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Cell-mediated immunity in idiopathic glomerulonephritis

M. SASDELLI, L. CAGNOLI,* P. CANDI, M. MANDREOLI,* E. BELTRANDI† & P. ZUCCHELLI* *Divisione di Nefrologia e Dialisi dell'Ospedale di Arezzo, * Divisione di Nefrologia e Dialisi and † Servizio di Laboratorio dell'Ospedale M. Malpighi di Bologna, Italy*

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SUMMARY

Various recent reports have suggested the presence of a functional defect of lymphocyte subpopulations in minimal-change nephropathy during the active phase. A probable role of inhibitory humoral factor(s) has been hypothesized. However, other authors have been unable to detect a significant difference between plasma from patients with nephrotic syndrome due to minimal-change nephropathy and plasma from other glomerulonephritis in the degree of inhibition of mitogen-induced lymphocyte transformation. In our study, T cell function, as measured by the response to PHA in autologous plasma, was depressed only in patients with minimal-change nephrotic syndrome and in patients with membranoproliferative glomerulonephritis. The lymphocyte function returned to normal when lymphocytes were cultured in homologous plasma. The lymphocyte responsiveness of patients with other glomerulonephritis with or without nephrotic syndrome was normal in both autologous and homologous plasma. Moreover, only plasma from patients with minimal-change nephropathy in the active phase and with membranoproliferative glomerulonephritis were able to induce inhibition of mitogenesis of lymphocytes from healthy donors. These data seem to confirm the presence of specific humoral inhibitory factor(s) in the plasma of these patients. Finally, preliminary findings seem to demonstrate an increase of the number of T_G cells in patients with minimal-change nephropathy in remission who relapse early in the subsequent follow-up.

INTRODUCTION

The important role played by the humoral immune system in the pathogenesis of glomerulonephritis is well known, while the involvement of cell-mediated immunity is doubtful. Minimal-change nephrotic syndrome is thought to be immunologically mediated (Shalhoub, 1974) and we previously found a consistent defect of the lymphocyte response to mitogens in this nephropathy which might be related to the presence of humoral inhibitory factors in accordance with other investigators (Sasdelli *et al.*, 1980; Moorthy, Zimmerman & Burkholder, 1976). These alterations were present only in the active phase of the disease, disappearing during remission (Sasdelli *et al.*, 1980).

Here we have studied a larger number of patients with minimal-change nephropathy, some of whom were included in our previous paper (Sasdelli *et al.*, 1980), and we have extended the study of lymphocyte subpopulations to several forms of idiopathic glomerulonephritis with or without nephrotic syndrome to clarify whether a cell-mediated immune disorder was detectable in these nephropathies.

Correspondence: Professor P. Zucchelli, Divisione di Nefrologia e Dialisi, Ospedale M. Malpighi, Via P. Palagi 9, 40138 Bologna, Italy.

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MATERIALS AND METHODS

Patients

One hundred and ninety-nine blood samples collected in heparinized containers were obtained from 171 patients with idiopathic glomerulonephritis. Some values were obtained from individual patients in different periods of the disease. However, we have included all data in our paper because for each of these patients the lymphocyte behaviour was constant during the various phases of the nephropathy. The diagnosis was established in all patients by history, clinical examination and renal biopsy. Renal specimens were studied by light microscopy and immunofluorescence in all patients and also by electron microscopy in 113 of them. The histological diagnosis was carried out according to Churg & Duffy (1973). Ninety-six patients had a nephrotic syndrome (NS) at the time of the study. Twenty-two blood samples were obtained from 15 patients with minimal-change nephropathy (MCN) who were in sustained remission after steroid treatment. In all patients serum creatinine was less than 1·5 mg/dl. At the time of the study no patients had been treated with immunosuppressive drugs or else the schedule had been stopped for at least 3 weeks beforehand.

The control group comprised 20 healthy age-matched volunteers with no evidence of renal or systemic disease.

The subjects studied are reported in Table 1.

Table 1. Patients studied in different renal histopathological groups

	Patient No.	Sample No.	Age (years) ($\bar{x}\pm$s.d.)	Serum albumin (g/dl) ($\bar{x}\pm$s.d.)	Serum cholesterol (mg/dl) ($\bar{x}\pm$s.d.)	Proteinuria (g/24 hr) ($\bar{x}\pm$s.d.)
Minimal-change nephropathy (MCN)						
With NS	21	26	22±17	2·2±0·6	404·5±105·1	7·2±3·3
In remission	15	22	25±16	3·9±0·3	180±36·9	0·11±0·14
Focal sclerosing glomerulonephritis (FSG)						
With NS	10	12	39±19	2·1±0·8	420±106·3	6·8±3·9
Without NS	9	12	28±8	3·6±0·2	185·4±21·4	1·4±0·9
Diffuse mesangial proliferation (DMP)						
With NS	16	17	37±18	2·2±0·5	417·2±96·5	7·4±3·1
Without NS	10	13	44±15	3·7±0·3	179·8±26·3	1·8±0·8
Membranous glomerulonephritis (MGN)						
With NS	21	21	49±8	2·3±0·6	409·1±102·7	7·6±4·2
Without NS	14	14	41±18	3·6±0·4	188·4±34·2	1·5±0·9
Membranoproliferative glomerulonephritis (MPGN)						
With NS	11	14	28±12	1·9±0·2	398±106	6·7±2·9
Without NS	9	10	24±13	3·8±0·5	175±22·2	1·7±0·6
IgA glomerulonephritis (IgA GN)						
With NS	17	18	33±12	2·2±0·5	416±98·9	7·3±2·9
Without NS	18	20	39±16	3·9±0·5	183·9±32·1	1·8±0·8
Total	171	199				
Normals	20	20	38±10	3·8±0·4	184·2±36·4	0·09±0·08

Methods

In all patients we studied the percentage and absolute number of T lymphocytes (E_t and E_a rosettes), B cells (EAC rosettes) and the lymphocyte function by PHA, Con A and PWM responsiveness in autologous plasma. Percentages of T cells with surface receptors for the Fc fragment of IgG (T_G cells) were evaluated in eight patients with MCN during NS and in 10 patients with MCN in remission. The intrinsic lymphocyte function was studied by culturing patients' lymphocytes in homologous plasma obtained from a pool of 20 healthy subjects of group AB.

A screening of inhibitory plasma factors was performed by evaluating the effect of patients' plasma on normal lymphocytes compared with the responsiveness of normal lymphocytes to mitogens in autologous plasma.

Lymphocyte separation. Lymphocyte suspensions, harvested from patients with idiopathic glomerulonephritis and healthy subjects, were isolated in Ficoll–Hypaque gradient as described by Böyum (1968). The cells floating at the interface were collected, washed three times in Hanks' solution (DIFCO) and incubated with carbonyl iron for 30 min at 37°C to remove phagocytic cells. This procedure gives a preparation of 99% small lymphocytes with a viability of approximately 95% cells, as revealed by the trypan blue exclusion test.

Plasma separation. The plasma was separated from heparinized venous blood obtained from patients and healthy volunteers and stored at −20°C until use.

Sheep erythrocyte total rosettes (E_t rosettes) assay. Sheep red blood cells (SRBC) treated with sulphydryl reagent 2-aminoethylisothiouronium bromide (AET, Merck) were used (Kaplan & Clark, 1974). AET-SRBC were prepared by incubating 1 vol of washed packed SRBC in 4 vol of freshly prepared AET solution, pH 8·5, for 20 min at 37°C with periodic shaking. Cells were washed four times at 4°C and 1% suspension in medium RPMI with fetal calf serum was prepared. Equal volumes of AET-SRBC suspension and isolated lymphocytes (2×10^6 cells/ml) were mixed and centrifuged at 200 *g* for 10 min at 4°C. The pellet was gently resuspended and 200 cells were counted and the percentage and absolute number of lymphocytes binding more than two erythrocytes was determined.

Sheep erythrocyte active rosettes (E_a rosettes) assay. Incubation of 0·1 ml of the lymphocyte suspension (5×10^6 cells/ml) and 0·1 ml of 20% fetal calf serum in Hanks' was performed, after addition of 0·1 ml of 0·5% SRBC, and the mixture centrifuged. The cell pellets were resuspended gently and the rosettes immediately counted (Wybran & Fudenberg, 1973).

Erythrocyte–antibody–complement rosettes (EAC rosettes) assay. A 5% suspension of SRBC was incubated for 30 min at 37°C with rabbit anti-sheep red blood cells (Ambozeptor, Behringwerke) diluted 1:2,000. Fresh human complement diluted 1:20 was added and the suspension incubated for 30 min at 37°C. Equal volumes of the prepared sheep cells (EAC) adjusted to a 1% solution and isolated lymphocytes (5×10^6 cells/ml) were mixed and incubated at 37°C for 20 min and then spun at 200 *g* for 5 min. After resuspension by vigorous mixing in a whirl-mixer, the percentage of EAC rosettes was calculated in the same manner as that described above for E rosettes.

T_G assay. The technique described by Ferrarini *et al.* (1975) was used to detect T cells with receptors for the Fc fragment of IgG (EA IgG-RFC). In brief, 2% SRBC were mixed with a suspension of ox erythrocytes (OxRBC) sensitized with anti-OxRBC rabbit IgG. One hundred microlitres of these indicator cells were then added to 100 μl of a lymphocyte suspension (2×10^6/ml). After centrifugation at 100 *g* for 5 min and incubation for 1 hr at 4°C, the pellet was resuspended and examined in a haemocytometer.

Preparation of antisera anti-ox erythrocytes (OxRBC). OxRBC stroma were prepared by lysis of ox erythrocytes in distilled water and 1 ml was injected in a rabbit every day for 3 weeks. Serum was then collected and the IgG anti-OxRBC were purified by DEAE cellulose chromatography. Immunoelectrophoresis of this fraction showed only a single line of IgG precipitation with anti-whole rabbit serum.

Lymphocyte transformation. The blastogenic response of lymphocytes to various mitogens (phytohaemagglutinin P, concanavalin A and pokeweed mitogen) was determined *in vitro* by incorporation of ^{3}H-thymidine in lymphocytes cultured in microtitre plates as described elsewhere (Sasdelli *et al.*, 1980). Culture medium consisted of 0·2 ml of medium TC 199 containing sodium

bicarbonate, penicillin and streptomycin with 10% plasma, either from patients with idiopathic glomerulonephritis or from a pool collected from healthy donors of group AB screened for atypical antibodies. Responding lymphocytes, 1×10^5 cells/ml, were stimulated with 1 μg/ml of phytohaemagglutinin P (PHA-P, DIFCO), 30 μg/ml of concanavalin A (Con A, Miles Yeda, Israel) and 100 μg/ml of pokeweed mitogen (PWM, GIBCO). Doses of various mitogens were selected from dose–response kinetic studies in 20 normal controls in whom maximum response was obtained with the above doses.

Cultures were incubated in triplicate in a 5% carbon dioxide humidified atmosphere at 37°C for 2 days, added to 0·25 μCi of ^{3}H-thymidine (sp. act. 25 Ci/mmol) and harvested after 24 hr. The cells were collected and washed on glass-fibre filters which were then dried. Counting was performed for 10 min in a liquid scintillation counter (Nuclear Chicago) using 10 ml of scintillation fluid. No correction for quenching was performed because the count efficiency was always constant both in controls and in patients. The arithmetic mean and s.e.m. of triplicate cultures were calculated and the net increase in c.p.m. was determined (net increase = c.p.m. stimulated − c.p.m. non-stimulated).

Screening of inhibitory serum factor(s). The blastogenic response of lymphocytes from nine healthy donors to the various mitogens was evaluated in both autologous plasma and plasma

Table 2. Lymphocyte function tested by ^{3}H-thymidine incorporation

	Mitogenic response to PHA (c.p.m. × 10^3)	
	Autologous plasma	Homologous plasma
Normals	115·7 ± 5·1	91·5 ± 5·6
Minimal-change nephropathy (MCN)		
With NS	64·1 ± 6·3*	82·7 ± 5·9
In remission	128·6 ± 8·7	
Focal sclerosing glomerulonephritis (FSG)		
With NS	82·2 ± 10·4	92·7 ± 13·9
Without NS	92·2 ± 10·5	
Diffuse mesangial proliferation (DMP)		
With NS	118·7 ± 7·7	102·6 ± 9·6
Without NS	133·5 ± 8·1	
Membranous glomerulonephritis (MGN)		
With NS	112·2 ± 6·2	102·7 ± 9·4
Without NS	108·3 ± 9·6	
Membranoproliferative glomerulonephritis (MPGN)		
With NS	92·7 ± 16·5†	91·2 ± 7·0
Without NS	79·8 ± 14·0†	
IgA glomerulonephritis (IgA GN)		
With NS	107·2 ± 7·5	90·5 ± 5·0
Without NS	115·3 ± 7·3	

Statistical significance derived from the Mann–Whitney *U*-test. Difference between each group and normals: * $P < 0{\cdot}001$; † $P < 0{\cdot}05$.

collected from patients with MCN and inhibited blastogenic response of lymphocytes in autologous plasma.

Statistical methods. All results are expressed as arithmetic means ± mean standard error of three individual cell cultures. The significance of difference between mean values was tested by Wilcoxon paired signed-rank test and Mann-Whitney *U*-test for non-parametric data.

RESULTS

The total number of lymphocytes with various lymphocyte markers showed no significant differences between the normal group and any of the other groups with or without NS for T lymphocytes, either E_t or E_a rosettes. A small but insignificant elevation in total number of EAC rosettes was apparent only in the patients with MCN with NS.

The mean values of the mitogenic response to PHA of lymphocytes from patients with renal diseases and normal subjects are shown in Table 2. T cell function, as measured by the response to PHA in autologous plasma, was significantly reduced in patients with MCN during NS ($P < 0{\cdot}001$) and in patients with membranoproliferative glomerulonephritis (MPGN) either with ($P < 0{\cdot}05$) or without NS ($P < 0{\cdot}05$) compared with the normal group. PHA lymphocyte responsiveness in autologous plasma from patients with MCN in remission was not statistically different from controls.

There was no difference between mean values of patients with diffuse mesangial proliferation (DMP), membranous glomerulonephritis (MGN), IgA glomerulonephritis (IgA GN) and controls. Some individuals with focal sclerosing glomerulonephritis (FSG) gave clearly lower values of PHA

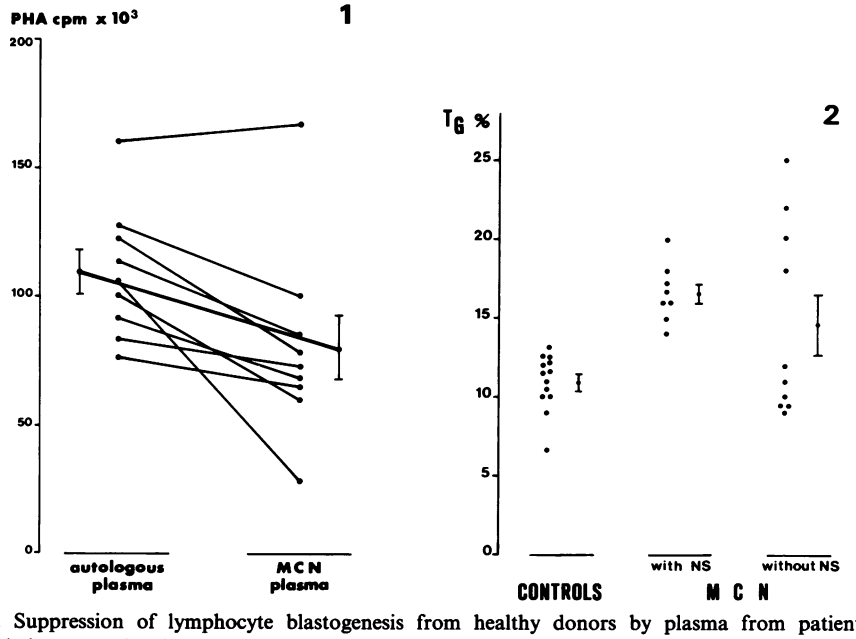

Fig. 1. Suppression of lymphocyte blastogenesis from healthy donors by plasma from patients with minimal-change nephrotic syndrome. The lymphocyte response to PHA is significantly reduced by MCN plasma during the active phase (mean value $80{\cdot}4 \pm 12{\cdot}5$ c.p.m. $\times 10^3$ compared with $109{\cdot}2 \pm 8{\cdot}5$ c.p.m. $\times 10^3$ of lymphocytes in autologous plasma; $P < 0{\cdot}01$). Statistical difference derived from Wilcoxon paired signed-rank test.

Fig. 2. Values of T_G in controls and MCN with and without NS. A significant increase of T_G cells is observed only during the active phase of MCN compared with controls (mean value $16{\cdot}6 \pm 0{\cdot}6$ s.e.m. versus $10{\cdot}9 \pm 0{\cdot}5$ s.e.m.; $P < 0{\cdot}001$). Statistical difference derived from Mann–Whitney *U*-test for non-parametric data.

Table 3. T_G values in MCN during remission and the incidence of relapse(s) in subsequent follow-up

T_G during remission (%)	Case No.	Sustained remission (No. of cases)	Subsequent relapses (No. of cases)
< 14·3	6	6	0
> 14·3	4	1	3

response than the healthy subjects but the mean value of these patients was not significantly different from that of controls.

A decrease in reactivity was also seen in Con A and PWM cultures of MCN lymphocytes in autologous plasma, but was not as marked as the decrease seen in PHA responsiveness. Mean values of mitogenic response to Con A and PWM had approximately the same pattern as the response to PHA compared with normal controls. There was no correlation between inhibition of PHA responsiveness and the degree of proteinuria, cholesterolaemia, albuminaemia, alpha-2-globulinaemia and C3 serum levels.

In order to distinguish between the cellular and the plasma components of *in vitro* lymphocyte response, we studied the lymphocyte function in homologous plasma obtained from a pool of normal subjects of group AB. No significant intrinsic cellular defect was evident since lymphocytes from patients with different glomerulopathies showed mean ^{3}H-thymidine incorporation values comparable to normals (Table 2).

Moreover, to examine the plasma effect, lymphocytes from healthy adult blood donors were incubated in both autologous plasma and in plasma of nine patients with MCN during the active phase. Fig. 1 outlines the results of these series: the lymphocyte response to PHA was significantly reduced by MCN plasma ($80{\cdot}4 \pm 12{\cdot}5$ c.p.m. $\times 10^3$ compared with $109{\cdot}2 \pm 8{\cdot}5$ c.p.m. $\times 10^3$ of lymphocytes in autologous plasma; $P < 0{\cdot}01$). During remission, the plasma of these patients no longer inhibited lymphocyte blastogenesis. The mean percentage of T_G lymphocytes was significantly higher in patients with MCN and NS than in controls ($16{\cdot}6 \pm 0{\cdot}6$ versus $10{\cdot}9 \pm 0{\cdot}5$; $P < 0{\cdot}001$) (Fig. 2). During sustained remission the individual values of T_G presented a considerable overlap with the values obtained in controls and in patients with the active phase of the disease. Preliminary observations seem to indicate that there is a correlation between percentages of T_G lymphocytes during remission and the subsequent follow-up of MCN: three out of four patients with high levels of T_G during remission showed a relapse of the NS within 3 months, while the six patients with normal T_G remained in remission (Table 3).

DISCUSSION

Various recent reports have suggested that the cellular immune system is involved in the pathogenesis of MCN (Plager & Stutzman, 1971; Mallick *et al.*, 1972; Shalhoub, 1974; Lagrue *et al.*, 1975; Eyres, Mallick & Taylor, 1976; Moorthy *et al.*, 1976). Shalhoub (1974) hypothesized that MCN is due to an uncontrolled proliferation of a T cell clone or T cell subclass and an increase in circulating levels of a thymic hormone or lymphokine capable of influencing glomerular permeability to proteins.

In accordance with other authors (Moorthy *et al.*, 1976) we previously reported (Sasdelli *et al.*, 1980) the presence of a functional defect of lymphocyte subpopulations in MCN. We found that lymphocyte response to PHA was reduced only during the active phase of MCN while the response returned to normal in both homologous and autologous plasma during remission. In addition, according to Moorthy *et al.* (1976), we observed that only plasma from patients with active MCN inhibited the mitogen-induced proliferation of normal lymphocytes, while plasma from patients with MCN in remission were no longer inhibitory. These data suggested the existence of a

functional T cell disorder related to the presence of a humoral inhibiting factor during the active phase of MCN. Iitaka & West (1979) were unable to detect a significant difference between sera from MCN patients and sera from patients with other forms of nephrotic syndrome in the degree of inhibition of mitogen-induced lymphocyte transformation. Therefore, they suggested that inhibition of mitogen-induced blastogenesis is related to the nephrotic syndrome *per se* and did not represent a particular pattern of MCN.

Beale *et al.* (1980) observed that T cell response as measured by the mitogenic effect of PHA was significantly reduced in nephrotic syndrome from different glomerulopathies while in mixed lymphocyte culture (MLC), sera from patients with active MCN were significantly more inhibitory than sera from patients with NS due to other causes. Thus they concluded that since the two groups of patients were comparable biochemically, the difference in degree of suppression may be due to a specific circulatory immunoregulatory factor present in patients with active MCN. Only MLC, which is a more sensitive assay for immunoregulatory factors, would be able to detect this specific feature of MCN.

Three basic questions arose from the analysis of all the reported data. The first question was to evaluate whether lymphocyte hyporesponsiveness in autologous plasma was a specific feature of the active phase of MCN or was also present in patients with NS from other nephropathies. In our study we confirm earlier reports (Moorthy *et al.*, 1976) that a defect of lymphocyte subpopulations is present in MCN during the active phase; moreover, similar behaviour is also noted in MPGN. In the latter, however, the PHA-induced response of lymphocytes is persistently reduced also in patients without NS, while in the former the response quickly returned to normal during remission. These results suggest that the inhibition of lymphocyte response to mitogens during MCN is correlated to disease activity, while in MPGN it is a constant feature of the glomerulopathy. We found no significant reduction in lymphocyte response to mitogens in any of the patients with other glomerular disease with or without NS. Our data therefore agree with those of Moorthy *et al.* (1976) and do not confirm those of Iitaka & West (1979) and Beale *et al.* (1980). The second question was whether the lymphocyte hyporesponsiveness in MCN and MPGN was due to a reduction in the number of T lymphocytes, to an intrinsic defect of lymphocytes *per se* or to a functional defect due to plasma inhibitory factor(s). In our experience, the total number of T lymphocytes, evaluated by E rosette test, was normal in all groups of patients studied with or without NS. The responsiveness of lymphocytes from patients with MCN and MPGN returned to normal in homologous plasma. Blastogenesis in autologous plasma of lymphocytes from patients with other glomerulopathies (with or without NS) was within the normal range. All these data seem to demonstrate that some inhibitory plasma factor(s) is present in MCN and MPGN and that this immunoregulatory factor(s) is specific and may play a role in the lymphocyte hyporeactivity found in these nephropathies.

Moreover, the finding that only sera from patients with MCN and NS and with MPGN were able to induce inhibition of mitogenesis of lymphocytes from healthy volunteers, seems to confirm the presence of specific humoral inhibitory factors in the sera of these patients. Diminished cellular response to mitogenic stimuli has been reported in protein malnutrition (Beatty & Dowdle, 1978) and related to increased alpha-2-globulinaemia and cholesterolaemia (Curtiss & Edgington, 1976). A large number of circulating substances capable of modifying lymphocyte responsiveness have recently been identified (Tomasi, 1977). However, since all groups of patients with NS presented comparable levels of protidaemia, cholesterolaemia, hyper-alpha-2-globulinaemia and hypoalbuminaemia, the hypothesis that reduced lymphocyte responsiveness to mitogens was due to these aspecific biochemical alterations seems unlikely. No correlation was found between C3 serum levels and lymphocyte hyporeactivity in MPGN. Recently, Tomizawa *et al.* (1979) reported the presence of inhibitory factors in active MCN plasma, relatively heat-stable, unadsorbable to charcoal powder, with a wide variety of molecular weight. However, further studies are needed to assess the true nature and biological importance of these apparently specific factors that, at present, remain for the most part unknown.

As regards the third question, we examined whether some abnormality in T cell subsets existed during MCN. Trompeter, Layward & Hayward (1978) found that T_G cells in blood from patients with steroid-sensitive nephrotic syndrome were normal and their number raised when steroids were

administered. They hypothesized that the abnormality of T_G cell number was not primarly related to the disease. On the contrary, in our cases, a significant increase in the number of T_G cells was observed during the active phase of MCN, while during remission the individual values of T_G cells presented a considerable overlap with the values obtained in the control group. No correlation was found between steroid therapy and T_G cell number. Furthermore, we found a good correlation between values of T_G cells during remission and the incidence of the relapse(s) in the subsequent follow-up of the disease: an increased number of T_G cells was recorded only in patients who relapsed in the subsequent 3-month period of follow-up.

T_G lymphocytes are supposed to represent a T cell subset with suppressor function, even though some indications now exist that many of the T_G cells may not be T cells at all (*Lancet*, 1980). Whatever the immunological significance of the lymphocyte subset identified as T_G cells, our data seem to suggest that they may be clinically important.

In conclusion, our findings show that a functional lymphocyte alteration is detectable only in the active phase of MCN and in MPGN and seems to be due to humoral inhibitory factors. An increase of the number of T_G cells is present in patients with MCN in remission who relapse early in the subsequent follow-up.

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