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Increase of IgA specific helper Tα cells in patients with IgA nephropathy

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SUMMARY

Patients with IgA nephropathy show an emergence of IgA dominant circulating immune complexes (CIC) as well as increased levels of serum IgA and/or IgA bearing peripheral blood lymphocytes. In order to elucidate immunological aberrations responsible for the increased IgA synthesis in such patients, quantitative and qualitative analysis was performed on Tα cells which have been recently identified as possessing IgA specific helper activity on human B cells. Three different methods were employed to quantitate Tα cells. These methods included a rosette formation of T cells with either bovine red cells coated with the IgA fraction of anti-bovine red cell antiserum or those coated with TNP and anti-TNP IgA antibody, and an analysis of T cells combined with fluorescein conjugated human IgA myeloma protein. Tα cells were sorted by a fluorescence activated cell sorter and co-cultured with a B cell rich fraction to evaluate whether there is a qualitative difference in IgA specific helper activity between patients and healthy adults. Tα cells were significantly increased in patients with IgA nephropathy while there were no significant changes in patients with chronic proliferative glomerulonephritis without mesangial deposition of IgA. There was no qualitative difference in IgA specific helper activity of Tα cells between patients and healthy adults. It is suggested that increased levels of Tα cells in patients with IgA nephropathy may be responsible for increased synthesis of IgA in such patients.

INTRODUCTION

IgA nephropathy is presumed to be a chronic glomerulonephritis which is mediated by IgA dominant immune complexes (Lowance, Mullins & McPhaul, 1977; Woodroffe *et al.*, 1980). Synthesis of IgA may be enhanced in patients with IgA nephropathy, because serum IgA (Witworth, 1976) and/or IgA bearing peripheral blood lymphocytes (Nomoto, Sakai & Arimori, 1979) are increased in such patients. There may be some familial factors which induce an increase in synthesis of IgA, because an increase in IgA bearing cells was observed not only in patients with IgA nephropathy but also in some family members of such patients (Sakai *et al.*, 1979b). The immunological mechanism of the familial increase in synthesis of IgA in this disease is yet to be elucidated. We have previously reported that IgA specific suppressor T cell activity is decreased in patients with IgA nephropathy although such decrease in suppressor T cell activity may be due to a secondary effect of increased levels of serum IgA and/or IgA-bearing lymphocytes (Sakai, Nomoto & Arimori, 1979a). Recently, we have reported that T cells with receptors for the Fc portion of IgA (i.e. Tα cells) are IgA specific helper T cells (Endoh *et al.*, 1981). The purpose of the present study

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was to determine whether Tα cells are increased in patients with IgA nephropathy, and whether there are qualitative changes in IgA specific helper activity of Tα cells in these patients.

MATERIALS AND METHODS

Individuals studied. Thirty patients with IgA nepropathy and 12 patients with chronic proliferative glomerulonephritis without mesangial deposition of IgA were examined. All patients were diagnosed by renal biopsies evaluated by light microscopy, electron microscopy and immunofluorescent staining. Fifteen age matched healthy adults served as controls.

Separation of T and B lymphocytes. Mononuclear cells were obtained from heparinized venous blood using phenol free heparin (Riker Laboratories, Northridge, California, USA) and isolated on Ficoll-Hypaque density gradient (Böyum, 1968). Adherent cells were depleted by incubation in plastic petri dishes at 37°C for 45 min. Mononuclear cells were then pelleted with sheep red blood cells (SRBC), and after 60 min the rosetting T cells were separated on another Ficoll-Hypaque gradient. The rosettes were treated with ice cold ammonium chloride Tris lysing buffer (Boyle, 1968), and T cells were separated from the SRBC on another density gradient. The B cell fraction obtained from the interface after the intial density gradient separation of E rosetting cells was deprived of residual T cells by rosetting again with SRBC and density gradient separation. The T cell fraction contained 99–100% of SRBC rosetting cells, and the B cell fraction contained 70–80% of surface immunoglobulin bearing cells detected by FITC conjugated anti-human IgG + IgA + IgM (Behringwerke AG, Marburg-Lahn, W. Germany, Lot No. 686H, F/P ratio 3·2). The B cell fraction contained 1–2% non-specific esterase positive cells but no E rosette forming cells.

Enumeration of Tα cells. Three methods were employed to enumerate Tα cells in the T cell fraction. In the first method, IgA fraction was obtained from rabbit anti-bovine red cells (BRC) antiserum (Cappel Laboratories, Cochranville, Pennsylvania, USA, Lot No. 13025) using an immunoadsorbent column of cyanogen bromide activated Sepharose 4B beads (Sigma, St Louis, Missouri, USA, Lot No. 94C-0066) conjugated with the IgG fraction of goat anti-rabbit IgA antiserum (Cappel Laboratories, Lot No. 13269). The purity of IgA eluted from the column was examined by immunoelectrophoresis. The purified IgA was then incubated with BRC, and the BRC coated with rabbit IgA antisera (IgA-BRC) were mixed with T cells, pelleted, and incubated on ice for 30 min. Rosetting cells (Tα) were counted by light microscopy. Viability of cells was examined by addition of 1% toluidine blue, and more than 99% of cells from all samples were viable. In the second method, BRC were initially coated with TNP (picryl sulphonic acid) (Sigma) and then incubated with MOPC-315 mouse myeloma IgA protein (Litton Bionetics, Kensington, Maryland, USA, Lot No. PG078) for 30 min on a rotator (Lum *et al.*, 1979). The MOPC-315-IgA-TNP-BRC were incubated with the T cell fraction, and rosette forming cells were counted by light microscopy. In the third method, IgA myeloma protein was purified from serum samples of a patient with IgA myeloma. After precipitation with sodium sulphate, the myeloma protein was purified by ion exchange chromatography on DEAE-cellulose (Sigma) followed by gel filtration on Sephadex G-200 (Pharmacia, Piscataway, New Jersey, USA). The purified protein was IgA (λ) which was free of contaminating IgG, IgM, and other serum proteins as determined by Ouchterlony analysis and immunoelectrophoresis. The myeloma protein was IgA1 as detected by an Ouchterlony plate using anti-human IgA1 or IgA2 antisera (Nordic Immunological Laboratories, Tilburg, The Netherlands, Lot No. 1080). Dissociation of α and light chains by gel filtration in acid buffer was not examined. The purified IgA myeloma protein was conjugated with fluorescein isothiocyanate (FITC) (Sigma) using the method of Kawamura (1977), and absorbed three times with mouse liver acetone powder (Sigma) (F/P ratio 1·8). T cells were incubated with FITC conjugated human IgA myeloma protein at 4°C for 30 min. T cells combined with FITC-IgA protein were counted by a fluorescence activated cell sorter (FACS II, Becton Dickinson Electronics Lab., Mountain View, California). The pressure of the sample input was 0·9 kg/cm^2. The cells were illuminated by a laser in 495 nm at 400 mV. The approximate rate of the cell flow was 1,000 cells/sec. The size of the cells was determined, and lymphocytes with positive fluorescence were gated for subsequent counting. FITC conjugated bovine serum albumin (BSA) (Sigma, Lot No. 85C-8011) was used to determine the

electronic gate to exclude cells with non-specific staining. Once this channel was chosen, the percentage of cells from the same cell population that was specifically stained by FITC-IgA was calculated by the following formula:

$$\% \text{ of specifically stained cells} = \frac{I-C}{T-C} \times 100$$

where I = the number of FITC-IgA stained cells from channel X to channel 1000; C = the number of FITC-BSA stained cells from channel X to channel 1000; and T = the total number of cells analysed.

Separation of the Tα cell fraction. Two methods were employed to separate the Tα cell fraction. In the first method, Tα cells were separated by rosette formation with MOPC-315-IgA-TNP-BRC which was described above. T cells were incubated with these coated BRC at 4°C for 1 hr, and separated by Ficoll-Hypaque density gradient. Cells obtained from the pellet were designated as 'Tα cell rich fraction', and those from the interface were termed as 'Tα cells poor fraction'. An aliquot of the Tα cell rich fraction was incubated in RPMI 1640 (GIBCO, Grand Island, New York, USA) in 5% CO_2/air at 37°C for 6 hr, and then incubated with MOPC-315-IgA-TNP-BRC. The percentage of rosette forming cells was calculated by light microscopy. In the second method, T cells were stained with FITC-IgA as described above, and then cells combined with FITC-IgA (i.e. bright cells) were sorted from those without FITC-IgA on their cell surface (i.e. dark cells). After the sorting, an aliquot of the bright cells was incubated with 150 μg/ml of trypsin (Sigma, Lot No. 72087) at 37°C for 15 min and washed four times with RPMI 1640 at 4°C. The washed cells were incubated in RPMI 1640 in 5% CO_2/air at 37°C for 6 hr, and stained with FITC-IgA again, and then FITC-IgA positive cells were counted by FACS.

Assay of helper activity in Tα cells. The Tα cell rich fraction obtained by either rosette formation with MOPC-315-IgA-TNP-BRC or sorting of FITC-IgA binding cells by FACS was co-cultured with the B cell rich fraction obtained from allogeneic patients or healthy adults. In parallel studies, the Tα cell poor fraction or dark cells were also co-cultured with B cells. An equal number of T and B cells was cultured at a concentration of 2×10^6 cells/ml with 10 μl of pokeweed mitogen (GIBCO) in RPMI 1640 containing 100 units/ml of penicillin and 100 μg/ml of streptomycin, and supplemented with 10% heat-inactivated and deaggregated (105,000 *g* for 1 hr) fetal calf serum (FCS; GIBCO, Lot No. A461622) in 5% CO_2/air at 37°C for 7 days. In other parallel studies, one fifth the number of Tα cells were added to B cells and cultured as described above to determine the effect of the amount of Tα cells on *in vitro* B cell activity. After 7 days of culturing, the percentages of cells with cytoplasmic immunoglobulins were determined by the method of Moretta *et al.* (1977). Briefly, cytocentrifugal preparations of the cultured cells were fixed in ice cold acetic acid (5%) and ethanol (95%), and then rehydrated in phosphate-buffered saline (PBS). The cells were stained at room temperature for 30 min with FITC conjugated heavy chain specific anti-human IgG, IgA, IgM antisera produced in goats (Meloy, Springfield, Virginia, USA). In some randomly selected experiments, FITC conjugated $F(ab')_2$ fragments of goat anti-human IgG, IgA or IgM (Cappel laboratories, Lot No. 10884, 10884, 11953) were used to stain the cytoplasmic immunoglobulins in the cultured cells. Slides were examined with an incident-type fluorescent microscope (Zeiss, Model 9901).

Statistical significance of the data obtained from these studies was evaluated by either the χ^2 test of unpaired *t*-test.

RESULTS

Table 1 shows the amount of Tα cells in the T cell rich fraction of peripheral blood lymphocytes obtained from patients with IgA nephropathy, patients with chronic proliferative glomerulonephritis without mesangial deposition of IgA, and healthy adults. Tα cells were significantly increased in patients with IgA nephropathy as determined by the three different methods employed in this study. Although there was no linear correlation, patients with IgA nephropathy showed an increase not only in the amounts of Tα cells but also in the levels of serum IgA and/or IgA bearing peripheral blood lymphocytes.

Table 2 indicates that Tα cells obtained from either patients with IgA nephropathy or healthy

Table 1. Amount of Tα cells and laboratory findings in patients with IgA nephropathy (IgA-N), patients with chronic proliferative glomerulonephritis without mesangial deposition of IgA (PGN) and healthy adults (control)

Item	Method	Mode	IgA-N ($n=30$)	PGN ($n=12$)	Control ($n=15$)
Amount of Tα cells	Rosette formation with BRC coated with IgA fraction of anti-BRC antiserum (%)	Range	2·47–8·84	n.d.	0·55–2·67
		Mean	6·17*		1·15
	Rosette formation with BRC coated with TNP combined with anti-TNP IgA (%)	Range	1·76–3·00	n.d.	0·61–1·97
		Mean	2·39*		0·97
	Fluorescence analysis of FITC conjugated IgA binding cells (%)	Range	0·66–1·44	0·17–0·98	0·05–0·88
		Mean	0·98†	0·27	0·25
Laboratory findings	Urinary protein (g/day)	Range	0–1·1	0–1·3	0
		Mean	0·6	0·7	0
	Serum creatinine (mg/dl)	Range	0·8–1·4	0·8–1·2	0·7–1·1
		Mean	1·0	0·9	0·9
	Serum IgA (mg/dl)	Range	228–478	139–279	144–260
		Mean	331*	231	210
	IgA bearing blood lymphocytes (%)	Range	4–18	0–4	0–4
		Mean	8·8*	3·1	2·8

*$P<0·001$; †$P<0·05$; n.d. = not done.

Table 2. *In vitro* activity of Tα cells in patients with IgA nephropathy (patient) and healthy adults (control)

Type	T cells; method	Source	B cells; source	Amount of IgA producing cells		
Tα	FACS	control	control	8·0%		
Tα	FACS	patient	control	8·5%	*	
Tα	FACS	control	patient	11·5%	†	†
Tα	FACS	patient	patient	12·0%	*	
Tα	rosette	control	control	1·4%		
non-Tα	FACS	control	control	2·4%		
non-Tα	rosette	control	control	1·3%		
no T cells were added			control	0%		

Each figure represents a mean value of triplicate samples

* $P>0·20$; † $P<0·05$.

adults induced an increase in IgA producing cells *in vitro*. There was no significant difference between the amount of IgA producing cells co-cultured with Tα cells from patients and those from healthy adults. ($P>0·20$). The B cell rich fraction obtained from patients showed a small but significant ($P<0·05$) increase in IgA producing cells over that from healthy adults.

DISCUSSION

Receptors on lymphocytes capable of binding the Fc portion of IgG (Dickler & Kunkel, 1972), IgM (Moretta *et al.*, 1975), IgE (Gonzalez-Malina & Spiegerberg, 1977) or IgA (Lum *et al.*, 1979) have been identified in human peripheral blood. Although T cells with Fcα receptors (i.e. Tα cells) has

been suggested to function as a polyclonal activator of B cells (Lum, Benveniste & Blaese, 1980), analysis of the function of pure Tα cells has been warranted. The results from our previous report (Endoh *et al.*, 1981) indicate that Tα cells show an IgA specific helper activity in B cells *in vitro*. To analyse the function of Tα cells, it was mandatory to obtain a pure Tα cell fraction. The Tα cell rich fraction obtained by rosette formation and subsequent density gradient centrifugation yielded a relatively crude population of Tα cells which induced polyclonal activation of human B cells *in vitro*. Such polyclonal activation of B cells (Lum *et al.*, 1980) might be due to contaminating cells other than Tα cells. These contaminating cells were non-rosette forming cells which might be sedimented during the separation of rosettes on Ficoll-Hypaque density gradient. In contrast to rosette formation, separation of the Tα cell rich fraction by FACS provided a relatively pure population of Tα cells which induced IgA specific helper activity *in vitro* (Endoh, *et al.*, 1981). Although sorting of cells bound with FITC conjugated human IgA myeloma protein does not exclude the existence of cells with ligands for the Fab portion of that IgA, bright cells (i.e. IgA binding T cells) showed that they formed rosettes with MOPC-315-IgA-TNP-BRC even before treatment with trypsin to remove FITC-IgA (Endoh *et al.*, 1981).

An increase in Tα cells in peripheral blood from patients with IgA nephropathy has been demonstrated by three different procedures. Measurement of Tα cells by FACS yielded lower values than other procedures, probably due to a high setting of the electronic gate to exclude any cells with non-specific fluorescence. Although there was no linear correlation, patients with IgA nephropathy showed an increase in both Tα cells and levels of serum IgA and/or IgA bearing peripheral blood lymphocytes. Patients with chronic proliferative glomerulonephritis without increased levels of serum IgA and/or IgA bearing lymphocytes did not show an increase in Tα cells in their peripheral blood. Serial studies are warranted to examine whether changes of the levels of serum IgA and/or IgA bearing lymphocytes parallel those of Tα cells in patients with IgA nephropathy.

Tα cells obtained from either patients with IgA nephropathy or healthy adults show IgA specific *in vitro* helper activity in the B cell rich fraction. There was no qualitative difference in the IgA specific helper activity of Tα cells between patients with IgA nephropathy and healthy adults. A spontaneous increase in IgA production in lymphocyte cultures without addition of Con A treated T cells observed in our previous report (Sakai *et al.*, 1979a) might be due to an increase in Tα cells in those cultures. It is concluded that an increase of Tα cells in patients with IgA nephropathy may be responsible for an increased synthesis of IgA in such patients.

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