Increased dimeric IgA producing B cells in the bone marrow in IgA nephropathy determined by in situ hybridisation for J chain mRNA

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Abstract

Aim—To investigate the possible role of the systemic IgA immune system in the pathogenesis of IgA nephropathy

Methods—J chain mRNA expression in the IgA cells of the bone marrow was studied. Bone marrow trephine biopsy specimens from seven patients with IgA nephropathy and seven matched controls were examined by (1) non-isotopic in situ hybridisation (ISH) and (2) combined immunofluorescence and non-isotopic ISH to identify the plasma cell type. Serum polymeric IgA was also determined using standard high pressure liquid chromatography and sandwich enzyme linked immunosorbent assay.

Results—Non-isotopic ISH revealed a similar number of J chain mRNA positive cells/unit length in biopsy specimens from patients (16·5 ± 2·7 cells/mm) and controls (17·7 ± 2·4 cells/mm). Combined immunofluorescence and ISH revealed a greater proportion of J chain mRNA positive IgA cells in patients (7·6 ± 1·45%) compared with controls (3 ± 0·8%). Serum polymeric IgA was similar in both patients (91 ± 22 mg/l) and controls (77 ± 24 mg/l).

Conclusion—These data suggest that excess production of dimeric IgA occurs in the bone marrow in IgA nephropathy.

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Keywords: IgA nephropathy, J chain, bone marrow.

IgA nephropathy is a common form of glomerulonephritis characterised by the mesangial deposition of IgA1.1–3 The molecular nature of the deposited IgA1 remains contentious but there is good evidence to support the view that a significant proportion of it is dimeric (J chain positive).4–6 Mesangial deposition of dimeric IgA is thought to occur based on the large body of evidence detailing abnormal polymeric IgA biology in IgA nephropathy.7

IgA is the immunoglobulin of mucosal defence. The elevated concentrations of serum polymeric IgA8 and the clinical association of mucosal infection and macroscopic haematuria in IgA nephropathy led to the view that mesangial IgA originated in the mucosal IgA system, and that the deposited immunoglobulin resulted from an exaggerated IgA response, either innate to the mucosal system or secondary to environmental influences.9–11

Two tissues from the mucosal immune system have been available for study in IgA nephropathy—tonsils and intestinal mucosa. The intestinal mucosa contains a smaller proportion of IgA plasma cells in IgA nephropathy compared with normal mucosa,12,13 and our previous work on duodenal IgA plasma cell J chain mRNA expression has suggested a shift away from dimeric IgA production at this site.13 These data suggest that the site of hyperresponsive IgA biology in IgA nephropathy is unlikely to be the intestinal lamina propria. In contrast, increased numbers of IgA cells have been identified in tonsils14–17 and increased J chain expression has been reported in tonsillar IgA cells.14–16 Although these reports were based on potentially non-specific antibody techniques, we have recently confirmed this finding using in situ hybridisation for J chain mRNA (unpublished data). There is therefore good evidence for a shift towards dimeric IgA production in the tonsils in IgA nephropathy. However, it is difficult to accept that the tonsils are the only site of deranged dimeric IgA function in IgA nephropathy, as although tonsillectomy may reduce the frequency of macroscopic haematuria (and also of mucosal infection), the procedure is not curative.

Furthermore, IgA nephropathy does occur in patients who have had a tonsillectomy as children. An alternative view proposed by van den Wall Bake et al18 is that mesangial IgA derives from the systemic (bone marrow) IgA system. This is supported by elevated IgA plasma cell counts in bone marrow aspirates from patients with IgA nephropathy. We have recently confirmed this finding in bone marrow trephine biopsy specimens from patients and matched controls.19 Although bone marrow IgA cell J chain expression in IgA nephropathy has been studied,20 two colour immunofluorescence techniques were used, using a J chain antibody which others have found to be non-specific, demonstrating a tendency to bind to immunoglobulin light chains (Dr M Kerr, University of Dundee, Scotland, unpublished observations).

Here, we describe an in situ hybridisation study of J chain mRNA expression within the bone marrow IgA plasma cells of patients with IgA nephropathy to address whether the bone marrow is an additional source of excess dimeric IgA production in IgA nephropathy. The concentrations of polymeric IgA within the serum of patients and controls were also determined to assess any potential correlation between serum concentrations and marrow cell profile.
Methods

Bone marrow trephine biopsy specimens were obtained from seven patients with confirmed IgA nephropathy (six men and one woman, mean age 45 years, range 34–59 years). None of the patients had macroscopic haematuria at the time of biopsy, six had microscopic haematuria and two had proteinuria. All patients had normal serum creatinine concentrations.

Control samples for bone marrow studies were derived from age and sex matched volunteers (mean age 45-9 years, range 34–59 years), undergoing minor surgery under general anaesthetic.

Collection of bone marrow biopsy specimens from a small number of controls and patients was approved by the Leicester Health Authority Ethical Committee. Sample collection was from willing volunteers who gave informed consent. These samples were identical with those studied previously in that biopsy specimens from the patients had increased numbers of IgA and IgA1 cells.19 Biopsy was performed under local anaesthetic (2% lignocaine to skin and periosteum). Bone marrow samples were taken from the posterior superior iliac spine. After collection, the trephine specimens were immersed in 10% formal saline for 48 hours. Following formal saline fixation, all specimens were decalcified (EDTA, pH 7.0-0.48 hours), processed and embedded in paraffin wax as for normal histology. Processed samples were then coded. A blood sample was taken at the time of biopsy and the serum stored.

PROBE AND PROBE LABELLING

Deoxylignucleotides (unlabelled sequences were kindly donated by Pathway Services Ltd, Leicester, UK) complementary to J chain mRNA were 3’ end labelled with the nucleotide analogue digoxigenin-11-dUTP using a labelling kit (Boehringer Mannheim, Mannheim, Germany). The labelling reaction was performed at 37°C for two hours in 100 μl volumes. The labelling reaction mixture was as follows: 200 ng probe cocktail; 10 μl 10 mM MnCl2; 2-3 μl 1 mM digoxigenin-11-dUTP; 20 μl 5 x buffer; and 1 μl terminal deoxynucleotidyl transferase. Labelled probe was then purified through Sephadex G50 spin columns and labelling confirmed by test filters. All deoxylignucleotides were 30 bases long; this length has been established as a practical compromise among hybrid stability, hybrid specificity, cost of synthesis and purification, and tissue penetration efficiency.22

NON-ISOTOPTIC ISH

The protocol for non-isotopic ISH on decalcified tissue was adapted from techniques we have previously reported for use with paraffin wax embedded, non-decalcified specimens.23-25 RNase free reagents and glassware (diethylpyrocarbonate (DEPC) treated; Sigma, Poole, Dorset, UK) were used throughout. Slides were dewaxed with fresh xylene (2 x 3 minutes) and rehydrated through industrial methylated spirit (IMS) 99% (2 x 3 minutes). Pretreatments included protein digestion with proteinase K (2–10 μg/ml). Samples were post-fixed with 0.4% paraformaldehyde in 1 x phosphate buffered saline (PBS)/DEPC for 10 minutes, and then acetylated with 0.25% acetic anhydride. Slides were then washed in DEPC water and returned to 1 x PBS/DEPC. Slides were hybridised overnight at 37°C after a 15 minute “hot-start” at 90°C (to destroy endogenous alkaline phosphatase). Hybridisation solution contained 20 ng/ml probe, 600 mM NaCl, 50 mM Tris (pH 7.5), 0.2% bovine serum albumin, 1% sodium dodecyl sulphate (SDS), 1% polyvinylpyrrolidone (40 kDa), 1% Ficoll (400 kDa), 0.1% sodium pyrophosphate, 5 mM EDTA, 10% dextran sulphate, and 30% formamide.

Posthybridisation washes were as follows: 2 x standard saline citrate solution (SSC; 150 mM NaCl, 15 mM trisodium citrate, pH 7.0-0.30% formamide (2 x 10 minutes); 2 x SSC at room temperature (2 x 10 minutes); and 15 minutes in neat sheep serum. Slides were incubated with alkaline phosphatase labelled sheep polyclonal digoxigenin antibody (Boehringer Mannheim) diluted 1 in 600 in sheep serum for 30 minutes. Specimens were then washed in blocking solution, propidial alcohol buffered saline, 0.1% Triton-X-100, 3% bovine serum albumin (2 x 5 minutes), immersed in buffer 3 (0.1 M Tris/HCl (pH 9-5), 0.1 M NaCl, 0.05 M MgCl2) (1 x 10 minutes), and in substrate solution overnight (44 μl nitroblue tetrazolium (NBT) (Sigma, 75 mg/ml in 70% dimethylformamide), 33 μl 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma, 50 mg/ml in dimethylformamide) in 10 ml buffer 3). Finally, sections were washed in running tap water for 10 minutes, counterstained with Mayer’s haematoxylin and mounted in aqueous mounting medium.

COMBINED IMMUNOFLUORESCENCE AND NON-ISOTOPIC ISH

The protocol for combined immunofluorescence and non-isotopic ISH was adapted from that reported previously for use on non-decalcified paraffin wax embedded tissue23 and was identical with the non-isotopic technique described above up to the detection step. A fluorescein isothiocyanate (FITC) labelled polyclonal rabbit antihuman IgA antibody (Dako, High Wycombe, UK) was used (diluted 1 in 20 in 10 mM Tris/HCl (pH 7.5)) in conjunction with the antidigoxigenin antibody. Specimens were then incubated with a secondary antirabbit polyclonal anti-FITC (Dako) (diluted 1 in 50 in blocking solution) for 30 minutes. Alkaline phosphatase development was then performed. Slides were finally incubated with an FITC labelled polyclonal swine antirabbit antibody (Dako) diluted 1 in 25 with blocking solution. After final washing, uncounterstained slides were mounted in aqueous mounting medium.

IgA plasma cells were then visualised under ultraviolet light (490 nm, green). Coexpression of J chain mRNA could then be determined by alteration to 1% field illumination, under which J chain mRNA positive cells could be observed (blue/black).
CONTROLS AND EXPERIMENTAL DESIGN
Specificity of the J chain mRNA signal was confirmed by the use of appropriate negative controls on each section as previously listed. These were omission of probe, omission of digoxigenin antibody, RNase Al pretreatment controls, random oligonucleotide cocktail similarly labelled (unlabelled sequences kindly donated by Pathway Services Ltd), in addition to a non-homologous oligonucleotide cocktail with the same G–C content as the J chain probe.22–25

SERUM POLYMERIC IgA
Serum IgA was measured by radial immunodiffusion as previously reported.19 Percentage monomeric and polymeric IgA concentrations in serum samples were estimated. Serum samples were passed through a Superose 6 gel filtration column on a high pressure liquid chromatography system using phosphate buffer, and fractions containing polymeric and monomeric IgA were collected. This procedure was calibrated using commercially purified IgA myeloma proteins (The Binding Site, Birmingham, UK). IgA concentrations in the fractions were measured by sandwich enzyme linked immunosorbent assay as described previously.26

MICROSCOPY
Ultraviolet microscopy was performed using a Nikon Optiphot microscope. After initial study with non-isotopic ISH alone all J chain mRNA positive cells within each biopsy specimen were counted. The length of each biopsy specimen was then measured directly using an eyepiece graticule and low magnification. After simultaneous non-isotopic ISH and immunofluorescence each IgA cell within the specimen was examined in turn in a systematic manner under ultraviolet light (490 nm) with the aid of a grid-graticule. Expression of J chain mRNA was then determined by viewing the cell under bright field illumination. Cells were counted by a single observer on coded specimens, thus blinding the observer to the source of each specimen.

STATISTICS
Data were analysed using the unpaired t test and Wilcoxon/Mann-Whitney U analysis. Unless stated, figures and results are expressed as mean ± standard error of mean. Clearly, it should be remembered that the small sample size means that there is a low statistical power to detect a difference between the groups.

Results
NON-ISOTOPIC ISH
Well defined NBT/BCIP sites of hybridisation were seen in cells in all control and patient bone marrow trephine specimens. Background staining was negligible and morphology was well preserved on all occasions. Negative controls did not yield a signal on any occasion. In contrast to both duodenal mucosa13 and tonsil (manuscript submitted), in which the distribution of J chain mRNA expressing cells demonstrated a predictable distribution, marrow J chain mRNA positive cells have an apparently random distribution (fig 1).

Enumeration of the absolute numbers of J chain mRNA positive cells within each biopsy specimen revealed a similar number of cells per unit length in both control (17·7 ± 2·4 cells/mm) and patient (16·5 ± 2·7 cells/mm) samples (p = NS) (fig 2).

COMBINED NON-ISOTOPIC ISH AND IMMUNOFLOUORESCENCE
Monomeric (cytoplasmic IgA positive, J chain mRNA negative) and dimeric (cytoplasmic IgA positive, J chain mRNA positive) IgA cells were readily distinguished using this technique (fig 3). A greater proportion of IgA plasma cells were J chain mRNA positive in patient (7·1 ± 1·45%) compared with control (3 ± 0·8%) (fig 4).
Bone marrow J chain expression in IgA nephropathy

SERUM POLYMERIC IgA

Serum IgA concentrations from the controls and patients studied here have been reported previously. In the samples from these 14 subjects polymeric IgA represented between 1 and 6% of serum IgA concentrations. Although the patients generally had higher values of polymeric IgA (91 ± 22 mg/l) compared with controls (77 ± 24 mg/l), this was not statistically significant.

Furthermore, we could not demonstrate a correlation between serum polymeric IgA and the percentage (or absolute numbers) of J chain mRNA positive IgA plasma cells in bone marrow trephine biopsy specimens. There was no statistical correlation either individually or when the groups were combined.

Discussion

We have previously described a technique for the simultaneous identification of mRNA by non-isotopic ISH and cytoplasmic protein by immunofluorescence in paraffin wax embedded and archival tissue. We have now extended this technique for use on frozen tissue (manuscript submitted) and on decalcified specimens. ISH on decalcified tissue is hampered by the fact that mRNA tends to degrade during the decalcification process. Our preliminary studies suggested that decalcification with EDTA at neutral pH gave much better results than decalcification with acid, confirming the previous findings of other authors. We also found that non-isotopic ISH on bone marrow trephine biopsy specimens produced a significant amount of background staining, which was only eliminated after the addition of an acetylation step to the specimen pretreatment protocol and incubation of the antidigoxigenin antibody with sheep serum during the detection step. We have found the technique robust and reliable on tissue up to four years old.

This is the first report of the demonstration of J chain mRNA in bone marrow by ISH. We have shown that although absolute numbers of J chain mRNA positive cells are similar in patients and controls, there is an increase in the proportion of IgA cells which express J chain mRNA in patients. We have recently reported an excess number of IgA and IgA1 cells in the bone marrow biopsy specimens studied here.

Although the expression of J chain mRNA is not exclusively associated with the production of polymeric IgA in other immunocytes, experimental studies in a Chinese hamster ovary cell expression system (JD Atkin et al, manuscript submitted) suggest that J chain mRNA is essential for the production of dimeric IgA, in contrast to multimeric (hexamer) IgM. Transfection experiments in a mouse myeloma cell line, J558L with an α heavy chain, both with and without the J chain binding terminal cysteine residues support this proposal (JD Atkin et al, manuscript submitted). Our results therefore suggest a shift towards dimeric IgA1 production in the marrow in IgA nephropathy.

Figure 4. Scatter plot of the percentage of J chain mRNA positive IgA B cells in bone marrow trephine biopsy specimens from seven patients with confirmed IgA nephropathy and seven matched controls. — mean.
We found no correlation between the proportion of dimeric IgA marrow cells and serum polymeric IgA. The reason for this is unclear but may relate to the fact that some serum polymeric IgA exists as aggregated (J chain free) monomers. Any association between dimer producing IgA cells and serum dimeric IgA may therefore be masked by the presence of J chain free IgA macromolecules. Alternatively, the bone-marrow-concentrator in polymeric IgA in IgA nephropathy may derive from another site—for example, tonsils or spleen, or the lack of any association may simply reflect the small numbers of patients studied. Although it would have been preferable to study a larger population, bone marrow biopsy specimens can only be taken from willing volunteers with informed consent. Not unexpectedly, the number of volunteers was small.

Nevertheless, this study provides new information about abnormal dimeric IgA biology in IgA nephropathy. The findings mirror those in the tonsil (manuscript submitted) and are therefore opposite those in the mucosal lamina propria. IgA is a very T cell dependent isotype. J chain mRNA expression is orchestrated by T cell products. The abnormal patterns of IgA and J chain mRNA expression in these three tissues may reflect an abnormality of T cell control, perhaps as a result of defective T cell trafficking or innate abnormalities of a T cell population. We recently reported an abnormality of IgA affinity maturation in IgA nephropathy, another T cell dependent function. 6

Under normal circumstances the mucosal and systemic IgA systems are believed to be related but distinct. 7 We have now described abnormalities in J chain mRNA expression in both systems. These findings add to the number of abnormalities of both systems described by others. 8-10 Collectively, these findings raise the possibility that in IgA nephropathy there is an imbalance in the putative mucosa–marrow immune axis, well recognised in mice 11 but yet to be characterised in man, resulting in an exaggerated overlap between the two systems.

In conclusion, combined non-isotopic ISH and immunofluorescence can be used to detect simultaneously mRNA and cytoplasmic protein, respectively, in decalcified specimens. We have used this method to demonstrate an abnormal shift towards dimeric IgA production within the systemic IgA immune system. This underlines immune abnormalities within the bone marrow in IgA nephropathy and highlights the bone marrow as a possible source of the raised serum concentrations of polymeric IgA and deposited dimeric IgA, which is characteristic of this form of glomerular disease.

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20 van der Wall Bake AWL, Daha MR, Evers-Schouten J, van ES LA. Serum IgA and the production of IgA by peripheral blood and bone marrow lymphocytes in patients with primary IgA nephropathy: evidence for the bone marrow as the source of mesangial IgA. Am J Kid Dis 1988;12:410–14.
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