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The bone marrow as production site of the IgA deposited in the kidneys of patients with IgA nephropathy

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SUMMARY

Patients with primary IgA nephropathy have increased plasma levels of polymeric IgA1 and deposits of IgA1 in their kidneys. The origin of this material is unknown. The production of IgA and its subclasses was investigated in the bone marrow of 14 patients and 19 controls using two colour immunofluorescence and tissue culture. Patients had an increase in the percentage of plasma cells containing IgA (45·8 ± 7·2 mean ± s.d.) compared to controls (40·1 ± 10·5) ($P = 0·08$). IgA plasma cells containing subclass IgA1 were significantly ($P < 0·01$) increased in patients (89·9 ± 2·7%) compared to controls (84·1 ± 6·7%). Correspondingly IgA plasma cells containing subclass IgA2 were significantly decreased ($P < 0·01$) in patients (7·4 ± 3·0%) compared to controls (13·5 ± 5·9%). Production of IgA in bone marrow culture in patients was increased (1,684 ± 1,151 ng/culture) compared to controls (1,087 ± 937), but this difference was not significant ($P = 0·2$). However, in patients the IgA1 subclass contributed significantly ($P < 0·01$) more to the IgA synthesis in culture (ratio of IgA1 over IgA: 0·96 ± 0·02) than in controls (ratio 0·90 ± 0·06). These findings suggest that the bone marrow may well be the site of long-term overproduction of the IgA1 found in the circulation and mesangial deposits in IgA nephropathy.

Keywords Immunoglobulin A nephropathy Immunoglobulin A subclasses Bone marrow *in vitro* Ig synthesis immunoglobulin production

INTRODUCTION

Primary IgA nephropathy is characterized by the deposition of IgA in the glomerular mesangium (Berger *et al.*, 1967). Most investigators now agree that the IgA deposited in the glomeruli is of the subclass IgA1 (Conley, Cooper & Michael, 1980; Valentijn *et al.*, 1984). At least part of the deposited IgA is in a J-chain containing polymeric form, capable of binding to secretory component (SC) *in vitro* (Béné, Faure & Duheille, 1982; Tomino *et al.*, 1982; Valentijn *et al.*, 1984). The recurrence of the disease in renal allografts suggests that the glomerular IgA is derived from the circulation (Berger *et al.*, 1975). The finding of increased serum levels of polymeric IgA restricted to the subclass IgA1 in patients with IgA nephropathy supports this concept (Lopez Trascasa *et al.*, 1980; Valentijn *et al.*, 1983; Valentijn *et al.*, 1984). It is still not clear whether the polymeric IgA1 circulates as part of antigen–antibody complexes. Recent evidence suggests that circulating immune complex-like material in these patients may not only consist of polymeric IgA1, but also monomeric IgA1 complexed with IgG and C3 (Czerkinsky *et al.*, 1986).

Abnormalities of immune regulation have been found in patients with IgA nephropathy by several groups of investigators. Peripheral blood mononuclear cells produce more IgA than cells of healthy controls (Bannister *et al.*, 1983; Egido *et al.*, 1983; Feehally *et al.*, 1986; Waldo, Beischel & West, 1986). The IgA produced contains a larger fraction of polymers (Egido *et al.*, 1982).

The origin of the abnormal polymeric IgA1 in the circulation of patients with IgA nephropathy remains unknown. The mucosa-associated or secretory immune system in these patients has been the subject of several studies. The isotype distribution of immunoglobulin-producing cells in the intestinal mucosa of patients did not differ in comparison with that of controls (Westberg *et al.*, 1983). On the other hand tonsils of patients contain more cells producing polymeric IgA than in controls (Béné *et al.*, 1983; Egido *et al.*, 1984). Subclass distribution was not studied in any of these investigations.

The bone marrow is the most important source of plasma IgA in healthy human beings (Hijmans, Schuit & Hulsing-

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Hesselink, 1971; Radl *et al.*, 1974; Turesson, 1976; Kutteh, Prince & Mestecky, 1982; Alley, Nash & McDermott, 1982). The bone marrow produces predominantly monomeric IgA1 (Kutteh *et al.*, 1982; Crago *et al.*, 1984). In view of the importance of the bone marrow as a production site of plasma IgA we conducted a study comparing the bone marrow in patients with IgA nephropathy with healthy controls. Our hypothesis is that a disturbance in immune regulation in patients with IgA nephropathy results in an increased production of IgA1 in the bone marrow, leading to abnormally elevated levels in the circulation and secondary deposition in the kidney.

MATERIALS AND METHODS

Patients

The study protocol was approved by the ethical committee of Leiden University Hospital, and all individuals gave informed consent. Fourteen patients with biopsy proven IgA nephropathy were studied, 11 males and 3 females with a mean age of 32·4 years (range: 18–44). Patients with Henoch Schönlein purpura, systemic lupus erythematosus or liver disease were excluded. Kidney function was normal in 12 patients. One patient had mild renal failure (creatinine clearance 48 ml/min), and another was treated by haemodialysis. As controls we used 18 otherwise healthy individuals undergoing lumbar spine neurosurgical procedures ($n=16$), or serving as donors for allogeneic bone marrow transplantation ($n=2$), and one patient with intracapillary proliferative glomerulonephritis without deposition of mesangial IgA. The mean age of the controls, 12 males and 7 females, was 41·4 years (range: 16–59).

The bone marrow samples were obtained by gentle aspiration of 1–2 ml in syringes pre-filled with 2 ml Hanks' Balanced Salt Solution (HBSS), to which 1% of preservative free heparin had been added. Samples were taken from the posterior iliac crest under local anaesthesia in the patients, or general anaesthesia in the controls. Neither patients nor controls had experienced mucosal infections in the 2 weeks preceding the study.

Preparation of bone marrow cell suspensions

The bone marrow samples were diluted with 10 ml HBSS with heparin and 5% heated fetal calf serum (FCS) (Gibco Ltd, Paisley, Scotland). The supernatant layer with the fat containing bone marrow particles was harvested after centrifugation at 2,500 *g* for 5 min. This procedure was carried out to avoid as far as possible the admixture of peripheral blood cells. The bone marrow cells were freed from the particles by repeated vortexing, and washed twice in HBSS plus FCS. The cell suspension was adjusted to 4×10^6 nucleated cells per ml, for the preparation of cytospin slides. Before culture cells were washed once more in complete culture medium: RPMI 1640 (Flow Laboratories, Zwanenburg, The Netherlands) supplemented with 10% heated FCS, 2 mM L-glutamine, 12 mM $NaHCO_3$, 20 mM HEPES buffer (Sigma Chemical Company, St Louis, MO), penicillin (100 U/l), and streptomycin (0·1 mg/ml) (Sigma). The cell suspension was filtered over cotton wool to remove aggregates before culture.

Immunofluorescence reagents

Goat anti-human total Ig conjugated to tetramethylrhodamine isothiocyanate (TRITC) (lot 485) was obtained from Nordic Laboratories (Tilburg, The Netherlands). Mouse monoclonal antibodies to human IgA (clone 69-6.3.2) coupled with FITC, and to IgA1 (clone 69-11.4) or IgA2 (clone H5-512) coupled with TRITC were prepared and tested for specificity as previously described (Haaijman *et al.*, 1984; Valentijn *et al.*, 1984).

Immunohistochemistry

Cytospin preparations were made with 50 μl of the bone marrow cell suspension. After air drying the slides were fixed in 95% ethanol plus 5% glacial acetic acid at −20°C for 20 min. They were washed three times in phosphate buffered saline (PBS) pH 7·6 for 10 min before incubation with an appropriate dilution of the fluorescent antibodies in PBS at room temperature for 30 min. After the slides had been rinsed in PBS twice for 15 min, a drop of glycerol plus 10% (v/v) PBS, containing 22·5 mg/ml of the anti-fading compound 1,4-diazabicyclo (2,2,2) octane (Sigma Chemical Company), was applied.

Fluorescence microscopy and cell counting

Fluorescent cells were counted in a Leitz Orthoplan microscope equipped with filters for selective observation of green or red fluorescence. Cell enumerations were performed by two independent observers throughout the study. All cytospin slides were number coded, and neither of the observers was aware of the code representing patients or controls. For the determination of the percentage of IgA containing immunocytes, at least 300 cells containing diffuse red cytoplasmic fluorescence for total Ig were examined for concomitant green fluorescence representing IgA. For the determination of the IgA subclass distribution at least 200 cells with green (IgA) staining were examined for red (subclass) fluorescence. The final percentages were calculated as the mean of the results of both independent observers.

Tissue culture

Bone marrow nucleated cells were cultured in duplicate for 7 days at 1×10^6 cells/ml in complete culture medium in round botton 12 × 75 mm polystyrene test tubes (Falcon 2058, Becton Dickinson & Co, Cockeysville, MD) containing 1 ml of the cell suspension in a 5% CO_2 in moist air atmosphere at 37°C. At the end of the incubation period the cell-free supernatant was stored at −20°C until the ELISA determination of immunoglobulin content. Bone marrow cultures were not performed in two of the control subjects.

ELISA

Flat-bottomed polystyrene microtest plates (Titertek, Flow Laboratories, Zwanenburg, The Netherlands) were coated with an appropriate dilution of a capturing antibody in carbonate buffer pH 9·6 for 2 h at 37°C. For the determination of IgM and IgA isotype specific rabbit antibodies were employed (Dakopatts, Copenhagen, Denmark). For the IgG assay the capturing antibody was rabbit anti-human F(ab), prepared in our own laboratory. In the IgA subclass ELISA the primary antibody was a subclass specific mouse monoclonal antibody (clone 69-11.4, described above, for IgA1; for IgA2 we utilized a new clone 184-3.1, produced and characterized along the same lines as the above-mentioned clone H5-512). After three washes with PBS containing 0·05% Tween 20 (PBS-Tween), culture supernatants diluted 1:2 to 1:64 in PBS-Tween containing 1% heated

newborn calf serum (PBS-Tween-NBCS) were applied to the wells, and incubated at 37°C for 1 h. The wells were washed with PBS-Tween three times and a second antibody coupled to biotin diluted appropriately in PBS-Tween-NBCS was applied for 1 h at 37°C for the detection of bound human immunoglobulin. IgM and IgG were detected with mouse monoclonal antibodies (clones HB 57 and HB 43, American Type Culture Collection, Rockville, MD) and IgA with the above-mentioned clone 69-6.3.2. Both IgA subclasses were detected with isotype specific rabbit anti-human IgA, prepared in our own laboratory. After three washes with PBS-Tween, streptavidine conjugated to horseradish peroxidase (Zymed, Sanbio BV, Uden, The Netherlands) in PBS-Tween-NBCS was applied to the wells for 1 h at 37°C. The wells were washed three times with PBS-Tween, and substrate (*o*-phenylenediamine, Sigma) conversion was read at 492 nm according to conventional methods. Concentrations in test samples were calculated from standard curves obtained in each assay with a dilution series of human plasma containing known concentrations of IgG, IgA, IgM and IgA subclasses. Calculations were made at sample dilutions yielding an OD in the steep linear part of the calibration curve. All determinations were performed in duplicate for each supernatant, and the final concentration was expressed as the mean for duplicate cultures. Total contents of IgA1 and IgA2 were calculated by multiplying the measured ratio of the subclass over the sum of both subclasses with the measured total IgA content.

Statistical analysis

Differences in the mean percentages of cells positive for a given isotype were assessed with Student's *t*-test. Differences in the amounts of immunoglobulin produced in culture, and in their ratios were assessed with a Mann–Whitney *U*-test.

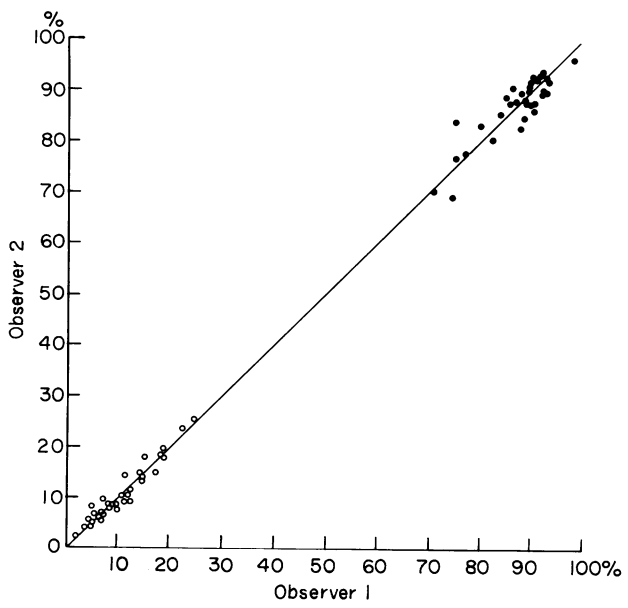

Fig. 1. Interobserver correlation of immunofluorescence of the percentages of IgA subclass positive plasma cells. The drawn line represents the ideal of identical results in the two observers. (●) IgA1/IgA; (○) IgA2/IgA.

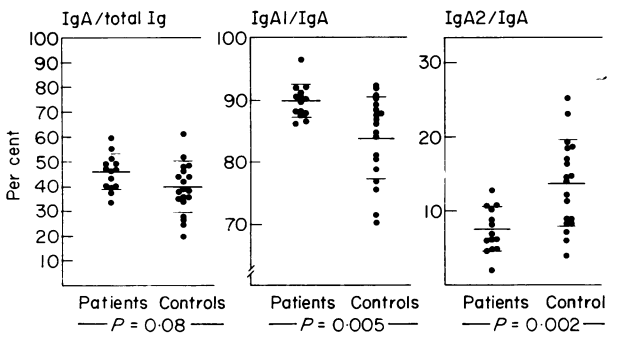

Fig. 2. Percentages of isotype positive bone marrow plasma cells in patients *vs* controls (horizontal bars denote mean ± 1 s.d.).

RESULTS

Percentage of IgA and subclass positive cells

The percentages of Ig containing cells as determined by two independent observers correlated well for IgA over total Ig ($r = 0{\cdot}96$), IgA1 over IgA ($r = 0{\cdot}89$) and IgA2 over IgA ($r = 0{\cdot}96$). The interobserver correlation for both IgA subclasses is shown in Fig. 1. The sum of the IgA1 and IgA2 percentages was close to 100% in all of the individuals studied. For the entire group of patients and controls the sum averaged 97·4 ± 2·7% (range: 92·1–103·6).

The individual percentages for patients and controls are graphically represented in Fig. 2. The mean percentage of IgA positive cells was higher in patients (45·8 ± 7·2) versus controls (40·1 ± 10·5), although this difference did not reach statistical significance. Patients had a significantly ($P = 0{\cdot}005$) higher fraction of IgA1 containing cells (89·9 ± 2·7 *vs* 84·1 ± 6·7%), and a significantly ($P = 0{\cdot}002$) lower fraction of IgA2 containing cells (7·4 ± 3·0 *vs* 13·5 ± 5·9%).

Production of Ig in vitro

Only the results of unstimulated cultures are given, as PWM stimulation resulted in a decrease of immunoglobulin production in most individuals, as has been described previously (Alley, Nash & McDermott, 1982). There were no significant differences between patients and controls in the amounts of IgM, IgG or total IgA produced. IgA1 production was increased, and IgA2 production decreased in patients, but these differences failed to reach statistical significance (Table 1). The ratio of IgA1 to total IgA production was significantly ($P = 0{\cdot}002$) higher in patients than controls (Table 2). The *in vitro* production of IgA1 expressed as a percentage of IgA production correlated significantly ($r = 0{\cdot}70$, $P = 0{\cdot}0001$) with the percentage of IgA1 containing plasma cells as determined by double

Table 1. Production of immunoglobulin in culture

	IgM	IgG	IgA	IgA1	IgA2*
Patients ($n = 14$)	362 (355)	1,718 (1,310)	1,684 (1,151)	1,626 (1,125)	58 (44)
Controls ($n = 17$)	360 (514) ($P = 0{\cdot}32$)	1,395 (962) ($P = 0{\cdot}56$)	1,087 (937) ($P = 0{\cdot}20$)	1,011 (903) ($P = 0{\cdot}17$)	76 (47) ($P = 0{\cdot}26$)

* Results expressed as mean ng produced per culture (s.d.).

Table 2. Ratio of isotype production in culture

	IgA:IgG	IgA:IgA+IgM+IgG	IgA1:IgA*
Patients ($n=14$)	1·32 (1·35)	0·44 (0·17)	0·96 (0·02)
Controls ($n=17$)	0·77 (0·38)	0·36 (0·12)	0·90 (0·06)
	($P=0·14$)	($P=0·15$)	($P=0·002$)

* Results expressed as mean ratio of ng produced (s.d.)

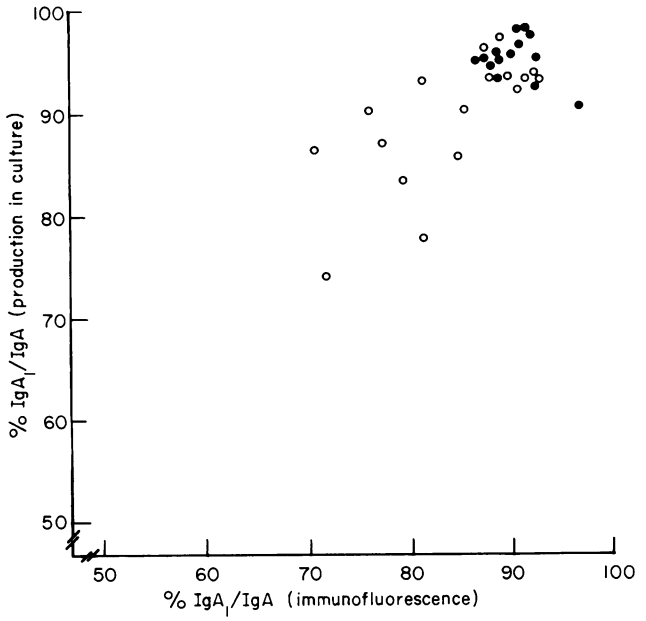

Fig. 3. Correlation of immunofluorescence method and bone marrow culture in determining the fraction of IgA1 in total IgA. (●) Patient; (○) control. $r=0·70$; $P=0·0001$.

immunofluorescence in the same individual (Fig. 3). In contrast to controls, all patients with IgA nephropathy had high levels of IgA1 production expressed as a percentage of IgA production, correlating with the higher percentages of IgA1 containing plasma cells determined by immunofluorescence.

DISCUSSION

Employing two different techniques, we demonstrate a significant shift in the IgA subclass distribution towards IgA1 in the bone marrow in patients with IgA nephropathy. Although the percentage of total IgA plasma cells is increased in patients, this difference is not statistically significant, possibly due to the relatively small size of our patient group. Considering that the bone marrow is the prime source of plasma immunoglobulin (Hijmans *et al.*, 1971; Radl *et al.*, 1974; Turesson, 1976; Alley *et al.*, 1982; Kutteh *et al.*, 1982), the subclass shift found in this study suggests that the IgA1 in the circulation and mesangial deposits in patients may well be largely derived from the bone marrow. The difference in IgA subclass distribution found in this study could theoretically be the result of the slight differences in age and sex distribution in our study populations. However, no correlations between IgA subclass plasma cells or production and age or sex could be found in our control group, nor in a recent study of 30 normal bone marrow trephines (Lenormand & Crocker, 1987).

The frequent occurrence of exacerbations in IgA nephropathy manifested by gross haematuria following mucosal infections has led to the hypothesis that the IgA found in the mesangial deposits is derived from the circulation and is produced locally in the mucosa associated lymphoid tissues. Studies on the intestinal mucosa, however, failed to find any significant difference in patients compared to healthy controls (Westberg *et al.*, 1983). Moreover the considerable percentages of IgA2 plasma cells found in human mucosal tissues (Kett *et al.*, 1986) argue against this theory of local mucosal production, as IgA2 is conspicuously absent in the mesangial deposits. Two groups of investigators found an increase in the content of IgA plasma cells in the tonsils of patients with IgA nephropathy (Béné *et al.*, 1983; Egido *et al.*, 1984). IgA subclass distribution was not determined in these studies. In our opinion it is unlikely that the usually not enlarged tonsils in these patients are the sole or most important source of the abnormal circulating and mesangial IgA1. A second argument against a pivotal role for the tonsils in primary IgA nephropathy is the observation that the disease may well occur in patients who previously underwent tonsillectomy.

Theoretically two explanations are possible for the selective deposition of IgA1 in the glomerular mesangium. The first possibility is that patients with primary IgA nephropathy have an exaggerated immune response encompassing both IgA subclasses, but that for reasons unknown only IgA1 is trapped in the mesangium. The finding in the serum of a selective increase of IgA1 polymers (Valentijn *et al.*, 1984) and immune complexes containing predominantly IgA1 (Czerkinsky *et al.*, 1986) argues against this explanation. The second possibility is that a lymphoid organ containing predominantly the subclass IgA1 is involved in the exaggerated immune response in patients with IgA nephropathy. Apart from the bone marrow with its high content of IgA1 (Crago *et al.*, 1984) evaluated in this study, the respiratory tract associated lymphoid tissues remain a possible origin of the elevated levels of IgA1 in the circulation, and the IgA deposited in the glomerular mesangium. The nasal mucosa, tonsils and bronchi contain 75–95% IgA1 subclass plasma cells (Kett *et al.*, 1986; Burnett, Crocker & Stockley, 1987). Due to the invasive procedures required, this organ system is difficult to study in humans. Moreover the mucosal immune system and bronchus associated lymphoid tissues in particular are an unlikely source since evidence is lacking that they contribute in any significant amount to the circulating pool of IgA (Brown *et al.*, 1982).

Integrating the findings in the tonsils of patients (Béné *et al.*, 1983; Egido *et al.*, 1984) with the findings of the present study, we propose that patients with IgA nephropathy have an abnormality of the mucosa-bone marrow axis. Evidence for the existence of such an axis, linking mucosa-associated and systemic lymphoid tissues through the exchange of lymphoid cells, has been found in experimental animals (Van Snick, 1981; Elson & Ealding, 1984; Alley, Kiyono & McGhee, 1986). Considering the differences found between the murine and the human IgA system, one must however be careful in extrapolating these findings to man. Repeated antigenic stimulation at a mucosal surface, most frequently via upper respiratory tract infections, would lead to local activation of B-lymphocytes that

will then recirculate and localize in mucosal tissues and the bone marrow to differentiate into IgA1 producing plasma cells. The bone marrow localization of activated B-lymphocytes becomes more significant after repeated immunizations and in the later phases of the immune response (Benner, Hijmans & Haaijman, 1981; Alley *et al.*, 1986). It is our view that the accumulation of IgA1 producing plasma cells in the bone marrow of patients with IgA nephropathy is responsible for the chronic overproduction of IgA1 antibodies that are involved in the formation of IgA deposits in the mesangium.

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