Immune regulation in IgA nephropathy

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Chapter 2

Deficient IgAl immune response to nasal cholera toxin subunit B in primary IgA nephropathy

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Summary

Twelve IgA nephropathy (IgAN) patients and 18 controls were immunized with novel protein antigens, cholera toxin subunit B (CTB) via the nasal route and keyhole limpet hemocyanin(KLH) subcutaneously. Antibody secreting cells and antibody response in body fluids were determined by ELISPOT assay and ELISA, respectively. Analysis of variance showed, in contrast to controls ($p<0.001$), no CTB-specific IgA response in the nasal washes of patients with IgAN. Significantly lower numbers of CTB-specific antibody-secreting cells in peripheral blood ($p<0.001$)and CTB-specific antibodies in plasma ($p<0.005$) were found in IgAN, both restricted to the IgAl subclass. The proportions of CTB-specific IgAl-secreting cells in bone marrow aspirates correlated significantly with the corresponding ratios in plasma, with significantly lower values ($p<0.005$)in IgAN as compared to controls. These results support the existence of a 'mucosa-bone marrow axis' in humans, but no dysregulation of this axis was found in IgAN. The deficient mucosal IgA immune response to CTB observed in this study after primary mucosal immunization indicates that patients with IgAN have a defective immune response when challenged intranasally. These patients may depend on more frequent and/or prolonged antigen encounter at mucosal sites before efficient mucosal immunity is established. Repeated seeding of antigen-specific cells to secondary lympoid organs could result secondarily in the relative hyperresponsiveness found in IgAN upon reactivation by parenteral immunization.

Introduction

Primary IgA nephropathy (IgAN) is a common form of primary glomerulonephritis with a varied spectrum of clinical presentations, leading to progressive renal failure in a substantial proportion of patients [1;2]. The disease is characterized by deposits of IgAl in the glomerular mesangium [3-8]. The mesangial IgA has been found to consist at least in part of polymeric IgA (p-IgA) [5;9-11]. Although increased plasma levels of IgAl and IgAl-containing immune complexes are thought to be of pathogenetic importance, the mechanism of the mesangial deposition remains unclear [12]. The increased concentration of plasma IgAl appears to be the result of an increased production of this isotype by the bone marrow [13-16]. The macromolecular IgA found in the circulation also contains predominantly monomeric IgAl (m-lgA1) [17;18]. The pathogenetic significance of p-lgA in IgAN is still subject of controversy. In children p-IgA levels correlate with bouts of macroscopic hematuria [19;20], but in adults the elevation of serum p-IgA and its correlation with disease activity is less clear [5;6;15,21-27].

Although it seems obvious that the stimulus for IgA production is of mucosal origin, the responsible agent in IgAN and the site of immunization remain to be elucidated. On the basis of the association of episodes of macroscopic hematuria with infections of mainly the upper respiratory tract [1], it is assumed that in IgAN the mucosal immune system is stimulated by microbial antigens. However, previous studies of IgA responses, both after systemic and oral secondary immunization, in patients with IgAN have provided conflicting results [28-34]. The mucosal immune response after nasal immunization with novel or recall (viral)antigens has not been studied in patients with IgAN.

Systemic immunization in humans results in the transient appearance in the peripheral blood of B cells capable of spontaneous antigen-specific antibody production [35-37]. These anti-body-secreting cells are considered to represent migrating B cells on their way to their final destination in systemic lymphoid tissues, including lymph nodes, spleen and bone marrow [38]. The appearance of antigen-specific antibody-secreting cells is followed by a rise

in specific serum antibodies produced mainly by bone marrow plasmocytes [38]. Antigen-specific antibody-secreting cells in peripheral blood have also been detected after intranasal immunization [39-41], extending the evidence for the concept of a common mucosal immune system [42;43]. According to this concept, B cells activated at mucosal inductive sites, migrate via the circulation to local but also to remote mucosal effector sites, where they undergo final differentiation into plasmocytes.

Exposure of a mucosal surface to non-viable or live microbial antigens (vaccines or infection) may result in a local and a systemic immune response. Except for live antigens, an IgA response is not regularly induced in the systemic compartment [44]. Cholera toxin and its B subunit (CTB) have been shown to induce not only strong mucosal IgA responses but also serum IgA (and IgG) antibodies [45]. This is explained by the ability of the B subunit to bind avidly to GM 1 ganglioside, its natural ligand present on microfold cells overlying the mucosa associated lymphoid tissue (MALT) [46].

The routes of immunization and types of antigens that might induce an effective immune response in the human bone marrow have not been clarified. Repeated exposure to mucosal antigens may induce B-cells to migrate to mucosal and non-mucosal lymphoid tissues such as the lymph nodes and spleen [47]. Upon rechallenge, memory B cells may leave the lymph nodes and disseminate the secondary response to the bone marrow where they differentiate into plasma cells that secrete p-IgA antibodies [30]. The existence of such a 'mucosa-bone marrow axis' is suggested by studies in experimental animals [48-50]. However, there are no data available yet to support this axis in humans.

The high IgAl serum levels in patients with IgAN could either be a primary hyper-responsiveness of the mucosal immune system or a compensatory reaction of the systemic compartment to a hypothetical hyporesponsivity of the MALT. To investigate the hypothesis of a dysregulated 'mucosa-bone marrow axis' in patients with primary IgA nephropathy we studied the immune response in both compartments of the IgA immune system after primary and booster intranasal immunization. This study indicates that patients with IgAN are not hyper- but hyporesponders when they are immunized intranasally with CTB.

Materials and methods

Human subjects

The study protocol was approved by the Ethical Committee of the Leiden University Hospital. All individuals gave informed consent. Twelve patients (11 males, mean age 36.5 years; range 26 to 52 years) with biopsy proven IgAN were studied. None of the patients had clinical or laboratory evidence of Henoch-Schönlein purpura, systemic lupus erythematosus, liver disease or received immunosuppressive therapy. Kidney function was normal or mildly impaired (creatinine clearance >80ml/mim). None of the patients had macroscopic hematuria or proteinuria >2g/24hours. As controls 18 healthy volunteers, (12 males, mean age 28.4 years; range 21 to 40 years) were recruited. Neither patients nor controls had received the whole cell/B subunit, parenteral whole cell cholera vaccine or had had clinical cholera previously. There were no symptoms or signs of mucosal infection in the two weeks preceding or during the study period. All subjects completed the study.

Immunization protocol

Individuals were given 0.33 mg of recombinant CTB (provided by JH) [37] per immunization intranasally by spray and 250 µg KLH (Calbiochem, La Jolla, CA, USA) subcutaneously. Two identical doses of CTB and KLH were given as booster immunization on days 14 and 28, respectively. Serum samples and peripheral blood mononuclear cells (PBMC) were obtained on days 0, 7, 21, 35 and 42. Two weeks after the second booster immunisation (day 42) bone marrow samples were obtained from the posterior iliac crest [13]. Nasal washes were collected on days0, 7, 21 and 35. Both antigens were well tolerated and no side-effects were noted.

Cells

Venous blood was collected in sterile, heparinized syringes. PBMCs were isolated by centrifugation using a standard Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO, USA) density gradient. Bone marrow nucleated cells (BMNC) were processed as previously described [13]. After lysis of

contaminating red blood cells and three washings, the cells were placed in complete tissue culture media consisting of RPMI 1640 medium supplemented with L-glutamine (Gibco, Breda, The Netherlands) and 10% heat-inactivated fetal calf serum (ΔFCS) at a concentration of 5 x 10^6 cells/ml. The number of viable cells was established by trypan blue exclusion in a hemocytometer. The immunoglobulin-producing cells were assayed immediately in the enzyme-linked immunospot (ELISPOT) assay.

Body fluids

Serum samples were obtained from fresh venous blood after overnight fast. At the same time nasal wash effluent was collected after installation of 3 x 1 ml of sterile normal saline into each nostril with the neck extended, remaining still for one minute. Samples were immediately placed on ice, centrifuged (1200 rpm for 10 min at 4°C) and the supernatants collected. Samples were stored frozen at -20°C until assayed.

ELISPOT assay

Total immunoglobulin and antigen-specific antibody-secreting cells were enumerated using the ELISPOT technique as described previously [51]. For the enumeration of immunoglobulin-secreting cells, the wells of nitrocellulose-bottomed, 96-well Millititer HA plates (Millipore, Bedford, MA, USA) were coated with heavy chain specific, affinity purified goat $F(ab')_2$ fragments against human IgA, IgG or IgM (Jackson, West Grove, PA, USA). The capture antibodies for enumeration of immunoglobulin-producing cells of the two IgA subclasses were mAb 69-11.4 (specific for IgAl) and mAb 16-512-H5 (specific for IgA2). The quality, specificity and general usefulness in different body fluids of these mAbs has been described [5;52;53].

Purified CTB (5 μg/ml) and KLH (20 μg/ml) were used as the coating reagents in the antigen-specific ELISPOT. For the detection of CTB-specific antibody-secreting cells, individual wells of the Millititer HA plates were precoated with 100 μl of 6 μM GM1 ganglioside (Sigma) in phosphate buffered saline, pH 7.4 (PBS) overnight at 4°C. GM1 precoated wells were subsequently coated with 100 μl of CTB overnight at 25°C. The next day

remaining binding sites were saturated with 150 µl of culture medium containing 10% ΔFCS for two hours at 37°C in a humidified atmosphere with 5% CO_2. Between the different incubations the wells were washed three times and soaked for at least five minutes using sterile PBS. After discarding the blocking solution, 100 µl of cell suspension was dispensed into the wells at four different cell densities (range, $6.25 \cdot 10^5$/ml to $5 \cdot 10^6$/ml) in duplicate. After incubating the cells for four hours at 37°C and 5% $C0_2$, the plate was washed three times with PBS and three times with PBS containing 0.05% Tween 20 (PBST). Biotinylated heavy chain-specific, affinity purified F(ab')2 fragments against human IgA, IgG or IgM (Tago, Burlingame, CA, USA), appropriately diluted, were added as secondary antibody.

Antigen-specific antibodies of the IgA subclasses were detected by mAb 69-11.4 (for IgAl) and mAb 16-512-H5 (for IgA2). After overnight incubation at 4°C the wells containing the mAbs were incubated with affinity purified, biotinylated goat anti-mouse IgG for two hours at 37°C. After washing and soaking with PBST six times, extravidin-conjugated alkaline phosphatase (Sigma) was added to the wells to bind the secondary antibody for 60 minutes at room temperature. Following a final wash (PBST and PBS 3 times, respectively) and soak in PBS the nitrocellulose-bottomed wells were exposed to a chromogen substrate solution consisting of 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (BCIP; Bio-Rad Lab, Richmond, CA, USA) and p-nitroblue tetrazolium chloride (NBT; Bio-Rad) in 0.1 M $NaHCO_3$ plus 1 mM $MgCl_2$, pH9.8. When spots reached maximal intensity, generally between 45 and 60 minutes, the reaction was stopped by thoroughly rinsing the plates with water. Spots were counted under a stereomicroscope with x 40 magnification and expressed as number of spot-forming cells (SFC) per 10^6 PBMCs or BMNCs added to the wells. Appropriate control wells (GM1 ganglioside, culture medium, irrelevant antigen) showed no spots. In preliminary experiments we determined the optimal intranasal dose of CTB needed to induce the appearance of CTB-specific ASC. In a group of five volunteers a dose of either 0.33 or 1 mg induced an apparently maximal response with a peak between five and nine days. Based on these data we selected a dose of 0.33 mg CTB for nasal immunization and a sampling period of seven days [43] after each immunization.

ELISA

Plasma and nasal washes were tested for total and antigen-specific antibodies of the various isotypes by enzyme-linked immunosorbent assay (ELISA). 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. Reagents were heavy chain specific, affinity purified goat $F(ab')_2$ fragments against human IgA, IgG or IgM (Jackson, West Grove, PA, USA). In the IgA subclass ELISA the primary antibodies were subclass specific mAb 69-11.4 (IgAl) and 16-512-H5 (IgA2). In the CTB-specific ELISA the polystyrene plates were coated with 100 µl of CTB (2.5 µg/mI) or KLH (10 µg/ml) overnight at room temperature. After three washings with PBST, non-specific binding sites were blocked with PBST containing 1% bovine serum albumin.

Appropriate serial dilutions of serum or nasal wash samples were added to duplicate wells and incubated for two hours at 37°C. Samples obtained at the different time points after immunization were investigated in the same ELISA plate. Nasal wash anti-CTB antibody levels were corrected for the influence of dilution, which occurred when the specimen was obtained, by dividing the antibody level by the total level of the corresponding isotype by ELISA or the albumin concentration as determined by rate nephelometry (Array Rate Nephelometer; Beckman, Brea, CA, USA). In the ELISA for total immunoglobulin serial two fold dilutions of a normal human serum pool (NHS) with known concentrations of IgA, IgAl, IgA2, IgG and IgM served as a standard [53]. In the vaccine-specific ELISA's titers were expressed in arbitrary units per ml (AU/ml), relative to a standard serum yielding high optical density (OD) values in ELISA for a certain isotype. A different standard serum was chosen for each isotype and used in a dilution series on each EL1SA plate. The standard sera yielded increasing OD values in a dose dependent fashion to values usually over 2.000.

Bound total immunoglobulin was detected by heavy chain-specific, affinity-purified goat $F(ab')_2$ fragments against human IgA (for lgA, IgAl and lgA2), lgG and IgM coupled to biotin (Tago). Vaccine-specific antibodies were detected by optimal dilutions of biotinylated goat anti-human IgA,

IgG, IgM (Tago) and mAb 69-11.4 (IgAl) or mAb 16-512-H5 (IgA_2) as secondary antibodies and incubated for two hours at 37°C. The anti-IgA subclass mAbs were detected by biotinylated, affinity-purified goat anti-mouse IgG. Consecutive incubations followed with streptavidine 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% H202. Between each step the wells were washed three times (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 or titers 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 ELISA-titers and the SFC-numbers showed a skewed distribution and were transformed logarithmically prior to analysis. The two way ANOVA with repeated measurements was used to study the effect of both group (patient vs. control) and time after immunization. Post hoc comparisons were made using Scheffe's procedure. Simple linear regression was used to determine correlation between the number of SFC and antibody concentrations or titers. Significance was accepted at the 0.05 level. Results are expressed as geometrical mean ±SEM.

Results

Antibody in nasal washes after intranasal immunization

The CTB-specific IgA antibody response in nasal washes after intranasal immunization was determined by specific ELISA. Samples obtained at the

different time points for each subject were examined in the same ELISA plate relative to an internal standard and expressed in arbitrary units per ml (AU/ml). Pre-immunization titers of CTB-specific IgA were all in the lower linear parts of the standard curves and not significantly different between the two groups. The local IgA immune response after immunization for each individual was defined as the fold increase in titer relative to baseline.

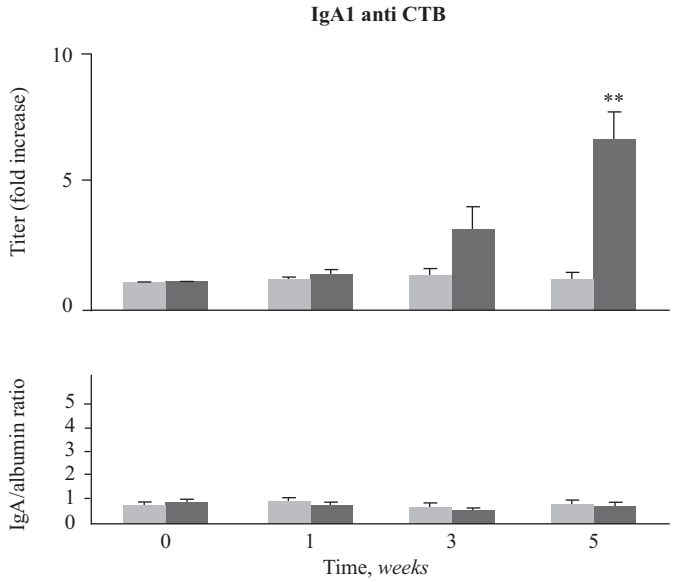

Figure 1. **CTB-specific IgA immune response in nasal wash.** IgA immune response in nasal wash effluents of patients with IgAN (□) and controls(■) following immunization with CTB intranasally on days 0, 14 and 28. Response is defined as the fold increase in the ratio of CTB-specific IgA titer/total IgA1 (AU/µl) relative to the preimmunization ratio. Results are expressed as geometrical means ±SEM, and showed a deficient local immune response in patients with IgAN, reaching significance (**$p<0.0005$) following the third immunization as compared to controls. No significant differences were found with respect to the total IgA1/albumin ratios (µg/µg) between groups or with time, indicating results were not biased by dilutional differences in obtaining the specimen.

To standardize for dilutional differences in obtaining the specimen, the titers of CTB-specific IgA antibodies (AU/ml) in the nasal washes were related to

the corresponding concentration of total IgA (μg/ml). The IgAl-anti CTB over total IgAl ratio (AU/μg) after each challenge relative to pre-immunization values is plotted against time in Figure 1. In contrast to controls, patients with IgAN showed no local IgA immune respons after the first (day 14) and second (day 28) rechallenge. This difference was significant ($p<0.0005$) after the second rechallenge as compared to controls. The total IgA over albumin ratios did not vary significantly in time and no significant differences between the two groups were found (Figure 1). This indicated that the results were not biased by the method used to standardize for differences in dilution, occurring when samples were obtained.

Circulating anibody-secreting cells after subcutaneous or intranasal immunization

The frequencies of antigen-specific spot forming cells (SFC) occurring in peripheral blood were measured before and after intranasal (CTB) and subcutaneous (KLH) immunization in patients with IgAN and controls by ELISPOT assays.

The numbers of SFCs per 10^6 PBMC are plotted against time for CTB in Figure 2 and for KLH in Figure 3. Analysis of variance revealed a significant ($p<0.0001$) increase in the numbers of specific IgM, IgG, IgA, IgAl and IgA2 SFCs in peripheral blood both after intranasal immunization with CTB (Figure 2) and subcutaneous immunization with KLH (Figure 3).

After primary intranasal immunization with CTB the numbers of CTB-specific IgM or IgG SFCs were not significantly different between patients and controls ($p=0.85$ and $p=0.23$, respectively). However, the number of CTB-specific lgA SFCs was significantly ($p<0.005$) lower in patients. The number of CTB-specific SFCs of the IgAl subclass was significantly ($p<0.0001$) lower in patients, while no significant ($p=0.84$) difference was found between the groups with respect to CTB-specific SFC of the lgA2 subclass. The number of CTB-specific IgA2 SFCs rose significantly in time, but was strikingly low compared to the IgAl response in controls. The IgAl-ratio (IgAl/{IgAl+IgA2}) in patients was also significantly ($p<0.005$) lower in patients. Comparable results were found after the first and second booster

immunization (Figure 2). Since peak responses have been shown to occur earlier after booster immunization [54], our results obtained after the second (day 21) and third (day 35) dose probably reflect the descending limb of the immune response.

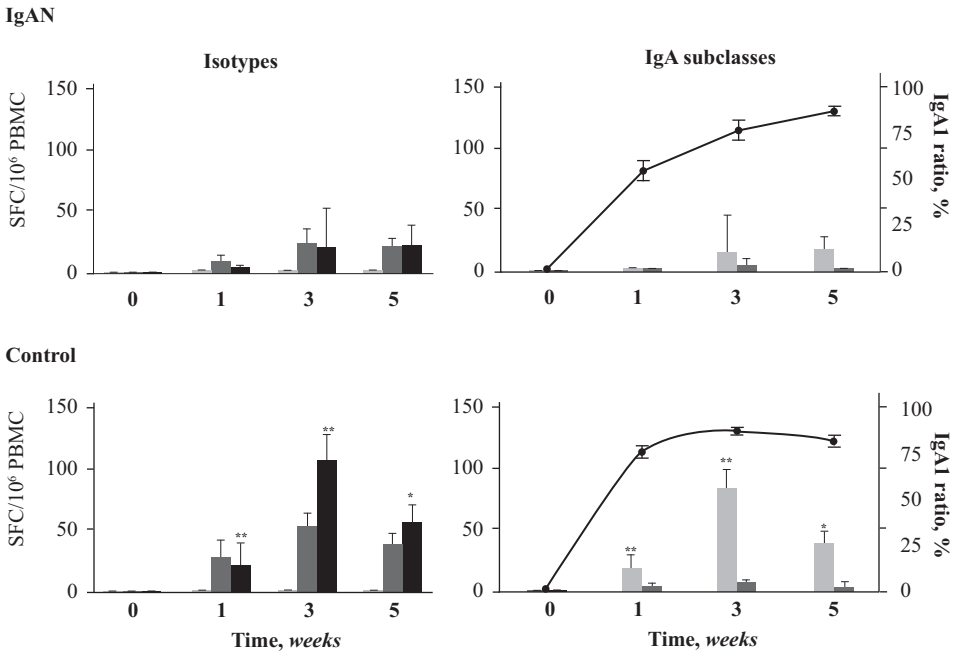

Figure 2. Nasal immunization with CTB. Frequencies of IgM (left panels, ■), IgG (left panels, ■), IgA (left panels, ■), IgAl (right panels, ■) and IgA2 (right panels, ■) isotypes of CTB-specific spot forming cells (SFCs) in controls and patients with IgAN measured in PBMCs isolated before immunization or seven days following each immunization with CTB intranasally on days 0, 14 and 28. The ratios (●) of IgAl SFCs over {IgAl + IgA2} SFCs are also shown in the right panels. Results, expressed as geometrical means ±SEM, showed significantly ($p<0.0001$) lower numbers of CTB-specific SFCs inpatients with IgAN, restricted to the IgAl subclass. p values from Scheffe's procedure are *$p<0.05$, **$p< 0.005$).

After primary subcutaneous immunization with KLH the numbers of KLH-specific IgM (p=0.27), lgG (p=0.13), IgA (p=0.93), IgAl (p=0.81) and IgA2 (p=0.26) SFCs were not significantly different between patients and controls.

Subsequent booster immunizations also yielded no significant differences in the numbers of KLH-specific SFCs between the two groups (Figure 3).

The numbers of total immunoglobulin secreting cells of the different isotypes showed no significant differences between patients and controls during the course of time (Figure 4).

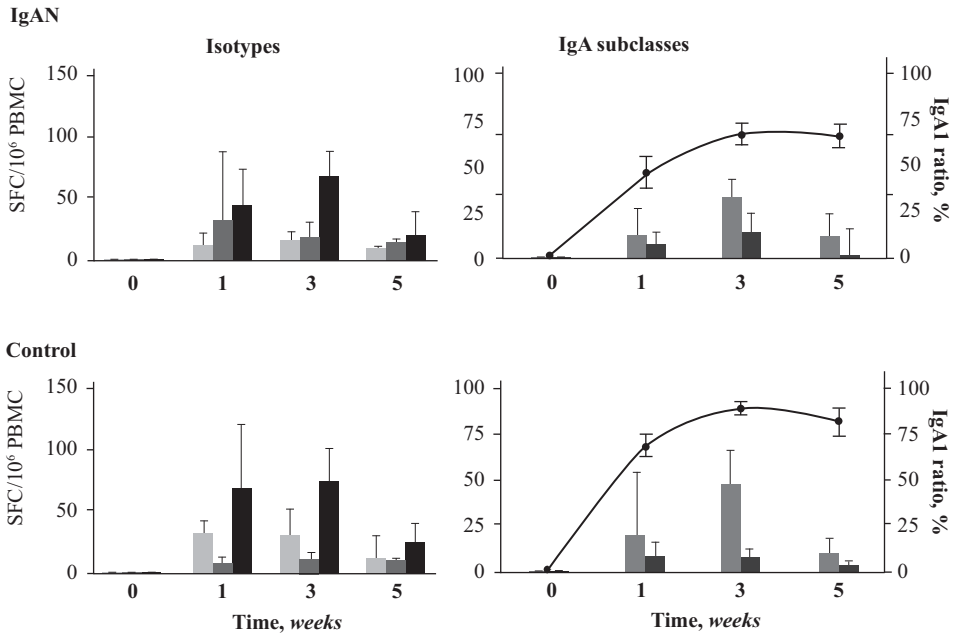

Figure 3. Subcutaneous immunization with KLH. Frequencies of IgM (left panels, ▒), IgG (left panels, ■), IgA (left panels, ■), IgA1 (right panels, ■) and IgA2 (right panels, ■) isotypes of KLH- specific spot forming cells (SFCs) in controls and patients with IgAN measured in PBMCs isolated before immunization or seven days following each immunization with KLH subcutaneously on days 0, 14 and 28. The ratios (●) of IgA1 SFCs over {IgA1 +IgA2} SFCs is also shown in the right panels. Results, expressed as geometrical means ±SEM, showed no significant differences in KLH-specific SFCs of the various isotypes between patients with IgAN and controls.

Specific antibodies in plasma after systemic or intranasal immunization

The antigen-specific antibody response in plasma after intranasal (CTB) and subcutaneous (KLH) immunization was determined by specific ELISAs.

Samples obtained at the different time points for each subject were examined in the same ELISA plate relative to an internal standard and expressed in arbitrary units per ml (AU/ml). Pre-immunization titers of CTB-specific and KLH-specific isotypes were all in the lower linear parts of the standard curves and not significantly different between the two groups. The antibody titers for each individual was expressed as the fold increase in titer relative to baseline and plotted against time.

Analysis of variance showed a significant ($p<0.0001$) increase in the IgG, IgA (Figure 5) and IgAl (Figure 6) titers against both CTB and KLH after intranasal and subcutaneous immunization, respectively.

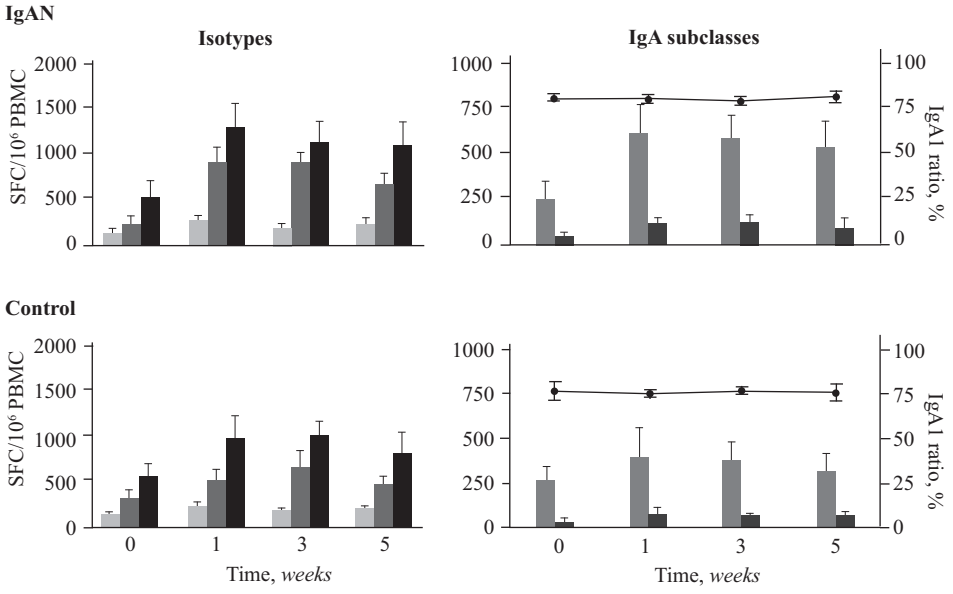

Figure 4. Total immunoglobulin secreting cells. Frequencies of total IgM (left panels, ■), IgG (left panels, ■), IgA (left panels, ■), IgAl (right panels, ■) and IgA2 (right panels, ■) spot forming cells (SFCs) in controls and patients with IgAN measured in PBMCs isolated before immunization or seven days following each immunization on days 0, 14 and 28. The ratios (●) of IgAl SFCs over {IgAl + lgA2} SFCs is also shown in the right panels. Results, expressed as geometrical means ±SEM, showed no significant differences in immunoglobulin-secreting cells of the various isotypes between patients with IgAN and controls.

Comparison between the two groups showed a significantly ($p<0.001$) lower CTB-specific IgA immune response in plasma after nasal immunization in patients with IgAN (Figure 5). The CTB-specific IgAl immune response was also found to be significantly ($p<0.005$) lower in patients (Figure 6). No differences were found between the groups with respect to the CTB-specific IgM, IgG and IgA2 immune responses.
After subcutaneous immunization with KLH no significant differences were found in the KLH-specific immune responses between patients and controls. The results for IgA and IgG are plotted against time in Figure 5.

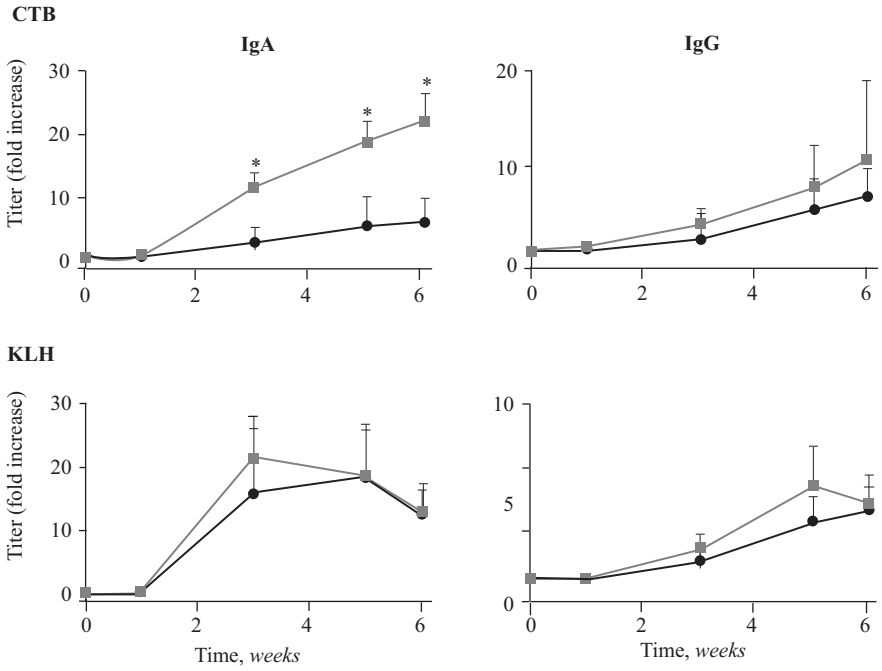

Figure 5. Antigen-specific immune response in plasma. Specific IgA (left panels) and IgG (right panels) immune response in plasma of patients with IgAN (●)and controls (■) following immunization with CTB (intranasal) and KLH (subcutaneous) on days 0, 14, 28. Response defined as fold increase in titer (AU/ml) relative to preimmunization. Results, expressed as geometrical means ±SEM, showed a significantly ($p<0.001$) lower CTB-specific IgA immune response in plasma in patients with IgAN as compared to volunteers. No significant differences were found between the groups with respect to the CTB-specific IgG and KLH-specific IgA and IgG responses. (P-values from Scheffe's procedure are *$p<0.05$, **$p<0.005$).

Correlation between ASC in bone marrow and antibodies in plasma

Correlation between the number of total immunoglobulin and CTB-specific SFCs in bone marrow aspirates and the corresponding concentration or titer in plasma was examined two weeks after the last immunization (day 42). At this time virtually no CTB-specific SFCs were found in the peripheral blood. The lack of correlation between PBMC and BMNC indicated that there was no significant contamination of the bone marrow aspirates by peripheral blood.

Analysis by simple linear regression showed that there was a significant correlation between the number of IgA ($r=0.67$; $p<0.005$) and IgAl ($r=0.76$; $p<0.001$) SFCs in the bone marrow (ELISPOT) and the concentration of IgA and IgAl in plasma (ELISA) on day 42, respectively. Patients with IgAN had significantly higher numbers of total IgA ($p<0.05$) and IgAl ($p<0.01$) SFCs in bone marrow aspirates and significantly higher concentrations of IgA ($p<0.01$) and IgAl ($p<0.005$) in plasma. These results are shown in Figure 7A.

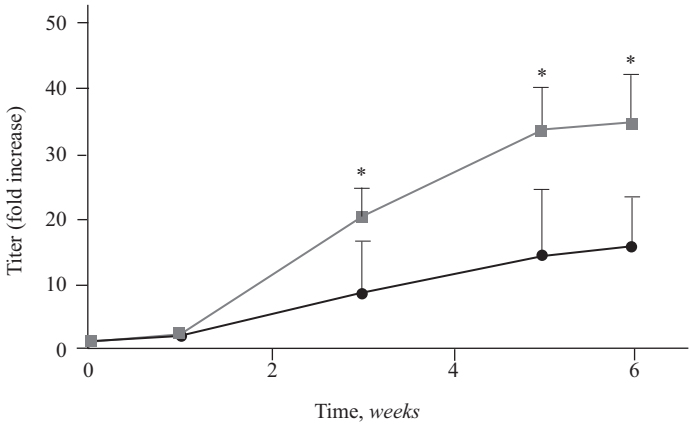

Figure 6. CTB-specific IgAl immune response in plasma. IgA1 immune response in plasma of patients with IgAN (●) and controls (■) following immunization with CTB intranasally on days 0, 14, 28. Response defined as fold increase in titer (AU/ml) relative to preimmunization. Results, expressed as geometrical means ±SEM, showed a significantly ($p<0.001$) lower CTB-specific IgAl immune response in patients with IgAN. p values from Scheffe's procedure are *$p<0.05$.

There was also a significant correlation between the proportion (SFC/SFC) of IgA-antiCTB/total-lgA (r=0.68; p<0.001) and lgAl-antiCTB/total-IgAl (r=0.62; p<0.001) in the bone marrow (ELISPOT) and the IgA-antiCTB/total-IgA and IgAl-antiCTB/total-IgAl ratios (AU/µg) in plasma (ELISA) on day 42. Patients with IgAN had significantly lower ratios of CTB-specific IgA/total-IgA (p<0.05) and CTB-specific lgA/total-IgAl (p<0.005) in bone marrow aspirates, and also significantly lower ratios of IgA-antiCTB/total-IgA (p<0.005) and IgAl-antiCTB/total-IgAl (p<0.005) in plasma. These results are shown in Figure 7B.

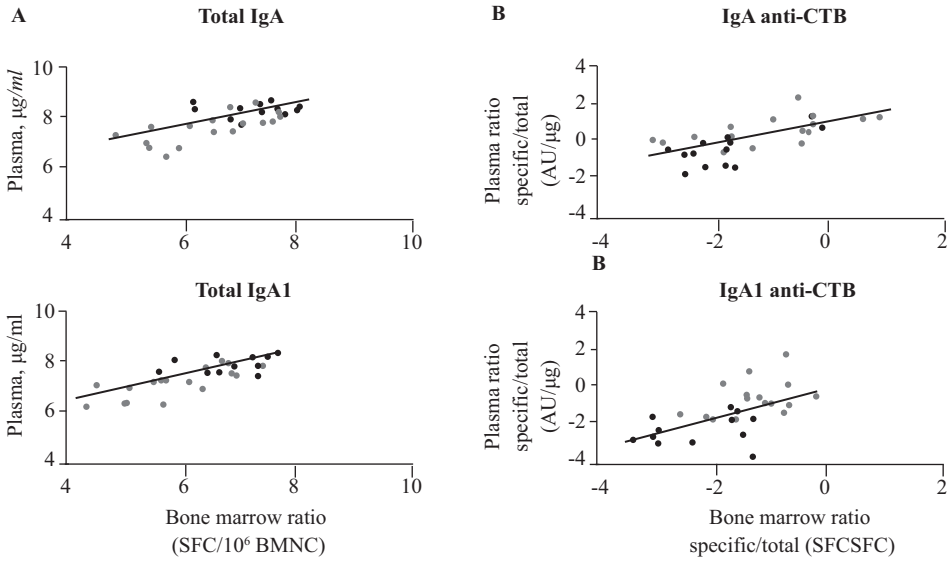

Figure 7. Correlation between bone marrow and plasma after nasal immunization. (**A**) Correlation between the number of total IgA (r=0.67, p<0.005) or IgAl (r=0.76, p<0.001) SFCs in bone marrow aspirates (SF/l0^6 BMNC) and plasma concentration of IgA or IgAl (µg/ml) two weeks after the last immunization. Results (transformed to their natural logarithms) showing significant correlation between bone marrow and plasma, with significantly higher total IgA and IgAl (p<0.01)values in IgAN (●), both in bone marrow aspirates and plasma. (**B**) Correlation between IgA-anti-CTB/total-IgA (r=0.68, p<0.001)and IgAl-anti-CTB/total-IgAl (r=0.62, p<0.001)ratios (SFC/SFC) in bone marrow aspirates and corresponding ratios (AU/µg) in plasma. Results (natural logarithms) showed significantly (p<0.005) lower CTB-specific IgA and IgAl values in patients with IgAN (●), both in bone marrow aspirates and plasma.

Discussion

Elevated plasma levels of IgAl are thought to play an essential role in the pathogenesis of IgAN [14], and are the consequence of an increased production, in which the bone marrow may be the predominant site [13;15;16]. The clinical association of exacerbations of the disease with upper respiratory tract infections suggests that the trigger for the increased IgAl production is frequently in the nasopharynx [1]. Furthermore, a significantly higher proportion of IgAl-producing cells was found in the tonsils of patients with IgAN [55-57]. In contrast, the histology, the percentage of IgA plasma cells, and the IgA subclass distribution in the small bowel were found to be normal [58;59] or showed a reduced percentage of IgA plasma cells [60] as compared to controls. However, it is still unclear how upper respiratory infections can induce an overproduction of IgAl in the bone marrow of IgAN patients. Such a sequence of events would require a link between the mucosal and systemic compartments of the IgA immune system through a hypothetical "mucosa-bone marrow axis" [13;15]. Evidence for the existence of such an axis has so far only been reported in experimental animals [48-50].

The present study is the first, to our knowledge, to provide evidence for the existence of a mucosa-hone marrow axis in humans. Both in the healthy controls, and in the IgAN patients we have demonstrated in this study the presence of specific IgAl antibody-producing cells in the bone marrow after nasal antigen presentation. This suggests that mucosal presentation of antigens in humans not only leads to a dissemination of the immune response to distant mucosal sites as defined in the concept of the common mucosal immune system [42;43] but also to the systemic compartment of the IgA system, especially the bone marrow, the predominant site of plasma IgA production in humans [61]. In the current study we chose CTB as the antigen because of its strong immunogenicity. Apart from a small previous study [37], this is the first study demonstrating the good mucosal immunogenicity of nasal immunization with CTB in humans. The frequency of responders (>4-fold increment in plasma titer) in the control group was 16%, 94% and 100% after the first, second and third immunization, respectively. The CTB-specific secretory IgA response

in controls was almost exclusively of the IgAl subclass as was described previously for serum antibodies after oral immunization with CTB [54]. Such an antigen would provide us the best opportunity to demonstrate the communication of a mucosal antigen presentation to a bone marrow immune response.

In the light of previous immunization studies in IgAN patients, which have provided evidence of both a normal [29;31;33;34]and an increased [30-32] IgA immune response, sometimes limited to the relevant subclass IgAl [30] or polymeric forms [31], we had expected that the IgAl anti-CTB response would be higher in our patients than in the controls. Moreover, our hypothesis was that especially the immune response in the patients' bone marrow after nasal immunization would be significantly higher, reflected by increased numbers of IgAl antibody-secreting cells, and increased levels of plasma IgAl antibodies. Surprisingly, we found significantly lower immune responses in the IgAN patients, not only in the systemic compartment (bone marrow and plasma), but also locally in the nasal secretions, and in the transient circulating antibody secreting cells. Therefore no apparent dysregulation of the mucosa-bone marrow axis was found in patients with IgAN.

As an internal control, all subjects were concurrently systemically immunized with KLH. In contrast to the decreased immune response to nasal CTB, the systemic immune response to parenteral KLH was not significantly different in patients compared with healthy controls. These unexpected findings raise the question why there is a discrepancy in the induced immune response to nasal CTB and parenteral KLH in patients in the present study, while some earlier studies with recall antigens using the oral [32] or parenteral [30;31] immunization route have suggested an exaggerated systemic IgA immune response in IgAN patients. A possible reason is that the antigens employed in the current study are both "neo-antigens" to which our subjects had in all probability not been previously exposed. This factor makes the current study different from the published immunization studies in which recall antigens were used. A hypothesis explaining the different findings using neo-antigens could be that IgAN patients have a deficient primary nasal immune response, resulting in a delayed activation of immunocompetent cells. Interestingly,

recall nasal priming with tetanus toxoid and subsequent parenteral boostering resulted in lower serum IgAl antibodies (and also a smaller increase) in IgAN patients before and after parenteral rechallenge [34]. An indication of IgA-specific suppression after oral recall immunization has also been reported. Elevated serum IgA antibodies prior to immunization decreased to normal levels afterward, suggesting some degree of *in vivo* hyporesponsiveness in patients with IgAN [33]. Such a deficient primary immune response may then lead to persistence or recurrence of the antigenic stimulus in patients, whereas healthy individuals succeed in effective elimination or exclusion of antigen. The resulting ongoing or repeated stimulation of the immune response may eventually lead to overproduction of IgA antibodies in the systemic compartment, and an increased number of memory cells. The previously reported increased IgA immune response to recall antigens may be the reflection of this increased level of immunological memory [30-32].

It is relevant to note that all of the earlier studies that found evidence for a significantly higher systemic IgA [32] or IgA1 [30] 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 [30]. The higher levels of naturally occurring antibodies to certain types of antigens and the high total serum IgA in IgAN suggest that a wide range of antigenic specificities are activated in these patients. The most consistent observation has been that the abnormalities in the IgA system in patients with IgAN are predominantly or exclusively restricted to the IgAl subclass. This restriction could be explained by the chemical nature of the immunogen, or a second explanation would be that the IgAl response is determined by the site of exposure to the antigen [44]. This would mean that patients with IgAN are more heavily exposed, in frequency or duration, to antigens that induce an IgAl antibody response. On the other hand, in IgAN, mucosal abnormalities have so far mainly been found in the oropharyngeal region [55-57].

A third possibility is a selective dysregulation of IgAl-producing B-lymphocytes in patients with IgAN. It is tempting to speculate that the deficient mucosal and systemic IgAl response we found in IgAN patients with

nasal CTB is the consequence of inadequate antigen presentation (such as by dendritic cells) or an abnormality in T-cell regulatory mechanisms governing the common mucosal immune system and seeding of primed B-cells to the systemic compartment.

In summary, the data indicate that patients with IgAN have a deficient primary mucosal immune response to intranasally administered CTB, limited to the IgAl subclass. Future studies will have to show whether the current findings also pertain to other antigens. Studies are in progress to elucidate whether there is a discrepancy between the induced mucosal and systemic IgA immune response to repeated nasal challenge by viral recall antigens in patients not primed by previous parenteral immunization.

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