Clin. exp. Immunol. (1987) **70**, 110–115

Impaired immunoglobulin G production in minimal change nephrotic syndrome in adults

H. YOKOYAMA, H. KIDA*, T. ABE, Y. KOSHINO, M. YOSHIMURA & N. HATTORI *First Department of Internal Medicine, School of Medicine, Kanazawa University, and *Department of Internal Medicine, Kanazawa National Hospital, Kanazawa, Japan*

(Accepted for publication 29 April 1987)

SUMMARY

Serum IgG, T and B cell subsets, cytoplasmic IgG positive cells (cBγ) and IgG in the medium (cIgG) of a 5 day culture of peripheral lymphocytes in both stimulated and non-stimulated (spontaneous) conditions with pokeweed mitogen (PWM) were studied in 30 adult patients with minimal change nephrotic syndrome (MCNS). In the nephrotic phase (11 patients), surface IgG positive B cells (sBγ) and spontaneous cBγ increased ($P < 0{\cdot}05$), whereas PWM-stimulated cIgG did not increase, and serum IgG decreased significantly ($P < 0{\cdot}05$). The cBγ/sBγ ratio calculated as an index of IgG synthesis in B cells increased spontaneously ($P < 0{\cdot}05$), but did not increase under PWM-stimulation. The cIgG/cBγ ratio as an index of IgG secretion from each matured B cell, reduced in both spontaneous and stimulated conditions ($P < 0{\cdot}05$, $P < 0{\cdot}01$, respectively). In the phase of unstable remission maintained by steroid therapy (10 patients), these parameters tended to normalize and the OKT4/OKT8 ratio decreased ($P < 0{\cdot}05$), while the ratio remained unchanged in the nephrotic phase. However, after discontinuation of steroid (nine patients), spontaneous cBγ and the spontaneous cBγ/sBγ ratio were again increased, and the cIgG/cBγ ratio decreased ($P < 0{\cdot}05$) as observed in the nephrotic phase. These results suggest that B cells in patients with MCNS both in the nephrotic state and stable remission after discontinuation of steroid are activated spontaneously, but the secretory process of IgG from the matured cells is impaired, and that steroid improves these abnormalities.

Keywords minimal change nephrotic syndrome immunoglobulin G T cell subsets B cell subsets

INTRODUCTION

As the causes of a marked decrease in serum immunoglobulin (Ig) G during the nephrotic phase of minimal change nephrotic syndrome (MCNS), impaired differentiation of B cells from IgM-producing to IgG-producing cells and the loss of serum IgG into urine have been reported (Giangiacomo *et al.*, 1975). However, as reported previously by Dall'Aglio *et al.* (1984) and from our laboratory (Tani *et al.*, 1982; Yokoyama *et al.*, 1985), both surface IgM-positive and IgG-positive B cells were increased in the peripheral blood. In addition, Beale *et al.* (1983) pointed out an increased production of IgG, IgA and IgM in the culture of mononuclear cells isolated from peripheral blood of nephrotic patients with MCNS. However, in the nephrotic phase, asymmetric depression of the serum level in IgG subclasses (Shakib *et al.*, 1977), low serum IgG levels even after

Correspondence: Hitoshi Yokoyama, MD, First Department of Internal Medicine, School of Medicine, Kanazawa University, 13-1 Takara-machi, Kanazawa, Ishikawa 920, Japan.

a fairly long period of remission and low response of cultured lymphocytes to stimulation by pokeweed mitogen (PWM) were reported (Heslen *et al.*, 1982; Herrod *et al.*, 1983), indicating the presence of impaired IgG production.

In this study, we investigated the processes of differentiation, maturation and IgG secretion of B cells in an attempt to clarify the characteristics of the altered IgG-producing system in MCNS and the effects of corticosteroids on the alterations.

MATERIALS AND METHODS

Patients. Thirty patients with histologically proven MCNS, 19 males and 11 females, ages ranging from 16 to 65 with a mean of 26 years, were studied. Histological diagnosis was confirmed based on light and immunofluorescence microscopic observations. Ten healthy males, ages ranging from 24 to 34 with a mean of 27 years, served as the controls.

Clinical definition. Clinical conditions were classified into the following three categories: (1) nephrotic syndrome: condition with daily urinary protein excretion of more than 3·5 g and serum albumin less than 30 mg/ml; (2) unstable remission: disappearance of urinary protein and normalization of serum albumin level, while requiring steroid therapy for maintaining remission; (3) stable remission: remission lasting for 6 months or longer after discontinuation of steroid therapy. Examinations during the nephrotic phase were carried out before institution of the following steroid therapy.

Treatment. The patients were treated with prednisolone in a daily dose of 40 mg (0·6–1·0 mg/kg/day) for 4 to 8 weeks, followed by gradual tapering to a maintenance dose of 10–20 mg a day or every other day in accordance with clinical improvement. Immunosuppressive drugs were not prescribed.

Cell separation. Mononuclear cells were isolated from heparin-treated peripheral venous blood by Ficoll-Paque (Pharmacia) gradient centrifugation (400 ***g***, 30 min), and were freed of erythrocytes with 10 mM Tris–0·83% ammonium chloride buffer (pH 7·65). The cells were washed five times with RPMI 1640 medium (Gibco), and adjusted to 5×10^6 cells/ml. Preparations were more than 95% viable by the 0·25% trypan blue exclusion test.

T cell subsets. These were identified by indirect immunofluorescence technique using monoclonal antibodies OKT3, OKT4 and OKT8 (peripheral T; T3, inducer/helper; T4 and suppressor/cytotoxic; T8, Ortho; Reinherz *et al.*, 1979a, b; 1980). It was performed as previously described, and the percentage of positive cells was recorded (Yokoyama *et al.*, 1985).

B cell subsets. Surface Ig positive cells identified by the direct immunofluorescence technique using FITC-conjugated anti-human IgG + IgA + IgM (sB), IgM (μ-chain; sBμ) and IgG (γ-chain; sBγ) rabbit IgG/F(ab′)$_2$ antibody (Behringwerke) were regarded as B cells. The false positivity due to cells bearing Fc receptors was eliminated by a treatment with acetate-buffered saline (pH 4·0) for 1 min (Kumagai *et al.*, 1975).

Cell culture. The final culture medium consisted of RPMI 1640 supplemented with 20% heat-inactivated fetal bovine serum, 50 IU/ml penicillin, 50 μg/ml streptomycin, 250 μg/ml amphotericin-B and 2 mM L-glutamine (Gibco). The cells prepared as above were cultured in this medium with PWM (10 μg/ml, Gibco; stimulated) and without (spontaneous) at a concentration of 5×10^5/ml in duplicate in the lymphocyte culture tube (Nunclon No341113, Nunc) at a final volume of 1 ml for 5 days at 37°C in a humidified atmosphere of 5% CO_2 in air. In preliminary studies the cytoplasmic IgG positive cells in both the normal and MCNS patients reached a maximum level on the 5th day, and the IgG secretion at the 7th day. Cell viability at the end of the culture was at least 80%.

Cytoplasmic Ig positive cells (cBγ). The harvested cells were washed twice with 10 mM PBS (pH 7·4) after being counted in a haemocytometer, and then smeared on the non-fluorescent glass slide using a cytocentrifuge. The smear was dried, and fixed with ethanol supplemented with 5% glacial acetic acid at −20°C for 20 min, rehydrated in cold PBS, and stained with FITC-conjugated anti-human IgG (γ-chain) rabbit serum (Behringwerke). The absolute numbers of cytoplasmic fluorescence-positive cells (cBγ) were calculated in each tube (Kearney & Lawton, 1975).

Table 1. T and B lymphocyte subsets in minimal change nephrotic syndrome

		Control ($n=10$)	Nephrotic phase ($n=11$)	Unstable remission ($n=10$)	Stable remission ($n=9$)
T3	(%)	67·4±1·7	64·4±2·1	60·3±2·3**	65·9±1·6
T4	(%)	41·6±1·7	40·2±2·4	33·0±1·6***	41·3±1·2
T8	(%)	24·6±1·5	23·6±2·4	26·0±1·6	21·9±1·5
T4/T8 ratio		1·75±0·13	1·93±0·28	1·35±0·16*	1·96±0·15
sB	(%)	10·6±0·6	18·0±1·8***	13·2±2·2	12·0±1·2
sBμ	(%)	4·2±0·5	5·9±0·9*	4·2±0·5	3·4±0·6
sBγ	(%)	3·8±0·6	7·1±1·1**	5·8±0·6*	4·3±0·5

* $P<0·05$; ** $P<0·01$; *** $P<0·005$, comparisons between the controls and each clinical phase, mean±s.e.m.

T3, T4 and T8: OKT3, OKT4 and OKT8 positive T lymphocyte subsets, respectively. sB, sBμ and sBγ: total B lymphocyte, surface IgM, and IgG positive B lymphocyte subsets, respectively.

IgG concentration in the supernatant (cIgG). The cell-free supernatant, stored at −70°C, was analysed by solid-state quantitative immunofluorescence assay (Immuno-Fluor IgG, Bio-Rad) using a microplate fluorometer (MTP22-F, Corona Electric Co.). The minimum level of detection was 50 ng/ml. Pre-culture concentration of IgG in the supernatant of the cell suspension was below the range of measurement.

Serum IgG. This was measured with a laser nephelometer (Behringwerke).

Statistical significance was determined by Student's *t*-test.

RESULTS

T and B cell subsets. In the nephrotic phase, T cell subsets did not differ from those of the controls, but during unstable remission on steroid therapy both T3 and T4 were decreased

Table 2. Immunoglobulin G synthesis in minimal change nephrotic syndrome

	PWM	Control ($n=10$)	Nephrotic phase ($n=11$)	Unstable remission ($n=10$)	Stable remission ($n=9$)
cBγ	(−)	3·05±0·43 vs***	5·58±0·77** vs NS	2·67±0·48 vs***	4·43±0·64* vs*
cBγ	(+)	6·57±0·84	6·70±1·03	5·91±0·78	6·30±0·84
cIgG	(−)	161±32 vs*	170±42 vs NS	146±32 vs*	112±25 vs***
cIgG	(+)	415±102	193±47*	264±73	246±75
serum IgG (mg/dl)		1325±80	506±97***	1140±79	1310±101

Comparisons were carried out between the controls and each clinical phase, and between PWM (−) and PWM (+) group as shown in 'vs'.

* $P<0·05$; ** $P<0·01$; *** $P<0·005$; NS, not significant; mean±s.e.m.

PWM (+) or (−): culture with or without pokeweed mitogen.

cBγ, cytoplasmic Ig positive cells ($\times 10^3$/well).

cIgG, supernatant IgG after 5 day culture (ng/ml).

Table 3. B lymphocyte transformation and IgG secretion in minimal change nephrotic syndrome

	PWM	Control ($n=10$)	Nephrotic phase ($n=11$)	Unstable remission ($n=10$)	Stable remission ($n=9$)
cBγ/sBγ	(−)	15·2±1·7 vs***	22·9±3·9* vs NS	8·9±1·7** vs***	21·0±2·6* vs*
cBγ/sBγ	(+)	37·0±3·6	24·6±5·5*	19·9±2·9***	30·2±3·0
cIgG/cBγ	(−)	62·0±16·1 vs NS	26·9±7·4* vs NS	50·6±8·4 vs NS	26·4±4·9* vs NS
cIgG/cBγ	(+)	60·3±13·1	22·4±3·8**	47·3±17·8	24·6±5·7*
cIgG/sBγ	(−)	8·7±1·3 vs**	6·5±1·7 vs NS	5·4±1·0* vs*	5·2±1·5* vs*
cIgG/sBγ	(+)	20·9±4·4	6·8±1·9***	7·7± 1·6**	10·6±3·6*

Comparisons were carried out between the controls and each clinical phase, and between PWM(−) and PWM(+) group as shown in 'vs'.

* $P<0·05$; ** $P<0·01$; *** $P<0·005$; NS, not significant; mean±s.e.m.

PWM (+) or (−): culture with or without pokeweed mitogen.

cBγ/sBγ, %; cIgG/cBγ, pg/cell; cIgG/sBγ, pg/cell.

significantly ($P<0·01$) and T8 tended to increase, leading to a significant reduction of the T4/T8 ratio ($P<0·05$, Table 1). However, in stable remission, the values again returned to the range of the controls. By contrast, sB, sBμ and sBγ were increased significantly in the nephrotic phase ($P<0·005$, $P<0·05$ and $P<0·01$, respectively), and gradually returned to normal ranges according to clinical remission. Steroid therapy produced no apparent change.

Cytoplasmic IgG positive cells. cBγ was increased spontaneously in both the nephrotic phase and the phase of stable remission, but tended to decrease during unstable remission on steroid therapy (Table 2). PWM stimulation increased cBγ significantly in the unstable and stable remissions to the level of the controls, but in the nephrotic phase cBγ was already increased spontaneously to the comparable level of the controls with no further increase by the stimulation.

IgG concentration in the supernatant. The spontaneous cIgG level in each phase was comparable to that of the controls. PWM stimulation increased the level in the stable and unstable remissions, but the increments were apparently small compared to those of the controls, and in the nephrotic phase cIgG was not changed (Table 2).

Serum IgG. The serum IgG level was markedly low in the nephrotic phase compared to that of the controls, but returned to the range of the controls according to clinical remission (Table 2).

Maturation and IgG secretion of B cells. See Table 3. The rate of B cell maturation from sBγ to cBγ was estimated from the cBγ/sBγ ratio in each tube. The ratio was spontaneously increased in the nephrotic phase and the phase of stable remission, but was markedly decreased in the unstable remission put on the steroid therapy. PWM stimulation increased the ratio apparently in the unstable and stable remissions, but the ratio was lower compared to that of the controls. In the nephrotic phase, it was not changed by the stimulation.

The cIgG/cBγ ratio of each cultured tube was calculated as an index of IgG secretion from individual cBγ. It was apparently decreased in both the nephrotic phase and the phase of stable remission, but was comparable to the controls in the unstable remission. PWM stimulation did not affect the ratio in any phase of the disease or in the controls.

IgG production by individual sBγ estimated using the cIgG/sBγ ratio was decreased or tended to decrease in each disease phase without stimulation. Furthermore, the ratio was not affected by the PWM stimulation in the nephrotic phase, but was increased in the both unstable and stable remisson.

DISCUSSION

Although loss of IgG into urine was considered to be a primary cause of decrease in serum IgG in the

nephrotic phase of MCNS, the decrease was reported to reflect underlying abnormalities of humoral immunity, which were indicated by low serum IgG levels even during remission or the impaired responsiveness of IgG production *in vitro* to PWM-stimulation (Giangiacomo *et al.*, 1975; Heslen *et al.*, 1982; Herrod *et al.*, 1983). However, contradictory results have also been reported, in which *in vivo* production of immunoglobulins, especially IgG production, was either normal or increased (Andersen *et al.*, 1968; Gitilin, Janeway & Farr, 1956) and spontaneous IgG production *in vitro* was also increased (Beale *et al.*, 1983).

In order to explain these apparently discrepant results, surface IgG and cytoplasmic IgG positive B cells and IgG secretion in each cultured tube were analysed. As for the process of differentiation in B cells, both sBμ and sBγ were increased in the nephrotic phase as reported earlier from our laboratory (Yokoyama *et al.*, 1985). These data as well as the similar observation by Dall'Aglio *et al.* (1984) indicate that B cells have already differentiated from IgM-producing to IgG-producing cells, although Giangiacomo *et al.* (1975) had reported impairment of the process in the nephrotic phase.

Additional increases in spontaneous cBγ and the cBγ/sBγ ratio indicate that the B cell maturation from sBγ to cBγ was accelerated and the cells have already undergone stimulation *in vivo*. Thus, the PWM-stimulation did not induce a further increase of cBγ and cBγ/sBγ ratio in the nephrotic phase. On the contrary, no increase in cIgG and reduction of the cIgG/cBγ ratio indicate an impairment of IgG secretion by cBγ during the nephrotic phase. In addition, the cIgG level under PWM-stimulation was significantly lower compared to that of the controls, and PWM-stimulation did not affect IgG secretion from individual cBγ. Beale *et al.* (1983) also reported that PWM stimulation failed to increase or suppressed immunoglobulin secretion of mononuclear cells from MCNS patients in relapse. Similar impairments were also observed in the phase of stable remission in this study, suggesting that spontaneous accelerations of B cell maturation as well as impaired IgG secretion may be fundamental alterations in this disease.

In patients who achieved unstable remission during the steroid therapy, the cBγ/sBγ ratio was notably decreased, but the cIgG/CBγ ratio was improved, indicating that the steroid therapy suppresses B cell maturation from sBγ to cBγ in one hand, but improves IgG secretion in the other. In these patients, both T3 and T4 decreased, and T8 increased with a consequent decrease in the T4/T8 ratio as reported previously in adults by us and in children by Feehally *et al.* (1984) and Yokoyama *et al.* (1985). These changes of T cell subsets suggest that the alterations of B cell maturation and IgG secretion in MCNS might be attributed to the regulatory functions of T cells, mediated by T cell-replacing factors such as B cell specific growth factors and B cell differentiation factors (Melchers & Andersson, 1986).

In summary, it has become evident that, as the fundamental changes of IgG synthesis in MCNS, B cells are activated spontaneously, but the secretory process of IgG from them is impaired, and that the IgG-producing system as a whole is not capable of compensating for the loss of IgG in urine in the nephrotic phase.

It is also suggested that steroid therapy induces both the inhibition of B cell maturation in association with reduction of the T4/T8 ratio and the recovery of IgG secreting ability in MCNS.

This work was supported by a Research Grant (glomerular lesions) from the Intractable Disease Division, Public Health Bureau, Ministry of Health and Welfare, Japan.

REFERENCES

ANDERSEN, S.B., JARNUM, S., JENSEN, H. & ROSSING, N. (1968) Metabolism of γ-globulin in the nephrotic syndrome in adults. *Scand. J. clin. Lab. Invest.* **21,** 42.

BEALE, M.G., NASH, G.S., BERTOVICH, M.J. & MACDERMOTT, R.P. (1983) Immunoglobulin synthesis by peripheral blood mononuclear cells in minimal change nephrotic syndrome. *Kidney Int.* **23,** 380.

DALL'AGLIO, P., MERONI, P.L., BARCELLINI, W., BRIGATI, C., CHIZZOLINI, C., DE BARTOLO, G., MIGONE, L. & ZANUSSI, C. (1984) Altered expression of B lymphocyte surface immunoglobulins in minimal change nephrotic syndrome and focal glomerulosclerosis. *Nephron* **37,** 224.

FEEHALLY, J., BEATTIE, T.J., BRENCHLEY, P.E.C., COUPES, B.M., HOUSTON, I.B., MALLICK, N.P. &

POSTLETHWAITE, R.J. (1984) Modulation of cellular immune function by cyclophosphamide in children with minimal-change nephropathy. *N. Engl. J. Med.* **310,** 415.

GIANGIACOMO, J., CLEARY, T.G., COLE, B.R., HOFFSTEN, P. & ROBSON, A.M. (1975) Serum immunoglobulins in the nephrotic syndrome. *N. Engl. J. Med.* **293,** 8.

GITILIN, D., JANEWAY, C.A. & FARR, L.E. (1956) Studies on the metabolism of plasma proteins in the nephrotic syndrome. I. Albumin, γ-globulin and iron-binding globulin. *J. clin. Invest.* **35,** 44.

HERROD, H.G., STAPLETON, F.B., TROUY, R.L. & ROY, S. (1983) Evaluation of T lymphocyte subpopulations in children with nephrotic syndrome. *Clin. exp. Immunol.* **52,** 581.

HESLEN, J.M., LAUTIE, J.P., INTRATOR, L., BLANC, C., LANGRUE, G. & SOBEL, A.T. (1982) Impaired IgG synthesis in patients with the nephrotic syndrome. *Clin. Nephrol.* **18,** 144.

KEARNEY, J.F. & LAWTON, A.R. (1975) B lymphocyte differentiation induced by lipopolysaccharide. I. Generation of cells synthesizing four major immunoglobulin classes. *J. Immunol.* **115,** 671.

KUMAGAI, K., ABO, T., SEKIZAWA, T. & SASAKI, M. (1975) Studies of surface immunoglobulins on human B lymphocytes. I. Dissociation of cell-bound immunoglobulins with an acid pH or at 37°C. *J. Immunol.* **115,** 982.

MELCHERS, F. & ANDERSSON, J. (1986) Factors controlling the B-cell cycle. *Ann. Rev. Immunol.* **4,** 13.

REINHERZ, E.L., KUNG, P.C., GOLDSTEIN, G. & SCHLOSSMAN, S.F. (1979a) A monoclonal antibody with selective reactivity with functionally mature human thymocytes and all peripheral human T cells. *J. Immunol.* **123,** 1312.

REINHERZ, E.L., KUNG, P.C., GOLDSTEIN, G. & SCHLOSSMAN, S.F. (1979b) Further characterization of the human inducer T cell subset defined by monoclonal antibody. *J. Immunol.* **123,** 2894.

REINHERZ, E.L., KUNG, P.C., GOLDSTEIN, G. & SCHLOSSMAN, S.F. (1980) A monoclonal antibody reactive with the human cytotoxic/suppressor T cell subset previously defined by a heteroantiserum termed TH_2. *J. Immunol.* **124,** 1301.

SHAKIB, F., HARDWICKE, J., STANWORTH, D.R. & WHITE, H.R. (1977) Asymmetric depression in the serum level of IgG subclasses in patients with nephrotic syndrome. *Clin. exp. Immunol.* **28,** 506.

TANI, Y., KIDA, H., ABE, T., TOMOSUGI, N., SAITO, Y., ASAMOTO, T. & HATTORI, N. (1982) B lymphocyte subset patterns and their significance in idiopathic glomerulonephritis. *Clin. exp. Immunol.* **48,** 201.

YOKOYAMA, H., KIDA, H., TANI, Y., ABE, T., TOMOSUGI, N., KOSHINO, Y. & HATTORI, N. (1985) Immunodynamics of minimal change nephrotic syndrome in adults T and B lymphocyte subsets and serum immunoglobulin levels. *Clin. exp. Immunol.* **61,** 601.