three times with PBST.

Optical density (OD) was measured at 415 nm on a microplate reader (Bio-Kinetics Reader EL 312e, Biotek Instruments Inc., Winooski, VT, USA). Concentrations were obtained by interpolation on the standard curves using a four parameter modeling procedure (KinetiCalc, EIA Application Software). The final concentrations in each sample were calculated as the mean of the results at the proper sample dilutions yielding ODs in the linear parts of the calibration curves.

Statistical analysis

All statistical calculations were performed using the SPSS for Windows Release 6.0 software package. The concentrations of IgA and IgA subclasses in body fluids (serum, saliva) and the amounts of immunoglobulin produced in the respective cultures were not normally distributed and were consequently transformed logarithmically prior to analysis. Comparisons between groups for the different culture conditions and the various types of stimulation by cytokines were performed by analysis of variance (ANOVA) for repeated measures, followed by Scheffe’s procedure. P values < 0.05 were considered statistically significant. Results were expressed as their geometric means ± SEM. Data on the the ratio’s of the two IgA subclasses were normally distributed, and were consequently not transformed and analysed by (two-tailed) t-tests for independent samples and expressed as arithmetic means ± SEM. Comparisons within each group for the various types of stimulation by cytokines were performed using (two-tailed) t-tests for paired samples.

Results

IgA, IgA1, and IgA2 in biological samples

Salivary concentrations obtained for IgA and IgA subclasses were not significantly different in healthy volunteers and patients with IgAN. For IgA: 204 ± 36 vs 188 ± 50 µg/ml (P = 0.68); for IgA1: 147 ± 26 µg/ml vs 149 ± 22 µg/ml (P = 0.73); for IgA2: 75 ± 25 vs 76 ± 16 µg/ml (P = 0.96), respectively. The IgA1/(IgA1 + IgA2) ratios were also not significantly different, 0.64 ± 0.03 in controls and 0.63 ± 0.03 (P = 0.91) in patients. After correction of these values and expression of the concentrations of IgA, IgA1, and IgA2 relative to albumin again no difference was observed between patients and controls. The concentrations of IgA1 and IgA2 in saliva correlated significantly with each other (r = 0.67; P < 0.001) (Figure 1).

Patients with IgAN had significantly higher serum concentrations of IgA (1.88 ± 0.26 mg/ml vs 1.09 ± 0.08; P < 0.001) and IgA1 (1.65 ± 0.26 mg/ml vs 1.05 ± 0.14 mg/ml; P < 0.01) in their sera, while no significant difference was found for IgA2 (0.25 ± 0.02 mg/ml vs 0.20 ± 0.02 mg/ml; P = 0.17).

In vitro immunoglobulin isotype production

IgA was the main immunoglobulin isotype produced by unstimulated PBMCs after the 14-day culture period. No significant differences in the relative proportions of monocytes (CD14\(^{+}\)/CD45\(^{-}\)), T cells (CD3\(^{+}\)/CD4\(^{+}\)/CD8\(^{+}\)), and B cells (CD20\(^{+}\)) were found between patients and controls (data not shown). The amounts (µg/ml) of IgA (P = 0.89), IgA1 (P = 0.95), IgA2 (P = 0.13), IgG (P = 0.78) and IgM (P = 0.12) in unstimulated culture supernatants were not significantly different between patients and controls (Table 1).

Both anti-CD3 and L-CD40L stimulations resulted in significantly (P < 0.001) higher, and approximately equal, production of the respective immunoglobulin isotypes. No significant differences in IgA, IgA1, IgA2, IgG, and IgM production were found between the two groups independent of the stimulation employed (Table 1). The IgA1/(IgA1 + IgA2) ratio after the 14-day culture period was 0.50 ± 0.05 in unstimulated and in stimulated cultures.

![Fig. 1. Relation between IgA subclass levels in whole saliva.](Image)

Relation between IgA subclass levels in whole saliva. The IgA1 (x-axis) and IgA2 (y-axis) concentrations (µg/ml) in whole saliva from patients with IgAN (closed circles) and controls (open circles) showed a significant correlation (r = 0.67; P < 0.001). No significant difference in local IgA subclass synthesis was found between the two groups.
Sensitivity to addition of cytokines

In healthy volunteers addition of IL2 or IL10 to anti-CD3 (Figure 2, Table 2) or CD40-activated (Figure 3, Table 2) cultures resulted in the production of significantly higher amounts of IgA ($P<0.001$), IgA1 ($P<0.001$) and IgA2 ($P<0.001$). The combination of IL10 and IL2 significantly enhanced the IgA ($P<0.001$), IgA1 ($P<0.001$) and IgA2 ($P<0.001$) production further in both culture systems compared to results obtained with IL2 or IL10 alone. Addition of TGF-β to 14-day cultures with immobilized anti-CD3 or L-CD40L resulted in a significant ($P<0.001$) inhibition of the secretion of not only IgA, but of all immunoglobulin isotypes (Table 2, Figure 4).

In patients with IgAN L-CD40L activated cultures produced significantly less IgA1 ($P<0.005$) and IgA2 ($P<0.005$) after addition of IL-10 compared to controls (Figure 3). Compared to controls, concentrations of IgA in cultures with exogenous IL-2 or IL-10+IL-2 tended to be lower in patients, but the differences did not reach statistical significance (Table 2). Comparison of these cytokines within each group was as follows: in healthy volunteers, addition of IL2 or IL-10 to the cultures led to a significantly ($P<0.001$) increased production of IgM, IgG, IgA1, and IgA2 over the medium control (Table 2; Figures 2 and 3). In contrast, in patients with IgAN no significant differences were found for IgA1 ($P=0.18$) and IgA2 ($P=0.26$) in the response to IL-2. These results were independent of the type of stimulation, immobilized anti CD3 or L-CD40L, employed.

Addition of TGF-β to the cultures showed no significant differences in IgA, IgA1, IgA2, IgG, or IgM synthesis between patients and controls. However, significant differences were found, both in anti-CD3 and L-CD40L activated cultures, when IL-10 together with TGF-β was added (Figure 4). IgM was the predominant immunoglobulin synthesized, with significantly lower IgM ($P<0.05$), IgG ($P<0.05$), IgA1 ($P<0.05$) and IgA2 ($P<0.05$) concentrations in patients with IgAN (Table 2, Figure 4). The combination of TGF-β+IL10+IL2 did not give significant differences in IgM, IgG, IgA1, IgA2 production between patients and controls. However, IgA (2.59±0.13 vs 4.33±2.22), IgA1 (1.85±0.48 vs 3.12±1.31) and IgA2 (1.40±0.70 vs 3.03±1.31) production tended to be lower in patients.

None of the different conditions resulted in a selectively enhanced production of one of the two IgA
Table 2. Growth factor-induced immunoglobulin isotype production by PBMCs in T cell-specific or B cell-specific activated cultures

<table>
<thead>
<tr>
<th>Culture</th>
<th>Growth factor</th>
<th>IgA (μg/ml)*</th>
<th>IgG (μg/ml)*</th>
<th>IgM (μg/ml)*</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>IgAN</td>
<td>Control</td>
<td>IgAN</td>
</tr>
<tr>
<td>OKT3</td>
<td>Medium</td>
<td>1.24 ± 0.30</td>
<td>1.26 ± 0.34</td>
<td>1.70 ± 0.72</td>
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<tr>
<td></td>
<td>IL-2</td>
<td>1.82 ± 0.91b</td>
<td>1.88 ± 0.51b</td>
<td>2.04 ± 1.42</td>
</tr>
<tr>
<td></td>
<td>IL-10</td>
<td>2.14 ± 0.67</td>
<td>2.53 ± 1.10</td>
<td>5.93 ± 5.23</td>
</tr>
<tr>
<td></td>
<td>IL-10 + IL-2</td>
<td>2.97 ± 1.49</td>
<td>3.79 ± 1.57</td>
<td>6.15 ± 3.42</td>
</tr>
<tr>
<td></td>
<td>TGF-β</td>
<td>0.30 ± 0.08</td>
<td>0.33 ± 0.07</td>
<td>0.24 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>TGF-β + IL-10</td>
<td>0.66 ± 0.20d</td>
<td>1.16 ± 0.26</td>
<td>1.10 ± 0.42d</td>
</tr>
<tr>
<td></td>
<td>TGF-β + IL-10 + IL-2</td>
<td>1.18 ± 0.56</td>
<td>1.54 ± 0.56</td>
<td>2.10 ± 2.03</td>
</tr>
<tr>
<td>CD40L</td>
<td>Medium</td>
<td>1.69 ± 0.99</td>
<td>1.76 ± 1.15</td>
<td>1.25 ± 0.41</td>
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<tr>
<td></td>
<td>IL-2</td>
<td>2.66 ± 0.86e</td>
<td>2.97 ± 1.66c</td>
<td>2.84 ± 1.55</td>
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<tr>
<td></td>
<td>IL-10</td>
<td>4.60 ± 1.38</td>
<td>7.17 ± 4.24</td>
<td>4.10 ± 1.54</td>
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<tr>
<td></td>
<td>IL-10 + IL-2</td>
<td>9.95 ± 5.38</td>
<td>13.45 ± 8.12</td>
<td>9.24 ± 4.34</td>
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<tr>
<td></td>
<td>TGF-β</td>
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<td>0.57 ± 0.13</td>
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<tr>
<td></td>
<td>TGF-β + IL-10</td>
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<td>1.74 ± 0.40</td>
<td>0.67 ± 0.25d</td>
</tr>
<tr>
<td></td>
<td>TGF-β + IL-10 + IL-2</td>
<td>2.59 ± 1.03</td>
<td>4.33 ± 2.22</td>
<td>2.15 ± 0.95</td>
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</tbody>
</table>

*Results (n=20 in each group) were expressed as their geometric means ± SEM in μg/ml. **P=0.15 (IgAN) and P<0.05 (controls) vs medium alone (two-tailed paired sample t-test). **P=0.10 (IgAN) and P<0.05 (controls) vs medium alone (two-tailed paired sample t-test). **P<0.05 (Scheffe’s procedure: IgAN vs controls). **P=0.07 (Scheffe’s procedure: IgAN vs controls).

Discussion

Reports of recurrent IgA deposits in normal kidneys transplanted in IgAN recipients provide evidence that the basic abnormality in this condition lies within the IgA immune system rather than in the kidney [1]. Further evidence came from the observation that the IgA deposits disappear when a graft containing mesangial IgA deposits is accidently transplanted in a recipient not suffering from IgA nephropathy [2]. Patients with primary IgA nephropathy have elevated plasma levels of IgA1 and IgA1-containing macromolecular complexes and produce more IgA1 in their bone marrow than healthy controls [3,4]. The observation, that increased IgA1 levels in IgAN are restricted to the systemic compartment of the IgA immune system is confirmed in the present study. Both patients and controls had an IgA subclass ratio of 0.63 in their saliva, that is almost identical to measurements in parotid secretions [18] and cellular subclass ratio found in normal parotid glands [19] with the same monoclonal antibodies. The two-compartment-model of the IgA immune system [6] is supported by the observation that extracorporeal irradiation of blood in a patient with chronic lymphocytic leukemia led to a dramatic decrease in secretary but not in serum IgA [20]. In accordance with this concept, we found an IgA subclass production by PBMCs very similar to the situation in the mucosa with an IgA1/(IgA1 + IgA2) ratio of approximately 0.50. No significant differences were found between IgAN and controls, irrespective of the culture system used or growth factor added.

We recently provided evidence for the existence of a ‘mucosa-bone marrow axis’ in humans [21], extending previous evidence for a link between the mucosal and systemic compartments of the IgA immune system found in experimental animals [22–24]. This suggests that mucosal presentation of antigens in humans not only leads to a dissemination of the immune response to local and distant mucosal effector sites as defined in the concept of the common mucosal immune system, but also to the systemic compartment of the IgA system, especially the bone marrow, the predominant site of plasma IgA production in humans [6,7]. It is relevant to note that all of the earlier studies that found evidence for a significantly higher systemic IgA [3] or IgA1 [4] immune response, also reported higher preimmunization levels in IgAN patients. When the peak levels of IgA antibodies after immunization are related to these already elevated baseline levels, the relative increase in titers is not augmented in patients [4].

In the present study we assessed the capacity of the precursors of IgA producing cells in peripheral blood to secrete the different immunoglobulin isotypes. No significant differences in IgA or IgA subclass synthesis between IgAN and controls were found in unstimulated, T cell-specific (anti-CD3) or B cell-specific (L-CD40L) activated cultures. Accordingly, using the ELISPOT-assay, comparable numbers of spontaneous antibody secreting cells of the different isotypes have been described in patients with IgAN and controls [21,25]. The ubiquitous presence of CD40 on B lymphocytes and the fact that L-CD40L mimics the in vivo interaction between activated T cells and B cells has been well established [11]. Our results did not reveal an abnormal sensitivity to anti-CD3 mediated T cell activation or selective dysregulation of IgA- or IgA1-producing B-lymphocytes in patients with IgAN. These data cannot be compared with previous studies,
Fig. 3. L-CD40L-activated cultures. Culture wells containing $5 \times 10^3$ irradiated L-CD40L cells and $5 \times 10^4$ PBMCs per well were cultured with medium and with addition of IL-2 (20 U/ml), IL-10 (50 ng/ml), or both. At the end of the 14-day culture period, supernatants were assessed for IgA1 and IgA2 ($\mu$g/ml) and IgA1/(IgA1 + IgA2) ratios in 20 patients with IgAN (black bars) and matched healthy volunteers (open bars). (*$P < 0.005$ from Scheffe’s procedure; $P$-values in graph: paired sample two tailed $t$-test versus medium alone).

Fig. 4. L-CD40L-activated cultures with exogenous TGF-$\beta$. Wells containing $5 \times 10^3$ irradiated L-CD40L cells and $5 \times 10^4$ PBMCs per well were cultured with medium, TGF-$\beta$ (1 ng/ml) or TGF-$\beta$ in combination with IL-10 (50 ng/ml). At the end of the 14-day culture period, supernatants were assessed for IgA1 and IgA2 ($\mu$g/ml) and IgA1/(IgA1 + IgA2) ratios in 20 patients with IgAN (black bars) and matched healthy volunteers (open bars). (*$P < 0.05$ from Scheffe’s procedure).

The cytokine-induced synthesis of both IgA1 and IgA2 showed several differences between patients and healthy controls. First, addition of IL-2 to the culture media significantly increased the production of IgA1 and IgA2 in controls, but not in IgAN patients. This is not a novel finding and was described previously on a per cell basis using the ELISPOT technique [25]. Secondly, we found that in the L-CD40L system, exogenous IL-10 led to significantly lower levels of both IgA subclasses in patients with IgAN. A decreased sensitivity to IL-10 was not found in the anti-CD3 activated cultures, probably due to different routes of B cell activation in both culture systems. The consequence of the presence of other cells (mainly monocytes) and inherent interactions (monokines and/or T cell related cytokines) is likely to influence the results differently with exogenous growth factors in anti-CD3 or L-CD40L activated cultures. Since several cells including T lymphocytes and monocytes can produce IL-10, we can only speculate on its cellular source. No selective influence of IL-2 and IL-10, alone or in combination, was found on the IgA1 or IgA2 synthesis, irrespective of the stimulation of PBMCs either by L-CD40L cells or by immobilized anti-CD3.

Further differences between patients with IgAN and healthy volunteers were found in cultures with exogenous TGF-$\beta$ in combination with IL-10. In agreement with previous studies using PWM-stimulated human PBMCs we found that TGF-$\beta$ suppressed the synthesis of all three isotypes [13,25] and of both IgA subclasses in this study. Compared to cultures with TGF-$\beta$ alone, co-culture with IL-10 induced a significantly higher
production of predominantly IgM, and to a lesser degree also of IgG, IgA1, and IgA2 in healthy volunteers. In contrast, PBMCs from patients with IgAN synthesized significantly lower amounts of IgM, IgG, IgA1, and IgA2. An in vivo preactivated state may predominantly affect the B lymphocytes destined to produce IgA1 or IgA2 in IgAN, since both IL-2 or IL-10 significantly increased the synthesis of IgG and IgM in both groups. Theoretically there are at least three explanations for this difference between patients and controls. First patients with IgAN may have less naive circulating B lymphocytes, since TGF-β in combination with IL-10 has been demonstrated to induce IgA class-switching of naive human B cells in a system that requires the interaction of CD40 and CD40L [13]. In the context of the results of the present study, this suggests that patients with IgAN have preactivated B cells committed to IgA production in their peripheral blood. The decreased sensitivity to exogenous IL-2 and IL-10 found in the present study supports this explanation. The alternative explanations, namely a deficient response to IL-10 or a defect in the IgA class-switching, are less likely in the context of the data obtained in the present study with IL-10 and TGF-β alone. Furthermore, recent data did not show functional defects in the IgA class-switch at the molecular level in IgAN [26].

Although B cells were found to have shifted to a preactivated state, both immature and more differentiated IgA precursor were equally divided between IgA1 and IgA2. Therefore, in peripheral blood, we found no evidence for a selective dysregulation of IgA1-producing B lymphocytes in patients with IgAN. Several findings support the hypothesis that the type of antigen and the duration of the response determine the regulation of the expression of IgA subclasses, or the synthesis of pIgA and mIgA, respectively [7]. Apparently, at least quantitatively, the abnormalities in the IgA immune system in IgAN are predominantly restricted to the IgA1 subclass in the systemic compartment. The previously reported increased IgA immune response to recall antigens may be the reflection of an increased level of immunological memory, since these studies also reported significantly higher primunimmunization levels in patients with IgAN [3,4]. When the peak levels of IgA antibodies after immunization were related to these already elevated baseline levels, the relative increase in titres is not augmented in patients [4]. We recently reported a deficient IgA1 immune response to primary nasal immunization with a neoeantigen [21]. Repeated or prolonged exposure, before adequate mucosal immunity is established, at predominantly ‘IgA1-sites’ such as the nasal associated lymphoid tissue (NALT) [6] may eventually lead to overproduction of systemic IgA1 via the mucosa-bone marrow axis [21–24]. That such a state of increased memory responsiveness exists is supported further by the rapid development of macroscopic haematuria following infection of nasopharyngeal mucosal surfaces.

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