Immune regulation in IgA nephropathy

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“Je overschat zo gemakkelijk je eigen aandeel en
vergeet wat je geworden bent uitsluitend door anderen”
Dietrich Bonhoeffer

Ter nagedachtenis aan mijn vader

Contents

Abbreviations

APRIL	a proliferation-inducing agent
ASGP-R	asialoglycoprotein receptor
BAFF	B-cell activating factor
BDCA	blood dendritic cell antigen
BlyS	B lymphocyte stimulating protein
CTB	cholera toxin subunit B
DC	dendritic cell
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot assay
FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
GFR	glomerular filtration rate
GM-CSF	granulocyte/macrophage colony stimulating factor
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
HMW	high molecular weight
IgA	immunoglobulin A
IgAN	IgA nephropathy
IL	interleukin
KLH	keyhole limpet hemocyanin
MALT	mucosa associated lymphoid tissue
MBL	mannose-binding lectin
MCP	monocyte chemoattractant protein
MIF	macrophage inhibitory factor
mIgA	monomeric IgA
MHC	major histocompatibility complex
NHS	normal human serum
PBMC	peripheral blood mononuclear cell
pIgA	polymeric IgA
pIgR	polymeric Ig receptor
SIgA	secretory IgA
TGF	transforming growth factor
TNF	tumor necrosis factor

Chapter 1

General introduction

1. Natural history and clinical description of IgA Nephropathy

IgA nephropathy (IgAN), first described by Berger and Hinglais in 1968, is the most common primary glomerulonephritis worldwide [1-3]. The disease is characterized by mesangial deposits of IgA, predominantly polymeric IgA (pIgA) of the IgA1 subclass, and found on immunofluorescence staining of kidney sections. Co-deposition of complement factor C3 together with properdin is often present [4;5]. Next to IgA, IgG is also present in a substantial percentage of the biopsies, while IgM is only rarely found. On light microscopy the picture can vary from slight mesangial hypertrophy to extra capillary proliferation of glomeruli, with sclerosis and interstitial fibrosis.

IgAN occurs at any age, but most commonly the age of onset is in the second or third decade of life. Males are more often affected than females. The overall male:female ratio is 2:1. No exact data on incidence rates are available, but incidence rates seem to be higher in certain countries like Japan, Australia and Southern Europe. In the United Kingdom, Canada and the United States of America incidence rates seem to be lower [6]. Whether these differences are determined by geographical or genetic factors is not clear. Besides it is important to realise that data on incidence rates depend on referral rates and on biopsy policy, which might be different in certain countries.

Most patients with IgAN present with microscopic hematuria with or without mild proteinuria. About 40% of patients have episodes of macroscopic hematuria. This is sometimes preceded by infections, most commonly upper respiratory tract infections. This phenomenon is called synpharyngitic hematuria [7;8]. Other infections like gastro-intestinal or urinary tract infections have also been reported to precede macroscopic hematuria [9;10]. Proteinuria is common and can vary from mild proteinuria to nephrotic syndrome. There is still no specific treatment that influences mesangial IgA deposition. Patients with proteinuria of >1 gram per 24 hour and those with hypertension should be treated with angiotensin-converting enzyme inhibitors and angiotensin receptor blockers. The use of prednisone and other immunosuppressive agents should be limited to those patients who present with acute renal failure and crescentic glomerulonephritis [11].

IgAN has been considered a benign disease for a long time, but nowadays it is clear that eventually 30-40% of patients will develop renal failure. In Western Europe and the United States of America 7-10% of the patients on renal replacement therapy suffer from IgAN [12]. The severity of histological lesions is correlated with renal function, especially severe sclerosis and tubulointerstitial damage correlate with poor outcome [13]. Next to histological lesions, unfavourable outcome is associated with persistent hematuria and proteinuria of more than 1 gram per 24 hours, decreased glomerular filtration rate (GFR) at the time of the diagnosis, and hypertension. Although several laboratory tests have been suggested to correlate with clinical outcome, so far no reliable biomarker has been identified to predict outcome in IgAN [14-16]. Recurrence of IgAN after renal transplantation is very common, suggesting that IgAN is a systemic disease [17]. Another argument that IgAN is a systemic disease comes from the observation that IgA depositions disappear from a kidney of an IgAN patient, after transplantation of this kidney to a non IgAN patient [18].

2. Characteristics of IgA

2.1 Human IgA immune system

The human IgA system is complex, as human IgA is produced at many different sites in the body and exists in different forms. The majority of IgA is produced by mucosal tissue and secreted as secretory IgA (SIgA). A smaller amount of IgA is produced by plasma cells in the bone marrow and appears in the circulation. These different compartments, the mucosal and the systemic, are linked by the so called mucosa-bone marrow axis and do not function independent from each other [19].

There are two subclasses of human IgA, IgA1 and IgA2. The main structural difference between the subclasses is the 18-amino acid hinge region, which is lacking in IgA2 [20]. IgA2 has two different allotypes IgA2m1 and IgA2m2 [20]. Both IgA1 and IgA2 exist in monomeric (mIgA) and in polymeric forms (pIgA). PIgA consists of dimeric IgA (dIgA), but also larger

molecules exist. Dimeric IgA consist of two IgA molecules linked with a 21 kD joining protein, the J chain. IgA and J chain are co synthesized by plasma cells and polymers are assembled before secretion [21;22]. The composition of pIgA is variable and may consists of complexes of IgA and the FcαRI/ CD89, IgA immune complexes and IgA-fibronectin complexes [23;24]. As mentioned before, IgA at mucosal tissues consists mainly of SIgA, which consist of dimeric IgA with secretory component, derived from the mucosal epithelium (Figure 1).

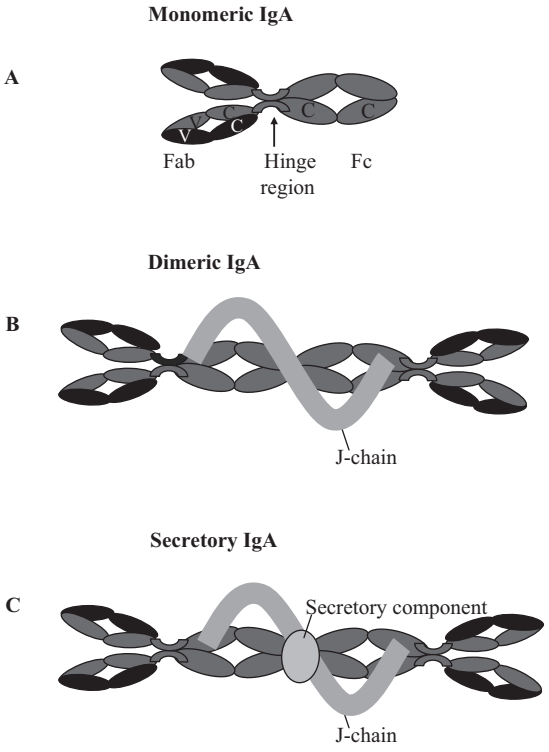

Figure 1. Different forms of human IgA. Human IgA consists of molecules with different sizes and structures. Monomeric IgA (mIgA) is a single IgA molecule with 2 heavy chains (grey), both containing 1 variable (V) and 3 constant (C) domains, and 2 light chains (black), each containing 1 variable and 1 constant domain. This results in a constant part of the molecule (Fc) and an antibody binding part (Fab), connected to each other by the hinge region of the molecule (A). Dimeric IgA (dIgA) consists of two single IgA molecules, joined by J-chain (B). Secretory IgA (SIgA) consists of dimeric IgA connected to secretory component (C).

Although IgA is mainly produced at the mucosa, IgA is also present in the systemic compartment in a concentration of 2-3 mg/ml. Serum IgA exists mainly as IgA1 (90%) and is predominantly monomeric IgA. Like other immunoglobulins in serum, IgA is produced by plasma cells in the bone marrow. The function of systemic IgA is not well understood. It might be that IgA is involved in the regulation of immune responses in an anti-inflammatory way [25]. On the other hand the complex of the IgA receptor CD89 with IgA has probably pro-inflammatory effects. These complexes have been shown to be present in IgAN patients and in an animal model their presence was associated with IgA deposition and glomerulonephritis [26;27].

The function of the mucosal compartment is better understood and will be discussed more extensively in the following paragraph.

2.2 Mucosal IgA compartment

IgA is the major immunoglobulin in mucosal secretions and has an important function in mucosal defence against bacterial and viral infections. IgA at mucosal surfaces is mainly dimeric and secreted as secretory IgA (SIgA). Before secretion dIgA is bound to the polymeric Ig receptor (pIgR), located at the basolateral site of the mucosal epithelium. Then IgA is transported to the mucosal surface, where it is secreted together with a proteolytically cleaved part of the pIgR (secretory component).

Both subclasses IgA1 and IgA2 are produced at the mucosa. The distribution of the two subclasses varies at different mucosal sites. In the nasal mucosa the relative contribution of IgA1 is 93% and in the bronchial mucosa 75%. In the proximal gastrointestinal tract the relative contribution of IgA1 is high, 83% at the gastric mucosa, and low in the distal part, 36% in the colon [28]. Although the major part of SIgA is present in secretions of the gastrointestinal tract and the respiratory tract, small amounts of SIgA are also found in the circulation [29;30]. The potential role of SIgA in the pathogenesis in IgAN will be discussed in more detail in chapter 5.

There is a cross talk between mucosal sites in the human body. This means that an immune response induced at one mucosal site will result in an immune response with the same antigen specificity at another mucosa and

even might induce a clear systemic immune response. The different mucosae can be seen as a common mucosal immune system [31]. On the other hand in many cases a mucosal immune response will not give rise to a systemic immune response, this phenomenon is called mucosal tolerance [32].

3. Mesangial IgA in IgA Nephropathy

3.1 Mechanisms of IgA deposition

The hallmark of IgAN is the presence of mesangial IgA depositions. The mechanism or mechanisms by which IgA accumulates are so far incompletely understood. Deposition of IgA in IgAN cannot simply be explained by high serum levels of IgA, as other diseases with high levels of serum IgA do not show deposition of IgA in the mesangium.

The binding of IgA to the mesangium can either be specific, directed against certain antigens or by specific receptors, or the binding can be non-specific. So far no auto-antibodies directed against specific mesangial antigens have been described in IgAN [33]. Therefore IgAN is not considered an auto-immune disease.

In vitro studies showed binding of IgA to human mesangial cells (HMC) [34-36]. It has been suggested that specific IgA receptors, like the FcαRI receptor, CD89, are present on HMC and responsible for the binding of IgA to these cells. However, so far no CD89 has been detected on HMC [37]. Also other receptors like the asialoglycoprotein receptor or the transferrin receptor have been suggested to be involved in the binding of IgA in IgAN [38;39]. Although these receptors are present on HMC, no abnormalities in the expression or the function of one of these receptors has been shown in IgAN patients.

The size distribution of circulating IgA and IgA-containing immune complexes (IgA-IC) seems to be involved in the mechanism of IgA deposition. In animal models aggregates of mainly polymeric IgA induced glomerular inflammation, whereas monomeric IgA did not induce inflammation [40]. In IgAN patients levels of IgA-IC are elevated [24;41]. It is likely that the

higher serum levels of IgA-IC will promote mesangial deposition through size dependent trapping [42].

The possible role of altered glycosylation of IgA1 as a factor involved in the mechanism of deposition will be discussed in paragraph 4.

3.2 Consequences of mesangial IgA depositions

Once IgA is present in the mesangium it may have an effect on the renal tissue via various routes. IgA can activate the complement system via the alternative pathway and via the mannose binding lectin (MBL) route [43;44]. MBL activation by IgA is restricted to polymeric IgA, which is the main type of mesangial IgA [44]. Recently it has been shown in renal biopsies that IgA deposits co-localize with MBL and that activation of the complement system via the MBL route is associated with more severe glomerular injury [45]. Activation of the complement system via either route will eventually result in formation of the terminal complex C5b-9, which can activate mesangial cells to produce inflammatory mediators and growth factors. Of the different growth factors, transforming growth factor-β (TGF-β) and platelet derived growth factor (PDGF) have been suggested to be involved in progressive inflammation in IgAN [23].

4. IgA in IgA Nephropathy

Levels of plasma IgA1 are elevated in about half of the IgAN patients [46;47]. The elevated IgA concentration seems to be the result of higher production of IgA by plasma cells in the bone marrow [48-51]. The fact that other diseases with high serum IgA concentrations, like multiple myeloma or HIV, are not associated with renal IgA depositions supports the idea that the higher IgA1 concentrations are not the only cause of mesangial IgA deposition in IgAN. Next to the higher concentrations of IgA1 qualitative changes of IgA in IgAN patients have been described. The most important change in IgA is the glycosylation pattern of IgA1 in IgAN patients. IgA of IgAN patients contains a reduced galactosylation of the O-linked glycans in the hinge

region [52-56]. The hinge region consists of 17 amino-acids, of which 6 are 0-linked glycosylation sites. The same abnormality in glycosylation pattern is found in IgA produced in vitro by tonsillar lymphocytes, suggesting that tonsils might contribute to the abnormally glycosylated IgA in serum [57;58]. IgA glycosylation takes place in the golgi apparatus. Recently specific glycosyltransferases have been described, but so far no abnormalities in expression or in function were demonstrated in IgAN [3]. A more extensive discussion on the role of glycosylation is given in chapter 7. In the urine of IgAN patients immune complexes containing aberrantly glycosylated IgA have been described. These complexes were not present in patients with other glomerular diseases [59]. Recently it has been suggested that a lectin-binding assay, based on the detection of undergalactosylated IgA1, might have potential as a non-invasive diagnostic test for IgA nephropathy [60].

Our hypothesis is that the quantitative and qualitative abnormalities of IgA in IgAN patients are the result of a disturbed regulation of the immune response. Therefore we focussed on different processes of the immune response, in particular of the primary immune response of IgAN patients in comparison with controls.

5. Production of IgA

5.1 Regulation of IgA production

Production of IgA antibodies is a tightly regulated process, and is controlled at several levels. Naïve B cells express surface IgM/IgD and have to go through a process of clonal expansion, isotype switching, affinity maturation and differentiation before IgA plasma cells have developed. The process of class switch recombination (CSR) occurs by looping out and deletion of segments of the DNA [61;62]. Induction of Ig secretion can be mediated by both T cell-dependent and T cell-independent antigens, operating via different mechanisms. The T cell dependent mechanism is mainly dependent on the CD40-CD40-ligand interaction. In the hyper IgM syndrome a genetic alteration of CD40L results in the absence of IgA and IgG [63;64]. For IgA production

cytokines like transforming growth factor β (TGF-β), IL-2 and IL-10 have been shown to be involved [65]. The T cell-independent route of Ig class switching can be induced by Dendritic cells (DC), via expression of surface molecules like B lymphocyte stimulator protein (BlyS, also called BAFF or TALL-1) and a proliferation-inducing ligand (APRIL), both members of the TNF-TNF-R family, like CD40-CD40L [66].

5.2 General characteristics of dendritic cells in immune regulation

Dendritic cells (DC) are professional antigen presenting cells that play a critical role in the initiation and regulation of immune responses [67;68]. An important characteristic of DC is their potency to migrate from blood to tissues and to lymph nodes. DC are abundantly present in the skin and in mucosal tissue of the gastrointestinal tract and the respiratory tract [69]. Besides, DC are present in most peripheral organs, including the kidney [70;71]. In most tissues DC are present in an immature state. Immature DC have a high capacity to capture antigens [67]. After capturing antigens, DC can migrate to lymphoid tissue, like lymph nodes. As a consequence of antigen uptake DC will maturate and differentiate in distinctive ways, dependent on the type of stimulus that is given to the DC. After maturation DC express co-stimulatory molecules and adhesion molecules on their cell surface and are able to present antigens to T cells in the context of major histocompatibilty (MHC) molecules. This process then leads to T cell activation, proliferation and infiltration to the site of inflammation. DC are actively involved in the immunological response and additionally play a crucial role in maintenance of self tolerance. In the thymus DC can present self-antigens in the context of MHC and T cells with a too high affinity are deleted (negative selection) [72]. So DC are involved and decisive in different immunological processes. On the one hand DC induce immune responses directed against bacteria's and viruses, on the other hand DCs are involved in prevention of auto-immunity.

In humans different subsets of DC can be distinguished, based on different phenotypic characteristics: 1) Langerhans cells and interstitial DC, both belonging to the myeloid lineage and 2) plasmacytoid DC, which are thought to be derived from a lymphoid precursor. Langerhans cells, which

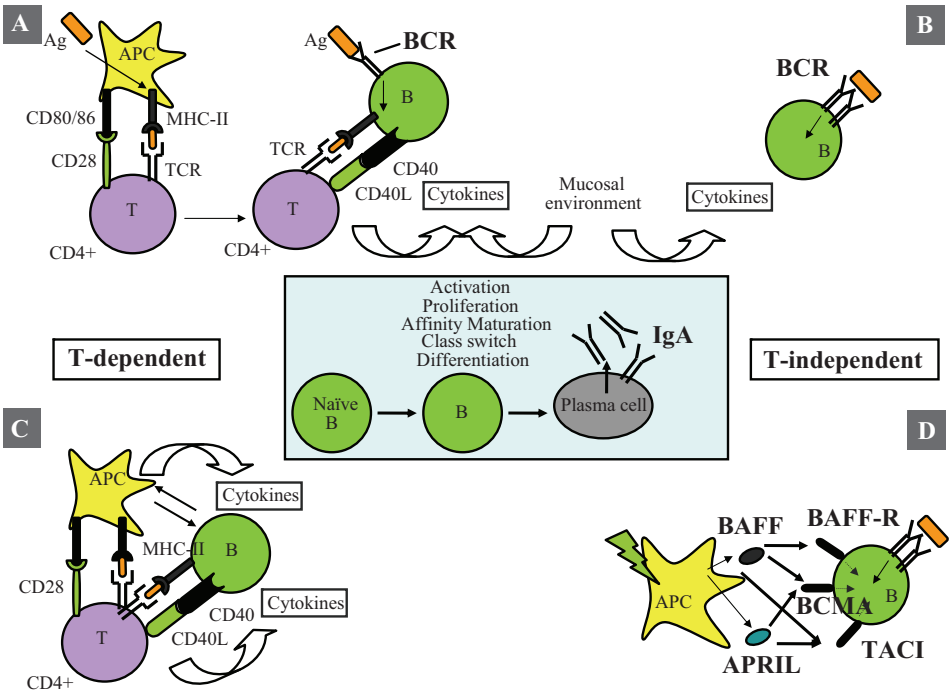

Figure 2. Basic principles of the regulation of IgA production. A naïve B cell has to undergo a process of activation, proliferation, somatic hypermutation and affinity maturation, class switch recombination and differentiation, before is has become an IgA producing plasma cell. This process can occur either T cell dependent or T cell independent.

In the traditional model of T cell dependent activation, an antigen presenting cell (APC), takes up antigen (Ag), processes this and presents peptides in the context of MHC II to CD4+ T cells. This mechanism is dependent on co-stimulatory molecules like CD80/86 and CD28. Activated T cells secrete a variety of immunostimulatory factors including cytokines and start to express CD40L. The subsequent interaction of activated T cells and B cells through CD40-CD40L and the cytokine milieu, possibly also derived from the mucosal environment, will together determine the production of IgA. In this model, Ag specificity is determined by the fact that B cells take up Ag through surface Ig expression (BCR) and present this in class II to recruit T cell help (A). In the T cell independent response, specific B cells will be activated through local cytokines, in combination with an Ag-specific activation signal through the B cell receptor (B).

More recently it has become clear that APC can also have a direct role in B cell activation. In a three cell model of B cells, T cells and APC, the latter cell type can deliver both contact and

soluble mediators to the CD40-activated B cells (C). In the T cell independent route, activated APC, for instance through TLR signalling, increase their expression of TNF family members BAFF and APRIL. These ligands show a complex interaction with three potential receptors expressed on B cells: TACI, BCMA and BAFF-R. In combination with cross-linking of B cell receptors, these signals can regulate IgA switch and differentiation into antibody producing cells (D).

express the surface marker langerin and CD1a are located mainly in epithelial layers. Toll Like Receptor (TLR) signalling interstitial DC, expressing the C-type lectin DC-SIGN and/or the blood dendritic cell antigen 1 (BDCA-1) are located in the dermis and in most peripheral organs. Plasmacytoid DC are characterized by expression of BDCA-2 and not only present in lymphoid organs, close to high endothelial venules, but are also attracted to sites of inflammation [73-75]. These two different subsets of DC, myeloid derived DC and plasmacytoid DC, have different functions, that are only partially characterized, and might be dependent on the activation status and the localisation of the DC [70].

5.3 Role of DC and B cell activation

As mentioned above, regulation of IgA production can occur both in a T cell dependent and a T cell independent manner. In both cases, DC seem to have a critical role (Figure 2). DC can induce an immune reaction by presenting antigens to T cells, leading to activation of B cells. The interaction between B cells and T cells has been studied extensively and is mainly dependent on MHC and the CD40-CD40-ligand interaction [76]. Upon activation DC can produce several chemokines, leading to recruitment of B cells to the place of inflammation [77].

Next to this T cell dependent effect of DC on B cells, there is a direct interaction between B cells and DC, which is T cell independent. This has been shown in an *in vitro* model, in which in vitro generated DC were co-cultured with B cells in the presence of a CD40L transfected cell line [78;79]. Addition of *in vitro* generated DC to human B cells resulted in a three to six fold increase in the recovery of viable B cells within a week [80]. In the cultures DC and B cells cluster and in the presence of IL-10 and TGF-β, DC

skewed isotype switching of naïve B cells towards IgA_1 and IgA_2 producing plasma cells [79]. Which factor or factors are responsible for the direct effect of DC on B cells is unknown. DC produce several cytokines like IL-12, and the IL-12R is, although expressed in the main human B-cell subsets, functional in naïve B-cells [81].

The TNF superfamily member B lymphocyte stimulator (BlyS) also known as BAFF is known to promote B cell survival and differentiation. BlyS is expressed on most myeloid cells, including DC and could be the cytokine by which DC regulate B cell function [82-84].

Other factors by which DC can have a direct effect on B cells are retinoic acid (RA) which acts in a synergistic way with interleukin-6 (IL-6) or IL-5. In mice intestinal DC induced T cell-independent expression of IgA and gut-homing receptors on B cells [85].

6. Scope of the thesis

IgAN is a complex disease. The present thesis investigates the possible contribution of various players of the immune response in the pathogenesis of IgAN. As the regulation of the IgA immune response in IgAN patients seems to be disturbed and is likely to be involved in the pathogenesis of IgAN, we focussed on various components of the immune response. In particular we have focussed on DC in primary immune responses in IgAN. Immunization studies in IgAN patients have shown conflicting results. Both hyper and hypo IgA responses have been reported, dependent on the antigen used and the route of immunization. In chapter 2 we describe a study in which IgAN patients are simultaneously immunized with two neo-antigens via two different routes. The differences in the immune response are studied in a quantitative and in a qualitative manner. IgAN patients appeared to have a clear IgA hypo response after nasal immunization as compared with controls. We postulated that the observed differences in IgA production could be caused by either different numbers of mucosal DC or by changes in different subsets of DC in IgAN patients.

In chapter 3 we describe an immuno-histological analysis of nasal biopsies from IgAN patients and controls. The nasal biopsies were studied for the presence of DC and different subsets of DC. As the number of mucosal DC in IgAN patients appeared not to be lower than in controls, we postulated that the DC could be less effective in inducing an IgA immune response. Therefore we studied in an in vitro system whether DC of IgAN patients differ in their capacity to induce IgA production by naïve B cells. This study is described in chapter 4. It was found that DC from IgAN patients are less effective in inducing IgA production in naïve B cells. These studies suggest that DC surface molecules are responsible for the difference in IgA production that was found between IgAN patients and controls.

IgA deposits in IgAN consist mainly of polymeric IgA1. Moreover in a minority of cases SIgA can be detected in mesangial deposits. As SIgA is colocalized with MBL and associated with more hematuria and more proteinuria, SIgA might have a pathogenic role in the development of IgAN. Chapter 5 is a general review on the role of SIgA in IgAN. In chapter 6 we describe the size distribution of antigen-specific IgA after immunization in IgAN patients and in control persons. Both mucosally induced and systemically induced immune responses were studied in IgAN patients and in controls. Besides we investigated whether antigen-specific SIgA could be detected in the circulation, after mucosal immunization. It appeared that the size distribution of antigen-specific IgA was mainly polymeric. No differences between patients and controls were observed. In both groups we were able to detect low concentrations of antigen-specific SIgA in the circulation.

The glycosylation pattern of IgA in IgAN patients differs from the normal glycosylation pattern of IgA and is also very likely to be involved in the pathogenesis of IgAN. Since the function of IgA is strongly determined by its glycosylation pattern, and since additional recent information on this issue was published, chapter 7 summarizes and discusses these findings in the context of the studies described in the recent thesis.

Finally chapter 8 summarizes the different studies and outlines the relevance of these studies in the context of the pathogenesis of IgAN.

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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.

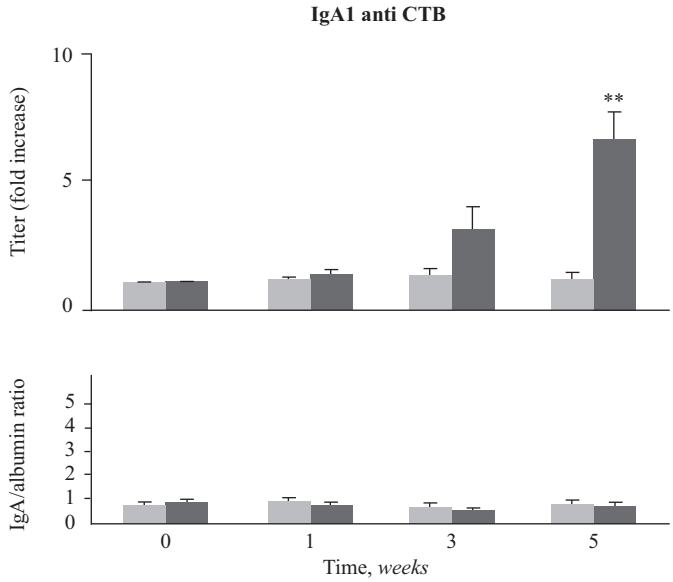

Figure 1. **CTB-specific IgA immune response in nasal wash.** IgA immune response in nasal wash effluents of patients with IgAN (▫) and controls(■) following immunization with CTB intranasally on days 0, 14 and 28. Response is defined as the fold increase in the ratio of CTB-specific IgA titer/total 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.

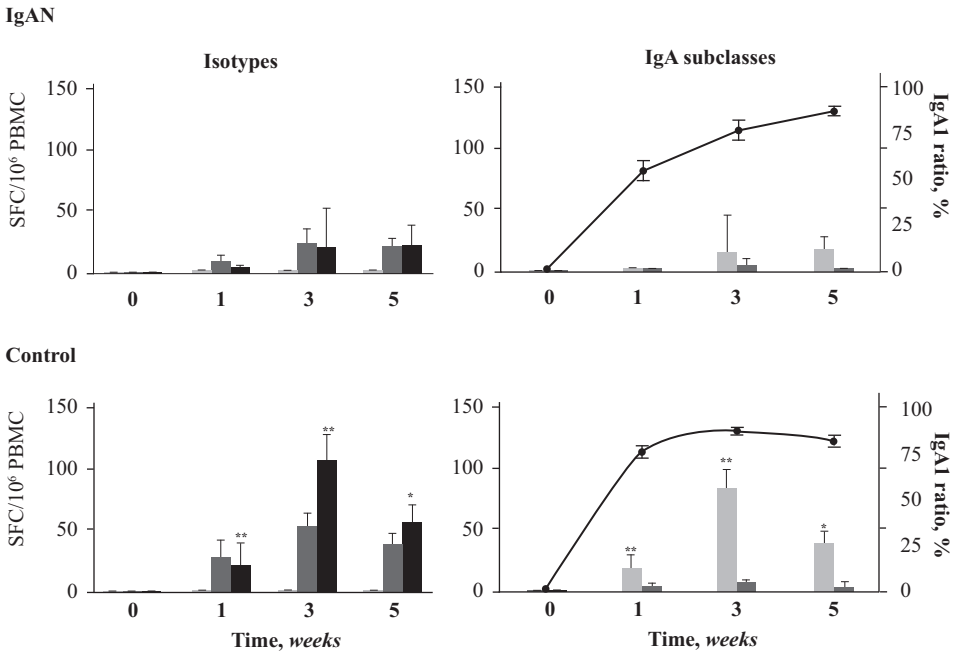

Figure 2. Nasal immunization with CTB. Frequencies of IgM (left panels, ■), IgG (left panels, ■), IgA (left panels, ■), IgAl (right panels, ■) and IgA2 (right panels, ■) isotypes of CTB-specific spot forming cells (SFCs) in controls and patients with IgAN measured in PBMCs isolated before immunization 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).

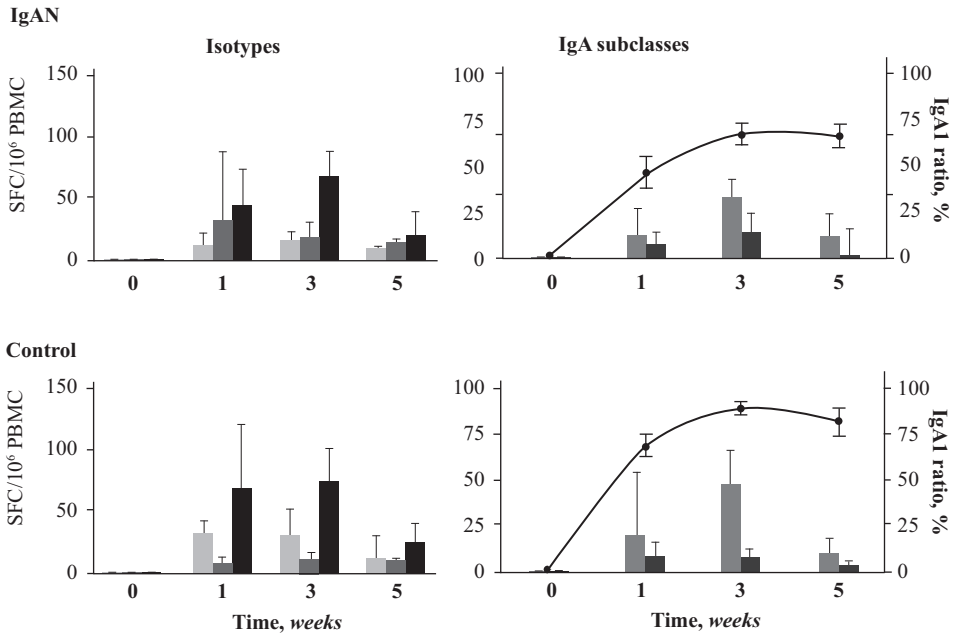

Figure 3. Subcutaneous immunization with KLH. Frequencies of IgM (left panels, ▒), IgG (left panels, ■), IgA (left panels, ■), IgA1 (right panels, ■) and IgA2 (right panels, ■) isotypes of KLH- specific spot forming cells (SFCs) in controls and patients with IgAN measured in PBMCs isolated before immunization 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.

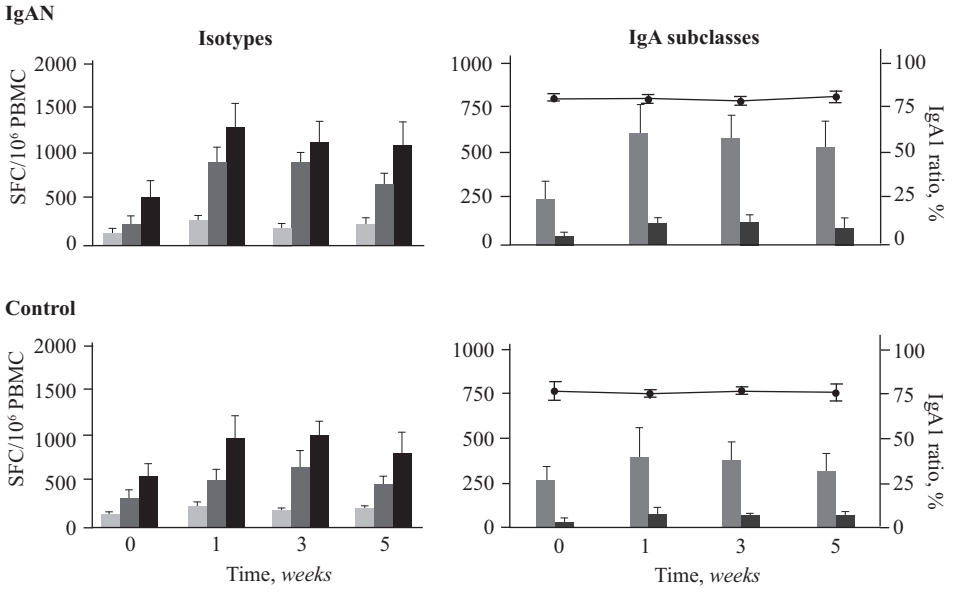

Figure 4. Total immunoglobulin secreting cells. Frequencies of total IgM (left panels, ■), IgG (left panels, ■), IgA (left panels, ■), IgAl (right panels, ■) and IgA2 (right panels, ■) spot forming cells (SFCs) in controls and patients with IgAN measured in PBMCs isolated before immunization or seven 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.

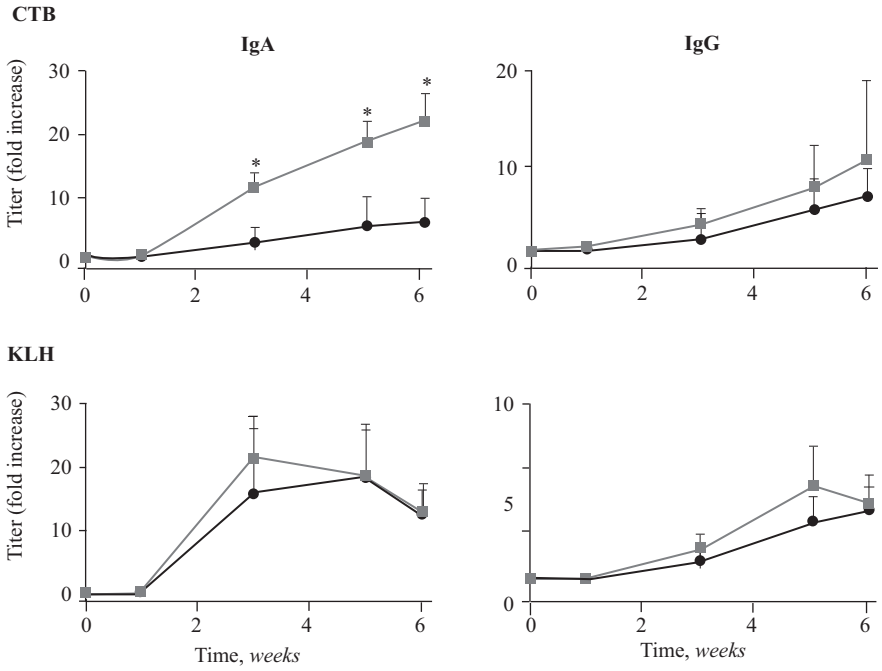

Figure 5. Antigen-specific immune response in plasma. Specific IgA (left panels) and IgG (right panels) immune response in plasma of patients with IgAN (●)and controls (■) following immunization with CTB (intranasal) and KLH (subcutaneous) on days 0, 14, 28. Response defined as fold increase in titer (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.

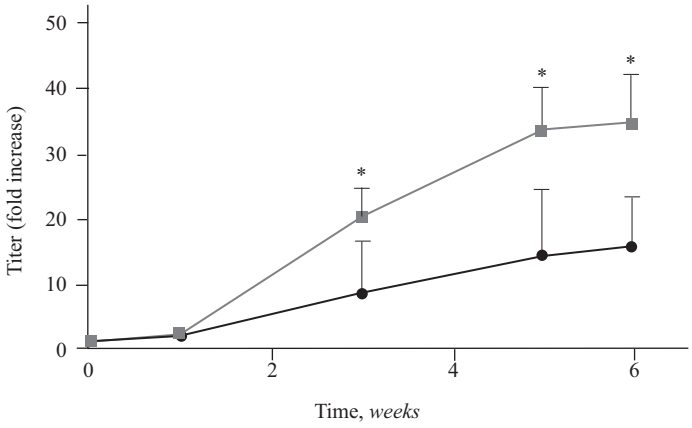

Figure 6. CTB-specific IgAl immune response in plasma. IgA1 immune response in plasma of patients with IgAN (●) and controls (■) following immunization with CTB intranasally on days 0, 14, 28. Response defined as fold increase in titer (AU/ml) relative to preimmunization. Results, expressed as geometrical 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.

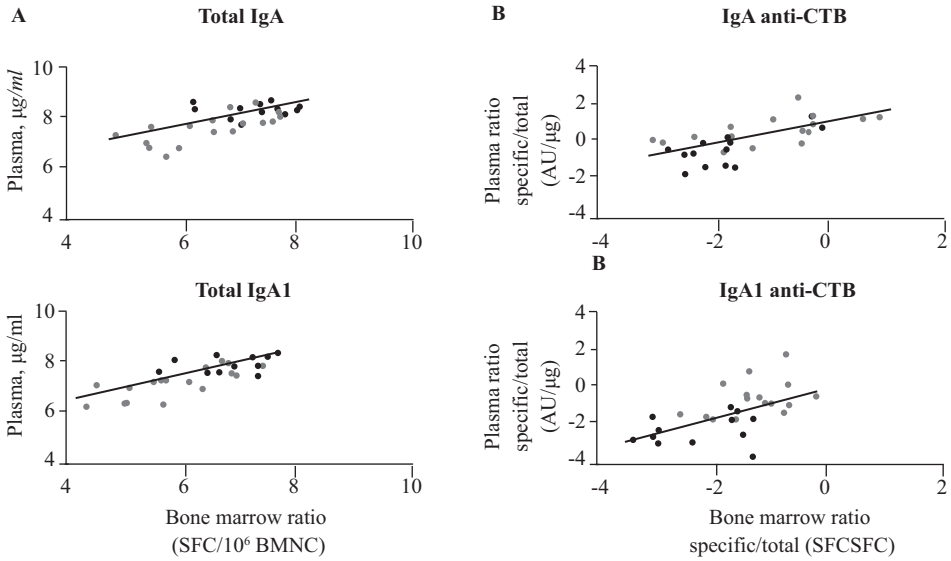

Figure 7. Correlation between bone marrow and plasma after nasal immunization. (**A**) Correlation between the number of total IgA (r=0.67, p<0.005) or IgAl (r=0.76, p<0.001) SFCs in bone marrow aspirates (SF/l0^6 BMNC) and plasma concentration of IgA or IgAl (µg/ml) two weeks after the last immunization. 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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Chapter 3

Immuno-histological analysis of dendritic cells in nasal biopsies of IgA Nephropathy patients

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Summary

IgA nephropathy (IgAN) is the most common primary glomerulonephritis worldwide. Intranasal vaccination of patients with IgAN has shown mucosal and systemic IgA hyporesponsiveness. Here we investigated whether this IgA hyporesponse in IgAN patients can be explained by reduced numbers or altered subset distribution of dendritic cells (DCs) in nasal mucosa.

Eighteen IgAN patients and 18 healthy volunteers were recruited for this study. Nasal biopsies were taken, after local anaesthesia, from the lower edge of the inferior turbinate. Staining for different subsets of DCs was performed, using specific monoclonal antibodies. To detect myeloid dendritic cells we used CD1a, DC-SIGN and BDCA-1 as a marker and for plasmacytoid DCs we used BDCA-2. DC-cell numbers in the epithelium and in lamina propria were counted separately and expressed as positively stained cells per mm^2.

Both myeloid and plasmacytoid DC could be demonstrated in nasal biopsies. Quantification showed that IgAN patients contained significantly more DC-SIGN positive cells in the lamina propria compared to controls. In addition in IgAN patients we observed more CD1a positive cells in the epithelium. No differences in BDCA-1 and BDCA-2 positive cells were found between patients and controls. The number of positively stained cells in the epithelial layer correlated strongly with the number of positively stained cells in the lamina propria.

Patients with IgAN have higher numbers of CD1a positive cells in the epithelial layer and more DC-SIGN positive cells in the lamina propria. Therefore the earlier observed IgA hyporesponsiveness in IgAN patients after mucosal vaccination cannot be explained by lower numbers of nasal DCs.

Introduction

IgA nephropathy (IgAN) is the most common primary glomerulonephritis worldwide [1-3]. The disease is characterized by depositions of IgA1 in the mesangium of the kidney [4]. Recurrence of IgAN after renal transplantation is very common, indicating that next to renal factors, specific characteristics of IgA itself (quantitative and/or qualitatively) play a role in the pathogenesis [5]. IgA serum titers are increased in 50% of the patients, but the fact that other diseases with higher serum IgA titers, like HIV infection, are not associated with IgA depositions in the kidney, shows that higher serum levels of IgA are not the only cause of IgAN [6;7]. Alternatively, qualitative alterations in IgA such as differences in glycosylation, are thought to contribute to disease pathogenesis [8;9].

Although the pathogenesis of the disease is not clear, there are reasons to believe that immune responses at mucosal surfaces play an important role. This idea stems from the observation that an upper respiratory tract infection in about 40% of IgAN patients leads to aggravation of their complaints in the form of increased blood levels in urine (synpharyngitic hematuria) [10]. It has been proposed that tonsils are responsible for the increased IgA levels in the serum [11]. Retrospective studies suggest that tonsillectomy is correlated with a favourable clinical course of the disease [12]. Several immunization studies, using different antigens and different routes of administration have shown conflicting results [13;14]. Simultaneous mucosal and systemic vaccination in IgAN patients with two neoantigens lead to an impaired IgA response upon the mucosal challenge, whereas no differences were seen after systemic vaccination [15;16].

Given the role of dendritic cells (DCs) as professional antigen presenting cells involved in the initiation of immune responses and antibody formation, there is some suggestion that aberrant DC function contributes to IgAN. Not only do they link innate and adaptive immunity, but DCs might also be decisive via the interaction with T and B cells for the induction of effector immune responses or tolerance [12;17;18]. DCs have a direct effect on B cells and can induce isotype switching and enhance immunoglobulin

production [19;21]. In the presence of CD40 activated naïve B cells, DC can skew isotype switching towards IgA [19]. Recently we have shown in an in vitro model that DC of IgAN patients induce less IgA production by naïve B cells compared to DC from control persons [22].

Different subsets of DCs have been described, based on phenotypic characteristics. There are two major subsets of DCs, namely myeloid DCs and plasmacytoid DCs. The myeloid DCs are found at two sites in tissues: Langerhans cells, which express the surface marker langerin and CD1a are located mainly in the epithelial layer, and interstitial DCs, expressing the C-type lectin DC-SIGN and/or the blood dendritic cell antigen 1 (BDCA-1). Plasmacytoid DCs are characterized by expression of BDCA-2 and /or CD123 [23-25]. These two different subsets of DCs have different functions, that are only partially characterized, and might be dependent on the activation status and the localisation of the DC [2;26]. In addition interactions between subsets of DCs and cells that are locally present, may determine the outcome of an immune response. The effect of the mucosal microenvironment on DC response, and thereby on IgA production is still unclear.

Based on the described hyporesponsiveness upon mucosal challenge of IgAN patients, and the central role of DCs in initiating immune responses, and our observation that DC from patients with IgAN induce less production of IgA by B cells, we hypothesised that IgAN patients in addition might have reduced numbers of DCs or that IgAN patients may have an altered composition of certain DC subsets at mucosal sites. To answer this question, nasal biopsies of 18 IgAN patients and 18 control persons were stained for specific DC subset markers and the results analysed with respect to DC cell numbers and subset composition.

Materials and methods

Selection of IgAN patients and control persons

Patients with biopsy proven IgAN were selected for entry in this study. Control persons in this study are persons with no underlying immunological

disease, who visited the ear nose throat department for a surgical nasal septum correction and were asked for permission for a nasal biopsy during this operation. Neither patients nor controls were using immunosuppressive drugs at the time of the study nor in the three months before biopsies were taken. No infections or macroscopic hematuria were present at the time of the study.

Mean age of the patients was 51 years (range 23-71) versus 39 years (range 10-59) of the controls (n.s.). The male: female ratio was 78% versus 22% in the IgAN patient group and 72% versus 28% in the control group. Renal function was stable in most of the patients. Mean creatinine clearance was 78 ml/min (range 24-136). Mean proteinuria was 1.3 gram per litre (range 0-4.7). Neither the IgAN patients nor the control persons had an infection of the upper respiratory tract at the time of biopsy.

The study was approved by the ethical committee of the Leiden University Medical Centre. All individuals gave informed consent.

Nasal biopsies

One nasal biopsy was taken from each person. All biopsies were taken by the same investigator. Local anaesthesia was achieved by applying a cotton wool carrier with 50-100 mg of cocaine and 3 drops of adrenaline (1:1000) under the inferior turbinate, without touching the biopsy site. Biopsy specimens were taken from the lower edge of the inferior turbinate by using a Gerritsma forceps [27] and embedded in Tissue-Tek II OCT compound. The biopsies were immediately frozen and stored at -150 ^{o}C.

Immunohistochemical staining

Each tissue specimen was cut into 6µm-thick sections. All stainings were developed with the immunoalkaline phosphatase method, as previously described [28]. All cryosections that were accepted for evaluation had an undamaged basal membrane of at least 1 mm length and a surface of the lamina propria of at least 1 mm^2. The mean length of the basal membrane was 3.8 mm and the mean surface of the epithelial layer was 3.0 mm^2. Multiple fields were counted and the number of cells is expressed as positively stained cells per mm^2. As a negative control we replaced the primary antibody by

mouse IgG. Different subsets of DCs were stained using specific monoclonal antibodies. Myeloid dendritic cells were stained using mouse monoclonal antibodies directed to CD1a (Leu 6, Becton Dickinson, San Jose CA), anti DC-SIGN (CD209, DC-SIGN 1 R&D) or BDCA-1 (clone AD5-8E7; Miltenyi Biotec). Plasmacytoid DCs were stained with monoclonal antibodies directed to BDCA-2 (clone AC144; Miltenyi Biotec).

Biopsies were coded and counted for positively stained cells, independently by two investigators for each antibody, as has been described previously [28]. Positively stained cell numbers in the epithelium and in the subepithelial lamina propria, 100 μm deep along the basal membrane, were counted, using a light microscope (Zeiss, Jena, Germany) with an eye piece graticulate at a magnitude of 400x and were expressed as positively stained cells per mm^2.

Double staining

For double staining of BDCA-1 or BDCA-2 with DC-SIGN, acetone fixed sections were incubated with 0.1% H_2O_2 and 0.1% NaN_3 in PBS for 30 min at room temperature (RT) to block endogenous peroxidase activity. The slides were blocked with PBS containing 1% PBS and 5% heat inactivated normal human serum (NHS) for 45 minutes at RT and subsequently incubated with antibodies directed against BDCA-1 or BDCA-2 in PBS with 1% BSA and 1% NHS overnight at RT. Antibody binding was detected using a HRP labeled goat-anti-mouse antibody (Dako, Glostrup, Denmark) in PBS with 1% BSA and 1% NHS for 60 min at RT. Next, the slides were blocked again with PBS containing 1% PBS and 5% NHS for 45 minutes followed by an overnight incubation with antibody against DC-SIGN in PBS with 1% BSA and 1% NHS at RT. The presence of DC-SIGN antibody was detected by incubation with a rabbit-anti-mouse IgG2b specific antibody (Jackson, Suffolk, UK) in PBS with 1% BSA and 1% NHS at RT followed by detection with a biotinylated goat-anti-rabbit antibody (Dako, Glostrup, Denmark) in PBS with 1% BSA and 1% NHS and amplified with a streptavidin biotin complex containing alkaline phosphatase (Dako, Glostrup, Denmark). Eventually, development with Tyramide-FITC in tyramide buffer (NEN™ Life Science Products)

for 30 min at RT was used to detect the presence of BDCA-1 or BDCA-2 positive cells. For detection of DC-SIGN positive cells, Fast Red TR salt and naphtol tablets (both from Sigma-Aldrich, Zwijndrecht, The Netherlands) were dissolved according to manufacturers instructions. For development, equal amounts of Fast Red TR and naphtol solutions were mixed and added to sections for 15 minutes at RT. Slides were mounted with mowiol.

Statistical analysis

Statistical analysis was performed, using the Mann-Whitney test. The Spearman correlation coefficient was calculated to determine the correlation between the different subsets of DCs. P values of <0.05 were considered significant.

Results

Demonstration of DC subsets in nasal biopsies

We collected nasal biopsies from 18 IgAN patients and 18 control persons. Biopsies were analysed by hematoxylin eosin (HE) staining to check the quality of the tissue and confirm the presence of an epithelial layer (Figure 1A).

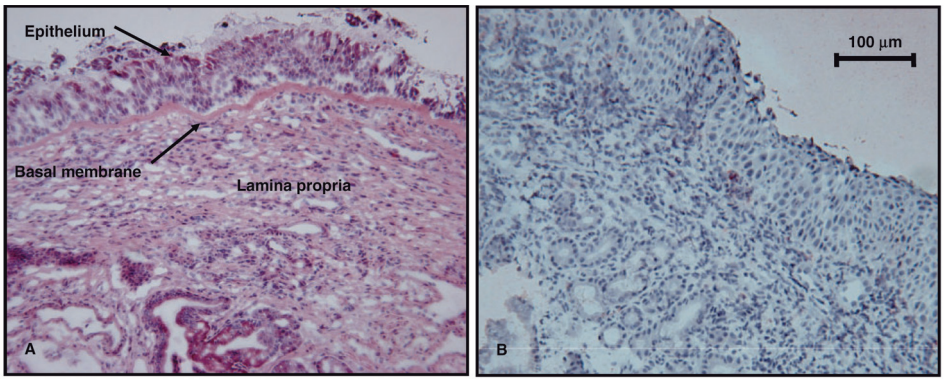

Figure 1. HE staining and negative control of nasal mucosa. Light microscopic section of nasal mucosa, stained with hematoxylin eosin at a magnification of 100x (A). The outer epithelial layer is divided from the lamina propria by the basal membrane. Negative control of a biopsy stained for BDCA-1 (B).

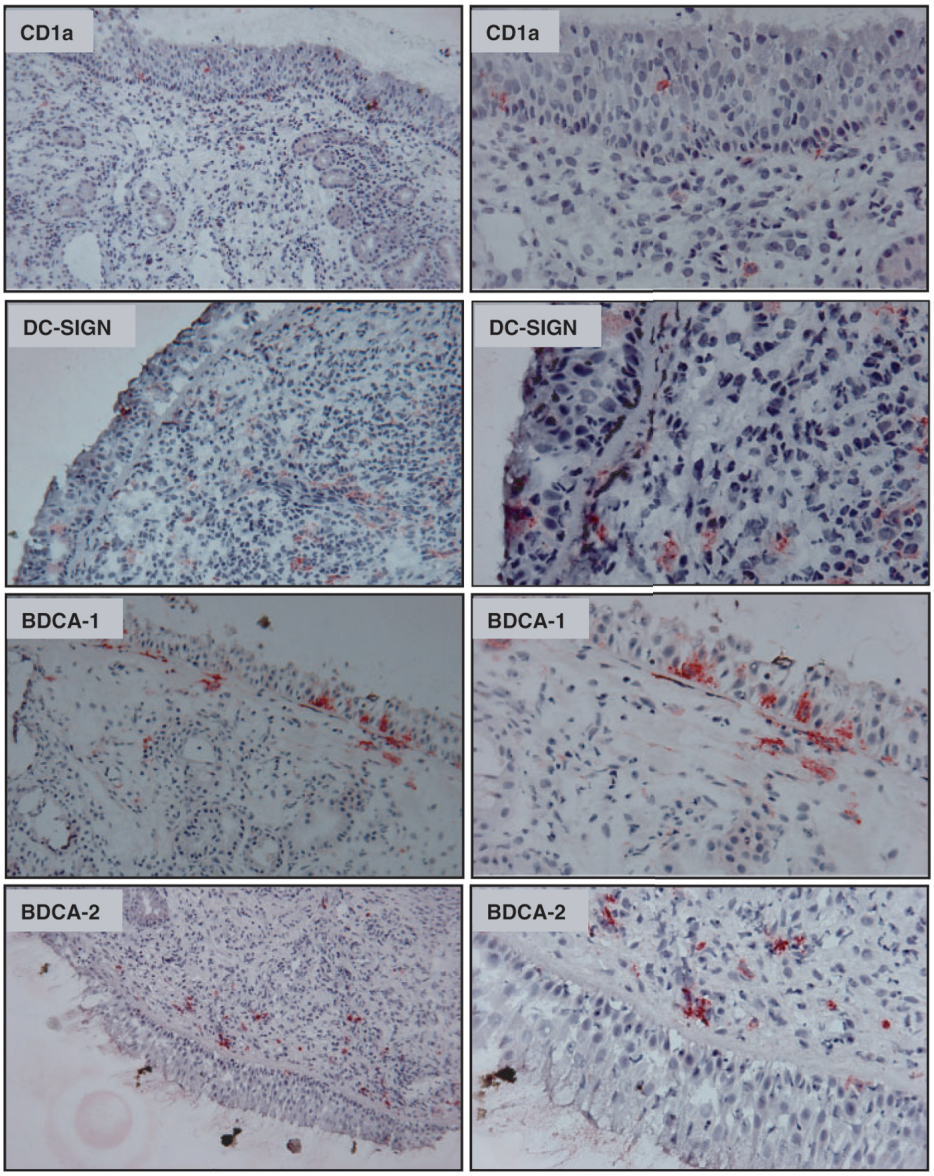

Figure 2. Expression of DC-SIGN, CD1a, BDCA-1 and BDCA-2 in nasal mucosa. Cryosections of nasal mucosa were stained for expression of DC-SIGN, CD1a, BDCA-1 and BDCA-2 as described in *material and methods*. The left panel shows cryosections at an original magnification of 100x and the right panel at a magnification of 250x. CD1a positive cells are more frequently located in the epithelial layer. DC-SIGN positive cells and BDCA-2 positive cell are mainly located in the lamina propria. Shown are representative pictures of IgAN patients.

Cryosections were specifically analysed for the presence of DCs using specific monoclonal antibodies. To identify myeloid DCs, cryosections were stained for CD1a, DC-SIGN and BDCA-1. To quantify plasmacytoid DCs, cryosections were stained for BDCA-2. After staining, the number of cells was counted and related to the surface tissue area and compared to the negative control stainings (Figure 1B).

Positive cells are coloured red and were counted by two investigators independently. In all cryosections an epithelial layer, with a variable thickness was visible. The epithelial layer is separated from the lamina propria by the basal membrane. In the lamina propria, glands and vessels are visible, which were excluded from the analysed surface. In both the epithelial layer and the lamina propria DCs are present. Pictures of representative cryosections with an original magnification of 100x (left panel) and 250x (right panel) are shown in figure 2.

Quantification of myeloid dendritic cells in nasal biopsies

Different subsets of myeloid DCs were identified, using CD1a, DC-SIGN and BDCA-1 as markers. Biopsies were stained for BDCA-1, which is a broadly expressed marker for myeloid DCs. Compared to CD1a and DC-SIGN the absolute number of positively stained cells was highest for BDCA-1. No differences in BDCA-1 positively stained cells between epithelial layer and lamina propria were present. The number of positively stained cells did not differ between the control group and the IgAN patients (Figure 3).

The number of DC-SIGN positive cells was higher in the lamina propria than in the epithelial layer. Moreover the number of DC-SIGN positive cells in the lamina propria was significantly higher in IgAN patients than in the control group (median 71.8 vs. 41.6 $p=0.022$). In contrast no significant differences were found in the number of DC-SIGN positive cells in the epithelium between the two groups (Figure 3).

In both the control group and in the IgAN patient group the number of CD1a positive cells was higher in the epithelial layer than in the lamina propria. The number of CD1a positive cells in the epithelial layer was significantly higher in IgAN patients than in control persons (median 72.8 vs

20.2, p=0.009). In contrast the number of CD1a positive cells in the lamina propria did not differ between the IgAN patient and the control group (Figure 3). We also looked at the actual ratio numbers of DC between the different compartments. No significant differences in tissue distribution of positively stained cells was present between the two groups with respect to the different myeloid markers (data not shown).

Quantification of plasmacytoid dendritic cells in nasal biopsies

Plasmacytoid DCs were stained using BDCA-2 as a surface marker. The number of positively stained cells in the lamina propria was higher than in the epithelial layer. No differences were found in the number of BDCA-2 positively stained cells between the IgAN patients and the control persons, neither in the epithelial layer, nor in the lamina propria (Figure 4). With respect to BDCA-2, no difference in actual ratio of numbers of DC between different sites was present between the IgAN patients and the controls. The number of plasmacytoid DC in these nasal biopsies was lower than the number of myeloid DC.

Correlations between the number of positively stained cells

To determine whether CD1a, DC-SIGN, BDCA-1 and BDCA-2 positively stained cells in the epithelial layer are correlated with the number of positively stained cells in the lamina propria, we calculated Spearman correlation coefficients. For all stainings the number of positively stained cells in the epithelial layer appeared to be correlated to the number positively stained cells in the subepithelial lamina propria. The correlation coefficient varied from 0.49 to 0.69 with p values of maximal 0.0017 (Figure 5). In both the control group and in the IgAN patients this correlation was found.

We also investigated the correlation between the different subsets of DCs. In the epithelial layer there was a correlation between $CD1a^+$ cells and $BDCA\text{-}1^+$ cells and a correlation between $DC\text{-}SIGN^+$ and $BDCA\text{-}1^+$. In the lamina propria no correlation between CD1a and BDCA-1 was found. Furthermore a correlation between epithelial BDCA-1 and epithelial BDCA-2 was present and between epithelial BDCA-1 and lamina propria BDCA-2 . (Table 1).

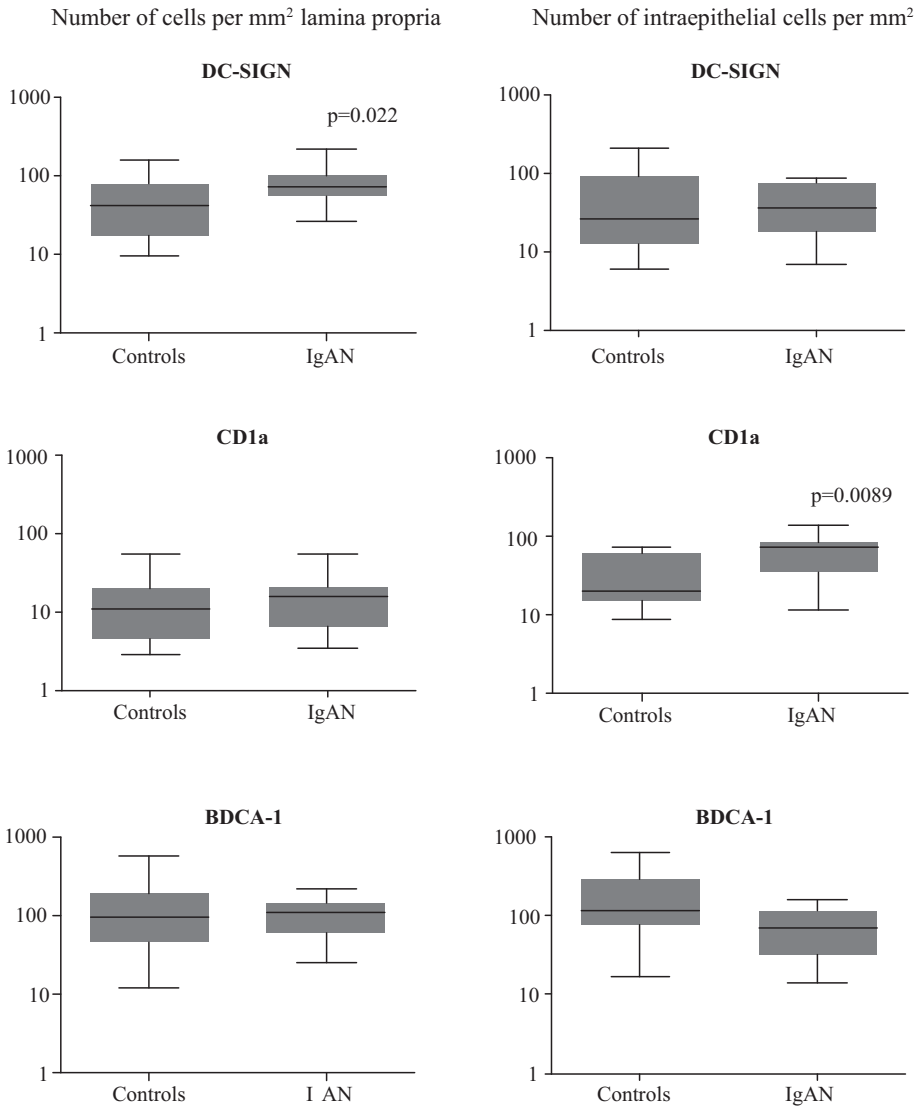

Figure 3. Number of different subsets of myeloid DCs in nasal mucosa of controls and IgAN patients. Cryosections of nasal biopsies from control persons and IgAN patients were stained for DC-SIGN, CD1a and BDCA-1 respectively. Positive cells in the epithelial layer (right panel) and in the lamina propria (left panel) were counted as described in materials and methods section. Bars represent median ± interquartile range and the smallest and greatest values in the distribution. Statistics were performed using the Mann-Whitney test. p values of <0.05 were considered significant.

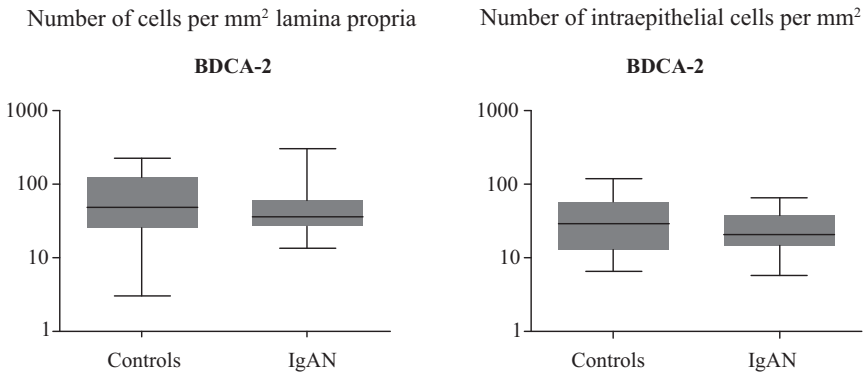

Figure 4. Number of plasmacytoid DCs in nasal mucosa of controls and IgAN patients. Cryosections of nasal biopsies from control persons and IgAN patients were stained for BDCA-2, a marker for plasmacytoid DCs. Positive cells in the epithelial layer and in the lamina propria were counted as described in materials and methods sections. Bars represent median ± interquartile range and the smallest and greatest values in the distribution. Statistics were performed using the Mann-Whitney test.

Table 1. Correlation of different subsets of DCs in nasal mucosa. Nasal mucosa was stained for the presence of different subsets of DCs. Spearman correlation coefficients were determined to calculate statistical relations between the different DC molecules. p values of <0.05 were considered significant.

	DC-SIGN epithelium	CD1a epithelium	BDCA-1 epithelium	BDCA-2 epithelium
DC-SIGN-E	x	n.s		
DC-SIGN-LP	r=0.69 ***	n.s	n.s.	n.s.
CD1a-E	n.s	x		
CD1a-LP	n.s	r=0.49 ***	n.s.	n.s.
BDCA-1-E	r=0.50 ***	r=0.39 *	x	
BDCA-1-LP	n.s	r=0.38*	r=0.60 ***	n.s.
BDCA-2-E	n.s	n.s	r=0.38 *	x
BDCA-2-LP	n.s	n.s	r=0.50 **	r=0.55 ***

* p<0.05 E=epithelium

** p< 0.01 LP=Lamina Propria

*** p<0.002

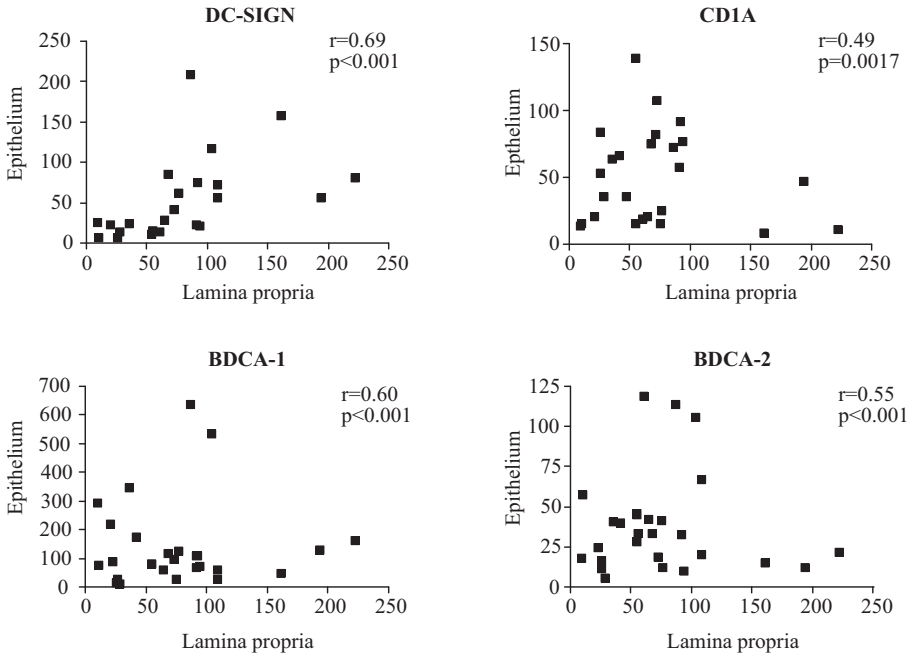

Figure 5. Correlation between DC-SIGN$^+$, CD1a$^+$, BDCA-1$^+$ and BDCA-2$^+$ cells in epithelial layer and lamina propria. Cryosections of nasal biopsies were stained for DC-SIGN, CD1a, BDCA-1 and BDCA-2, as described in materials and methods section. The number of positively stained cells in the lamina propria and epithelial layer are plotted. Spearman correlation coefficients were determined to calculate statistical relations. Filled squares represent DC counts of an individual biopsie.

Double staining for DC-SIGN and BDCA-1 or BDCA-2

To further investigate the correlations between the different markers of DCs, we performed double stainings for DC-SIGN and BDCA-1 or BDCA-2. When staining for two myeloid DC markers, DC-SIGN and BDCA-1 we found a substantial proportion of double positive cells. However also single positive cells were observed (Figure 6A). When double staining for DC-SIGN and BDCA-2 was investigated, we found no overlap in staining (Figure 6B). This is compatible with the fact that these two markers are expressed on DC subsets proposed to be derived from different lineages (myeloid versus lymphoid respectively).

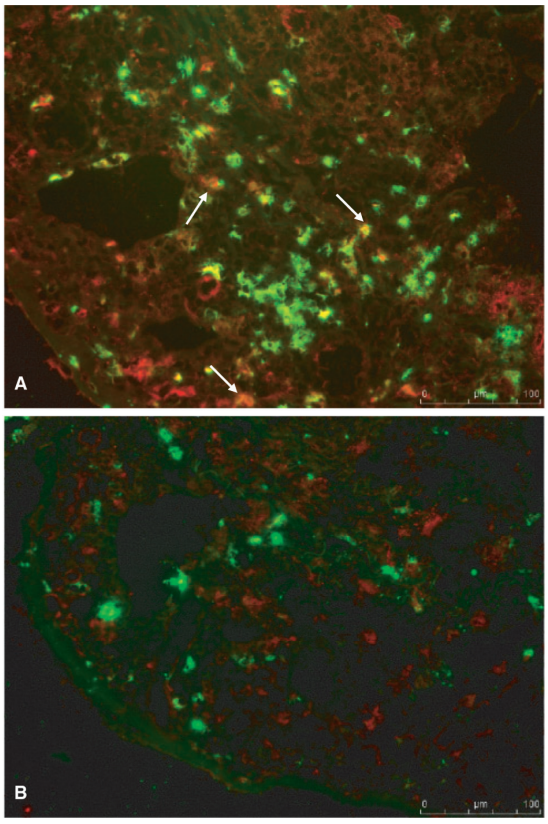

Figure 6. **Nasal biopsies contain DC-SIGN+ BDCA-1+ cells.** Cryosections of nasal biopsies were stained for DC-SIGN (green) and BDCA-1 (red) (A) or BDCA-2 (red) (B) as described in materials and methods sections. White arrows indicate DC-SIGN+ BDCA-1+ cells. Original magnifications were x200.

Discussion

In the present study we investigated the nasal mucosa of IgAN patients for the presence of different subsets and numbers of DCs, as previous experiments suggested an aberrant immunological response in these patients. After mucosal vaccination with a neoantigen, the specific IgA immune respons was hampered in IgAN patients in comparison with controls, whereas no

differences were found in the antigen specific IgG and IgM responses [15]. In the same study no differences were found in immunoglobulin production after simultaneous systemic vaccination with another neoantigen Keyhole Limpet Hemocyanin, (KLH). Therefore we wanted to address the question whether IgAN patients have reduced numbers of DCs in the nasal mucosa, or have a different composition of specific subsets of DCs. To this extent, nasal biopsies were taken from IgAN patients and controls and were stained for the presence of different subsets of DCs. Our results indicate that IgAN patients did not have reduced numbers of DCs, and even showed higher numbers of intra-epithelial $CD1a^+$ cells and DC-$SIGN^+$ cells in the lamina propria.

DCs as professional antigen presenting cells are present in the epithelial layer and form a continuous network [29]. DCs can migrate and after antigen uptake and processing can induce immune responses. DCs have direct effects on many different cells. It is well known that DC can activate naïve T cells, which subsequently can induce B cell responses. However also direct effects of DC on B cells are described, leading to isotype switching and enhanced immunoglobulin production [19;30;31]. These effects are dependent on CD40-CD40L interaction [32]. Recently it has been reported that DC can also induce Ig production by B cells in a CD40 independent way, through activation of BAFF and APRIL [33;34].

In the current study we investigated the number of nasal DCs of IgAN patients and a control group. Our hypothesis, that the number of nasal DCs in the IgAN patients was lower than the number of nasal DCs in the control group, appeared not to be true. As a matter of fact the number of CD1a positive cells in the nasal epithelial layer and the number of DC-SIGN positive cells in the nasal lamina propria were even significantly higher in the biopsies of the IgAN patients. This means that the formerly observed IgA hyporespons after nasal vaccination can not be explained by a reduced number of antigen presenting cells.

The different subsets of DCs in human are not fully functionally characterized and so far most knowledge about their function is from *in vitro* experiments. Dubois et al. have described an increase of IgA production via stimulation of naive B cells by CD34-derived interstitial DC, but no effect

on IgA production after stimulation with Langerhans cells which are derived from a CD1a positive precursor [31]. This suggests higher IgA production when DC-SIGN positive and BDCA-1 positive DCs are present. IgAN patients appeared to be hyporesponders after mucosal vaccination. Whether this hyporesponse is related to the higher numbers of CD1a positive DCs in the epithelial layer remains speculative. An alternative explanation for the mucosal hyporesponsiveness of IgAN patients, could be that DC are functioning well, but that there are defects in the B or T cells. Recently it was shown that $CD4^+$ T cells of patients with IgAN show an aberrant expression of homing receptors, potentially contributing to altered mucosal immune responses [3].

In vivo the type of DC that captures and presents an antigen to T cells is probably decisive whether a processed antigen gives rise to an immune response or is tolerated. In the pathogenesis of pulmonary diseases like asthma and chronic obstructive pulmonary disease DCs play a central role [36]. As DCs in the upper respiratory tract are in contact with many antigens each day, a dysfunction of this complex system might contribute to the pathogenesis of diseases in which the mucosa is involved, such as IgAN. The additional effect of local tissues on the function or phenotype of DCs *in vivo* is also unknown. It has recently been described by Allam et al. that mucosal myeloid $CD1a^+$ DCs differ from their skin counterparts by the expression of the high affinity receptor for immunoglobulin E [37].

Recently we have studied the capacity of monocyte-derived DCs from IgAN patients and control persons to induce IgA production in an *in vitro* model. We found a reduced capacity of DCs from IgAN patients to increase IgA production by naïve B cells, when DCs were cultured in the presence of IL-10 and CD40L [22]. Whether the increased number of CD1a and DC-SIGN positive DCs in the nasal biopsies of IgAN patients are a kind of compensation for the reduced functional capacity to increase IgA production is only speculative. It would be interesting to study functional activities of mucosal DC of IgAN patients. However techniques and availability of tissue are presently not sufficient to perform proper functional analysis. An alternative way to get more information on DC function could be further phenotypic analysis of tissue DC, preferably during antigen challenge.

In our study we found for all different stainings a strong correlation between the number of positively stained cells in the epithelial layer and in the lamina propria. We also calculated the correlation between different markers and found in the epithelial layer a strong correlation between $CD1a^+$ and $BDCA\text{-}1^+$ and a correlation between $DC\text{-}SIGN^+$ and $BDCA\text{-}1^+$ DCs. To further investigate this correlation we performed double staining for DC-SIGN and BDCA-1 and showed that there were double positive cells. This result is comparable with an analysis of DC subsets in human kidney in which we showed that many cells were double positive for DC-SIGN and BDCA-1 [38]. In cell suspensions of human pulmonary tissue, many $CD1a^+$ DC were also positive for BDCA-1 [36]. A weaker correlation was also present between BDCA-1 and BDCA-2 in the epithelial layer. Based on these observations we conclude that the correlations in DC staining we observed in our nasal biopsies are explained partially by double staining of the same cells and partially by local circumstances that result in the attraction of multiple DC subsets.

In conclusion the number of DCs in the nasal mucosa is not reduced in IgAN patients. We have shown that the number of CD1a positive epithelial DCs and the number of DC-SIGN positive cell in the lamina propria of IgAN patients in nasal biopsies is even significantly higher in comparison with control persons. Taken together with the observed IgA hyporesponse after mucosal vaccination with a neoantigen we therefore believe that reduced hyporesponsiveness of IgAN-DC may be the most probable cause for a mucosal hyporesponse in IgAN.

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Chapter 4

Dendritic cells of IgA nephropathy patients have an impaired capacity to induce IgA production in naïve B cells

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Summary

IgA nephropathy (IgAN) is the most common primary glomerulonephritis worldwide, characterized by mesangial IgA_1 deposits. We have previously demonstrated that IgAN patients have a hampered IgA immune respons after mucosal challenge with a neoantigen. Dendritic cells (DCs) are critically involved in the initiation of humoral immune responses, not only via activation of T helper cells, but also via direct effect on naïve B cells. The aim of this study was to investigate the capacity of DCs from IgAN patients to regulate IgA production.

DCs were generated by culturing monocytes for 7 days in the presence of IL-4 and GM-CSF. DCs from either IgAN patients (n=12) or controls (n=12) were cultured for 14 days with naïve B cells in the presence of CD40L-transfected mouse fibroblasts (L-CD40L cells) and medium with or without IL-2 or IL-10. Supernatants were tested for the presence of immunoglobulins by specific ELISA.

In the presense of CD40L and IL-10, DCs were able to increase immunoglobulin production by naïve B cells. DCs of IgAN patients induced significantly ($p=0.026$) less IgA production than DCs of control persons (2.30 µg/ml vs. 5.24 µg/ml), whereas no differences were found in the IgG and IgM production. When DCs were replaced by supernatant of CD40L-stimulated DCs of patients and controls, IgA production was increased, but no difference was seen between the two groups.

In the present study we show that DCs of IgAN patients have an impaired capacity to induce IgA production in naïve B cells, which might explain the observed IgA hyporesponse upon mucosal challenge with a neoantigen.

Introduction

IgA nephropathy (IgAN) is the most common primary glomerulonephritis worldwide [1-3]. Overall, IgAN is the cause of renal failure in ±10% of the people being on chronic renal replacement therapy in Western Europe and the United States. Patients usually present with microscopic hematuria and proteinuria. In renal biopsies, IgAN is characterized by IgA1 and complement component 3 (C3) deposits in the mesangium of the glomeruli [4;5]. After kidney transplantation, recurrent IgA deposits are found in the allograft in a high percentage of the cases. Moreover occasional accidental transplantation of a kidney of an IgAN patient lead to disappearance of IgA deposits [6;7]. Therefore it is most likely that the major cause of IgA deposition in the renal mesangium lies in IgA itself or the interaction between IgA and the mesangium.

The pathogenesis of IgAN is not clear. Several factors have been implicated to play a role in the formation of IgA immune deposits in the mesangium. Both quantitative and qualitative differences in IgA seem to be a factor involved in the pathogenesis [8;9]. Detailed biochemical analysis of IgA_1 suggests that underglycosylated IgA_1 is enriched both in serum and in mesangial deposits [10;11]. Moreover it has been suggested that glycosylation of IgA might be affected by the mode of B cell activation and the cytokine milieu [12].

A clinical observation is that upper respiratory tract infections frequently lead to an increase of hematuria (‘synpharyngitic hematuria’) [13;14]. Therefore a dysregulation of the mucosal immune response of IgAN patients might play a role in the pathogenesis [1]. Mucosal vaccination of IgAN patients with neoantigens was shown to lead to an impaired mucosal and systemic IgA response, compared with healthy persons [15;16]. In these studies no differences in the antigen specific IgG and the IgM responses were measured. These results seemed in conflict with the original idea that IgAN patients are hyperresponders after vaccination, as suggested by the higher serum IgA titers in most IgAN patients. The observation that systemic vaccination of IgAN patients with a recall antigen appeared to give rise to

a systemic and mucosal hyperresponse, might be explained by an increased level of immunological memory [17-19]. When we performed a simultaneous mucosal and systemic vaccination with a neoantigen, only the mucosal challenge showed a hyporesponse, whereas the systemic challenge was not different between patients with IgAN and controls [15]. This suggests that the mucosal immune regulation has specific regulatory features [20;21].

Immunoglobulin production is the result of a complex interaction between B cells, T cells and antigen presenting cells like dendritic cells (DCs). Naïve B cells are IgM/IgD positive and have to undergo a program of affinity maturation, isotype switch and plasma cell differentiation before high affinity IgG or IgA antibodies can be produced [22]. This process is tightly regulated by activated T helper cells through expression of CD40L/CD154 and the production of cytokines. In the hyper-IgM syndrome a genetic alteration of CD40L results in a deficit of circulating IgG and IgA [23;24]. For IgA production, cytokines like IL-10 and TGF-β have been shown to determine the isotype specificity [25]. More recently it has been shown in an *in vitro* model that DCs were capable to further increase IgA production in the presence of IL-10 and CD40L [26;27]. Therefore, DCs seem to have a direct effect on B cells, next to its well established role in the activation of T helper cells [28;29].

In the present study we investigated the capacity of DCs from IgAN patients to induce IgA production in naïve B cells *in vitro* and compared this with DCs from healthy volunteers. We found that DCs from IgAN patients induce less IgA production, whereas no differences for IgG and IgM were found.

Materials and methods

Selection of IgAN patients

Biopsy proven IgAN patients were selected for entry in this study. Patients did not take immunosuppressive drugs within a three months period before blood samples were taken. Age and sex matched healthy controls were selected.

No infections or macroscopic hematuria were present at the time of blood sampling.

Mean age of the patients was 42 years versus 39 years of the controls (Table 1). The male: female ratio was 83% vs 17% in both groups. Renal function was preserved and stable in most of the patients. Mean creatinine clearance was 78 ml/min. Only one patient had a creatinine clearance lower than 25 ml/min. Proteinuria was mild with a mean value of 1.3 gram per litre. The study was approved by the Ethical Committee of the Leiden University Medical Centre. All individuals gave informed consent.

Table 1. Characteristics of IgAN patients and control persons

	IgAN patients	**controls**
Number	12	12
Mean age (range)	42 (22-62)	39 (30-52)
Male/female	10/2	10/2
Years since diagnosis (range)	8 (1-22)	
Serum creatinine (μmol/ml)	135 (79-347)	N.D
Creatinine clearance (ml/min)	78 (19-117)	N.D
Proteinuria (g/L)	1.3 (0-1.9)	N.D
Erythrocyturia (0-4+)	2+	N.D

N.D is not determined

Generation of dendritic cells

Fifty ml of heparinized peripheral blood was collected from each IgAN patient and healthy control person. PBMC were isolated by Ficoll-Hypaque (Sigma, St. Louis, MO) density gradient centrifugation. Cells were washed three times, to remove Ficoll and thrombocytes. Subsequently monocytes were isolated by Percoll (Pharmacia, Uppsala, Sweden) gradient centrifugation and plastic adherence for 2 hours in six wells culture plates ($2.5x10^6$ cells/well; Costar, Cambridge, MA). Adherent monocytes were cultured for 7 days in RPMI 1640 containing 10% heat-inactivated FCS and penicillin/streptomycin (all from Gibco/Life Technologies, Breda, The Netherlands), in the presence of 5 ng/ml

GM-CSF (Leucomax, Novartis Pharma B.V., Arnhem, The Netherlands) and 10 ng/ml IL-4 (Peprotech, Rocky Hill, NJ) [30].

Analysis of cell surface phenotype by flow cytometry

Cells were harvested and washed in PBS containing 1% BSA and 1% heat-inactivated normal human serum and 0.02% NaN_3. FACS analysis was performed using mAb against CD1a (Leu-6) and CD14 (Leu-M3) (both Becton Dickinson & Co., Mountain View, CA) and CD86 (IT2.2; Pharmingen, San Diego) and CD209 (mouse anti-human DC-SIGN, AZN-DI, kindly provided by Prof. Yvette van Kooyk. Free University Medical Centre, Amsterdam, The Netherlands). Staining was visualized by using PE-conjugated goat-anti-mouse Ig (Dako, Glostrup, Denmark), assessed for fluorescence using a FACScan and analyzed with WinMDI software (Becton Dickinson & Co.).

Isolation of serum IgD+ ($sIgD^+$) B lymphocytes

B cells were isolated from tonsils by the Ficoll-Rosetting method [25]. Purified $sIgD^+$ B lymphocytes were separated using a preparative magnetic cell sorter (MACS; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the experimental procedure described in detail by Miltenyi et al. [31]. IgD was expressed on >99% of the $sIgD^+$ B cell population as assessed by FACScan analysis (Becton Dickinson & Co.).

B cell activation

All cultures were performed in Iscove's modified Dulbecco's medium (IMDM) glutamax (Gibco/Life Technologies) supplemented with 10% heat-inactivated FCS and ITS (Insulin 5 µg/ml, Transferrin 5 µg/ml and Selenium 5 ng/ml final concentration; Sigma). $1x10^4$ IgD^+ B cells were cultured in the presence of $1x10^4$ irradiated (33 Gy) DC and $0.25x10^4$ irradiated (75 Gy) murine L-cell fibroblasts stably transfected with human CD40L (L-CD40L cells) [32] in a final volume of 200 µl in 96-wells round bottom tissue culture plates (Costar) [26]. In each experiment cells were cultured in the presence of medium, IL-2 (20 U/ml) (Chiron, Emeryville, CA), or IL-10 (50 ng/ml)(Schering Plough, Dardilly, France). Each culture condition was performed in 5 fold. Conditions

without DC and/or without B cells served as negative controls. Supernatant was harvested after 14 days.

Generation of supernatant from dendritic cells

$5x10^4$ DCs were cultured for 72 hrs in combination with $1x10^4$ irradiated L-CD40L cells or $1x10^4$ L-Orient cells in a total volume of 500 µl in IMDM glutamax supplemented with 10% heat-inactivated FCS and ITS. Supernatant was harvested and stored at -20ºC until further use.

B cell experiments with supernatant of dendritic cells

All cultures were performed in IMDM glutamax supplemented with 10% heat-inactivated FCS and ITS. $1x10^4$ IgD^+ B cells were cultured in the presence of 50 µl supernatant of CD40L-stimulated or unstimulated DCs and $0.25x10^4$ irradiated (75 Gy) L-CD40L cells with or without IL-10 (50 ng/ml) in a final volume of 200 µl in 96-wells round bottom tissue culture plates (Costar). Each culture condition was performed in 5 fold. Conditions without supernatant and/or without B cells served as negative controls. Culture supernatant was harvested after 14 days and tested for Ig by ELISA.

Immunoglobulin quantification

IgA, IgA_1, IgA_2, IgG and IgM levels were determined by specific sandwich enzyme-linked immuno assay (ELISA), as described [15]. All ELISA's were performed in 96-well polystyrene plates (Greiner, Alphen a/d Rijn, The Netherlands). Optical density (OD) was measured at 415 nm on a microplate reader (Bio-Kinetics Reader EL 312e, Biotek Instruments Inc., Winooski, VT, USA).

IL-10 quantification

IL-10 was measured by a commercially available ELISA kit (Sanquin, Amsterdam, The Netherlands) and was used as indicated by the manufacturer.

Statistical analysis

Statistical analysis was performed, using students t-test, P values less than 0.05 were considered significant.

Results

Immunoglobulin production is dependent on the presence of CD40L and increased by dendritic cells

To be able to investigate the capacity of DCs of IgAN patients to modulate IgA production by naïve IgD^+ B cells, we established a culture system based on the presence of L-CD40L cells, IgD^+ naïve B cells and recombinant cytokines. Independent of the cytokines used, only very low concentrations of immunoglobulins were detected in the absence of CD40L. In the presence of CD40L the immunoglobulin production of all different isotypes was strongly upregulated (Figure 1). This Ig production is dependent on the presence of IL-10 and is not supported by IL-2. Next, monocyte-derived DCs were added to CD40L-activated B cell cultures. In the absence of exogenous cytokines, DCs did not significantly stimulate Ig production. However in the presence of IL-10 and also in the presence of IL-2 [33] DC stimulated production of IgA, IgG and IgM (Figure 2). This model allows the investigation of the B cell stimulating capacity of DCs of patients with IgAN.

No phenotypic differences between dendritic cells of IgAN patients and control persons

Immature DCs were generated by culturing monocytes from patients and controls in the presence of IL-4 and GM-CSF during 7 days. Monocytes are negative for CD1a and DC-SIGN and have high expression of CD14. Immature DCs have surface expression of DC-SIGN, a specific DC marker, and have high expression of CD1a and low expression of CD14 (Figure 3A). The phenotype of immature DC of IgAN patients and controls did not differ in expression of DC-SIGN (Figure 3B). Pilot experiments showed that individual contaminating B cells in the DC population could affect the outcome of Ig

production. This effect could be prevented by irradiation of (30 Gy) the DC population. Irradiation did not affect CD40L-induced functions of DC, like IL-10 production (Figure 4A) or CD86 expression (Figure 4B).

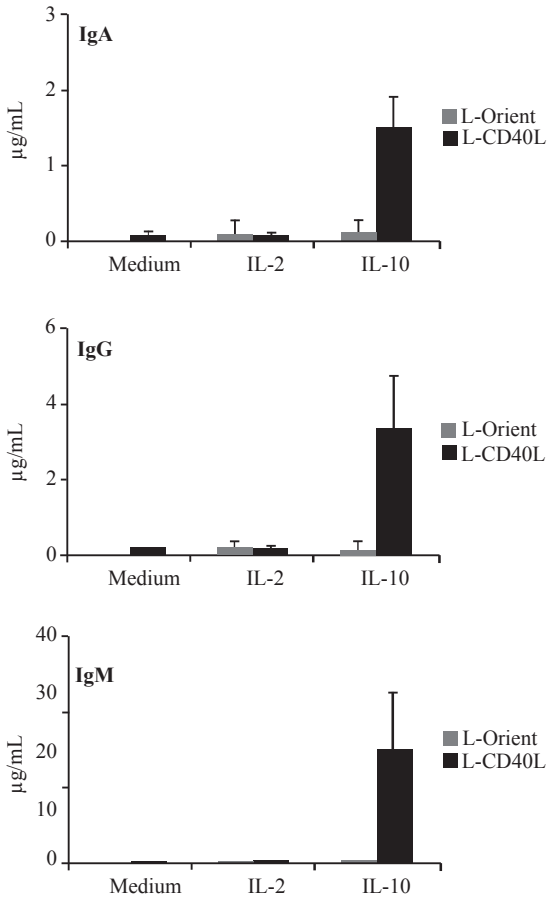

Figure 1. Immunoglobulin production is dependent on CD40L and IL-10. Highly purified IgD^+ B cells (10^4) were cultured with 75 Gy irradiated non-transfected L cells (L-orient) or CD40L transfected L cells (L-CD40L) ($0.25x10^4$) in the presence of IL-2 (20 U/ml) or IL-10 (50 ng/ml) as indicated. After 14 days supernatants were harvested and tested for IgA, IgG and IgM production using specific ELISA's. Data shown are the mean $\pm$SD of quadriple cultures and represents one out of 3 independent experiments.

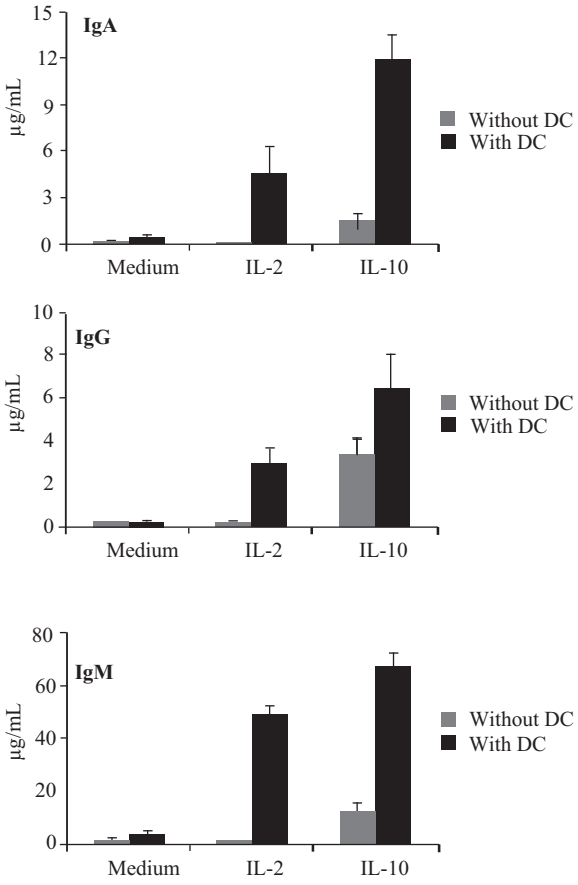

Figure 2. DCs increase Ig production by naïve B cells. Highly purified IgD+ B cells (10^4) were cultured with 75 Gy irradiated CD40L transfected L cells ($0{,}25x10^4$) in the absence of DCs (grey bars) or in the presence of DCs (10^4) (black bars), in the presence of IL-2 (20 U/ml) or IL-10 (50 ng/ml) as indicated. After 14 days supernatants were harvested and tested for IgA, IgG and IgM production using specific ELISA's. Data shown are the mean +SD of fivefold cultures and are representative of 3 independent experiments.

Co-culture of dendritic cells from IgAN patients with naïve B cells results in a reduced increase in IgA production

In order to compare the capability of DCs from IgAN patients with DCs from healthy volunteers in the induction of IgA switch in naïve B cells, monocytes were isolated from IgAN patients (n=12) and healthy volunteers (n=12).

Controls and IgAN patients were always tested in parallel with the same source of B cells and the same L-CD40L cells. Co-culture in the presence of IL-10 resulted in less IgA production induced by DCs from IgAN patients as compared to DCs from healthy control persons (5.34 µg/ml versus 2.34 µg/ml; p=0.026) (Figure 5A). No difference between patients and controls was observed when cocultures were performed in the presence of IL-2. To determine the specificity for the IgA regulation, also IgG and IgM were measured in the same supernatants. In all conditions, there were no differences between the IgAN patient group and the control persons with respect to IgG and IgM production (Figure 5B).

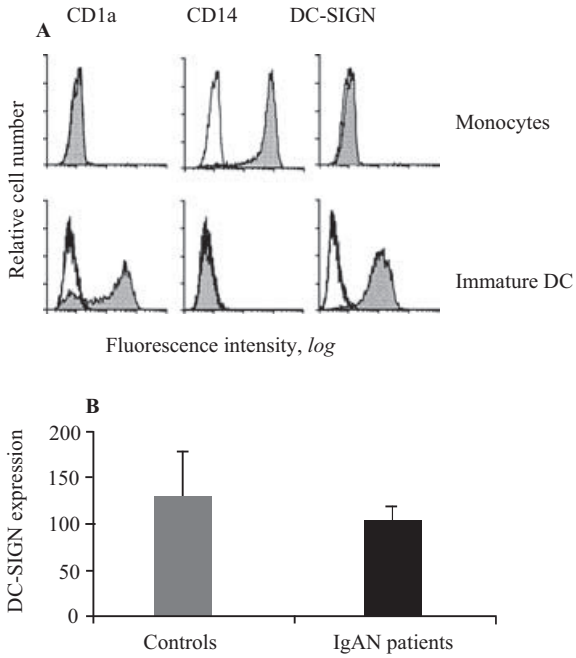

Figure 3. Analysis of CD1a, CD14 and DC-SIGN expression on monocytes and immature dendritic cells from controls and patients with IgAN. *(***A**) Surface expression of CD1a, CD14 and DC-SIGN on monocytes (upper panel) and monocyte derived DCs (lower panel) was determined using FACS analysis. Specific staining is represented by the filled histograms and control staining by the open histograms. Shown is a representative example of DCs from a control. (**B**) Surface expression of DC-SIGN on DC from control persons and DC from IgAN patients was determined using FACS analysis. Shown are the mean fluorescence intensity (MFI) ±SD of 5 control persons and 5 IgAN patients.

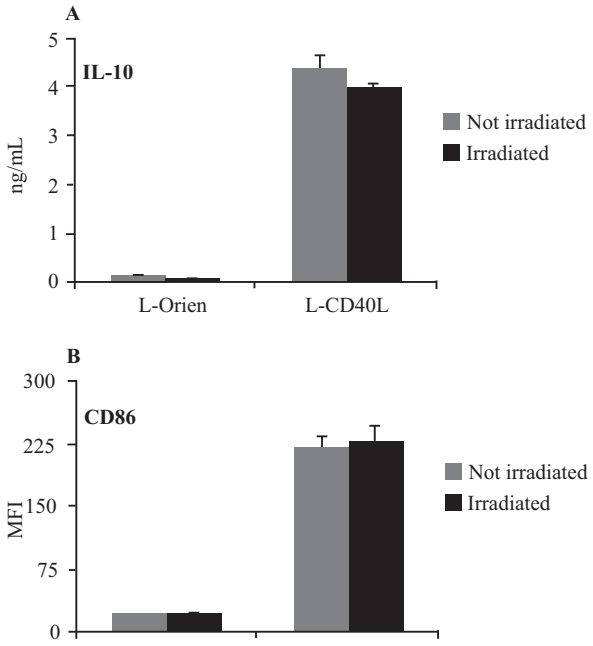

Figure 4. Irradiation of DCs does not influence CD40L induced IL-10 production and CD86 expression. (**A**) DCs ($5x10^4$) were either irradiated (33 Gy) or not irradiated and cultured for 72 hours in the presence of control L cells (left panel) or CD40L-transfected L cells (10^4) (right panel). IL-10 production was measured by specific ELISA. Shown are the mean $\pm$SD of quadriple cultures. (**B**) Surface expression on DCs of CD86 was determined by FACS analysis. Shown is the mean of three experiments.

In our analysis it was clear that there was a large individual variation. Therefore next to the mean absolute production of IgA we calculated the production of IgA induced by the patient-DC as a ratio of the response induced by control-DC in the same experiment. From this analysis it is clear that although the mean ratio of the 12 patients investigated is 0.43 ± 0.32, this ranged between a complete deficiency (ratio<0.05) and a normal response (ratio 1.05) (Figure 6).

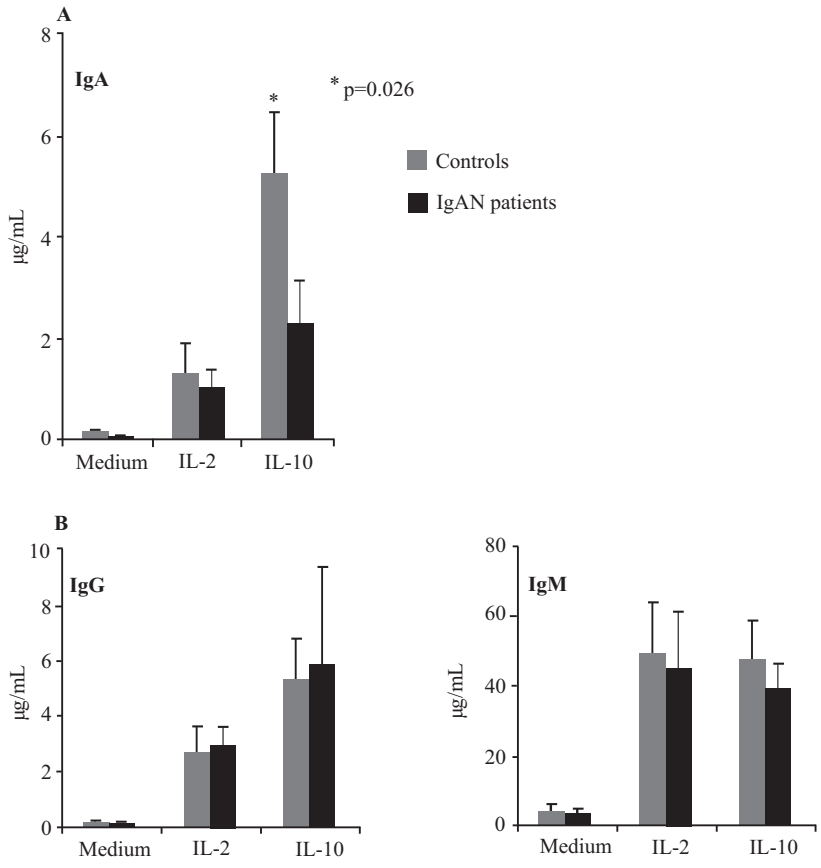

Figure 5. DC from IgAN patients induce less net IgA production compared with controls. (**A**) Highly purified IgD^+ B cells (10^4) were cultured with 75 Gy irradiated CD40L transfected L cells ($0.25x10^4$) in the presence of DCs from controls (n=12) or in the presence of DCs (10^4) from patients with IgAN (n=12), in the presence of IL-2 (20 U/ml) or IL-10 (50 ng/ml) as indicated. After 14 days supernatants were harvested and tested for IgA. Data shown are the mean ±SEM of fivefold cultures of 3 independent experiments. (**B**) IgG and IgM production was measured using specific ELISA's. Data shown are the mean ±SD of five fold cultures of 3 independent experiments.

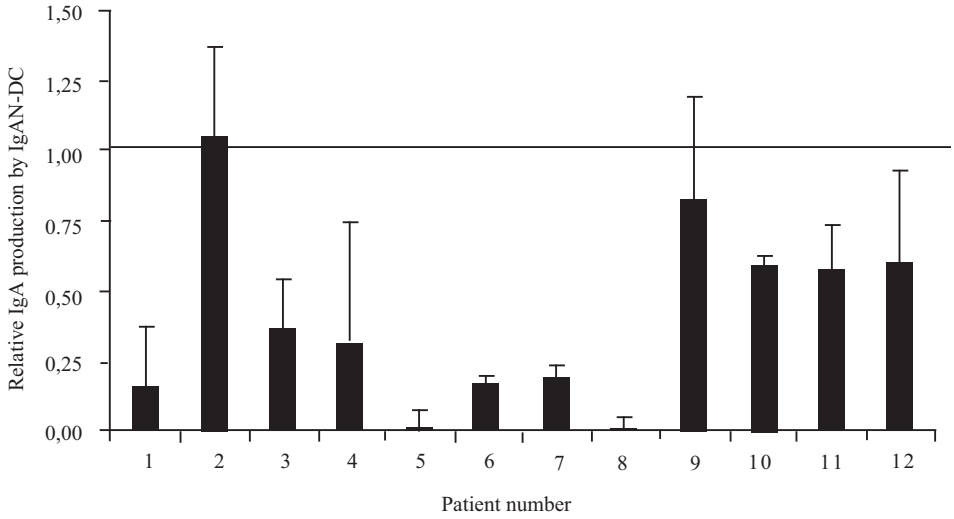

Figure 6. Ratio of IgA production induced by DCs from patients relative to controls. Mean individual IgA production induced by DC from patients with IgAN is divided by the mean IgA production induced by DC from control persons and expressed as a ratio. Shown are the mean +SD of five measurements.

Dendritic cells from IgAN patients induce less IgA_1 and IgA_2 production than DCs from healthy control persons

Next to the measurements of total IgA, also production of IgA subclasses, IgA_1 and IgA_2 were determined. Comparable with total IgA, the addition of DC together with IL-2 and IL-10 to naïve B cells induced both IgA_1 and IgA_2 production. In line with previous results and with the difference in serum concentrations of these subclasses, the production of IgA_1 was much stronger. As observed for total IgA, DCs derived from IgAN patients induced less IgA_1 as well as IgA_2 compared to controls, which was again restricted to the conditions with exogenous IL-10 (Figure 7).

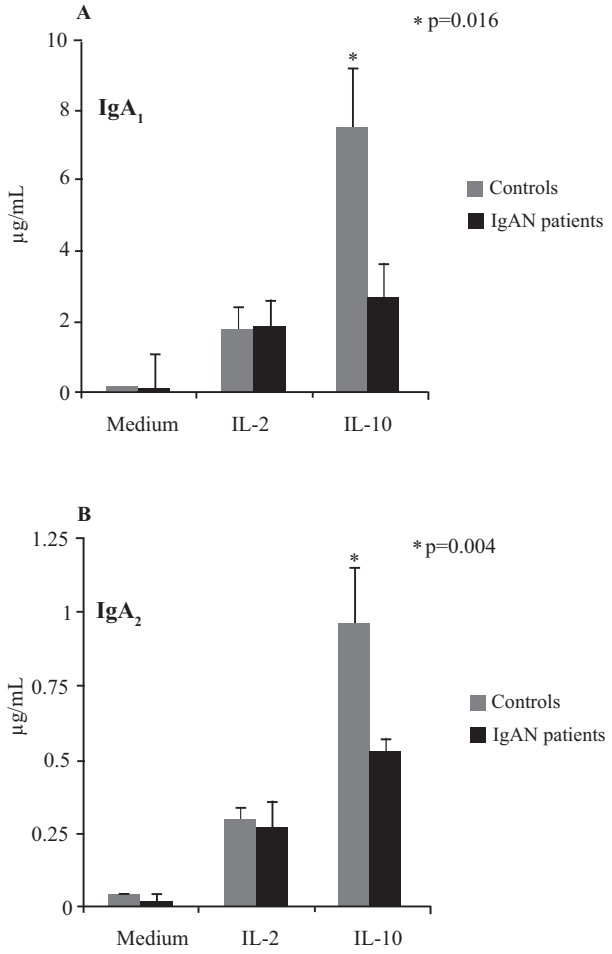

Figure 7. DCs from patients with IgAN induce less IgA_1 and IgA_2. Highly purified IgD^+ B cells (10^4) were cultured with 75 Gy irradiated CD40L transfected L cells ($0.25x10^4$) in the presence of DCs from control persons (grey bars) (n=12) or in the presence of DCs (10^4) from patients with IgAN (black bars) (n=12), in the presence of IL-2 (20 U/ml) or IL-10 (50 ng/ml) as indicated. After 14 days supernatants were harvested and tested for IgA_1 (**A**) and IgA_2 (**B**). Data shown are the mean ±SD of fivefold cultures of 3 independent experiments.

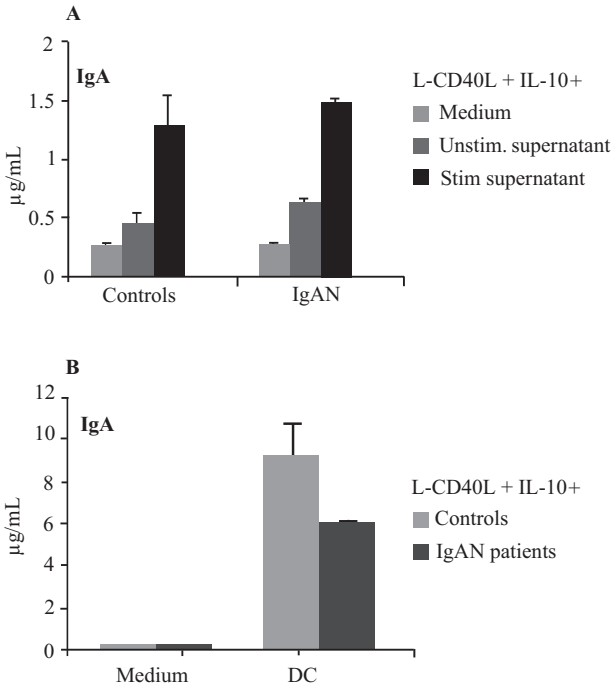

Figure 8. Supernatant of CD40L stimulated DCs can induce IgA switch, but to a lesser extent than DCs. (**A**) DCs ($5x10^5$) of control persons and IgAN patients were cultured in the presence of medium, control L-cells (unstimulated supernatant) or CD40L-transfected L-cells (stimulated supernatant) (10^5) in a total volume of 500 µl. After 72 hours supernatants were harvested and 50 µl of each supernatant was added to naïve B cells (10^4) in the presence of IL-10 (50 ng/ml). After 14 days supernatant was harvested and tested for IgA, using specific ELISA. (**B**) DCs (10^4) from the same control persons and IgAN patients were added to naïve B cells (10^4) in the presence of medium or IL-10 (50 ng/ml). After 14 days supernatant was harvested and tested for IgA, using specific ELISA.

Supernatant from cultured dendritic cells can induce IgA production in the presence of IL-10

In order to investigate whether a soluble factor, produced by DCs, is responsible for the differences in IgA production between the two groups, we generated supernatant from CD40L-stimulated DCs and from DCs cultured without CD40L. After 72 hours supernatant from this culture system was harvested

and added to naïve B cells in the presence of CD40L with or without IL-10. In the absence of exogenous IL-10, DC supernatant could not induce IgA production (data not shown). However in the presence of IL-10, especially supernatant of CD40L-activated DC could augment IgA production. However it should be noted that this effect is small compared to the effect of intact cells and importantly did not show a difference between controls and patients with IgAN (Figure 8A). In the same experiments, irradiated DCs were also investigated using the same B cells demonstrating again a hyporesponse of DC from IgAN patients (Figure 8B).

Discussion

In the present study, using an in vitro model, we show that monocyte-derived DCs of IgAN patients have a reduced capacity to induce IgA_1 and IgA_2 production in naïve B cells compared to DCs from control persons. This difference was seen provided that CD40L and IL-10 were present. In contrast, IL-2 also increased immunoglobulin production, but in this condition there was no difference in the B cell stimulatory capacity of DCs derived from IgAN patients or controls. No differences in IgG and IgM production were measured, independent of the different cytokines that were used. Addition of supernatant from CD40L stimulated DCs to naïve B cells resulted in Ig production, but did not result in a difference between controls and IgAN patients. This suggests that a DC membrane bound factor is responsible for the difference.

IgAN is a disease with a wide variety in clinical presentation and outcome. Some patients have a rapidly progressive renal deterioration leading to renal failure within a short time period, while others have stable kidney function for many years. Eventually about 30-40% of the patients will develop renal failure. It is generally accepted that genetic factors are involved in the pathogenesis of IgAN [34]. Not only familial cases of IgAN have been described, but also a linkage between locus 6q22-23 and IgAN has been described [35;36]. It is tempting to speculate that the variation in IgA

stimulatory capacity (Figure 6) might relate to some of these genetic factors. Alternatively, the in vitro hyporesponsiveness could potentially relate to the severity of IgAN. However in our study there was no correlation between clinical parameters like creatinine clearance or hematuria and the IgA inducing capacity (data not shown).

Vaccination studies have clearly demonstrated that patients with IgAN have a disturbed regulation of the mucosal immune response. Using CTB as a neoantigen we have previously shown that patients with IgAN show a specific IgA hyporesponsiveness, with normal IgG and IgM responses [15]. Importantly, simultaneous systemic administration of another neoantigen did not result in an IgA hyporesponse. These results indicate that the defects observed in IgA production are not generalized, but might be confined to the mucosal compartment. Regulation of IgA production is a complex process dependent on B cells, T cells and antigen presenting cells, but might also be affected by environmental mucosal factors. Previous in vitro studies investigating B cells or T cells of IgAN patients have not clearly pinpointed towards a molecular defect [37-39].

Total IgA production depends on IgA switch by IgD^+ B cells [26] and further proliferation and differentiation of these B cells. Class switch to IgA is a complex event, in which rearrangement of genes has to take place [40-42]. This process is dependent on the interaction between T cells and B cells and is influenced by many other factors. A key role in this process is the interaction between the TNF-R member, CD40 and its ligand [43]. This is demonstrated in the hyper-IgM syndrome in which a genetic alteration of the CD40L gene is responsible for low amounts of serum IgA, IgG and IgE [23;24]. Next to CD40L, IgA production is regulated by cytokines like IL-10 and TGF-β that act directly on B cells. Moreover as also shown in this study, DCs can further increase IgA production. Previous studies using similar IgD^+ tonsillar B cells have demonstrated that DCs augment the isotype switch process as proven by the increase in switch circles [44]. Although our system did not allow a direct investigation of the switch process, it is likely that this contributes to the increased IgA production. The nature of the DC signal promoting IgA production is at present not known. Although there is a B cell stimulating

factor in the supernatant of activated DC, our experiments with supernatant suggest that the difference between patients and controls seems to be caused by a membrane factor. Recently it has been shown that signalling through BAFF, another member of the TNF-R family, can also augment IgA switch in a CD40-independent manner. However, it was shown that BAFF acted in concert with anti-IgM B cell activation, but not in concert with CD40 signalling [45]. Moreover in preliminary experiments we were not able to show BAFF expression on normal monocyte derived DCs (data not shown). Therefore it is not likely that difference in BAFF expression can explain the observed in vitro differences in IgA production.

In the present study we have investigated monocyte-derived DC, and have tried to link the functional capacity of these cells to the observed mucosal hyporesponsiveness. At present it is unclear how these cells relate to mucosal DC populations. It will be a major technical challenge to investigate the functional capacities of mucosal DC directly, especially due to the limited access to mucosal tissues of these patients. However an intruiging observation in our study was that the defective IgA production, induced by DC, was only observed in the presence of IL-10. When DC were combined with IL-2, there were no functional differences between patients and controls. This suggests that there are at least two different mechanisms by which DC can increase IgA production by naive B cells. Until now there are no clues about the underlying molecular mechanisms. Similarly, it is not clear under which conditions in vivo IgA production is under the control of IL-2 or IL-10. Genetic inactivation of IL-10 in mice does not result in major B cell defects or reduced circulating Ig levels [46], whereas IL-2 deficiency does show dramatic reduction of circulating Ig levels [47]. In both cases, the mice develop chronic mucosal inflammation, suggesting a role in mucosal immune regulation [46;48]. However, it seems that expression of IL-10 is more widely distributed and that IL-10 has a more important role as an anti-inflammatory mediator [49;50]. It would be attractive to link the differential response in combination with IL-2 or IL-10, with the observed selective deficiency of a mucosal challenge, as discussed above [15]. Which local mucosal factors contributed to the earlier described IgA hyporespons after vaccination remains

unclear. It certainly needs further research to determine the precise role of DC and IL-10 in the regulation of mucosal IgA production.

In conclusion the current data strongly suggest that DCs of IgAN patients have an impaired capability to enhance IgA production. These data support the results from an earlier study in which mucosal vaccination was accompanied with an IgA hyporesponse [15]. The pathogenetic role of IgA hyporesponsiveness in IgAN could be that an initially inadequate clearance of an antigen leads to a prolonged immune reaction. This prolonged immune reaction could eventually lead to an increased serum IgA titer. Which molecular factors are responsible for the impaired IgA response and whether the impaired capacity to induce IgA switch is restricted to certain subsets of DCs, is still unknown and further studies are needed to resolve these questions.

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Chapter 5

The role of SIgA and complement in IgA-nephropathy

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Summary

IgA nephropathy (IgAN) is characterized by glomerular deposition of IgA, often together with complement components. This deposited IgA is mainly polymeric in nature. Although early studies suggested a role for local complement activation in the development of glomerular injury in IgAN, recent attention has focussed on the involvement of the lectin pathway of complement activation in the progression of renal disease in IgAN. Additionally, we have found that glomerular secretory IgA deposition may be one of the initiators of local complement activation in the kidney. In the present review we will discuss recent developments in this area and provide a model of how mucosal immunity and renal inflammation may be interconnected.

IgA Nephropathy

IgA nephropathy (IgAN) is the most common form of primary glomerulonephritis worldwide. The hallmark of this disease is the deposition of IgA1 in the glomerular mesangium [1;2]. These deposits are thought to be composed mainly of high molecular weight IgA1, sometimes together with IgG or complement components like C3 [3]. The disease shows a broad spectrum of clinical presentations, leading to progressive renal failure in a substantial proportion of patients. It has been reported that IgA deposits disappear after transplantation of a kidney from an IgAN patient into a non-IgAN patient [4]. Furthermore, after renal transplantation recurrent mesangial IgA deposition is observed in about 50% of patients [5]. These results strongly suggest that the basic abnormality of the disease lies within the IgA immune system rather than within the kidney. It is likely that several factors contribute to the development of IgAN, including the nature, glycosylation pattern and composition of IgA, dysregulation of the IgA immune response, and changes in the clearance of IgA from the circulation [6].

Serum levels of IgA are increased in approximately 50% of patients with IgAN [7]. Importantly, in other diseases associated with increased serum IgA, such as IgA myeloma, mesangial IgA deposition is not seen suggesting there is something particular about the IgA molecule in IgAN that promotes mesangial deposition. One of the most consistent changes seen in the circulating pool of IgA is aberrant IgA1 glycosylation, possibly due to a reduced activity of core 1 ß1,3-galactosyltransferase (reviewed by Jan Novak in this issue). This aberrantly glycosylated IgA has also been demonstrated in glomerular IgA deposits, suggesting such molecules have a predisposition to mesangial deposition compared to normally glycosylated IgA [8].

The increase in circulating IgA1 levels appears to be the result of an increased production of this isotype by the bone marrow [9] and a low clearance rate by the liver [9;10]. Mucosal pIgA plasma cell numbers are normal or even reduced in IgAN [11], and pIgA antibody levels in mucosal secretions are not increased and are sometimes lower than controls [12].

Interestingly, patients with IgAN often present with macroscopic hematuria following upper respiratory tract infections. Mucosal infection

or presentation of live micro-organisms leads to excess amounts of IgA in the systemic compartment with the propensity to induce glomerular injury. Immunization studies in IgAN, using different antigens and routes of administration, have produced conflicting results with respect to the systemic IgA response [12-14]. Mucosal and systemic IgA hypo-responsiveness to mucosal immunization with the neoantigen cholera toxin subunit B (CTB) has been reported [12]. Furthermore systemic antigen [12;15;16] challenge results in normal or increased titers of circulating pIgA1 antibodies [15;16] with increased levels of IgA in mucosal secretions of IgAN patients [17].

The fundamental result of these changes in the IgA immune system is a qualitative and quantitative alteration in circulating IgA. We believe these changes have a direct impact on the systemic clearance of IgA and the interaction of systemic IgA with glomerular mesangial cells. These events are the driving force for the local inflammatory response. Based on recent developments, this review will primarily focus on the role of secretory IgA (SIgA) and mannose binding lectin (MBL) in the pathogenesis of IgAN.

Immunoglobulin A

Immunoglobulin A (IgA) is the most abundantly produced immunoglobulin isotype, and plays a critical role in protecting the host against environmental pathogens at mucosal surfaces [18]. In humans, IgA in the circulation primarily consists of monomeric IgA (mIgA), and only 10-20% of the IgA is dimeric or polymeric IgA (pIgA). Furthermore, IgA consists of two subclasses namely IgA1 and IgA2. IgA1 has ten potential O-glycosylation sites and two N-glycosylation sites. IgA2 has no O-glycosylation sites but has two to three additional N-glycosylation sites. In vitro deglycosylation of IgA leads to self-aggregation, suggesting that underglycosylation of IgA may contribute to the generation of high molecular weight IgA [19].

In secretions, secretory IgA (SIgA) is generated during transcytosis of dimeric IgA (dIgA) by epithelial cells, ultimately leading to its association with the extracellular part of the polymeric Ig receptor (secretory component). Besides the presence of SIgA in the mucosa, low levels (10 µg/ml) of SIgA can be detected in serum [20;21].

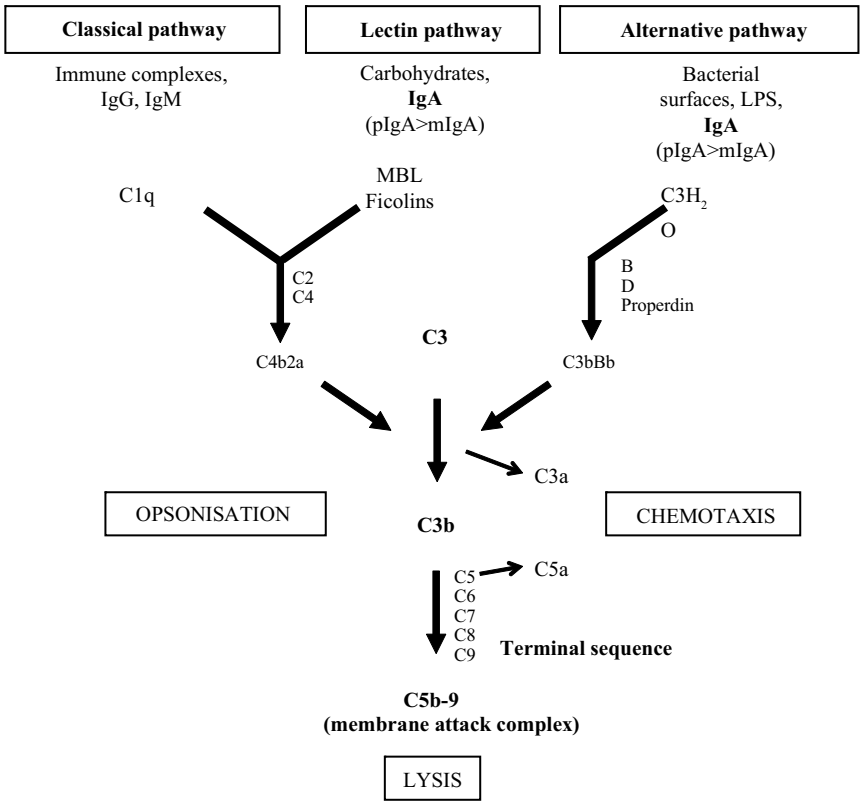

Figure 1. The three complement pathways of complement activation. IgA can activate both the lectin pathway and the alternative pathway. For both pathways polymeric IgA is a more potent activator than monomeric IgA. (MAC: membrane attack complex).

Complement in IgA Nephropathy

In IgAN deposits of IgA are commonly associated with the deposition of complement components, most often C3, the membrane attack complex (C5b-9) and properdin [22;23]. Furthermore, increased levels of split products of activated C3 have been observed in the circulation of patients with IgAN and associated with increased proteinuria and hematuria, suggesting involvement of the alternative pathway in IgAN [24]. Indeed, *in vitro* as well as *in vivo* studies, have shown that polymeric IgA can directly activate the alternative

pathway of complement, whereas monomeric IgA is a poor activator of the complement system [25;26] (Figure 1). The molecular basis for this difference between monomeric and polymeric IgA is not clear. Recently, the lectin pathway of complement, with the recognition molecules MBL, H-ficolin and L-ficolin, has been described [27;28]. MBL is able to bind directly to a number of micro-organisms, via carbohydrates expressed on their surface [29]. Upon binding to an activator, MBL activates the complement cascade via the lectin pathway, which plays a critical role in the first line of host defence against these pathogens. Furthermore, genetic polymorphisms in the MBL gene, resulting in low serum MBL levels and non-functional MBL, have a negative impact on several chronic diseases [30].

Evidence is accumulating that MBL and activation of the lectin pathway of complement can also be unfavourable for disease progression. This has been suggested for rheumatoid arthritis, and also for IgAN, based on renal biopsy studies demonstrating the presence of excess MBL in glomeruli in IgAN [31-33]. Furthermore, it has been shown that MBL is able to bind polymeric IgA, leading to activation of the lectin pathway *in vitro* [34]. However, there is no difference in the binding of MBL to IgA from healthy subjects or patients with IgAN [35]. The carbohydrate recognition domain of MBL is able to bind in a calcium-dependent way to a number of saccharides, such as D-mannose, L-fucose, and N-acetylglucosamine (GlcNAc). The binding of MBL by IgA is likely to be through the oligomannose structures present in the N-linked sugars of the heavy chains of polymeric IgA however this requires further confirmation [35].

Deposition of MBL in association with IgA, as a marker for lectin pathway activation, has been reported in a subpopulation of IgAN patients [31;32;36], but these findings have been questioned by others [37]. Furthermore, the relationship of glomerular MBL deposition with parameters of renal damage and complement activation via the lectin pathway is inconsistent between different studies. Recently our group described a renal biopsy series in which IgA was co-deposited with MBL in 25% of examined patients [38]. Furthermore, patients with MBL deposition exhibited more severe renal disease as compared to MBL-negative cases, suggesting an important role

for MBL in disease progression [38]. These results indicate that activation of the lectin pathway, initiated via MBL and possibly also L-ficolin, occurs in a subpopulation of IgAN patients, implicating MBL as a biomarker for disease progression in these cases [38]. There have been indications that MBL can be expressed by intrisic renal cells, but at present the relative contribution of these cells to deposited MBL is unclear. These results indicate that activation of the lectin pathway, initiated via MBL and possibly also L-ficolin, occurs in a subpopulation of IgAN patients, implicating MBL as a biomarker for disease progression in these cases [38].

These findings at the biopsy level emphasise the importance of further delineating the precise composition of IgA in mesangial deposits as ultimately this data will inform us about the mechanisms involved in IgA deposition and complement activation in IgAN. Local complement activation will result in cell injury and induction of an inflammatory cascade that contributes to disease progression. The impact of glycosylation has been reviewed in detail elsewhere [39] and will not be discussed further in this review. We concentrate on recent findings concerning the presence of SIgA in renal deposits.

Secretory IgA in IgA nephropathy

About 40% of patients with IgAN have recurrent episodes of macroscopic hematuria frequently preceded, one or two days earlier, by infections. Upper respiratory tract infections occur most frequently [40], but occasionally other infections have been implicated, including gastrointestinal and urinary tract infections. Mucosal immunization with a neo-antigen in healthy individuals leads not only to a localised mucosal immune response but also to an antigen-specific immune response in plasma, suggesting a close relationship between the mucosa and bone marrow. This response is reduced after immunization of patients with IgAN [12].

Production of secretory IgA (SIgA) is a specific process taking place at mucosal surfaces and occurs following binding of dimeric IgA (dIgA) to the polymeric Ig receptor (pIgR) and transcytosis of this IgA across the mucosal epithelium [41]. Epithelial IgA transport in the opposite direction has also been described, where SIgA binds selectively to microfold (M) cells

irrespective of their antigen-binding specificity, followed by transport of SIgA across the epithelium and targeting to subepithelial dendritic cells (DC) [42]. *In vitro* it has been demonstrated that human DC can bind and endocytose SIgA [43]. It has been suggested that this targeting of SIgA to DC may play an important role in mucosal immune regulation through modulation of DC activation [42].

Importantly, not all retrograde transport seems to be associated directly with DC uptake, because small amounts of SIgA can also be found in human serum [20;44]. Moreover, increased serum levels of SIgA have been reported in various diseases [45-48] indicating that SIgA may be a marker of clinical interest. Recently our group showed that in purified serum IgA preparations SIgA is found in high molecular weight IgA fractions and that the relative concentration of SIgA is higher in patients with IgAN as compared to controls [35]. In serum low concentrations of SIgA were measured but there were no differences in SIgA concentrations between patients with IgAN and healthy subjects. However, there was a correlation between hematuria in patients with IgAN and the serum SIgA concentration [21]. There also is evidence that systemic clearance of SIgA may be defective in IgAN [49].

Glycosylation of SIgA in IgA nephropathy

As mentioned previously, it is has been suggested that the glycosylation of IgA is an important pathogenic factor in IgAN. The predominance of IgA1 in mesangial deposits and the unusual hinge region of IgA1 with multiple O-linked glycosylation sites has stimulated a great deal of interest in changes to IgA1 glycosylation in IgAN. Indeed, both in serum but more importantly, also in the eluate of isolated glomeruli, a specific reduction of O-linked galactosylation has been observed [50-52]. Furthermore, with size fractionation of eluted proteins from kidney sections, it has been found that these deposits contain predominantly high molecular weight forms of IgA [53]. Recently, we demonstrated a 120-fold accumulation of SIgA, based on a comparison of the composition of serum and glomerular immunoglobulins, in IgA eluted from isolated glomeruli in IgAN [21].

Previous studies have shown a role for glycosylation of IgA in the binding and activation of mesangial cells [54-57]. Stimulation of mesangial cells with high molecular weight IgA leads to enhanced production of chemokines and cytokines, including IL-6, TGF-β, TNF-α, MCP-1, IL-8 and MIF [55;58;59]. Interestingly, SIgA binds better to mesangial cells than serum IgA, and binding of SIgA results in mesangial cell synthesis and release of increased amounts of IL-6 [21]. At present, it is unclear which mesangial cell IgA receptor binds SIgA.

The glycosylation of SIgA is different to serum IgA in several respects. Firstly, SIgA is a tetramolecular complex consisting of two IgA molecules, J chain and SC wrapped around the four α heavy chains. Modelling of SIgA suggests that the N-glycans of the α heavy chains can be masked by SC [60]. This may also result in altered exposure of the hinge region O-glycans. Moreover, specific analysis of the glycosylation of the IgA heavy chains present in SIgA, has demonstrated different N-glycan structures compared to that of serum IgA. Specifically, terminal GlcNAc residues are present in the majority of the N-glycans of SIgA [60]. The O-glycans of the hinge region of the α heavy chain of SIgA1 display a wide range of glycan structures, which for the major part are now characterized [60]. It would be very interesting to have precise information on the glycosylation of the α heavy chains of SIgA in IgAN, but at present such information is not available.

SIgA in IgAN biopsy specimens

To confirm the presence of SIgA in glomeruli in IgAN, our group stained kidney biopsy specimens from patients with IgAN for SIgA deposition. In 15% of cases positive staining for mesangial SIgA was observed [61]. In a separate study secretory component deposition was identified in 13 out of 191 IgAN renal biopsies, while all control biopsies were negative for secretory component [62]. In a Japanese study all IgAN biopsies studied were positive for secretory component, whereas normal kidneys were negative [63]. Interestingly, in this study there was an association between single nucleotide polymorphisms (SNPs) in the pIgR and the presence of IgAN [63]. The relationship between secretory component deposition and other molecules in

the glomeruli, and clinical parameters of the patients, has not been studied. In our study, double staining and confocal microscopy demonstrated remarkable co-localization of SIgA and MBL, supporting the previously reported strong correlation between SIgA and MBL in a subgroup of patients [61]. In addition, there was strong co-localization with C4d, suggesting local complement activation. An association between SIgA and MBL has been reported by other investigators [60]. Royle et al. suggested that disruption of the non-covalent interactions between secretory component and the IgA heavy chain, for instance at low pH, may lead to MBL binding and subsequent complement activation via the lectin pathway [60].

The notion of a pathogenic role of SIgA deposition in IgAN and the knowledge that mucosal sites are critical for the generation of SIgA raises interesting questions concerning the involvement of mucosal immune responses in IgAN.

The role of the tonsils in IgA nephropathy

The tonsils are located at the gateway of the respiratory and alimentary tract and belong to the mucosa-associated lymphoid tissue. The major function of the tonsils is as a first line of defence against viral, bacterial and food antigens. In IgAN tonsillar tissue contains more IgA-secreting B cells than healthy subjects and this increase is matched by a parallel increase in the number of dimeric IgA secreting cells [64]. Tonsils from patients with IgAN contain more IgA-producing cells compared to controls [65], and synthesise IgA1 which is less sialylated than serum IgA1 [66;67], suggesting that the tonsils may be a source of the IgA that deposits in IgAN.

Stimulation of the tonsils by ultra short wave has been shown to trigger acute changes in the urinary sediment in a subgroup of patients with IgAN but not patients with other renal diseases [68]. These patients had suffered more frequent episodes of macroscopic hematuria following upper respiratory tract infections and had higher levels of serum SIgA preceding tonsillar stimulation than those IgAN patients who did not respond to tonsillar stimulation [68].

Although no randomised controlled trials of tonsillectomy in IgAN patients have been reported it has been suggested that tonsillectomy can

improve renal outcome in some patients [69;70]. There is some retrospective evidence from Japan that tonsillectomy was associated with a favourable effect on long term renal survival in IgAN patients supporting the notion that the mucosal IgA immune system may have an important role in the pathogenesis of IgAN [71].

Immunization studies in IgAN patients

Immunization studies examining both systemic and mucosal (oral) secondary immune responses in IgAN have generated conflicting results with respect to serum and mucosal antigen-specific IgA responses [15;17;72-75]. In IgAN mucosal immunization with CTB resulted in an impaired mucosal and systemic antigen-specific IgA response compared with healthy subjects, whereas there was no difference in antigen-specific IgA responses following simultaneous systemic immunization with the neoantigen Keyhole Limpet Haemocyanin (KLH) [12].

Recently, Smith et al. described the O-glycosylation pattern of antigen-specific serum IgA1 against the systemic antigen tetanus toxoid (TT) and the mucosal antigen *Helicobacter pylori* (HP) [76]. In this study higher Vicia Villosa (lectin recognizing the Tn-antigen) binding was observed for IgA1 specific for HP as compared to TT. There were no differences between patients and controls. This suggests that IgA1 O-glycosylation may vary in different immune responses and may be determined at the site of antigen encounter. This also would imply that an altered balance in O-glycosylation pattern of IgA1 in IgAN patients could potentially be a consequence of a mucosal immune response rather than a generic defect in B cell O-glycosylation [76].

Recently we investigated the size distribution of antigen-specific IgA in serum and nasal washes after mucosal and systemic immunization [77]. Nasal washes contained mainly SIgA whereas serum IgA displayed the usual size distribution of serum IgA, being mainly monomeric. SIgA was detectable in these sera, and as expected restricted to the high molecular weight IgA fractions. We found that the antigen-specific IgA was found predominantly in the high molecular weight fractions, irrespective of the route of administration. Importantly, we were also able to demonstrate low but significant levels of

antigen specific SIgA in serum after intranasal vaccination, strongly suggesting a link between mucosal immune responses and circulating SIgA (figure 2).

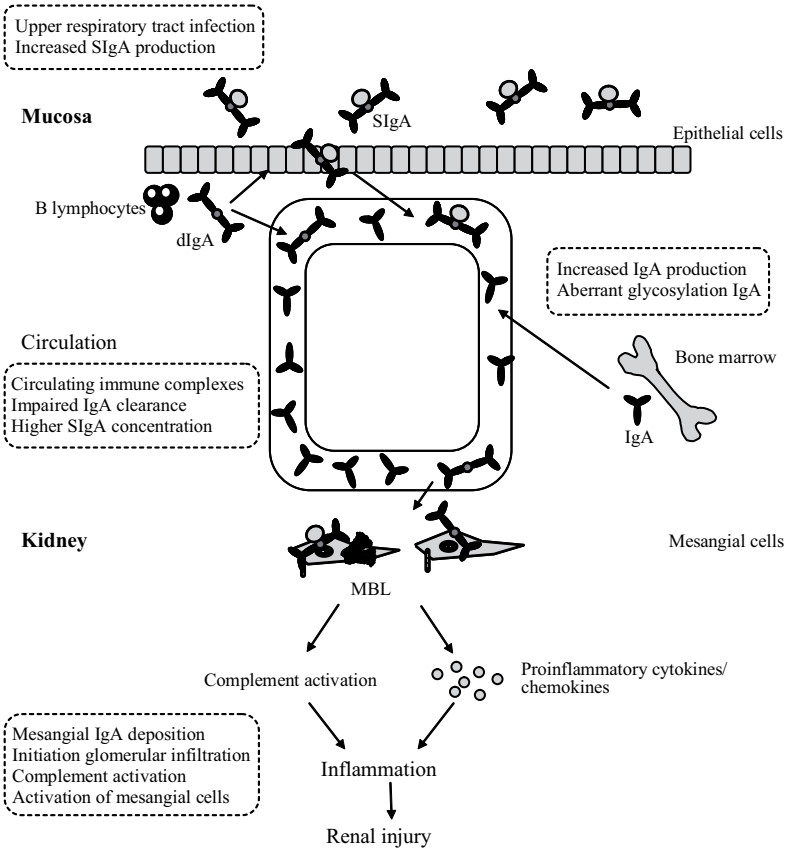

Figure 2. A model to link mucosal immune responses with deposition of SIgA in the renal mesangium. In IgAN three compartments are important, the mucosa, the circulation and the kidney. B lymphocytes present at mucosal sites will, upon activation, produce dIgA which will be predominantly secreted as SIgA across mucosal epithelial surfaces. Via an unknown mechanism, small amounts of SIgA enter the circulation and ultimately come into contact with mesangial cells. At the same time, both mucosal B cells and plasma cells in the bone marrow are producing increasing amounts of aberrantly glycosylated IgA which enters the circulation. The binding of SIgA and aberrantly glycosylated IgA1 leads to mesangial cell activation. SIgA deposition is also associated with mesangial deposition of MBL and activation of the complement system, resulting in more severe glomerular injury.

Conclusion

In this review we have discussed the potential role for MBL and SIgA in the pathogenesis of IgAN. Polymeric IgA is able to activate the alternative pathway but also by binding MBL activate the lectin pathway of complement. We also have discussed the mesangial deposition of MBL in IgAN and that this deposition is associated with more severe renal injury.

Patients with IgAN often experience macroscopic hematuria following upper respiratory tract infections. Although there is no obvious increase in serum SIgA in IgAN we have described a clear relationship between serum SIgA concentrations and risk of hematuria in IgAN. Moreover, compared to other serum immunoglobulins SIgA appears to deposit preferentially within glomeruli in IgAN and this is reflected by the SIgA staining observed in kidney biopsies of patients with IgAN. The presence of SIgA is strongly associated with co-deposition of MBL and the complement activation product C4d. Taken together, the data presented in this review support a role for SIgA and MBL in the pathogenesis of IgAN in a subpopulation of patients.

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Chapter 6

Secretory IgA responses in IgA nephropathy patients after mucosal immunization, as part of a polymeric IgA response

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Summary

Secretory IgA (SIgA), although generated at mucosal surfaces, is also found in low concentrations in the circulation. Recently, SIgA was demonstrated in mesangial deposits of patients with IgA nephropathy (IgAN), suggesting a role in the pathogenesis. This finding is in line with the belief that high molecular weight (HMW) IgA is deposited in the kidney. However there is little information on the size distribution of antigen-specific IgA in circulation upon mucosal challenge.

In this study we measured antigen-specific IgA, including SIgA, in serum following challenge of IgAN patients and controls via intranasal vaccination with a neoantigen, cholera toxin subunit B (CTB). We size fractionated serum and nasal washes to study the size distribution of total IgA, SIgA and CTB-specific IgA. Finally we compared the size distribution of antigen-specific IgA after mucosal immunization with the distribution upon systemic immunization.

A significant induction of antigen-specific SIgA was detectable in serum of both patients with IgAN and controls after mucosal immunization with CTB. Independent of the route of immunization the antigen-specific IgA response was predominantly in the polymeric IgA fractions, in both groups. This in contrast to total IgA levels in serum that are predominantly monomeric.

We conclude that mucosal challenge results in antigen-specific SIgA in the circulation, and that the antigen-specific IgA response in both IgAN patients and in controls, is of predominantly high molecular weight in nature. No differences between IgAN patients and controls were detected, suggesting that the size distribution of antigen-specific IgA in the circulation is not specifically disturbed in IgAN patients.

Introduction

IgAN is the most common primary glomerulonephritis worldwide, leading to renal insufficiency in 30-40% of patients [1-3]. The disease is characterised by deposits of polymeric IgA_1 in the mesangium of glomeruli [4;5]. The pathogenesis of IgAN is still not clear. Plasma IgA levels are elevated in about 50% of IgAN patients, but how this higher concentration of plasma IgA contributes to mesangial deposition remains unclear [4;6]. Qualitative changes of plasma IgA_1, such as the glycosylation pattern, are likely involved in the pathogenesis of IgAN [7;8]. IgA isolated from IgAN patients shows more terminal GalNAc (Tn antigen), which could contribute to deposition of IgA in the mesangium. Interestingly, after renal transplantation IgA depositions may appear in the renal allograft, suggesting, next to renal factors, a pathogenic role for serum IgA [9].

The mesangial IgA deposition in IgAN consists of IgA_1, together with complement factor C3 and sometimes IgG. Most of the deposited IgA consists of HMW IgA [10-12]. The composition of HMW IgA is diverse and may contain dimeric IgA, SIgA and complexes of IgA and the Fcα-receptor (CD89) and fibronectin [2;13]. Recently we showed that in approximately 25% of cases, mannose binding lectin (MBL) is present in renal biopsies of IgAN patients. The presence of MBL was associated with more histological damage and more proteinuria [14]. In about 15% of IgAN cases mesangial SIgA is present [15;16]. The presence of SIgA in renal biopsies showed a strong correlation with the presence of MBL and the complement degradation fragment C4d. Moreover, a strong colocalization of SIgA, MBL and C4d was observed [15]. A potential pathogenic role of SIgA is further supported by higher serum levels of SIgA in IgAN patients as compared to controls and the observation that higher levels of SIgA are correlated with more hematuria [12].

Several immunization studies have shown different results with regard to the specific serum and mucosal IgA responses in IgAN patients. Both hypo and hyper IgA responses have been described, dependent on the type of antigen and the route of administration of the antigen [17-22]. How these

differences in immune responses are related to the pathogenesis of IgAN is unknown. No data are available about antigen-specific SIgA in circulation after mucosal immunization. In addition, very little is known about the size distribution of IgA during primary immune responses in IgAN patients.

Therefore, we have investigated the presence of antigen-specific SIgA in the circulation after mucosal immunization. In addition we size fractionated sera and nasal washes collected from an earlier vaccination study in IgAN patients and controls, who underwent nasal mucosal and subcutaneous immunization with two different neoantigens. In the fractions we determined, in addition to total IgA and SIgA, the titers of antigen-specific IgA. As every individual was immunized with two different neoantigens via two different routes we were able to study whether the route of vaccination influences the size distribution of antigen-specific IgA. We conclude that after mucosal immunization antigen-specific SIgA appears in the circulation in both the patient and control group, and that antigen-specific IgA in serum is predominantly polymeric in both patients and controls, independent of the route of immunization. The amount of antigen-specific SIgA in IgAN patients and in controls is similar, suggesting that additional factors are involved in the pathogenesis of IgAN.

Materials and methods

Human subjects and immunization protocol

In an earlier vaccination study we investigated the primary immune response after simultaneous mucosal and systemic vaccination of IgAN patients and controls in a quantitative manner [17]. Participants were immunized with 0.33 mg of cholera toxin subunit B (CTB) intranasally by spray, and 250 μg of keyhole limpet haemocyanin (KLH) subcutaneously, repeated by two administrations of identical doses after 2 and 4 weeks, as described earlier [17]. None of the patients had clinical or laboratory evidence of Henoch-Schoenlein purpura, kidney function was normal or mildly impaired (creatinine clearance>80 ml/min) and none of the patients used corticosteroids or any

other immunosuppressive drug at the time of the study or at least 3 months before. Mean age of the IgAN patients was 40 years (range 30-47 yr). Healthy volunteers were recruited as controls with a mean age of 29 years (range 24-37 yr). The study was approved by the Ethical Committee of the Leiden University Medical Centre. All individuals gave informed consent.

From 20 controls and 11 IgAN patients, pre and post immunization samples, taken two weeks after the second booster immunization, i.e. 42 days from the start of the immunization protocol, were available for the assessment of total SIgA and antigen-specific SIgA. From six IgAN patients, all males with biopsy proven IgAN and 6 controls (5 males) sufficient amounts of material were present to perform a size separation of serum.

Quantification of IgA and antigen-specific IgA

Total IgA, SIgA and antigen-specific (S)IgA levels were determined by specific sandwich enzyme-linked immunosorbent assay (ELISA). Polystyrene 96-well plates (Greiner, Alphen a/d Rijn, The Netherlands) were coated with 100 µl/well of the capturing antibody, appropriately diluted in PBS. Total IgA was detected by heavy chain specific, affinity purified goat $F(ab')_2$ fragments against IgA (Jackson, West Grove, PA). SIgA was detected using a monoclonal antibody specific for secretory component (NI194-4; 3F8) as capturing antibody in a concentration of 2 µg/ml.

In the antigen-specific ELISA, plates were coated with 100 µl of CTB (2.5 µg/ml) (Sigma, St Louis, MO) or KLH (10 µg/ml) (Calbiochem, La Jolla, CA). Subsequently the plates were washed with PBS/0.05% Tween (PBST). Plates were incubated with appropriate dilutions of samples from IgAN patients and controls in PBS/1% BSA/0.05% Tween for 2 hours. Bound IgA was detected using mouse anti-human IgA (4E8) conjugated to biotin, followed by incubation with streptavidin conjugated to horseradish peroxidase (Zymed, Sanbio BV, Uden, The Netherlands). CTB-specific SIgA was detected by polyclonal sheep anti-human secretory component (5 µg/ml) (Nordic, Tilburg, The Netherlands), followed by rabbit anti-sheep conjugated to horseradish peroxidase (10 µg/ml) (Nordic). Enzyme activity of HRP was developed using ABTS (Sigma). Between each step the wells were washed three times with PBST.

Size fractionation of IgA

Serum and nasal washes, containing antigen-specific IgA, were size-separated with a HiLoad™ 16/60 HR200 Superdex prep grade gel filtration column (120 ml, Amersham Pharmacia, Roosendaal, The Netherlands), run in Veronal-buffered saline containing 2mM EDTA. Fractions were assessed for the presence of IgA, SIgA, antigen-specific IgA and total protein.

Percentages mIgA and pIgA in serum for each individual, were determined as follows: fractions containing pIgA (44-51 ml) or mIgA (52-60 ml) were pooled and assessed for total IgA and for antigen-specificity towards CTB and KLH. The percentages pIgA and mIgA were calculated by dividing the amount of pIgA by the sum of pIgA and mIgA together.

Statistical analysis

Statistical analysis was performed by the Student's T-test. Differences were considered significant when p values were less than 0.05.

Results

Antigen-specific Secretory IgA in serum

Using specific ELISA we tested pre and post immunization serum fractions of controls and IgAN patients for the presence of total SIgA. The mean $\pm$SEM of total SIgA concentrations in serum of the control group was 18$\pm$5.6 µg/ml before and 22.0$\pm$6.2 µg/ml after immunization (n.s.) (Figure 1A). In the IgAN group pre-immunization SIgA concentration was 23.3$\pm$8.6 µg/ml and post immunization 20.7$\pm$6.2 µg/ml (n.s.). Comparison of the SIgA concentration of the controls and patients revealed no significant difference, neither before, nor after immunization. For each individual there was a very high correlation between the total SIgA concentration before and after immunization (Control group $r=0.92$, $p<0.0001$, IgAN group $r=0.99$, $p<0.0001$), suggesting that SIgA levels are stable over time.

Next, we measured SIgA specific for CTB in serum before and after immunization. In both the control group and the patient group we were able

to detect a small, but significant increase in SIgA anti CTB after the second booster ($p<0.001$). The amount of CTB-specific SIgA was not significantly different between controls and IgAN patients (Figure 1B). In these samples we were not able to detect secretory IgM (data not shown). As a control, sera of non-immunized persons were tested for the presence of antigen-specific SIgA. These sera were negative for antigen-specific SIgA (data not shown).

Secretory IgA in nasal washes and serum

As the antigen-specific total IgA response in serum was strong [17] and there was only a slight increase in serum for antigen-specific SIgA, we were interested in the size distribution of the antigen-specific IgA. We size fractionated nasal washes and sera to test for total IgA and SIgA concentrations. In nasal wash only one peak of total IgA was found, exactly overlapping the SIgA peak, suggesting that most, if not all, IgA in nasal washes is SIgA (Figure 2A). In serum the profile of total IgA showed two distinct peaks, corresponding with polymeric IgA (pIgA) (44-51 ml) and monomeric IgA (mIgA) (52-60 ml) (Figure 2B). A high percentage of total IgA in circulation was monomeric, in accordance with previous observations. Secretory IgA was found in the polymeric IgA fractions.

Antigen-specific IgA in serum after mucosal immunization consists mainly of polymeric IgA

To determine the size of antigen-specific IgA, sera of 12 immunized persons (6 controls and 6 IgAN patients) were size fractionated as described in the methods section. Fractions were measured for total IgA, antigen-specific IgA and total protein. CTB-specific IgA concentrations were determined in the fractions, relative to an internal standard and expressed as arbitrary units. IgA anti-CTB was present in both pIgA and mIgA fractions, with higher levels in the polymeric IgA fractions (Figure 3A). The percentage pIgA anti-CTB was 57±21 in the control group and 63±24 in the IgAN patients (n.s) (Figure 3B). For total IgA the percentage pIgA was lower than the antigen-specific IgA in both the patient and the control group. The mean percentage pIgA of total IgA, was 33±2.9 in the control group and 32±3.6 in the IgAN group (n.s) (Figure 3C).

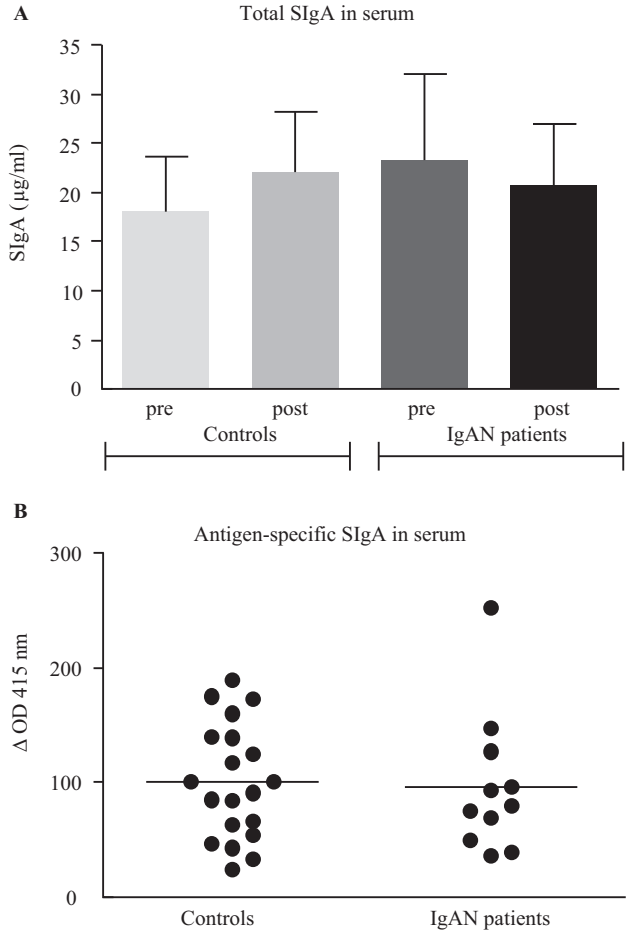

Figure 1. Total SIgA and CTB-specific SIgA in serum of controls and IgAN patients. Pre and post immunization sera of controls and IgAN patients were tested for total SIgA by ELISA (Figure 1A). CTB-specific SIgA was measured before and after immunization, and expressed by increase in OD x 1000. Shown are individual increases in OD values of 20 controls and 11 IgAN patients (Figure1B).

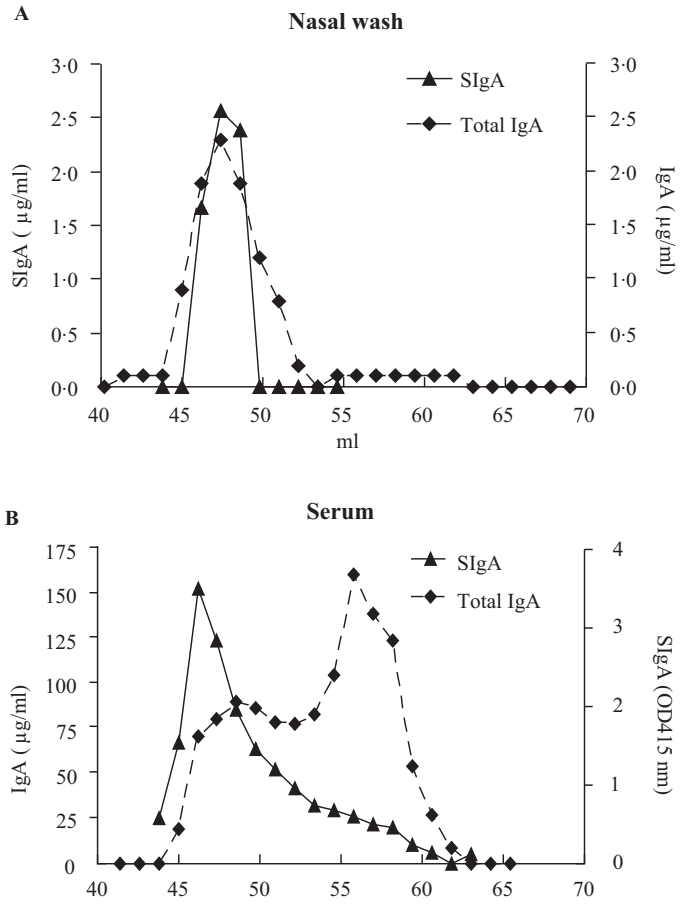

Figure 2. SIgA is present mainly in the high molecular weight fractions of IgA. IgA of nasal wash and serum was size fractionated with a HiLoad™ 16/60 HR200 Superdex prep grade gelfiltration column. Total IgA and SIgA was determined by specific ELISA. In nasal wash the total IgA peak and SIgA peak overlap (Figure 2A) In serum total IgA exists mainly of monomeric IgA and to a lesser extend of polymeric IgA. SIgA is present in the high MW fractions of IgA (Figure 2B). Shown are representative profiles of an IgAN patient.

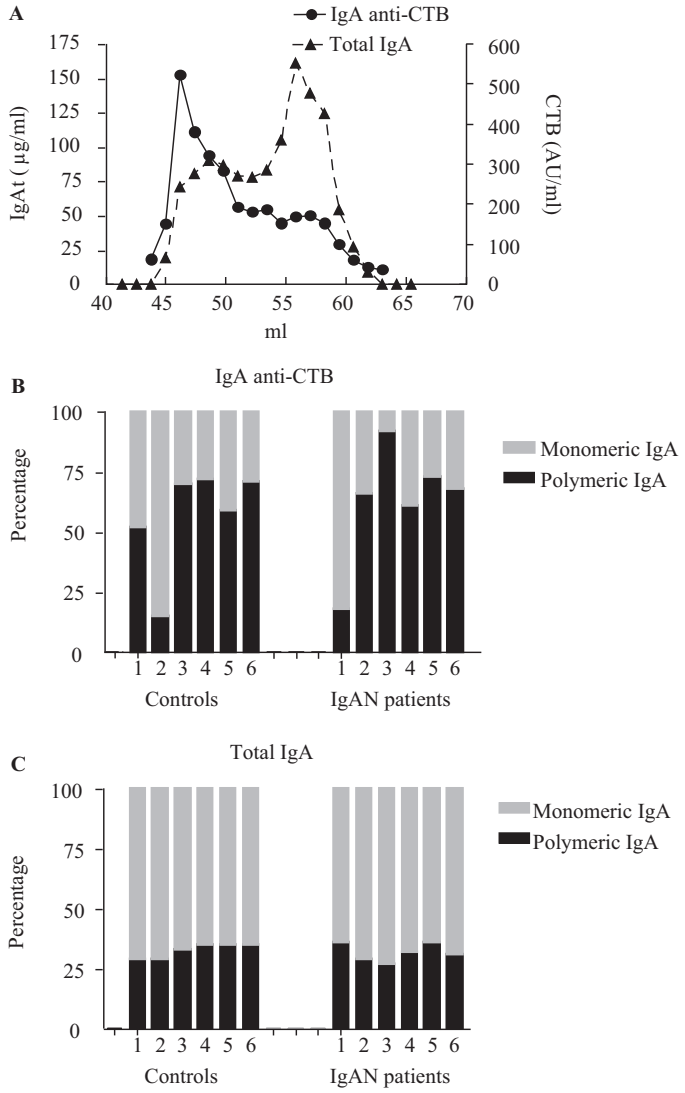

Figure 3. Percentage of pIgA and mIgA total IgA and CTB-specific IgA. IgA of serum was size fractionated with a HiLoad™ 16/60 HR200 Superdex prep grade gelfiltration column. Total IgA and CTB-specific IgA were determined by ELISA. Figure 3A shows a serum profile of total IgA and CTB-specific IgA of a representative IgAN patient. Concentrations of high molecular weight IgA (pIgA) and of low molecular weight IgA (mIgA) in serum were determined by ELISA for total and CTB-specific IgA. Figure 3B shows the percentages of CTB-specific mIgA and pIgA of 6 controls and 6 IgAN patients. In Figure 3C percentages total mIgA and pIgA are depicted of 6 controls and 6 IgAN patients.

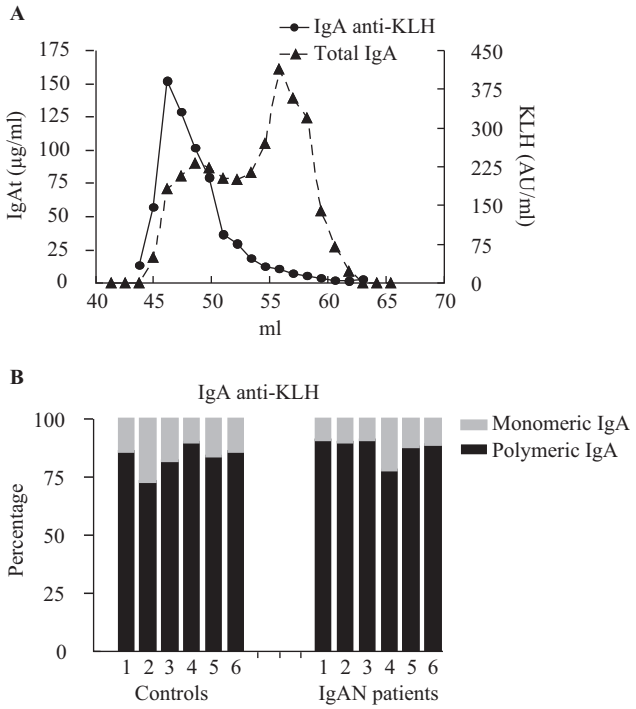

Figure 4. Antigen-specific IgA after systemic immunization consists almost exclusively of pIgA. IgA of serum was size fractionated with a HiLoad™ 16/60 HR200 Superdex prep grade gelfiltration column. Total IgA and KLH-specific IgA were determined by ELISA. Figure 4A shows a serum profile of an IgAN patient for KLH-specific IgA (black line) and for total IgA (dotted line). Concentrations of high molecular weight IgA (pIgA) and of low molecular weight IgA (mIgA) in serum were determined by ELISA for total and KLH-specific IgA. Figure 4B shows the percentages KLH-specific mIgA and pIgA of 6 controls and 6 IgAN patients.

Size distribution of antigen-specific IgA in serum after systemic immunization

To compare the size distribution of antigen-specific IgA after a mucosal challenge with a systemic immunization, fractions were also analyzed for the presence of IgA anti-KLH antibodies. In all samples (6 IgAN patients and 6 controls) IgA anti-KLH was detectable. IgA anti-KLH is almost exclusively present in the polymeric fractions (Figure 4A). The percentage pIgA anti-KLH

was 83±5.7 in the controls and 88±4.9 in the IgAN patients (n.s) (Figure 4B). In both groups the percentage pIgA was significantly higher after systemic vaccination as compared to mucosal vaccination.

Discussion

This is the first study showing induction of antigen-specific SIgA in serum, upon mucosal immunization. Besides this is the first study that compares the size distribution of antigen-specific IgA in serum following different routes of immunization. We demonstrate that the antigen-specific IgA response is mainly polymeric and independent of the route of immunization. The size distribution of antigen-specific IgA in patients with IgAN is not different from controls.

SIgA might play an important role in the pathogenesis of IgAN. This view is supported by the fact that higher concentrations of SIgA are present in serum of IgAN patients and by an association of higher SIgA concentrations with more pronounced hematuria [12]. Another argument for the involvement of SIgA in the pathogenesis of IgAN comes from the observation that in renal biopsies of IgAN patients in about 15% of cases SIgA can be detected [15;16;23]. The presence of SIgA is correlated with deposition of MBL and C4d. It has been described that renal injury is worse in patients with MBL deposition [14] Recently we showed that high concentrations of SIgA were present in IgA eluted from a removed allograft of an IgAN patient [15;16]. An additional argument suggesting a pathogenic role of SIgA in IgAN, is that about 40% of patients show a sudden increase in hematuria [24], within two days after an upper respiratory tract infection. It is tempting to speculate that this so called synpharyngitic hematuria is mediated by SIgA produced during a mucosal infection

In the present study we were able to show antigen-specific SIgA in plasma. As SIgA in serum is present in low concentrations we expected to find only very low concentrations of antigen-specific SIgA. The increase in antigen-specific SIgA was small but highly significant in both IgAN patients

and controls. SIgA is present in sera of humans in low concentration. The mechanism by which SIgA, produced at mucosal surfaces is transported to the circulation is not clear. This could be by leakage of SIgA or by active transport through the epithelial layer [25]. Whether SIgA in serum has a role in the immunological response is also a matter of debate. In our study all persons had low concentrations of antigen-specific SIgA in their serum. We recently showed that SIgA is present in renal biopsies of a small group of IgAN patients [15]. It would be very interesting to correlate the SIgA immune response with the absence or presence of SIgA in the biopsy. Unfortunately, this material is at present not available. Similarly, it has been shown that the glycosylation pattern of SIgA differs from serum IgA in several ways [26]. Whether the glycosylation patterns of (antigen-specific) SIgA differ between IgAN patients and controls and whether this influences deposition is at present not known and would require the design of a new vaccination study.

It has long been recognized that deposited IgA in IgAN mostly consists of HMW IgA_1 [10;11] Recently it has been demonstrated that HMW IgA has specific effector activities including MBL pathway activation of complement [14]. Therefore we have analyzed the size distribution of antigen-specific IgA upon simultaneous vaccination with two different neoantigens and show that the antigen-specific IgA response in both patients and controls is predominantly in the HMW fractions. Several immunization studies with different antigens and various routes of administration showed that IgAN patients have aberrant immune responses compared with control persons [18;19;22]. However there is only limited information about the size distribution of these specific IgA responses. After intramuscular vaccination with inactivated influenza virus, no differences in size distribution of antigen-specific IgA was found between IgAN patients and controls [19]. In the present study it appeared that mucosally administered CTB induced a clear mucosal and systemic immune response as described earlier [17]. Measuring the antigen-specific IgA response revealed that mucosally administered CTB induced an antigen-specific pIgA response and also to a lesser extent an antigen-specific mIgA response in serum. The antigen-specific IgA anti-KLH consists almost exclusively of polymeric IgA and a smaller fraction of monomeric IgA. Both IgAN patients and controls showed a similar capacity to induce these HMW responses.

In several immunization studies it appeared that most of the antigen-specific IgA was of the IgA1 subclass [17-19]. Although two studies showed higher IgA1:IgA2 ratios in IgAN patients [18;19] this finding was not shown in other studies [17;22]. It is important to realize that both the place of antigen presentation as well as the antigens used are of importance with respect of the subclass distribution. Overall there is a tendency of higher IgA1 :IgA2 ratio in IgAN patients after immunization. In the current study we were not able to differentiate between antigen-specific SIgA1 and SIgA2.

Here we describe for the first time the size distribution of antigen-specific IgA after simultaneously performed mucosal and systemic immunization with two different neoantigens. With regard to IgAN patients, size distribution of IgA after systemic recall immunizations has been described. Intramuscular vaccination with influenza virus showed higher monomeric IgA titers than that of polymeric IgA [19]. Systemic immunization with tetanus toxoid also showed predominantly monomeric IgA, but higher levels of polymeric IgA in IgAN patients than in control persons [21]. Whether these differences in size of antigen-specific IgA are determined by the type of antigen used in the different studies, or by differences between primary or recall immune responses is not clear.

In conclusion, in the present study we have investigated the size distribution of antigen-specific IgA responses upon mucosal and systemic immunization with a neoantigen. We observed that antigen-specific IgA responses were predominantly present in HMW IgA fractions, including antigen-specific SIgA. In view of the proposed pathogenic role of HMW IgA in IgAN, a more detailed analysis of antigen-specific IgA might be required to characterize the altered IgA response in patients with IgA nephropathy.

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Chapter 7

IgA1 glycosylation in IgA Nephropathy, as sweet as it can be

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Kidney International 2008, in press

The pathogenesis of IgA nephropathy (IgAN) is still not clear, but it is now well accepted that an aberrant glycosylation pattern of IgA is involved. This is supported by the fact that in IgAN mesangial deposits of IgA contain high concentrations of abnormally O-glycosylated IgA1, characterized by undergalactosylation. In the current issue Buck et al. [1] investigated whether this aberrant glycosylation pattern of IgA in IgAN patients is due to diminished activity or reduced gene expression of specific glycosyltransferases. They investigated the activity of the enzyme β-galactosyltransferase (C1Gal-T1) and its molecular chaperone (Cosmc) in purified B cells isolated from peripheral blood (PB) and bone marrow (BM) of IgAN patients and controls. They also measured gene expression in PB and BM cells and related this to the enzyme GalNAc-T2, which synthesizes the core O-glycan. Using this approach, no differences in O-galactosylation activity between IgAN patients and controls could be detected.

IgAN is a heterogeneous disease with a wide range in clinical presentations, varying from a-symptomatic microscopic hematuria to gross macroscopic hematuria and can have a variable clinical outcome ranging from normal glomerular filtration rate to end stage renal disease in up to 30% of cases. The histological hallmark of the disease is the presence of mesangial deposition of polymeric IgA1, in combination with complement factor C3 and often IgG or IgM. In IgAN patients the serum concentration of IgA is frequently increased compared with that in controls, but in addition IgA present in the circulation was shown to be abnormally O-glycosylated. Importantly, this undergalactosylated form of the IgA1 hinge region was also over-represented in biopsies of patients with IgAN [2]. Therefore, it is likely that this abnormally glycosylated IgA is involved in the pathogenesis of IgAN. The reduced galactosylation leads to increased exposure of the internal GalNAc (Tn antigen) that can be detected with specific lectins. Recently it has been suggested to use this specific characteristic for the development of an ELISA-based diagnostic test in IgAN [3]. This abnormal glycosylated IgA has a higher tendency to self-aggregate and form complexes with IgG antibodies directed against epitopes in the hinge region of IgA1. Besides, in patients with

Henoch-Schönlein purpura, a disease closely related to IgAN with a similar renal histological pattern, only those patients who had abnormal glycosylated IgA had renal involvement, whereas patients with normal glycosylated IgA did not have renal involvement [4].

Despite the importance of aberrantly glycosylated IgA in IgAN, the molecular mechanisms underlying these differences are not known. With the identification and characterization of the enzyme system responsible for O-linked glycosylation, including C1Gal-T1 and its molecular chaperone Cosmc, novel avenues of research have opened up. However, an important and intriguing observation is the fact that aberrant O-glycosylation seems to be absent in other serum proteins, whereas even for IgA it is generally accepted that only a small proportion of the IgA molecules show these alterations (Figure 1). For instance, GalNac specific lectins specifically react with high molecular weight fractions of serum IgA, which only constitute 10% of circulating IgA [5].

Since IgA1 glycosylation is determined within B lymphocytes, and only a fraction of the IgA1 seems to be affected, this suggest that alterations in glycosylation are only present in a subset of B cells. In humans, besides IgA the only other immunoglobulin that is O-glycosylated is IgD. Smith et al. have studied the glycosylation patterns of IgA1 and IgD in IgAN patients and in controls and observed that the abnormal underglycosylated pattern of IgA1 was not present in IgD [6]. As IgD is a marker of naïve B cells, this might suggest that deficiencies are restricted to certain differentiation stages of B cells. In the study of Buck et al. [1], enzyme activities were determined in purified B cells isolated by positive selection using anti-CD19 coated magnetic beads. As plasma cells are CD19 negative, this analysis might exclude the contribution of plasma cells. Although the number of plasma cells in the circulation will be low, in BM their number is expected to be higher. It is thought that IgA present in the circulation is produced mainly by plasma cells derived from the BM, suggesting that the absence of plasma cells in the analysis could be of importance.

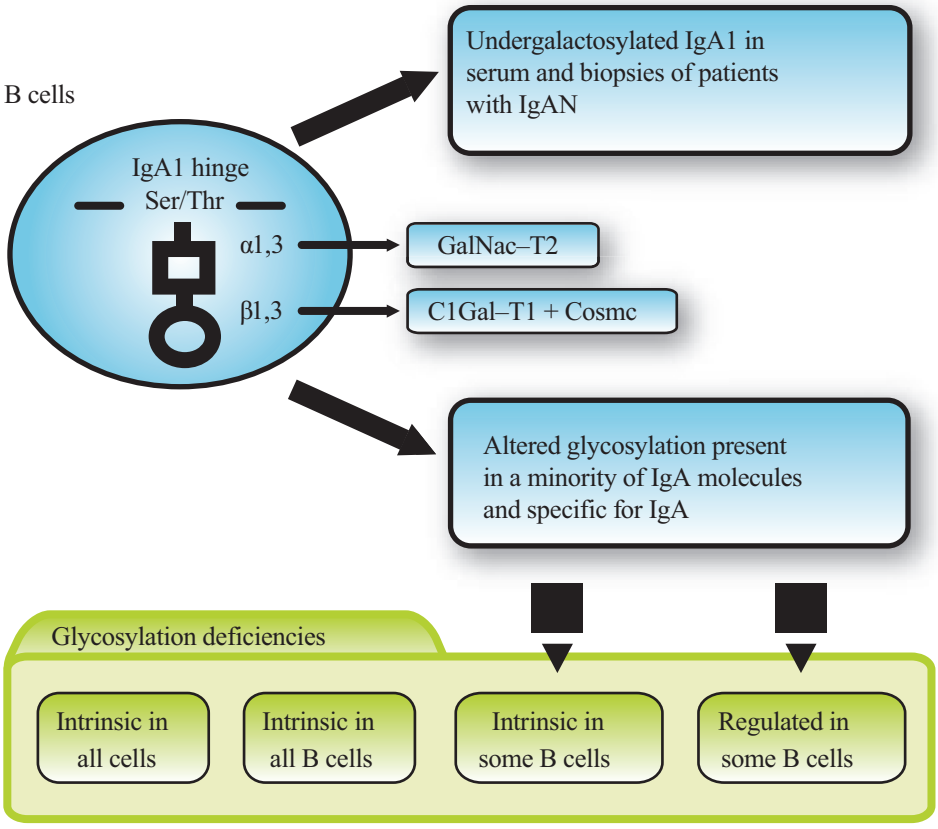

Figure 1. Schematic view on potential mechanisms leading to aberrantly glycosylated IgA. Undergalactosylated IgA1 is present in serum and biopsies of patients with IgAN. This glycosylation is controlled during the synthesis of IgA in B cells by the glycosyltranferases C1Gal-T1 and its molecular chaperone Cosmc and GalNAc-T2. As other proteins seem not to be affected and only some of the B cells in IgAN produce undergalactosylated IgA, generalized intrinsic defects in B cells or other cells can be excluded. Therefore, it is postulated that either the glycosylation is disturbed intrinsically in some B cells, or regulatory processes affect only some B cells during an ongoing immune response. In the latter case, environmental or cellular signals involved in B cell activation and IgA production might be involved.

The fact that the proposed deficiencies are only present in a subset of B cells (Figure 1), might suggest that genetic factors are not likely to be involved. Nevertheless, it was recently shown that polymorphisms in the gene for C1Gal-T1 were associated with susceptibility for the development of IgAN in a Chinese population [7]. A disease in which aberrant glycosylation patterns are present due to genetically determined factors is the so called Tn syndrome. This is a rare autoimmune disorder in which a somatic mutation in the Cosmc gene leads to incompletely glycosylated proteins in subpopulations of all cell lineages [8]. It is unclear whether such a mosaic pattern can contribute to the aberrant glycosylation in IgAN and so far no correlation between IgAN and the Tn syndrome has been described.

IgA present in the circulation is derived not only from the BM, but also from mucosal tissues. Interestingly, most IgA produced at the mucosa will be dimeric in nature, and can either be transported over epithelial barriers to generate secretory IgA (SIgA), or contributes to the high-MW IgA fraction in the circulation. Therefore, both high-MW IgA and SIgA, which can also be present in the circulation and in renal deposits [9], might represent a recent mucosal challenge. Buck et al. [1] investigated the activity of C1Gal-T1 and Cosmc and GalNAc-T2 in PB and BM samples taken from IgAN persons who had no macroscopic hematuria or intercurrent illness at the time of sampling. This suggests that no active immune reaction was present at the time of investigation, which might have masked a difference in glycosylation activity.

In a recent vaccination study, a direct comparison of glycosylation has been made of IgA directed against a mucosal antigen, *Helicobacter pylori* (HP), and against a systemic adjusted antigen, tetanus toxoid (TT) [10]. Both in IgAN patients and in controls the IgA1 against HP showed higher lectin binding, reflecting more undergalactosylated IgA than the IgA against TT. This strongly suggests that an aberrant glycosylation pattern in IgAN could not only be the result of an intrinsic defect in glycosylation mechanisms, but also might reflect differences in regulation of glycosylation (Figure 1). As immunoglobulin production is the result of a complex process, involving B cells, T cells, antigen presenting cells and local and systemic factors like

cytokines, each of these factors can potentially influence the glycosylation profile. Dendritic cells (DC) are professional antigen presenting cells which have direct effects on B cells, and under the influence of the mucosal environment can promote the generation of IgA producing cells [11]. We have shown a reduced capacity of DC of IgAN patients to induce IgA switch [12]. Whether the abnormal DC function also affects the glycosylation pattern in IgAN patients is so far unknown.

In conclusion it is clear that aberrantly glycosylated IgA plays an important role in the pathogenesis of IgAN. Glycosylation is a complex process, influenced by many factors, that can either be intrinsic or can differ in time and change due to locally determined circumstances. The study of Buck et al. [1] shows that overall there is no difference between IgAN patients and controls in C1Gal-T1 and Cosmc activity nor in gene expression, related to GalNAc-T2. Although this study's results might appear to be negative, it clearly brings IgAN research at a next level. It is conceivable that more detailed molecular analysis will be required to unravel the mechanisms contributing to undergalactosylation of IgA1. This should probably include developments in the direction of single cell analysis of IgA producing B cells or longitudinal analysis of actively developing IgA immune responses upon mucosal or systemic challenges.

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Chapter 8

General discussion and summary

Primary IgA Nephropathy (IgAN) was first described by Berger and Hinglais in 1968 [1]. Nowadays IgAN is recognized as the most common primary glomerulonephritis worldwide [2;3]. Initially IgAN was thought to be a disease with a benign character, but later it appeared that up to 35% of patients eventually develop end stage renal failure. In western Europe and the United States of America about 10% of patients undergoing renal replacement therapy have IgAN as underlying disease. So the impact of IgAN on individual patients and more general on healthcare systems is significant.

Fourty years after the first description by Berger, the pathogenesis of this disease is still not resolved. Generally patients develop IgAN in their early twenties, however it may also become obvious later in life. Men are more often affected than women with a ratio of 2:1. It is important to realize that most persons present with microscopic hematuria, without further signs or symptoms. About 40% of patients have macroscopic hematuria, sometimes starting shortly after an upper respiratory tract infection. Unfavourable clinical outcome is associated with high levels of proteinuria and uncontrolled hypertension. Furthermore a decreased glomerular filtration rate (GFR), at the time the diagnosis IgAN is made, correlates with bad outcome [4]. The only way to confirm the diagnosis IgAN, is to perform a renal biopsy. Differences in incidence rates suggest that next to environmental factors, like viral infections, genetic factors are involved in the pathogenesis [5]. Although no exact information about incidence of IgAN is known, the prevalence of IgAN seems to be higher in certain countries, such as Japan, Australia and southern Europe. In the United Kingdom, United States and Canada the prevalence rates are lower [4]. It is important to realise that these incidence numbers are influenced by variance in referral rates and kidney biopsy criteria. Autopsy studies from Japanese unselected persons show a prevalence of mesangial IgA depositions of about 16% , whereas in autopsy series of unselected German persons the prevalence was about 5%. [6;7].

So far no reliable diagnostic test, other than a renal biopsy, is available to predict the presence of IgAN. The disease is characterized by depositions of IgA, mainly IgA1, together with complement factor C3 and sometimes IgG in the renal mesangium [3;8]. Recurrence of IgAN after renal transplantation

is frequently observed, whereas on the other hand it has incidentally been described that IgA depositions disappear from a kidney of an IgAN patient when it was transplanted to a non-IgAN patient [9;10]. These observations contribute to the idea that the so far unresolved pathogenesis of IgAN involves the immune system rather than specific renal factors.

Next to immunological factors, genetic factors are likely to be involved in the pathogenesis of IgAN. Racial differences in susceptability to IgAN are observed, for instance the incidence of IgAN among African Americans is lower in comparison with Caucasians [11;12]. The strongest argument that genetic factors play a role in the pathogenesis of IgAN is that familial clustering of IgAN has been described [13-17]. Genome-wide linkage analysis of 30 families with IgAN, 24 from Italy and 6 from the United States, showed a close association of IgAN to chromosome 6q22-23 [18]. This locus has been named *IGAN1*. Whether this locus is involved only in familial IgAN or also in the isolated form of IgAN is not clear. Recently two other candidate loci, 4q26-31 and 17q12-22, have been identified as suggestive to be linked to IgAN in 22 Italian families with IgAN [19]. So far no specific genes in these loci influencing the pathogenesis of IgAN have been identified. Although Schena et al. reported a poorer prognosis of familial IgAN, no clinical parameters are recognized that distinguish sporadic IgAN from familial IgAN [15].

The variability of IgAN, in clinical presentation and outcome and in the histological picture of the renal biopsies, might reflect the variability in genetic background or genetic factors involved in the pathogenesis of the disease. On the other hand differences in environmental factors or geographical factors might be involved in the onset of IgAN or might influence the clinical outcome and could be responsible for the wide variability of IgAN.

Recently we identified a homozygous twin with biopsy proven IgAN, both presenting with microscopic hematuria in combination with hypertension. Interestingly both patients participated in the study described in chapter 4 and both appeared to have DC that induced only very little IgA production by naïve B cells. So far homozygous twins with IgAN have not been described.

In the past 4 decades several abnormalities in the immune system of IgAN patients have been described. Both quantitative and qualitative aspects of IgA seem to differ in IgAN patients in comparison with controls. Overall the serum IgA titer is increased in 50% of patients as compared to controls [20]. However, other diseases with higher IgA levels in the circulation, like multiple myeloma or HIV infection, are not associated with depositions of IgA in the kidney. Several immunization studies have shown aberrant immune responses in IgAN patients [21-23]. Next to this, several qualitative aspects of IgA seem to be different in IgAN patients. The most constantly described abnormality concerns the disturbed glycosylation pattern of IgA in IgAN patients [24;25]. In IgAN patients there is a reduction of galactosyl residues in the hinge region of serum IgA1 [26]. The exact mechanism how the glycosylation of IgA is disturbed, is so far not known. Recently several glycosyltransferases have been described, but no differences in enzyme activity, nor in gene expression are present in IgAN patients.

Chapter one as a general introduction outlines the clinical features of IgAN. Further, the human IgA system is described and the different structural aspects of IgA are discussed. It is important to realize that the human IgA system is unique and contains both IgA1 and IgA2, both existing in monomeric and polymeric forms. For this reason it is difficult to develop a representative animal model for IgAN. There are two compartments, namely the mucosal and the systemic compartment, where IgA is produced. The mucosal compartment seems to be the most important one. At mucosal sites throughout the body, IgA is produced in large quantities and is secreted as secretory IgA (SIgA). SIgA exists of dimeric IgA bound to secretory component and is involved in mucosal defence against viral and bacterial pathogens. Small amounts of SIgA however are present in the circulation. So far it is not clear what the physiological role of SIgA in the circulation is. In IgAN in about 15% of cases SIgA is present in the renal biopsies [27]. The presence of SIgA correlates with the presence of mannose binding lectin (MBL), suggesting that SIgA is involved in inflammation in the kidney [28]. Next to mucosally produced IgA, IgA is produced in the systemic compartment, in the bone marrow by plasma cells. The role of IgA in the circulation is less clear.

To investigate the immune response in IgAN over the years several immunization studies, using different antigens and different routes of antigen administration have have been performed. These studies have shown different results with respect to the IgA response in IgAN patients [21;23;29-32].

Chapter two concerns a study in which the IgA response in IgAN patients upon simultaneous stimulation with two different antigens, administered via two different routes is investigated. IgAN patients and controls were immunized with cholera toxin subunit B (CTB) applied at the nasal mucosa and with keyhole limpet hemocyanin (KLH) which was administered subcutaneously. Both CTB and KLH are considered as neoantigens and induce a primary immune response. In this study we also performed bone marrow biopsies. We were able to detect CTB-specific immunoglobulin producing cells in the bone marrow, providing evidence for the so called mucosa-bone marrow axis. This further supports the idea that the two compartments, mucosal and systemic, in which IgA is produced are immunologically connected.

It appeared that after mucosal immunization IgAN patients had a significantly, lower IgA response both at the nasal mucosa, as well as in serum. Besides the number of CTB-specific IgA producing cells in the bone marrow was significantly lower in the patient group than in the controls. No differences were found between patients and controls with respect to antigen-specific IgG or IgM. After subcutaneous immunization with KLH no differences were found between the two groups, neither in the antigen-specific IgA response, nor in IgG or IgM. This is the first study in which persons are simultaneously immunized with two different neoantigens via two different routes. An IgA hyporesponse in IgAN patients after mucosal immunization was also observed after oral immunization with live typhoid vaccine [30]. It is important to realize that in both studies people were immunized with a neoantigen, so the described results represent a primary immune response. In general many immune responses after viral infections will be caused by recall antigens, leading to secondary immune responses, which are partly dependent on immunological memory. The contrast of mucosal IgA hyporesponse and higher IgA levels in the circulation could be explained by the hypothesis that decreased mucosal clearance of antigens can lead to prolonged immune

reactions leading to higher levels of immunological memory. No IgA hypo response was observed after the systemic immunization with KLH. Which factor or which factors are responsible for the observed IgA hypo response after mucosal immunization is not clear from this study.

Dentritic cells (DC) are professional antigen presenting cells and are involved in the initiation of immune responses. Besides DC can have a direct effect on B cells and are capable of skewing immunoglobulin production by naïve B cells towards IgA1 and IgA2 [33]. Therefore we postulated that DC could be responsible for the differences in IgA response between IgAN patients and controls. To test our hypothesis, nasal biopsies of IgAN patients and control persons were taken and stained for the presence of DC and subsets of DC. In chapter three we describe the result of this study showing that the number of DC in the nasal mucosa of IgAN patients is not decreased. As a matter of fact we even found higher numbers of CD1a positive DC in the epithelial layer and higher numbers of DC-SIGN positive cells in the lamina propria.

As the number of nasal mucosal DC in IgAN patients was not reduced as compared to controls, and therefore could not be responsible for the earlier described IgA hyporesponse, we postulated that the DC might be less effective in inducing IgA production by naïve B cells. In chapter four we investigated in an *in vitro* model the functional capacity of DC to induce IgA production in naïve B cells. The model used in this chapter is first described by Fayette et al. [33]. In this model naive B cells are cultured in the presence of CD40L-transfected cells, which mimic activated T cells. By making use of these transfected cells MHC restriction is bypassed. In these experiments we made use of monocyte-derived DC, CD40L-transfected cells and naïve B cells. The cells were cultured in the presence of different cytokines, like IL-10 and IL-2. The only variable in the system was the source of the DC, which was either from an IgAN patient or from a control person. In these experiments it appeared that DC derived from IgAN patients showed a reduced capacity to induce IgA production in the presence of IL-10. Although the mean IgA production induced by DC from IgAN patients was strongly reduced, DC from some individual patients induced a near normal IgA production. This

might reflect that IgAN is a heterogeneous disease. No differences in IgG or IgM production where observed, independent of the different cytokines that were used. An experiment using supernatant of CD40 stimulated DC showed that the increase in immunoglobulin production induced by this supernatant was less than the induction by whole DC and that in this case no differences between patients and controls were present. This suggests that a membrane bound factor is responsible for the reduced functional capacity of DC from IgAN patients to induce IgA production by naïve B cells.

From the results of chapter 2,3 and 4 it can be postulated that the IgA hyporesponse in IgAN patients observed after mucosal immunization can at least partially be explained by a functional defect of DC in the nasal mucosa, which is caused by a molecule present at the cell surface of DC. The number of sub-epithelial DC-SIGN positive DC and epithelial CD1a-positve DC in the nasal mucosa appeared to be increased in IgAN patients. This could be a compensatory increase for the described reduced capacity to induce IgA production.

IgA depositions in IgAN contain high levels of polymeric IgA. Therefore we were interested in the size distribution of antigen-specific IgA. In chapter 6 we describe that the antigen-specific IgA response contains high levels of polymeric IgA, both after mucosal and after systemic immunization. No differences in size distribution of antigen-specific IgA were present between IgAN patients and controls. Next to the size distribution of IgA we were interested in the presence of antigen-specific SIgA after mucosal immunization. A pathogenic role for SIgA is suggested by the fact that about 40% of IgAN patients have episodes of macroscopic hematuria, often preceded by upper respiratory tract infections [34]. Besides it was shown that SIgA is present in renal biopsies in 15% of the cases. The presence of SIgA in renal biopsies correlates with the presence of MBL depositions. Although SIgA is mainly present at mucosal surfaces, in various secretions, low concentrations of SIgA are present in the circulation. In purified IgA, the relative concentration of SIgA is higher in IgAN patients than in control persons [35]. In the study described in chapter 6 we were able to detect small amounts of antigen-specific SIgA in the

circulation of both IgAN patients and controls, after mucosal immunization. This is to our knowledge the first study showing antigen-specific SIgA in the circulation and supports the hypothesis that SIgA has a pathogenic role in a group of IgAN patients.

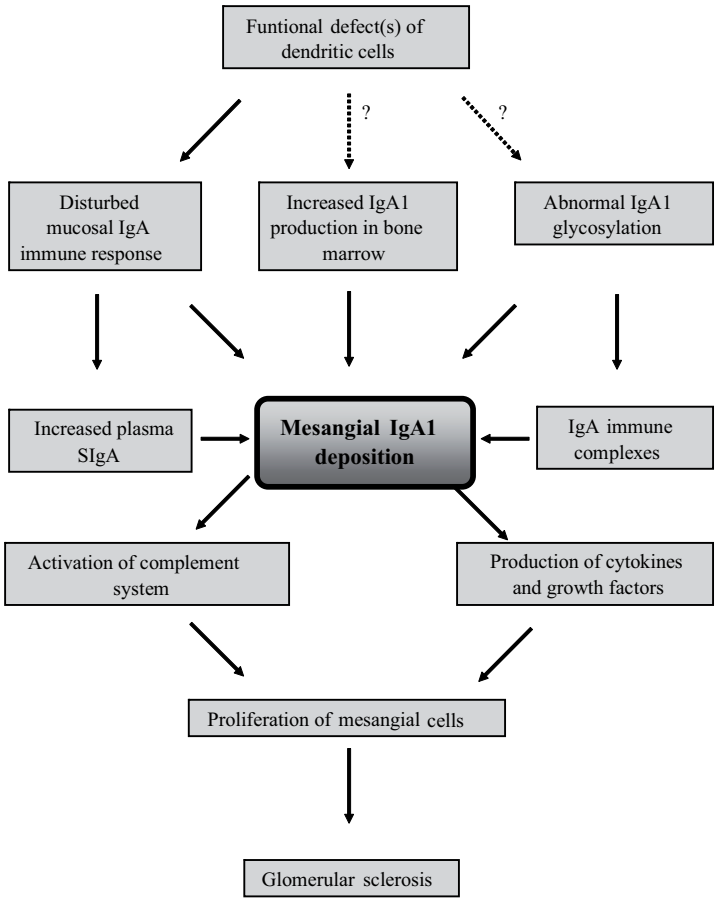

Figure 1. Overview of factors leading to mesangial IgA deposition and to progression of IgA nephropathy. A functional defect in DC might be responsible for the mucosal IgA hyporesponse and thereby to prolonged exposition to environmental antigens, eventually leading to higher levels of IgA memory cells and higher IgA plasma levels. Whether DC are directly involved in the glycosylation process of IgA and in the higher IgA production in the bonemarrow is speculative. The higher levels of SIgA in the circulation and the defective galactosylation of IgA will lead to mesangial deposition of IgA1. Once IgA is present in the kidney, this will lead to production of cytokines and growth factors and to activation of the complement system. Mesangial cells will proliferate, leading to glomerular sclerosis.

As IgA derived from IgAN patients is abnormally O-glycosylated , we discussed this issue in chapter seven. Importantly, this undergalactosylated form of the IgA1 hinge region was also over-represented in biopsies of patients with IgAN [36]. Therefore, it is likely that this abnormally glycosylated IgA is involved in the pathogenesis of IgAN. This abnormal glycosylated IgA has a higher tendency to self-aggregate and form complexes with IgG antibodies directed against epitopes in the hinge region of IgA1. Besides, in patients with Henoch-Schönlein purpura, a disease closely related to IgAN with a similar renal histological pattern, only those patients who had abnormally glycosylated IgA had renal involvement, whereas patients with normally glycosylated IgA did not have renal involvement [37]. SIgA has a different glycosylation pattern as compared to other forms of IgA [38]. Whether SIgA of IgAN patients is aberrantly glycosylated is at present not known.

IgAN is a heterogeneous disease, with a diverse and highly unpredictable outcome. The initial presentation can vary from microscopic hematuria with normal GFR, to gross macroscopic hematuria with end stage renal failure. Proteinuria can be absent, but can also be in the nephrotic range. Some patients remain stable for many years with a normal GFR, whereas others might develop end stage renal failure within a very short period of time. The histological hallmark of IgAN is the presence of IgA1 depositions in the renal mesangium, which is influenced by several factors (Figure 1). However, this picture can vary from slight mesangial hypertrophy with IgA1 depositions, to extracapillary proliferation with crescent formation. SIgA is present in renal biopsies in 15% of cases. Aberrant, undergalactosylated IgA1 seems to be involved in the pathogenesis. Since IgA1 glycosylation is determined within B lymphocytes, and only a fraction of the IgA1 seems to be affected, this suggests that alterations in glycosylation are only present in a subset of B cells. The question that arises is whether all these different clinical and histological pictures should be considered as one disease or that different characteristics represent a number of diseases with one common factor which is mesangial IgA deposition.

Further differentiation between IgAN patients, involving both immunological and histological investigations as well as clinical studies, is of importance to further unravel the pathogenesis of IgAN and eventually develop individual therapeutic strategies.

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Samenvatting

Het afweersysteem is er op gericht om bescherming te bieden tegen virussen en bacteriën, waaraan ieder mens dagelijks wordt bloot gesteld. Wanneer het lichaam hierdoor bedreigd wordt dan treedt een afweerreactie op, met als doel deze virussen en bacteriën onschadelijk te maken en te verwijderen. Tijdens deze afweerreactie worden cellen geactiveerd en worden uiteindelijk afweerstoffen (immuunglobulinen) geproduceerd, specifiek gericht tegen eiwitten op het oppervlakte van deze bacteriën of virussen. Er zijn 5 typen immuunglobulinen (Ig) namelijk, IgG, IgA, IgM, IgE en IgD. Deze afweerstoffen worden voornamelijk gemaakt door cellen in het bloed en in het beenmerg. IgA wordt echter ook in belangrijke mate gemaakt in de verschillende slijmvliezen (mucosa) van het lichaam, zoals in het slijmvlies van de luchtwegen en in het maag-darmstelsel. Soms is het immuunsysteem betrokken bij het ontstaan van een ziekte, zoals bij de ziekte IgA Nefropathie (IgAN).

IgAN is de meest voorkomende primaire glomerulonefritis in de wereld. Een glomerulonefritis is een ontsteking van glomeruli, de filterlichaampjes van de nieren. Per nier zijn er ongeveer 1 miljoen glomeruli aanwezig. De ziekte IgAN kenmerkt zich door de aanwezigheid van IgA in het steunweefsel (mesangium). van de glomeruli. Het ontstaansmechanisme (pathogenese) van IgAN is onduidelijk. De reden waarom IgA neerslaat en de factoren die dit beïnvloeden zijn slechts ten dele bekend. Genetische factoren lijken een rol te spelen bij het ontstaan van IgAN. In bepaalde families, waar IgAN vaak voor komt, blijkt een gen mutatie aanwezig. Echter in het grootste aantal gevallen zijn tot op heden geen genetische factoren gevonden die het optreden van IgAN verklaren. Naast genetische factoren zijn waarschijnlijk ook omgevingsfactoren betrokken bij de pathogenese van IgAN. Dit kan deels verklaren waarom de incidentie van IgAN geografisch sterke verschillen laat zien.

De ziekte openbaart zich vaak tussen het 20^{e} en 30^{e} levensjaar en komt vaker voor bij mannen dan bij vrouwen in een verhouding 2:1. Kenmerken van IgAN zijn bloed in de urine (hematurie), vaak gepaard met eiwitverlies

via de urine (proteinurie) en tevens is er vaak sprake van hoge bloeddruk. Een opmerkelijk verschijnsel dat bij een aantal IgAN patiënten optreedt, is dat er zichtbaar bloed in de urine verschijnt, in aansluiting op een infectie, vaak van de bovenste luchtwegen. Het beloop van IgAN kan sterk variëren. Ongeveer 30% van de patiënten zal uiteindelijk een totale nierinsufficiëntie ontwikkelen. Dit gebeurt meestal in de loop van vele jaren, maar kan ook in zeer korte tijd gebeuren. Om met zekerheid de diagnose IgAN te stellen is het noodzakelijk om een stukje nierweefsel onder de microscoop te beoordelen. Dit weefsel kan worden verkregen door middel van een prik in de nieren, een nierbiopsie, waarna met behulp van een specifieke kleuringmethode het IgA kan worden gevisualiseerd. Vaak zijn er naast IgA ook nog andere eiwitten in de nier aanwezig, zoals IgG en C3, een ander eiwit betrokken bij de activatie van een deel van het immuunsysteem. De aanwezigheid van deze eiwitten leidt tot een ontstekingsreactie in de nier, waardoor er beschadiging optreedt en uiteindelijk verlies van nierfunctie. In Nederland en andere westerse landen is IgAN de oorzaak van nierfalen bij ongeveer 10% van de patiënten die nierfunctie vervangende therapie ondergaan. In het bloed van IgAN patiënten is IgA vaak in verhoogde concentratie aanwezig. Dit is echter nog geen verklaring voor het feit dat IgA in de glomeruli neerslaat. Bij andere aandoeningen, waarbij IgA soms ook in sterk verhoogde concentratie in het bloed aanwezig is, zoals bij HIV of bij de ziekte van Kahler, slaat IgA niet neer in de nieren. Opmerkelijk is dat na een niertransplantatie in de getransplanteerde nier vaak opnieuw neerslagen van IgA worden aangetroffen. Een enkele maal is het ook gebeurd dat een nier, die van een IgAN patiënt afkomstig bleek te zijn, werd getransplanteerd naar een niet IgAN patiënt. In een dergelijke situatie blijkt het IgA uit de nier te verdwijnen. Dit is een argument voor het feit dat IgAN wordt veroorzaakt door karakteristieken van het IgA en niet door een intrinsieke afwijking in de nieren.

IgA komt in het lichaam in verschillende vormen voor. Er bestaan twee subklassen, namelijk IgA1 en IgA2, die zich van elkaar onderscheiden door verschillen in het verbindingsstukje (hinge) tussen het constante en variable gedeelte, waardoor ook bepaalde suikergroepen op het molecuul verschillend aanwezig zijn. Alleen de subklasse IgA1 wordt in de nieren aangetroffen.

Verder wordt er een indeling van IgA gemaakt, gebaseerd op verschillen in grootte. Zo bestaat er monomeer IgA (mIgA), dat uit 1 IgA molecuul bestaat, dimeer IgA (dIgA) dat uit 2 IgA moleculen bestaat, verbonden door een eiwit genaamd J-chain, en polymeer IgA (pIgA), dat een variabele afmeting heeft en bestaat uit meerdere IgA moleculen. Met name pIgA wordt in de nieren van IgAN patiënten aangetroffen. Zoals eerder genoemd wordt IgA voornamelijk geproduceerd door cellen die aanwezig zijn in de slijmvliezen en wordt uitgescheiden in secreta, zoals speeksel en darmsappen. Dit IgA onderscheidt zich van IgA geproduceerd in het bloed of in het beenmerg, door de aanwezigheid van secretoir component en wordt secretoir IgA (SIgA) genoemd. Hoewel SIgA voornamelijk in secreta voorkomt en daar betrokken is bij het neutraliseren van virussen en bacteriën komt SIgA ook voor in de circulatie. De rol van SIgA in het bloed is niet duidelijk. Bij IgAN patiënten is de concentratie van SIgA in het bloed hoger dan bij gezonde personen. Bij een laag percentage IgAN patiënten wordt SIgA eveneens in de nieren aangetroffen en is geassocieerd met meer hematurie en de aanwezigheid van ontstekingseiwitten in de nier.

IgA van patiënten met IgAN verschilt niet alleen kwantitatief van IgA van gezonde personen, ook kwalitatief. Er zijn belangrijke verschillen aangetoond in de besuikering (glycosylering) van het IgA molecuul. Bovendien blijkt dit anders geglycosyleerde IgA in verhoogde mate aanwezig in de nieren. Welke factoren verantwoordelijk zijn voor de verandering in glycosylering is onduidelijk.

Bij het op gang komen van een afweerreactie zijn verschillende cellen betrokken. In de eerste plaats zijn er cellen die een eiwit (antigeen) opnemen en dit in stukjes aan andere witte bloed cellen (T cellen) tonen, waardoor deze cellen geactiveerd kunnen raken. Deze antigeen presenterende cellen (APC) initiëren de immuunrespons. De belangrijkste APC is de dendritische cel (DC). Er zijn verschillende typen DC, die nog maar ten dele gekarakteriseerd zijn wat betreft hun functie. Een DC is ook in staat om een B cel (voorloper van een cel die immuunglobuline produceert) te laten veranderen in een plasmacel, een cel die een bepaald type Ig kan maken. Dit proces kan op

verschillende manieren plaatsvinden. Het kan door middel van activatie van bepaalde witte bloedcellen, T cellen, waarna deze T cel de B cel activeert en stimuleert tot Ig productie. Het kan echter ook door een direct effect van de DC op de B cel, waarbij de DC bepaalde signaaleiwitten afscheidt, die de B cel activeren, waarna proliferatie en differentiatie optreedt en er uiteindelijk een Ig producerende plasma cel ontstaat.

Het onderzoek, beschreven in dit proefschrift heeft zich gericht op verschillende aspecten van regulatie van de immuunrespons van IgAN patiënten. Hoofdstuk 2 gaat over een vaccinatie studie, waarbij IgAN patiënten en gezonde controle personen worden gevaccineerd met twee verschillende eiwitten, waarmee deze personen nog niet eerder in contact zijn geweest (neo-antigenen). Een dergelijke toediening leidt tot een zogenaamde primaire immuun respons. Het ene antigeen, Cholera Toxine B subunit (CTB) wordt toegediend op de slijmvliezen van de neus. Het andere eiwit, Keyhole Limpet Hemocyanin (KLH) wordt onder de huid toegediend. In totaal krijgen alle personen 3 maal deze eiwitten toegediend. Het blijkt dat de IgAN patiënten na de mucosale vaccinatie met CTB minder IgA gericht tegen het CTB maken dan de gezonde personen. Er wordt geen verschil gevonden tussen de beide groepen in de IgG en de IgM antistoffen gericht tegen CTB. Na de vaccinatie met KLH worden geen verschillen in concentratie van afweerstoffen gevonden tussen de beide groepen.

Aangezien het een primaire immuunrespons betreft, waarbij de DC een belangrijke rol speelt, hebben we gepostuleerd dat er minder DC aanwezig zijn bij IgAN patienten. Vervolgens hebben we gekeken naar de aanwezigheid van DC in de mucosa van de neus, de plaats waar het vaccin werd toegediend. Dit wordt beschreven in hoofdstuk 3. Het blijkt dat IgAN patiënten niet minder DC in de neus mucosa hebben dan de controle personen. Van bepaalde subtypen zijn er zelfs meer DC bij de IgAN patiënten aanwezig. Dit betekent dat de eerder gevonden IgA hyporespons niet verklaard kan worden door een tekort aan DC bij IgAN patiënten.

Om te onderzoeken of de aanwezige DC minder goed functioneren, hebben we gebruik gemaakt van een *in vitro* model. Dit model en de resultaten

van het onderzoek worden beschreven in hoofdstuk 4. In dit model wordt een primaire immuunrespons nagebootst. Daarbij wordt gebruik gemaakt van B cellen die nog niet eerder bij een immuun respons betrokken zijn geweest, zogenaamde naïeve B cellen, en van cellen die de geactiveerde T cel nabootsten. Aan deze cellen worden DC toegevoegd die zijn opgekweekt uit monocyten, voorlopercellen van DC, geïsoleerd uit het bloed van IgAN patiënten en van gezonde vrijwilligers. De enige variabele in dit model is de DC. Het blijkt dat onder deze gecontroleerde omstandigheden naïeve B cellen kunnen worden gestimuleerd om zich te ontwikkelen tot Ig producerende plasmacellen. Afhankelijk van de omstandigheden wordt er meer IgA, IgG of IgM geproduceerd. De aanwezigheid van DC verhoogt de productie van Ig in hoge mate. DC afkomstig van IgAN patiënten induceerden minder IgA productie dan DC afkomstig van controle personen. Er was geen verschil in de productie van IgG en IgM. Hoewel er onderling variatie was in het vermogen B cellen aan te zetten tot IgA productie tussen de verschillende IgAN patiënten was de gemiddelde IgA productie veel lager dan bij de controle personen.

De resultaten van de hoofdstukken 2,3 en 4 tonen dat DC van patiënten met IgAN funtioneel tekort schieten in het induceren van een primaire IgA immuunrespons. Onze hypothese is dat door het minder effectieve verloop van deze primaire mucosale IgA immuunrespons de totale expositieduur aan mucosale antigenen verlengd wordt, met als gevolg uiteindelijk een hogere concentratie afwijkend IgA in het bloed, wat kan bijdragen aan het neerslaan van IgA in de nieren.

Zoals eerder vermeld, wordt een belangrijk deel van de totale hoeveelheid IgA in de mucosa geproduceerd en dit wordt als secretoir IgA (SIgA) uitgescheiden in de diverse secreta. Bij de meeste IgAN patiënten is de concentratie van SIgA in het bloed verhoogd en bij 15% van de patiënten wordt in de nieren ook SIgA aangetroffen. In de hoofdstukken 5 en 6 wordt dieper ingegaan op de rol van SIgA. Hoofdstuk 5 is een overzichtsartikel, dat een algemene beschrijving geeft van de verschillende aspecten van SIgA en de rol van SIgA beschrijft in relatie tot IgAN. In hoofdstuk 6 tonen we aan dat er antigeen-specifiek SIgA kan worden aangetoond in het bloed van personen die mucosaal gevaccineerd zijn. Hierbij hebben we gebruik gemaakt

van de bloedmonsters van de personen uit de vaccinatie studie van hoofdstuk 2. Tevens hebben we gekeken hoe de verdeling in grootte is van de specifieke IgA respons na de mucosale en subcutane vaccinatie. Het is immers zo dat met name pIgA in de nieren neerslaat. Het blijkt dat zowel IgAN patienten als ook gezonde personen tijdens een primaire immuun respons voornamelijk pIgA produceren. Dit geldt voor de beide vormen van immunisatie. Deze studie is de eerste studie die het mogelijk maakt om de grootteverdeling van IgA moleculen na verschillende vormen van immunisatie met elkaar te vergelijken. Tussen beide groepen kunnen geen verschillen in grootteverdeling van het antigeen specifieke IgA worden aangetoond. Aangezien slechts een klein deel van het IgA uit het bloed neerslaat in de nieren is het goed mogelijk dat aanwezige subtiele verschillen in grootteverdeling van het IgA, niet gevonden zijn doordat de gebruikte methodes tekort schieten.

Hoofdstuk 7 beschrijft een ander belangrijk aspect van IgAN, namelijk de glycosylering (besuikering) van het IgA molecuul. In vele studies is inmiddels aangetoond dat de glycosylering een belangrijke rol speelt bij het ontstaan van IgAN. Patiënten met IgAN hebben IgA dat anders geglycosyleerd is in vergelijking met controle personen. Dit IgA heeft een verhoogde neiging tot complex vorming. Bovendien bevatten de neerslagen van IgA met name dit afwijkend geglycosyleerde IgA. Recent is er onderzoek gedaan naar enzymen die betrokken zijn bij dit glycosylerings proces. Het blijkt dat de aanwezigheid van deze enzymen en ook de gen expressie van deze enzymen niet verschillend is tussen patiënten en controles. Het is belangrijk te beseffen dat slechts een deel van het IgA van IgAN patiënten afwijkend geglycosyleerd is. Welke factoren bepalen hoe de glycosylering verloopt en welke factoren verantwoordelijk zijn voor het feit dat een deel van het IgA afwijkend geglycosyleerd is blijft nog onduidelijk.

Het bovenstaande toont aan dat bij IgAN patiënten gezocht moet worden naar afwijkingen in het immuunsysteem. Het onderzoek, zoals beschreven in dit proefschrift heeft zich gericht op de immuunrespons bij patiënten met IgAN, omdat met name de afwijkingen in de immuun respons van invloed kunnen zijn op het ontstaan van een verhoogde concentratie van afwijkend IgA in het bloed en daarmee van invloed kunnen zijn op het ontstaan van IgAN. Daar

het immuunsysteem een uitermate complex systeem is, waarbij vele eiwitten betrokken zijn en waarbij nog steeds nieuwe eiwitten en signaleringsroutes ontdekt worden, moge duidelijk zijn dat het oplossen van de pathogenese van IgAN een moeilijke zaak is, die verdergaand onderzoek noodzakelijk maakt

Curriculum Vitae

De schrijver van dit proefschrift werd geboren op 13 februari 1966 te Pijnacker. In 1984 behaalde hij het VWO ß diploma aan het Christelijk Lyceum Delft, te Delft. In datzelfde jaar begon hij met de studie Scheikunde aan de Rijksuniversiteit Leiden. In 1985 startte hij met de studie Geneeskunde eveneens aan de Rijksuniversiteit Leiden. In 1989 en in 1992 behaalde hij achtereenvolgens het doctoraal examen en het artsexamen. In deze periode werkte hij gedurende 8 maanden in het laboratorium Nierziekten (hoofd laboratorium Nierziekten: Prof.Dr. M.R. Daha). Aansluitend werkte hij van februari 1992 tot mei 1993 op de afdeling Nierziekten van het LUMC aan het project "mucosale immuun respons bij patiënten met IgA nefropathie" (hoofd afdeling Nierziekten: Prof.Dr. L.A. van Es). Van mei 1993 tot mei 1997 werkte hij als arts-assistent Interne Geneeskunde in het Medisch Centrum Haaglanden te Den Haag, alwaar in 1994 gestart werd met de opleiding tot internist (opleider Dr. E.J. Buurke). Vanaf 1997 werd de opleiding tot internist voortgezet in het LUMC en voltooid in 2000 (opleider Prof.Dr. A.E. Meinders). In 2000 startte hij met de opleiding tot nefroloog bij de vakgroep Nierziekten van het LUMC (opleider Prof.Dr. L.C. Paul). In deze periode werd onder begeleiding van Dr. C. van Kooten en Prof.DR. M.R. Daha ook gestart met het onderzoek beschreven in dit proefschrift.
Vanaf 1 oktober 2002 is hij werkzaam als internist-nefroloog in het Groene Hart Ziekenhuis te Gouda.

Nawoord

Het tot stand komen van dit proefschrift is het resultaat van een proces waaraan vele personen hebben bijgedragen. Graag wil ik dan ook iedereen die op welke manier dan ook betrokken is geweest bij het onderzoek hiervoor bedanken.

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