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Increased rates of polymeric IgA synthesis by circulating lymphoid cells in IgA mesangial glomerulonephritis

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SUMMARY

Recently we have described the existence of high levels of polymeric IgA, partially as immune complexes, in the serum and kidney from patients with IgA mesangial glomerulonephritis. As these patients often have macroscopic haematuria, following upper respiratory tract infections, our working hypothesis in this paper was that circulating lymphocytes from secretory tissues after viral stimulus could produce in these patients a large amount of polymeric IgA. To test it, peripheral blood lymphocytes (PBL) from patients and controls were cultured for seven days in the presence or absence of pokeweed mitogen (PWM). In cell culture supernatants immunoglobulin synthesis was measured by RIA and the proportion of polymeric and monomeric IgA was determined on Ultrogel Ac A22 column. There was no difference in spontaneous production of immunoglobulins between patients and controls. On the contrary, the IgA synthetized by PWM-stimulated PBL was significantly higher in patients than in controls. The percentages of IgA with molecular weight between 600,000 and 250,000 after supernate fractionation were significantly higher in patients than in controls. The true nature of polymeric IgA was confirmed by their ability to bind secretory component, the existence of covalent structures, and the decrease of the larger forms of IgA after reduction and alkylation. The percentage of IgA producing cells binding secretory component was significantly higher in patients than in controls (69 ± 21 versus 44 ± 27) after seven days of culture. IgM and IgG produced in patient culture were similar to controls. These results show that mitogen stimulated PBL from patients with Berger's disease synthetized a large amount of true polymeric IgA. It is suggested that a similar situation could occur *in vivo* after viral of other stimuli.

INTRODUCTION

IgA mesangial glomerulonephritis (Berger's disease) is a widely recognized entity, characterized by the presence in the glomerular mesangium of IgA, and frequently C3 in a granular pattern (Berger, 1969). Although the pathogenesis of this disorder is not clear, the high frequency of elevated serum IgA levels (Droz, 1976); and the recurrence of this nephropathy in transplanted kidneys (Berger *et al.*, 1975) has focused attention on serum IgA. In this regard we have recently demonstrated the existence of high serum levels of polymeric IgA, partially as immune complexes, in a large majority of patients with IgA mesangial GN (López-Trascasa *et al.*, 1980).

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The high frequency of macroscopic haematuria following upper-respiratory tract infections in these patients suggested the external secretions as a probable site of origin for that IgA. However, several authors have failed to detect secretory IgA in the glomeruli (Dobrin, Knudson & Michael, 1975; Whitworth *et al.*, 1976). Conflicting data has been observed concerning the class of IgA deposited (Andre *et al.*, 1980; Conley, Cooper & Michael, 1980). Recently it has been demonstrated that circulating cells have a potential to produce polymeric IgA with an equal distribution of IgA1 and IgA2 subclass (Kutteh *et al.*, 1980) supporting the validity of the specialized migratory route undergone by IgA precursor cells.

Taking into account that in the experimental murine model of IgA nephropathy, the presence of polymeric IgA containing IC was a prerequisite for the appearance of nephritis (Rifai *et al.*, 1979) and that in human IgA mesangial glomerulonephritis polymeric IgA was detected at the mesangial level (Egido *et al.*, 1980a, b; Sancho *et al.*, 1982). An approach to establish the source of the high levels of polymeric IgA found in these patients, independently of the IgA1 or IgA2 classes, was attempted.

In this paper we show that perypheral lymphoid cells (PBL) from patients with IgA mesangial glomerulonephritis in the presence of pokeweed mitogen (PWM) synthetized significantly more IgA than control subjects. Based on the ability of cytoplasmic IgA positive cells to bind secretory component (SC) intracellularly and on their biochemical properties, we have demonstrated that the IgA produced showed characteristics of polymeric IgA.

MATERIALS AND METHODS

Patients. The diagnosis of IgA mesangial glomerulonephritis was based on the presence of IgA in the glomerular mesangium with or without C3 and other immunoglobulins. Patients with clinical or biochemical evidence of liver disease, systemic lupus erythematosus, Henoch-Schönlein syndrome or other systemic diseases were excluded. Twenty patients with that diagnosis were studied. Medical students and staff members, matched for age and sex with patients with IgA glomerulonephritis, were used as controls.

Preparation of lymphocytes and cultures. Peripheral blood lymphocytes (PBL) were separated from heparinized blood (20 U preservative-free heparin/ml of blood) by Hypaque-Ficoll gradient centrifugation (Pharmacia Fine Chemicals, Uppsala) (Böyum, 1968). Preparations were 90–95% viable as assessed by trypan blue dye exclusion. The lymphocytes were washed twice with Hank's solution and once with RPMI 1640 medium. The last wash was found to be free of measurable immunoglobulins. The cells at a concentration of 2×10^6 lymphocytes/ml in RPMI 1640 medium, supplemented with 10% fetal calf serum, 2 mM glutamine, 50 μg/ml streptomycin and 100 U/ml penicillin, were incubated in the presence or absence of 10 μl/ml PWM at 37°C in a 5% CO_2 humidified atmosphere in 13×10 mm plastic culture tubes (Falcon Plastic Oxnard, California, USA). This amount of PWM employed was found to produce a maximal response in previous experiments. At the end of 7th day culture tubes were centrifuged at 400 ***g*** for 10 min and cells were employed to study the cytoplasmic receptors for SC, as described below. The amount of IgA, IgG and IgM secreted into the culture media was determined by a double antibody radioimmunoassay previously described (López-Trascasa *et al.*, 1980). Briefly, the antisera used in the RIA did not show cross-reactivity as assessed by immunoelectrophoresis and immunoprecipitation with labelled antigen and precipitation occurred only with the respective antigen-specific antiserum. Aliquots of IgA, IgG or IgM were labelled with ^{125}I using a chloramine–T procedure. One hundred microlitres of a rabbit anti-human heavy chain was used at a dilution which bound approximately 50% of the TCA-precipitable counts of the labelled immunoglobulins. The ^{125}I-Ag-Ab complex were precipitated by sheep anti-rabbit IgG added to equivalence with respect to rabbit IgG. After incubation and centrifugation the tubes were counted for radioactivity. A standard inhibition curve was constructed with unlabelled Igs. The assay conditions used allowed measurements of the Igs in the range of 10 to 300 ng/ml.

Isolation and purification of secretory component (SC) and anti-SC antibody. Human secretory component (SC) was isolated from human whey by affinity chromatography on IgM sepharose

absorbent (Underdown *et al.*, 1977). Purity of SC was tested by immunoelectrophoresis and double immunodiffusion with polyvalent antisera to normal human serum (Behring diagnostics) and to colostrom. SC obtained in these conditions contained trace amounts of lactoferrin but no IgA.

The purity and specificity of anti-SC (Dakopatts, Denmark) were proven as follows; immunoelectrophoresis and Ouchterlony double diffusion analysis showed no precipitation reaction against normal human serum IgA, IgG and both light chains, but reacted with free human SC and with human colostrum. This antiserum was further purified by passing it subsequently through a Sepharose 4 B column (Pharmacia Fine Chemicals, Uppsala) conjugated to human affinity chromatography IgA, IgG and both light chain types. The resulting antiserum gave a single precipitation line with SC.

Examination of lymphocyte cytoplasmic receptors for SC. The measurement of the affinity of the SC for IgA polymeric in cells and in supernates was based upon the methods of Brandtzaeg (1974) and Crago & Mestecky (1979), partially modified (López-Trascasa *et al.*, 1980; Egido *et al.*, 1980a; Sancho *et al.*, 1982). To examine cells for the presence of cytoplasmic receptors for SC, cells were first fixed with a 95% ethanol and 5% acetic acid solution for 1 hr at room temperature. After washing, the cells were incubated with purified SC in a moist chamber at room temperature and again washed with PBS. As shown previously (Egido *et al.*, 1980a; Sancho *et al.*, 1982) the optimal conditions for SC binding were used (150 μg/ml of SC and 30 min of incubation time). Slides were then incubated with rhodamine labelled anti-SC (Atlantic Antibodies, Scarborough, Massachusetts, USA), washed again and after incubated with F(ab') fragments of monospecific antibodies to human IgA, IgG and IgM conjugated to fluorescein isothiocyanate (Cappel Laboratories, Cochranville, Pennsylvania, USA). Two hundred cells were counted in the slides by two independent observers.

Column chromatography study. The conditions previously published by Kutteh *et al.* (1980) were followed. Cell culture supernates from patients and controls were fractionated by molecular gel filtration on a column of Ultrogel K 16/40 AcA 22 (LKB Instruments, Sweden) equilibrated in PBS 0·15 M pH 7·3, previously calibrated with Dextran blue (mw 2×10^6), Ferritin (450,000), Aldolase (150,000) and BSA (68,000). For chromatography in dissociating buffer the Ultrogel columns were equilibrated and recalibrated in 0·1 M sodium acetate buffer pH 4·1 and the sample was dialyzed against the same buffer before chromatography. In all cases the presence of IgA in the different fractions was assayed by RIA. In some cases supernates were reduced with 10 mM dithiothreitol 1 hr at 37°C and alkylated with 21 mM iodoacetamide in 0·2 M Tris, pH 8·4 15 min at 4°C (Kutteh *et al.*, 1980) before molecular filtration.

RESULTS

Cytoplasmic binding of SC

Cytoplasmic SC binding was studied at 7 days of culture in patients as well as in controls. At this time SC receptors were detected in the cytoplasm of 20% of the stimulated lymphocytes. The cytoplasmic Ig positive cells were around 40% at the end of the PBL culture. When we determined

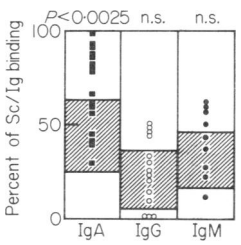

Fig. 1. Percentages of PWM-stimulated lymphocytes from patients with IgA mesangial GN simultaneously binding SC and immunoglobulins compared with controls. The dashed area represented the mean $\pm$ 1 s.d. of control values ($n = 13$).

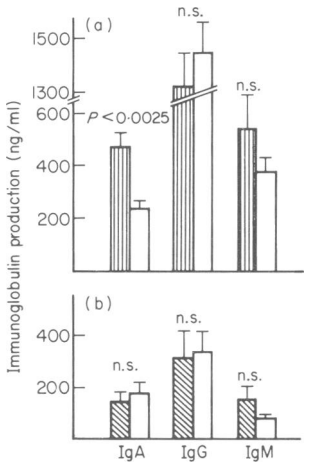

Fig. 2. Immunoglobulin production by non-stimulated (b) and PWM stimulated (a) PBL after 7 days of culture in 26 patients ■ ▧ and 15 controls □. Mean ± s.e.

the class of Ig produced by the SC binding cells, we found that even the majority contained IgA, cells producing IgM and IgG also join SC in patients as well as in controls. However, the binding SC/IgA ratio for cells was significantly higher in patients than in controls (69 ± 21, mean ± 1 s.d. versus 44 ± 27 respectively). No differences were found in the binding of SC/IgM or SC/IgG (Fig. 1). It is unclear why a certain population of cells containing intracellular IgG do bind SC. This phenomenon, observed by Crago & Mestecky (1979), was attributed to the possibility of concurrent production of SC-binding IgA or IgM in these cells. The differences observed between the percentages of IgG producing cells binding SC obtained by Crago & Mestecky (1979) and ours could be attributed to different technical procedures and/or reagents used.

Study of the amount of immunoglobulins produced by PWM-stimulated PBL

Taking into account that patients with Berger's disease have frequently high serum IgA levels, the *in vitro* immunoglobulin production was studied. As can be seen in Fig. 2, there was no statistically significant difference in spontaneous (non-stimulated) production of immunoglobulins between patients and controls. On the contrary, IgA production after PWM-stimulated PBL was significantly higher in patients (mean 470 ng/ml) than in controls (mean 240 ng/ml). No differences between amounts of IgM and IgG production were seen.

Biochemical characteristics of IgA produced by PWM-stimulated PBL

Supernates from 7 days cultures of PWM-stimulated PBL were pooled and fractionated on an Ultrogel AcA 22 column. A biphasic elution profile was obtained for IgA from culture supernates in patients as well as in controls. In both groups of subjects (patients and controls) there was a large proportion of IgA eluted in a position where polymeric IgA is expected to appear. The percentage of IgA comprised in these molecular weights was significantly higher in patients than in controls (Figs. 3 and 4). On the other hand the monomeric IgA produced by PBL culture in normal subjects was higher than in patients. There was a small amount of IgA (around 10%) found in the exclusion volume that could represent unspecific IgA aggregates produced during the technical procedures (Benveniste & Bruneau, 1979).

To confirm that the synthetized IgA with larger molecular weight was truly polymeric the following experiences were done: supernates stored at −20°C and thawed only once, then dialyzed against 0·1 M sodium acetate buffer, pH 4·1 and applied to a column that was equilibrated and recalibrated in the same buffer. As can be seen in Fig. 3 there was no significant changes of supernate IgA with molecular weight between 600,000 and 250,000 when treated at acid pH, what is in accordance with the covalent structure of polymeric IgA. To further study whether IgA was truly

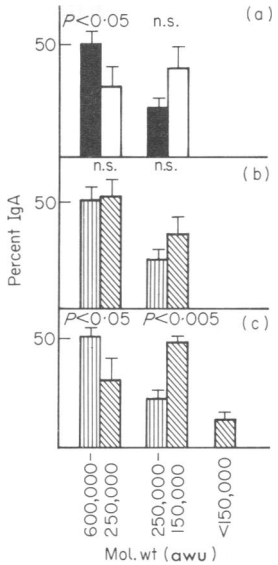

Fig. 3. Percentages of IgA synthesized by PWM-stimulated PBL according to their molecular weight in 7 patients and 7 controls (mean ± s.e.). Section a shows that the percentages of IgA with high molecular weight produced by patients were significantly higher than those of normal controls. Section b shows that there was no significant differences in the IgA percentages of patient's supernate before and after treatment at acid pH overnight, what is in accordance with the covalent structure of polymeric IgA. Section c shows that after reduction and alkylation of patient's supernate there was a decrease in the larger forms of IgA (600,000–250,000 awu), an increase in monomeric IgA (250,000–150,000 awu) and the appearance of smaller molecular weight fragments (> 150,000 awu). ■ = pH 7·3; □ = Controls; ▨ = pH 4·1; ■ = Red-AlK; ■ = IgA GN.

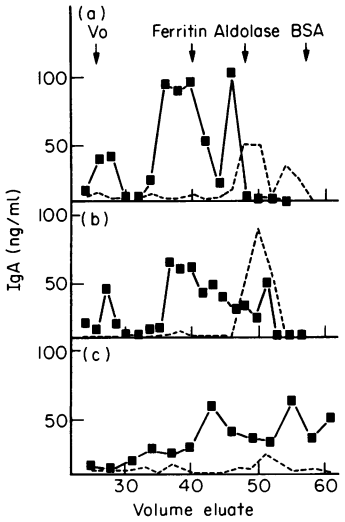

Fig. 4. Representative elution profiles of IgA produced by PWM-stimulated PBL in a patient and a control. Section a represents 7-day supernate fractionation on Ultrogel ACA 22 column eluted with PBS, pH 7·4 and assayed for IgA by RIA (patient (■) and control (. . .). Section b shows aliquots of the same supernates previously dialysed against and then eluted from the column with 0·1 M sodium acetate buffer pH 4·1. Section c depicts other aliquots reduced and alkylated, then fractionated and assayed for IgA. Arrows denote the elution profile of the Dextran blue (Vo), Ferritin (mol. wt = 440,000), Aldolase (mol. wt = 158,000) and BSA (mol. wt = 68,000) used as markers.

polymeric the affinity of the secretory component for this IgA was measured following the method of Brandtzaeg (1974) partially modified (Sancho *et al.*, 1982). To eliminate IgM, that could interfere with the assay by binding SC, the samples to be tested were previously incubated with an insolubilized anti-IgM sepharose 4B immunoadsorbent in antibody excess. After incubation of the IgA from the three peaks of Ultrolgel columns (Fig. 4) with ^{125}I-SC, the complexes formed were separated from free ^{125}I-SC by ultracentrifugation on 5–40% sucrose density gradients measuring the c.p.m. in each fraction (Sancho *et al.*, 1982). Only IgA comprised in the fractions where polymeric IgA was expected (second peak, elution volume between 32–45 ml, Fig. 4) was able to bind around 40% of ^{125}I-SC counted, similar percentage to that found in purified polymeric IgA from serum of these patients (López-Trascasa *et al.*, 1980) and in polymeric IgA from IgA myeloma (Brandtzaeg, 1974). Finally, supernates from patients and controls were reduced and alkylated before column filtration and the IgA was determined in the different fractions. As expected there was a decrease in the larger forms of IgA and an increase in monomeric IgA and in smaller molecular weight components (Figs. 3 and 4).

All these results suggest that the heavy IgA found in PBL supernates after 7 days of culture in the presence of PWM was a true polymeric IgA, and that patients with Berger's disease produce significantly larger amounts of that immunoglobulin than control subjects.

DISCUSSION

The data presented in this paper show that mitogen stimulated peripheral blood lymphocytes (PBL) from patients with IgA mesangial glomerulonephritis after 7 days of culture, synthetized a significantly higher amount of IgA, but not IgG or IgM, in relation to controls. These results are in agreement with the findings of a selective increase in IgA-bearing PBL (Sakai *et al.*, 1979) and a decrease of IgA specific suppressor T cell activity (Sakai, Nomoto & Arimori, 1979) in these patients. Furthermore, the study of culture supernates demonstrate that a large amount of the IgA produced present biochemical characteristics of true polymeric IgA. All these data, together with the elevated percentage of IgA producing cells capable of binding SC might explain the high serum levels of polymeric IgA often found in these patients (López-Trascasa *et al.*, 1980).

The nature and origin of the IgA deposited in the renal mesangium in patients with IgA mesangial glomerulonephritis has not been fully elucidated. Because this disease often follows infections of the upper respiratory tract it seemed probable that the source of that IgA was the external secretions. However, several studies have failed to reveal glomerular location of secretory IgA in Berger's disease (Dobrin *et al.*, 1975; Whitworth *et al.*, 1976), though others have occasionally found it (McCoy, Abramowsky & Tisher, 1974). Two recent papers have obtained conflicting data on this subject. Andre *et al.* 1980, found that glomerular IgA deposits consist predominantly of IgA2. By contrast Conley *et al.* (1980), have shown predominantly IgA1. Based upon the specific fixation of secretory component to J chain containing immunoglobulins (polymeric IgA and IgM), a technique successfully applied to study circulating B cells and intestinal epithelial cells (Brandtzaeg, 1976, 1978; Crago & Mestecky, 1979), we have shown that glomerular IgA is polymeric in the majority of these patients (Egido *et al.*, 1980a). This finding has been confirmed by Bene, Faure & Duheille (1980) using the same method.

Since less than 10% of IgA plasma cells in normal human bone marrow are capable of SC binding (Hijmans, Schuit & Hulsing-Hesselink, 1971; Radl *et al.*, 1974) whereas around 50% of IgA plasma cells in the intestinal mucosa and PWM-stimulated PBL culture for 7 days bound it (Brandtzaeg, 1973; Crago *et al.*, 1979), one must accept that the high levels of polymeric IgA found in these patients, and the polymeric IgA located in the mesangium, are originated in the migratory lymphocytes from the secretory tissues.

The increase in serum polymeric IgA in patients with Berger's disease in relation to normal subjects (López-Trascasa *et al.*, 1980) cannot be *a priori* exclusively explained by the high production of polymeric IgA by mitogen-stimulated PBL. Low levels of polymeric IgA found in serum of normal subjects are thought to be due to their rapid and effective clearance by the liver, probably through the SC receptors of the hepatocytes (Hall & Andrew, 1980, review).

Theoretically, a defect in IgA clearance by the liver, as probably have patients with alcoholic liver disease in which we have also observed high serum levels of polymeric IgA (Sancho *et al.*, 1981), could contribute to the serum findings in Berger's disease. Recently in another IgA related disease, dermatitis herpetiformis, in which IgA deposits are found in the skin, an impairment of Fc receptor function was found (Lawley, 1980).

It is possible that lymphocytes originated from secretory tissues in patients with Berger's disease produce high amounts of polymeric IgA following viral infections, just as occurred in PWM-stimulated PBL *in vitro*. That IgA, forming in part immune complexes, could theoretically saturate the SC (and probably Fc) receptors of the hepatocytes remaining longer in the circulation and therefore deposited in the glomeruli. In this context, Peppard *et al.* (1981) have recently demonstrated that, at least in rats, circulating complexes containing polymeric IgA are specifically removed through the liver.

The immunopathogenetic role of polymeric IgA in human IgA mesangial glomerulonephritis is unclear. In a recent animal model of IgA nephropathy, polymeric IgA was observed to be critical for renal deposition of complexes and induction of nephritic histological changes. Immune complexes formed either *in vivo* or *in vitro* with monomeric IgA failed to induce glomerulonephritis (Rifai *et al.*, 1979). The presence of polymeric IgA containing immune complexes in serum and kidneys from patients with Berger's disease (Egido *et al.*, 1980a) and alcoholic liver disease with IgA mesangial glomerulonephritis (Sancho *et al.*, 1982) suggest that this could also be the case in man. Furthermore, a clinical and histological improvement has been observed coincidentally with a diminution of serum polymeric IgA levels (Egido *et al.*, 1981a, b) in some patients with Berger's disease treated with phenytoin.

Although the intrinsic cellular mechanism of the increased rate of polymeric IgA synthesis mitogen-stimulated PBL in patients with Berger's diease are unknown our results further support the concept of polymeric IgA-linked nephritis (Egido *et al.*, 1980b).

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