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Expression of J chain mRNA in duodenal IgA plasma cells in IgA nephropathy

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Expression of J chain mRNA in duodenal IgA plasma cells in IgA nephropathy. Glomerular IgA in IgA nephropathy (IgAN) is at least in part polymeric, and is thought to derive from the mucosal IgA system in view of the association between mucosal infection and haematuria in this condition. To investigate this hypothesis, an *in situ* hybridization (ISH) technique was developed for the detection of J chain mRNA, the expression of which has been correlated with the secretion of high level polymeric immunoglobulin (pIg). Endoscopic duodenal biopsies from ten patients and matched controls were examined by: (i) two color immunofluorescence (IF); (ii) ISH; and (iii) combined ISH and IF, to permit simultaneous identification of plasma cell type. IF revealed a reduction in the percentage of IgA plasma cells ($P < 0.02$) and increased absolute numbers of IgG cells ($P < 0.02$) in patient biopsies. ISH demonstrated fewer J chain mRNA expressing plasma cells ($P < 0.005$) with lower signal intensity ($P < 0.002$) in patients' biopsies compared with controls. Combined ISH and IF confirmed a reduction in J chain mRNA-positive IgA plasma cells in the patient biopsies ($P < 0.02$). The reduction in J chain mRNA expression in duodenal IgA plasma cells in IgAN argues against the gastrointestinal lamina propria as the source of glomerular pIgA.

IgAN is the most prevalent form of glomerulonephritis in the Western world [1]. Approximately one-third of patients progress to end-stage renal failure [2]. The pathological hallmark of the condition is the predominant deposition of IgA in the glomerular mesangium [3]. Although the molecular nature of deposited IgA is controversial the balance of evidence suggests that it is, at least in part, polymeric (pIgA), since it contains J chain [4, 5] and has the capacity to bind secretory component [4, 6]. Renal biopsy elution studies also substantiate the polymeric nature of mesangial IgA [5, 7]. The source of the glomerular IgA remains unknown although strong circumstantial evidence points to the mucosal IgA immune system—the main source of pIgA. Many patients with IgAN experience episodic macroscopic hematuria [8] which may be spontaneous or coincide with mucosal infection, usually of the respiratory or gastrointestinal tracts. Increased circulating pIgA is found during such relapses [9–11]. A mucosal origin for glomerular IgA in IgAN is also suggested by increased levels of circulating IgA antibodies to food antigens and common respiratory and gastrointestinal pathogens [12–16], the presence of food antigens in association with glomerular IgA deposits also supports this view [17]. It has therefore been proposed that IgAN results from a poorly controlled mucosal immune response to environmental antigens, and that excess mucosally generated pIgA gains access to the circulation and becomes trapped in the mesangium [2, 18, 19].

Studies of a variety of human lymphoid tissues have reported abnormalities in IgA plasma cell count in IgAN [20–25], IgA cell counts, however, may not be relevant to the rate of pIgA production. Few studies have addressed the important question of J chain expression [22, 24].

J chain is the key to mucosal IgA secretory immunity as it is integral to the synthesis of functional pIgA. Although some studies have demonstrated that polymers may form *in vitro* in the absence of J chain [26, 27], other work suggests that J chain plays a crucial role in normal immunoglobulin polymerization [28–31] and secretion [32, 33]. It is also essential for transcellular transport to luminal surfaces, where polymeric Ig is in the front line of mucosal defence [34, 35]. The production of J chain is not controlled at the level of translation [28], but rather at the level of transcription [36]. Increases in pIg secretion have been shown to parallel rises in J chain mRNA [37] and an in *vitro* analysis of IgM production has revealed that the appearance of mRNA for J chain is a limiting factor in high level polymeric Ig secretion [32].

Some investigators have studied the expression of J chain in the tonsils of patients with IgAN using purely immunological techniques [22, 24], none has investigated J chain expression within the lamina propria, which is the main effector site of the mucosal IgA immune system. Evidence of increased J chain expression by mucosal IgA plasma cells would therefore provide strong support for the view that excess circulating pIgA and deposited glomerular pIgA are mucosally derived in IgAN.

Adequate investigation in this area has previously been hampered by the lack of readily available reliable anti-J chain antibodies, the relative inaccessibility of J chain within polymer structures, the difficulty of sample collection, and the absence of established techniques for the investigation of J chain gene expression within human lymphoid tissue.

We have developed an *in situ* hybridization technique which

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allows the detection of native mRNA species within plasma cells of a known isotype in routine pathology specimens. This method allows investigation of pIgA production in the gastrointestinal mucosa and elsewhere in IgAN, by studying J chain gene expression in individual IgA plasma cells.

Methods

Subjects

Endoscopic biopsies from the second part of the duodenum were obtained from ten patients with renal biopsy-proven IgAN who had no symptoms suggestive of gastrointestinal disease, (8 male, 2 female; mean age 47.8 years, range 32 to 62 years). Control biopsies were obtained in a similar manner from ten sex- and age-matched controls (mean age 49 years, range 36 to 65 years). Controls were individuals undergoing upper gastrointestinal tract evaluation in whom gastric and duodenal mucosae appeared macroscopically normal, and in which subsequent microscopic examination was also normal. No patient or control was receiving H_2 receptor blocking drugs or other agents known to interfere with mucosal function. No gastrointestinal pathology was identified in any control. Mean serum creatinine in the IgAN group was 121.4 μmol/liter (range 66 to 324 μmol/liter) and only two patients had serum creatinine raised above the reference range (170 and 324 μmol/liter, respectively). Three patients had reported macroscopic hematuria at some stage in their illness; however none had mucosal infection or macroscopic hematuria at the time of study, although 6 of 10 had microscopic hematuria and 4 of 10 had proteinuria. None was nephrotic. Although two individuals in the IgAN group had signs of mild gastro-esophageal reflux, they were asymptomatic. Endoscopies were performed entirely as part of the research protocol for this study with local ethical committee approval.

Two-color immunofluorescence

Endoscopic biopsy specimens were fixed in formol saline for 24 hours, then processed and embedded in paraffin wax as for routine histology. All samples were fixed and processed in the same manner. Four micrometer tissue sections were cut onto 3-aminopropyltriethoxysilane coated slides, and after drying overnight at 37°C sections were dewaxed using xylene and rehydrated through ethanol and ultra-pure water. Slides were immersed in 50 mM Tris-HCl buffer, pH 7.65, (Tris-HCl), then underwent proteolytic digestion with proteinase K (Boehringer Mannheim, Germany, 161519) in Tris-HCl, at a concentration of 2 μg/ml for one hour at 37°C. Sections were then washed in Tris-HCl and sequentially incubated with: (i) primary fluorescein isothiocyanate (FITC) labeled antibody for 30 minutes at room temperature [anti-IgA (Dako U.K. F316) 1:20, anti-IgG (Dako U.K. F315) 1:20, anti-IgM (Dako U.K. F317) 1:20 or unlabeled monoclonal anti-IgA_1 (Unipath U.K. M12011) 1:10]; (ii) secondary antibody for 30 minutes at RT [tetramethylrhodamine isothiocyanate (TRITC) labeled anti-heavy and light-chain antibody (Dako U.K. R320) 1:50 or FITC labeled rabbit anti-mouse antibody (Dako U.K. Z259) 1:10]; (iii) tertiary antibody for IgA subclass study [TRITC labeled anti-IgA (Dako U.K. R153) 1:50 for 7 minutes at room temperature]. Intervening washes were with Tris-HCl. Antibodies were diluted in Tris-HCl.

In situ hybridization

The *in situ* hybridization protocol for the detection of mRNA for immunoglobulin J chain and kappa light chain is fully described in a recent publication [38] and is adapted from previously documented methods [39, 40].

In summary, fixation, processing, dewaxing, rehydration and proteolytic digestion with proteinase K was performed as above using RNAse free solutions and glassware. RNAse pretreatment and "no hybridization" (probe omitted from hybridization solution) controls were run in parallel with the experimental slides. Hybridization was performed overnight at 37°C using digoxigenin labeled deoxyoligonucleotide probe cocktails. Probe concentrations of 200 pg/μl and 500 pg/μl were used for J chain and kappa light chain probes, respectively. Hybrids were detected using an alkaline phosphatase labeled sheep polyclonal anti-digoxigenin antibody. The detection sensitivity of labeled probe with this system was 100 fg/μl probe solution. Alkaline phosphatase was visualized with Fast red/naphthol AS-MX phosphate.

Combined in situ hybridization and immunofluorescence

The simultaneous demonstration of cytoplasmic immunoglobulin and native mRNA expression was achieved by joint incubation of the specimens with both an FITC labeled anti-human IgA antibody and the anti-digoxigenin antibody at the detection stage. The use of naphthol AS-MX phosphate in the demonstration solution produces a reaction product that is visible in bright field illumination and under ultraviolet (UV), at both 490 and 540 nm [41]. Hence both fluorochromes, the Fast red/naphthol phosphate product (mRNA) and FITC (immunoglobulin isotype) can be visualized together at 490 nm. Simultaneous visualization is illustrated in Figure 1. Figures 1A and 1B are the same field photographed sequentially under bright field illumination (Fig. 1A) and then UV (Fig. 1B). In Figure 1A cells expressing J chain mRNA have red cytoplasm (cells 1 and 2). Cells negative for J chain mRNA have a clear cytoplasm (cell 3). On viewing under UV (490 nm) J chain mRNA expressing cells which are negative for IgA (presumably IgM or IgG cells) appear red/orange (cell 1), IgA plasma cells negative for J chain mRNA appear green (cell 3), and the combined signal from IgA cells expressing J chain mRNA results in yellow fluorescence (cell 2) [38].

Probe labeling

The details of probe labeling are published elsewhere [38]. Cocktails of phosphoramidite deoxyoligonucleotides [42] were 3′ end labeled with the nonradioactive nucleotide analogue digoxigenin-11-deoxyuridine triphosphate using terminal deoxynucleotidyl transferase. Deoxyoligonucleotide design was based on previously published gene sequence data [43–46]. A cocktail of twelve oligonucleotides was used, each 30 bases in length. This length of oligonucleotide has been established as a practical compromise between hybrid stability, hybrid specificity, cost of synthesis and purification and tissue penetration efficiency [39].

Controls and experimental design

"No hybridization" and RNAse pretreatment controls were used for each specimen. Each specimen was also "probed" for

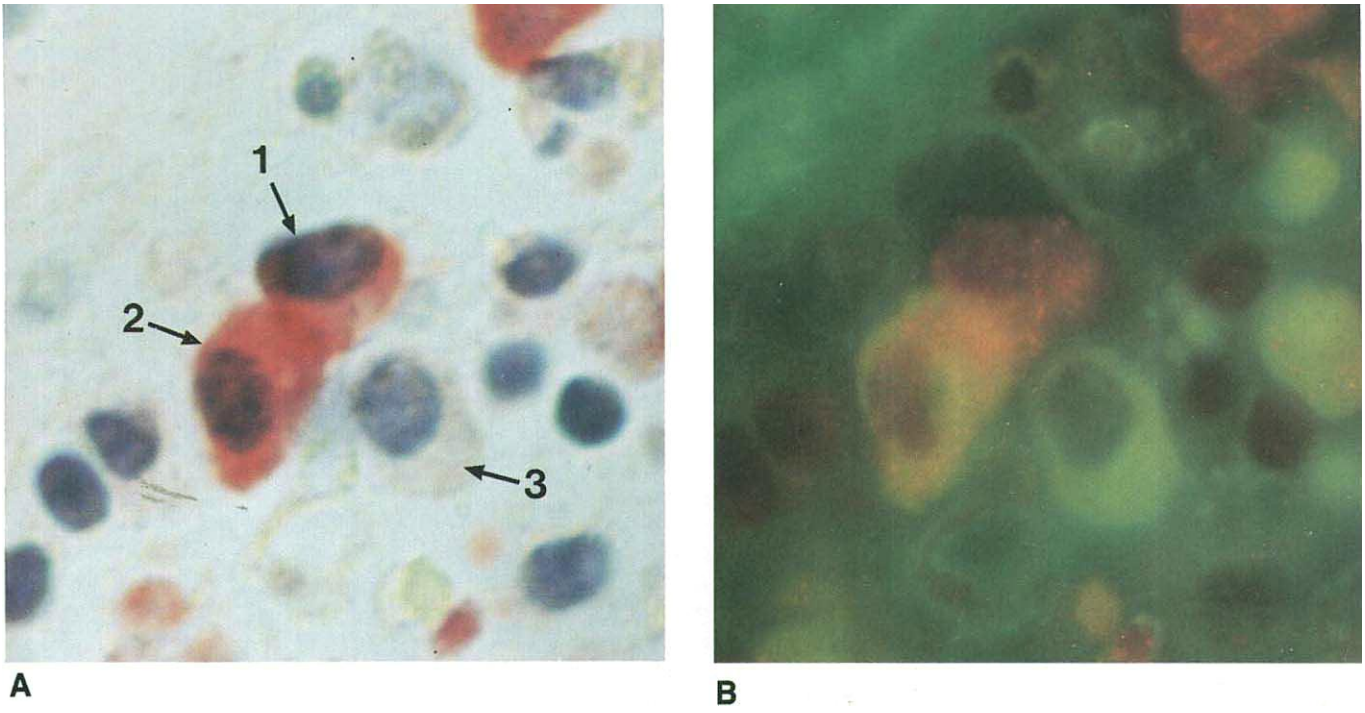

Fig. 1. *Simultaneous* in situ *hybridization and immunoglobulin detection by conventional immunofluorescence.* (A) *In situ* hybridization for immunoglobulin J chain mRNA. Bright field illumination. Hybrids were visualized with Fast red. Mayer's hematoxylin counterstain. Cells 1 and 2 are J chain mRNA positive, cell 3 is J chain mRNA negative. (B) The same field as in A but viewed under ultraviolet (490 nm). Combined J chain mRNA signal (red) and immunofluorescence for cytoplasmic IgA (green). Cell 1, J chain mRNA positive, IgA negative; cell 2, J chain mRNA positive, cytoplasmic IgA positive, (dual signal—yellow/ orange); cell 3, J chain mRNA negative, cytoplasmic IgA positive (×1000). Reproduced by permission of the Editor, Dr. J. Lilleyman of the *Journal of Clinical Pathology* published by the British Medical Journal Publishing Group.

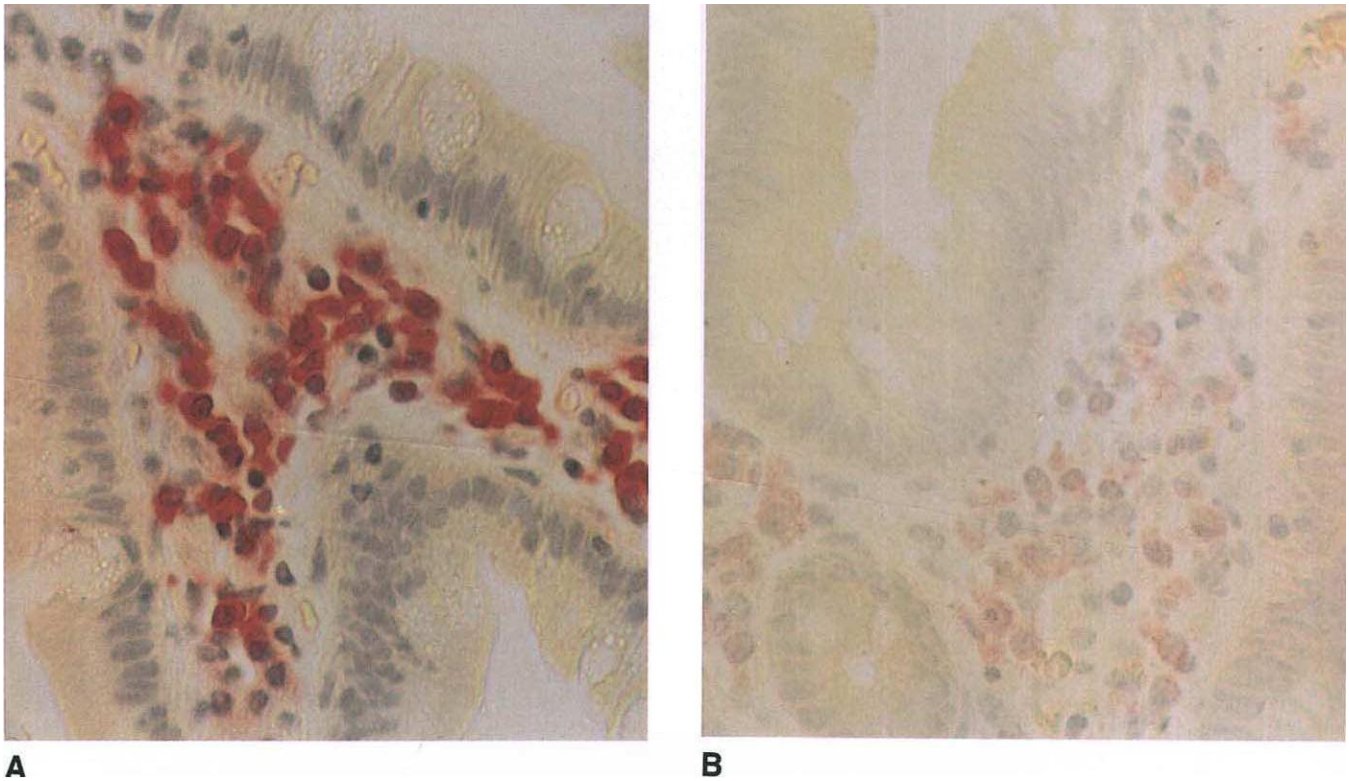

Fig. 4. In situ *hybridization for J chain mRNA using a digoxigenin labeled deoxyoligonucleotide probe on formol-saline fixed duodenal biopsies.* Fast red signal. Mayer's haematoxylin counterstain. (A) Control biopsy; (B) Patient biopsy (×400). Publication of these figures in color was made possible by Bayer plc, Berkshire, United Kingdom.

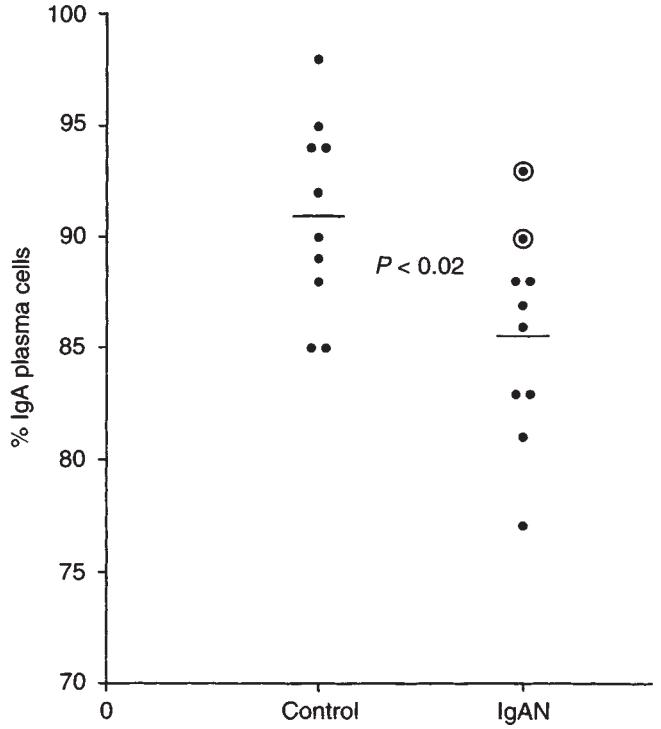

Fig. 2. *IgA plasma cells.* Proportion of IgA plasma cells in the duodenal lamina propria of patients with IgA nephropathy and controls. (⊙) Individual patients with serum creatinine above the normal range.

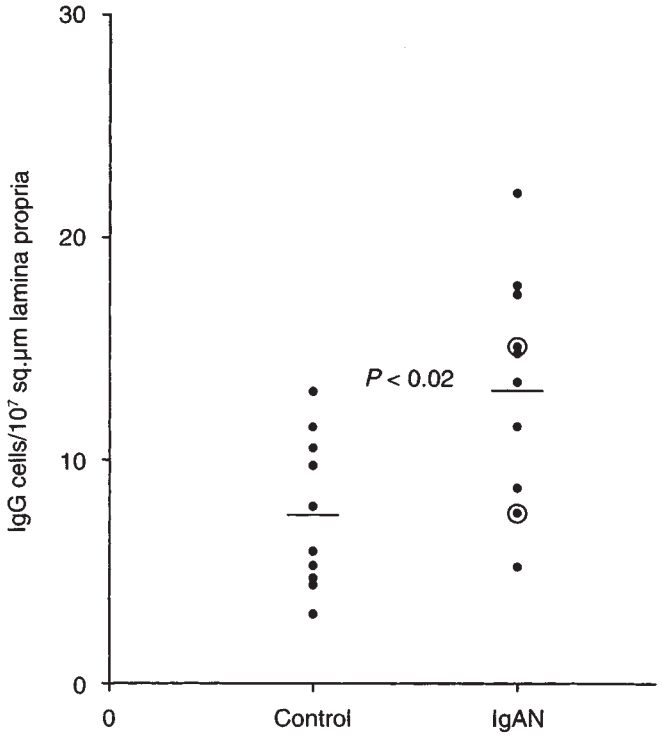

Fig. 3. *IgG plasma cells.* Absolute numbers of IgG plasma cells per unit area of the duodenal lamina propria in patients with IgA nephropathy and controls. (⊙) Individual patients with serum creatinine above the normal range.

the expression of known sequences which should not have been expressed in the tissue, namely parathormone and insulin [47, 48]. These probes had a similar G-C content to the J chain probe and acted as additional negative controls.

To assess poly-adenylated RNA preservation and determine the optimal proteolytic digestion for each sample, preliminary *in situ* hybridization studies were conducted after a range of proteolytic digests (1 to 10 μg/ml) using a digoxigenin labeled oligonucleotide poly-d(T) probe [40] and a digoxigenin labeled deoxyoligonucleotide cocktail for kappa light chain mRNA [38]. In this way the proteolytic treatment which would render maximum *in situ* signal intensity was defined for each specimen. *In situ* hybridization for J chain mRNA was then conducted after optimal proteolytic pretreatment on paired slides (patient and matched control) running back to back, in addition to controls as above.

To ensure that any differences between control and patient samples were not a reflection of a nonspecific alteration in plasma cell immunoglobulin chain mRNA expression in IgAN, the expression of kappa light chain mRNA in lamina propria cells was investigated by *in situ* hybridization alone and in combination with immunofluorescence [38]. Hence the expression of kappa light chain mRNA in duodenal IgA plasma cells acted as an "intracellular control" for the cells in which J chain mRNA expression was anticipated.

Microscopy

Bright field and ultraviolet microscopy was performed using a Nikon Optiphot microscope. Cell counts were performed on at least 500 cells on *in situ* sections and 200 cells on two-color immunofluorescent sections (counting error 3 and 9%, respectively) by a single observer unaware of the origin of the specimen. IgG cells were counted per whole section since IgG cells are very sparse within the lamina propria [49]. Absolute numbers were therefore related to the area of lamina propria on the specimen (measured directly from sections using an optical planimeter). All cell enumeration was performed on sections prior to counterstaining with Mayer's hematoxylin. Counterstaining was performed to confirm good preservation of morphology.

Confocal advanced light microscopy was performed on a MRC600 confocal laser scanning microscope using an Argon laser (maximum emission at 488 nm and 514 nm). Signal intensity in individual cells was assessed by utilization of the pixel intensity distribution facility of the microscope. Signal intensity was assessed on 50 of the brightest cells from each section, and the operators were unaware of the origin of each specimen.

Statistics

Data were analyzed with the unpaired *t*-test and Wilcoxon Mann-Whitney U analysis [50]. Unless stated otherwise, results are expressed as mean ± standard error of mean.

Results

Immunofluorescent studies

Immunofluorescence demonstrated a reduction in IgA plasma cells as a proportion of total plasma cells within the lamina propria of patients (mean 85.6 ± 1.5%) compared to controls

(mean 91 ± 1.8%; $P < 0.02$; Fig. 2). In contrast, an increase in absolute numbers of IgG plasma cells was identified in patient biopsies (mean 13.2 ± 1.6/10^7 sq · μm) compared to controls (mean 7.6 ± 1.1/10^7 sq · μm; $P < 0.02$; Fig. 3). There was no significant difference between patient and control biopsies in the proportion of IgM plasma cells or in IgA plasma cell subclass distribution (data not shown).

In situ hybridiation for J chain mRNA

Fast red visualized sites of hybridization were seen specifically staining the anticipated plasma cell population within the lamina propria of duodenal biopsies from both controls and patients. Sharp, clearly defined signal contrasted with negligible background staining. Morphology was well preserved with all specimens. Negative controls in which the probe was omitted from the hybridization solution, parathormone and insulin controls showed no signal on any occasion. RNAse A1 pretreatment also eliminated all signal. In contrast, pilot studies revealed that DNAse pretreatment did not influence positive results in any specimen, confirming that this method specifically identifies the presence of RNA.

Cells expressing J chain mRNA were seen throughout the lamina propria in control biopsies, but were maximally concentrated at the base of the villi (Fig. 4A). Although J chain mRNA positive cells were identified within the patient biopsies, in a similar distribution to that seen in the controls, the striking and unexpected difference was a uniform reduction in cell signal intensity (Fig. 4B). The contrast between patient and control samples was marked; the specimens could readily be assigned into two groups by observers unaware of the origin of each specimen with a <5% error rate. Enumeration of positive cells as a proportion of all cells with plasmacytoid morphology demonstrated lower numbers of positive cells in the patient biopsies (17 ± 4.8%) as compared to controls (39 ± 4.0%; $P < 0.005$; Fig. 5A). The fluorescent nature of the Fast red/naphthol reaction product lends itself to examination by confocal microscopy. The observed reduction in signal intensity from J chain mRNA positive cells within patient biopsies under bright field illumination, was semi-quantified by confocal microscopy: patients (mean pixel intensity value 54.7 ± 3.5 a.u.) versus controls (mean pixel intensity value 77.3 ± 5 a.u.), $P < 0.002$ (Fig. 6A).

In contrast, no difference in the expression of kappa light chain mRNA between controls and patients was identified, either in terms of positive cell enumeration or cell signal intensity (Figs. 5B and 6B).

Simultaneous in situ hybridization and immunofluorescence

Combined *in situ* hybridization and immunofluorescence allowed the identification of the plasma cell type in which J chain mRNA was identified. These studies revealed that in the lamina propria of patients with IgAN there is a reduction in the proportion of IgA plasma cells that are J chain mRNA positive in comparison to control biopsies (control median value 95%, patient median value 54%; $P < 0.02$; Fig. 7A).

No difference in the proportion of mucosal IgA plasma cells expressing kappa light chain was evident (Fig. 7B).

There were four patients in whom no J chain mRNA could be detected using this protocol (Figs. 5A and 7A). However, weak positive results were obtained using higher probe concentrations (400 to 600 pg/μl). There were no clinicopathological features, including the history of macroscopic hematuria, which distinguished these individuals from the other patients in the IgAN group. Serum creatinine was normal in all four cases.

Discussion

In situ hybridization has advantages over other nucleic acid assays since it allows identification of specific nucleic acid sequences in individual cells of a tissue within their normal topographical surroundings [51]. Our innovative adaptation of standard non-isotopic *in situ* hybridization methodology [38] allows not only a semi-quantifiable assessment of individual cell signal intensity, but also the immunofluorescent identification of the plasma cell type in which native mRNA is expressed. In addition our experience suggests that this method may be performed on routine or archival paraffin wax embedded material if such material is optimally fixed initially [39].

The studies presented here demonstrate for the first time immunoglobulin J chain gene expression by semi-quantitative non-isotopic *in situ* hybridization in human duodenal tissue, both in control and patient samples. Cells expressing J chain mRNA were identified throughout the lamina propria in both groups; however, they were distributed maximally as a core of cells within the proximal two-thirds of the villi.

Our combined IF and ISH studies provide new information concerning the J chain expression of duodenal IgA plasma cells in IgAN. In controls the great majority of duodenal IgA cells (median 95%) were J chain mRNA positive. These results compare favorably with previously reported figures for J chain expression in IgA cells of the distal ileum (derived from renal transplant donors) using purely immunological techniques, in which 97.3% of IgA cells were J chain positive [49]. However, J chain mRNA expression was significantly reduced in the IgA plasma cells of the duodenal lamina propria in patients with IgAN (median 54% positive) compared to controls, whereas the expression of kappa light chain mRNA was not, indicating that the observed down-regulation of J chain mRNA expression in duodenal IgA plasma cells in IgAN is specific for immunoglobulin J chain.

The most important marker of high level pIg secretion appears to be the levels of J chain mRNA [32]. Our findings therefore suggest that within the duodenal IgA plasma cells in IgAN there is a lower J chain mRNA copy number, reduced synthesis of J chain and a shift away from pIgA production and secretion. These data highlight a possible defect of IgA secretory immunity in IgAN, since specific mucosal defence is afforded by external transcellular transport of pIgA [52]. They also suggest in contrast to our initial hypothesis, that the lamina propria is unlikely to be the source of excess circulating IgA or deposited glomerular IgA in this condition.

The lamina propria abnormalities we have described could be secondary to the up-regulation of J chain expression and pIgA production at some other site, for example the tonsils or bone marrow. Both these tissues have been shown to contain greater numbers of IgA immunocytes in patients with IgAN compared to controls [21–25].

Alternatively, our findings may point to a fundamental flaw in mucosal immunity in IgAN, giving rise to a primary defect in mucosal antigen handling in this condition. Such a defect would reduce antigen exclusion and IgA-immune complex clearance

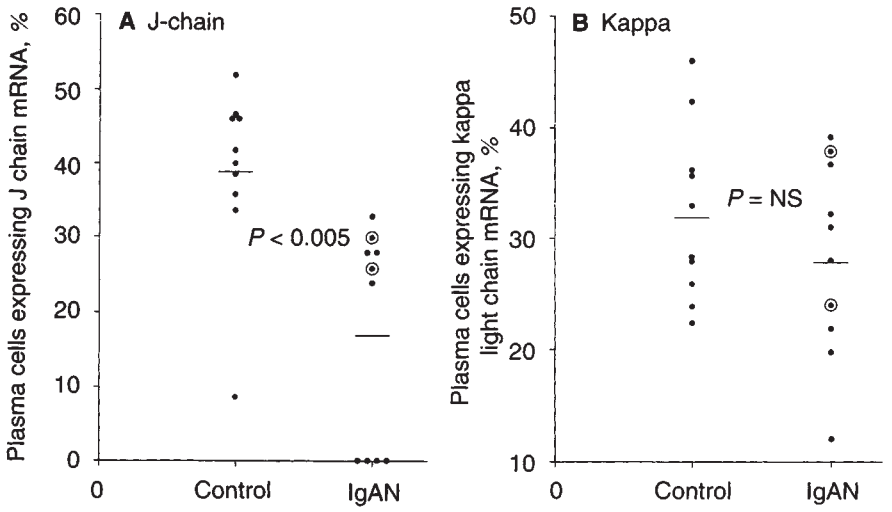

Fig. 5. *Proportion of cells of plasma cell morphology expressing mRNA species by* in situ *hybridization.* (⊙) Individual patients with serum creatinine above the normal range. (**A**) J chain mRNA; (**B**) kappa light chain mRNA.

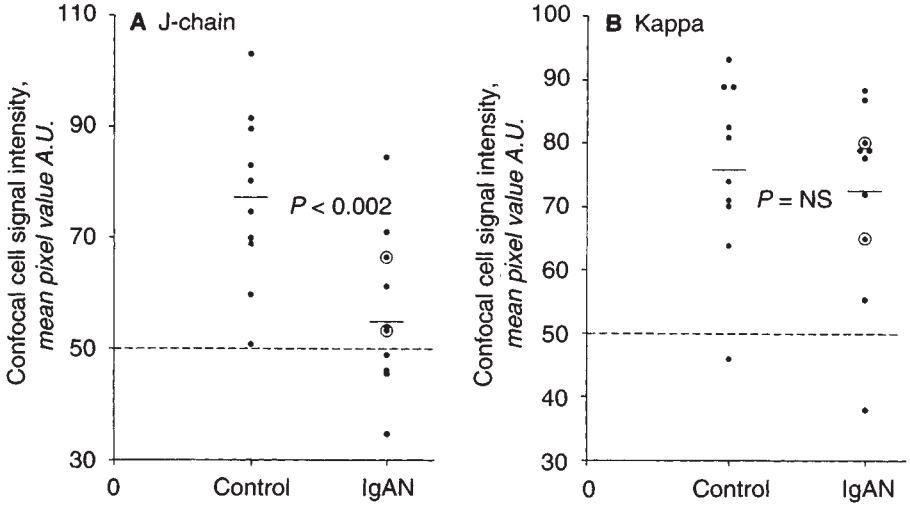

Fig. 6. *Mean positive cell signal intensity as measured by confocal microscopy.* Abbreviation is A.U., arbitrary units. Shaded area (≤50 A.U.) denotes values frequently obtained due to autofluorescence alone. (⊙) Individual patients with serum creatinine above the normal range. (**A**) J chain mRNA; (**B**) kappa light chain mRNA.

[53], allowing undue antigen penetration into lamina propria and ultimately the circulation. The systemic immune system would then be subject to a prolonged and excessive stimulation by antigens which under normal circumstances are excluded. The resulting immunoglobulin or immunoglobulin-antigen complexes would become potential species for glomerular deposition. This proposition is supported by many studies reporting increased circulating immune complexes in IgAN, frequently correlating with disease activity as defined by hematuria [9, 13, 54].

It has been suggested that the pattern of abnormalities we have demonstrated (reduced J chain but increased IgG expression) is associated with a general abrogation of oral tolerance [55]. Oral or mucosal tolerance is the T cell governed process which enables systemic (IgG) tolerance and mucosal (IgA) immunity to a luminal antigen to exist simultaneously [56]. There is experimental evidence from certain murine models of IgAN to support the link between defective IgA mucosal antigen handling, systemic IgG intolerance and glomerular immune deposits [57–59]. Although basic species differences in normal IgA immunology require animal models to be interpreted with caution, the present study provides evidence of a comparable defect in mucosal immunoregulation in IgAN in humans.

An early immunofluorescence study of the duodenal mucosa of only five patients with IgAN failed to demonstrate a significant difference in numbers of IgA, IgG or IgM cells [60]. Our immunofluorescence results confirm data of a later study which demonstrated that within the lamina propria in IgAN, the proportion of IgA cells is reduced and IgG cells increased [20]. Our study revealed a 5% reduction in IgA plasma cells and a near doubling of absolute numbers of IgG cells. This would equate to an increase in IgG cells of from 3 to 6% of total. Although this suggests a small reduction in the number of plasma cells in the lamina propria of patients, this was certainly not evident microscopically in our study or in that of Hene, Schuurman and Kater, in which the reduction of IgA plasma cells was mirrored by an equivalent increase in IgG and IgM cells [20].

Our initial ISH protocol revealed a significant difference between the proportion of plasmacytoid cells that express J chain mRNA in patients and controls. The proportion of J chain

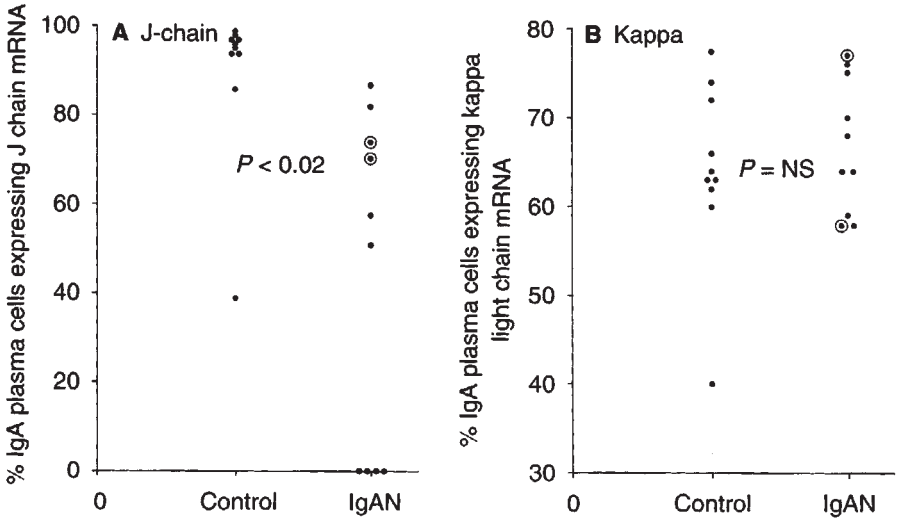

Fig. 7. *Combined* in situ *hybridization for immunoglobulin chain mRNA species within plasma cells expressing cytoplasmic IgA.* (⊙) Individual patients with serum creatinine above the normal range. (**A**) J chain mRNA; (**B**) kappa light chain mRNA.

mRNA positive cells was significantly less (in both controls and patients) than the results given by the simultaneous IF and ISH method which was able to identify functioning plasma cells. This disparity indicates that, at least in our hands, the identification of plasma cells using morphological criteria alone is insufficient, and stresses the importance of the use, in such studies, of a technique that can positively identify cells in which native mRNA is expressed.

The data presented here predict a reduction in sIgA in the jejunal juice; however, no such specimens were available for study. Although abnormalities of salivary sIgA have been reported [61], this appears to be a non-specific abnormality, being seen in a variety of different glomerulonephritides [62, 63]. This aspect of mucosal immunity in IgAN requires further study.

In summary, we have demonstrated a specific down-regulation of J chain mRNA expression in IgA plasma cells in the duodenal lamina propria in IgAN. This suggests that the ability of individuals with IgAN to deal with the usual environmental antigen load may be impaired. Our data suggest that the lamina propria component of the mucosal IgA system is not the source of glomerular pIgA in this condition, and that further investigation of IgA immunocyte J chain mRNA expression in both tonsil and marrow is required.

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