Spontaneous and pokeweed mitogen induced production of rheumatoid factor and immunoglobulins in type II essential mixed cryoglobulinaemia

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SUMMARY In order to evaluate functional lymphocyte defects in type II essential mixed cryoglobulinaemia (EMC) in vitro production of immunoglobulins (Ig) and rheumatoid factor (RF) has been studied in basal conditions and under pokeweed mitogen (PWM) stimulation in 15 patients and in 17 control subjects. The major finding was a significantly high basal and inducible production of RF by EMC lymphocytes as compared with the RF production in controls, while synthesis of polyclonal Ig was unaffected. A good correlation existed between in vitro production and serum levels of RF. Peripheral blood SmIg⁺ and Ia⁺ cells were also significantly increased. The possibility that EMC shares some pathogenetic mechanism with rheumatoid arthritis on the one hand and with lymphoproliferative diseases on the other is considered.

Key words: T lymphocyte subpopulations, immunoglobulin synthesis.

Type II EMC is a disease characterised by necrotising vasculitis with purpura, weakness, arthralgias, often glomerulonephritis, in the absence of underlying disorders.^{1–4}

Immunochemical analysis of circulating cryoglobulins shows the presence of at least two immunoglobulins, a monoclonal Ig, usually IgM, with rheumatoid factor activity, which binds to the polyclonal IgG to form a cold insoluble immune complex.² Although a defect of the cell mediated immunity has been described in this disorder,^{5–6} little is known about cellular mechanisms involved in the regulation of monoclonal cryo-RF production.

A spontaneous and inducible RF in vitro synthesis has been described in rheumatoid arthritis (RA), a closely related disease,⁷ but this RF is usually polyclonal and not cryoprecipitable.

These observations stimulated us to study the possibility that comparable alterations were present also in type II EMC, which is a peculiar autoimmune

disease, since the only detectable autoantibody (cryoprecipitable RF) is a monoclonal Ig. To do this we studied in vitro synthesis of Ig and RF in patients and controls, and evaluated possible alterations in lymphocyte subpopulations.

Patients and methods

PATIENTS

Fifteen patients (eight men and seven women) mean age 57 years (range 35–70), with mixed cryoglobulinaemia unassociated with any underlying disease were studied (Table 1). All had a history of purpura and arthralgias; 10 had diffuse proliferative glomerulonephritis proved by biopsy⁴ and seven hepatic involvement. Fourteen patients had IgG-IgM $\times$ cryoglobulins, with trace amount of IgA in three; one had IgG-IgG $\times$ cryoglobulins. No patient was receiving corticosteroids or immunosuppressive drugs, or both, at the time of the study nor in the previous eight weeks.

Seventeen normal subjects of comparable age and sex distribution formed the control group.

CRYOGLOBULINS

Isolation, purification, and immunochemical analy-

Table 1 Main clinical and laboratory data

Patient No	Age/sex	Cryoglobulins (cryocrit %)	C3* (%)	C4* (%)	IgG* (mg/dl)	IgA* (mg/dl)	IgM* (mg/dl)	IgM RF* (µg/ml)
1	35/M	IgG-IgA-IgM κ (61)	103	5	1070	263	261	226
2	66/F	IgG-IgM κ (10)	80	48	1310	419	339	35.5
3	58/M	IgG-IgM κ (40)	57	5	700	144	208	285
4	63/F	IgG-IgM κ (4)	65	42	1830	135	271	26
5	59/F	IgG-IgM κ (22)	64	67	1430	77	217	15
6	56/F	IgG-IgA-IgM κ (32)	40	5	1130	220	339	12
7	60/F	IgG-IgM κ (26)	69	5	963	144	414	20
8	53/M	IgG-IgM κ (14)	69	20	1190	135	123	37.2
9	70/M	IgG-IgM κ (12)	87	5	1190	118	840	476
10	52/M	IgG-IgM κ (5)	75	5	1370	42	280	7.8
11	64/F	IgG-IgM κ (1)	130	5	1230	347	212	34
12	44/M	IgG-IgM κ (90)	69	20	1560	135	1595	5.3
13	63/M	IgG-IgA-IgM κ (30)	70	25	1120	320	650	50
14	52/F	IgG-IgM κ (2)	97	58	1080	82	190	13
15	66/M	IgG-IgG κ (16)	33	5	1690	543	234	2

*Normal values: C3 65–140%; C4 43–146%; IgG 800–1800 mg/dl; IgA 90–450 mg/dl; IgM 70–280 mg/dl; IgM RF <8 µg/ml. SI conversion: mg/dl×0.01=g/l.

sis of cryoglobulins were performed as previously described.⁸

SERUM IMMUNOGLOBULINS

Serum Igs were determined by radial immunodiffusion using commercially available plates (Behringwerke).

COMPLEMENT

C3 and C4 were measured quantitatively with commercially available immunodiffusion plates (Behringwerke). Results were expressed as the percentage of pooled normal human serum.

CELL SEPARATION

Mononuclear cells were obtained from peripheral blood by centrifugation on a gradient of Ficoll-Hypaque (Biotest Laboratories). The cells thus obtained were washed three times in sterile phosphate buffered saline before further processing.

LYMPHOCYTE MEMBRANE MARKERS

A panel of monoclonal antibodies (Ortho Ph Corp) was used to identify T cell subsets and Ia⁺ cells. B

lymphocytes were identified with a fluorescein isothiocyanate labelled F(ab)2 goat antihuman Ig antiserum (Meloy Labs). Indirect and direct immunofluorescence techniques were used as described elsewhere.^{9 10}

CULTURE CONDITIONS

Cells were cultured at a concentration of 10⁶/ml (10⁹/l) in complete medium (RPMI 1640+10% fetal calf serum+2 mM L-glutamine; all reagents from Flow Laboratories) without antibiotics. The cultures were run in duplicate; one set in basal conditions, the other stimulated with 20 µg/ml PWM (Flow). The cultures were kept in a humidified atmosphere at 37°C with 5% CO₂ for seven days. On the eighth day they were collected and centrifuged at 2000 rpm for 15 minutes. The supernatants were then deep frozen, and the pelleted cells were discarded.

IMMUNOGLOBULIN ASSAY

An enzyme linked immunosorbent assay (ELISA)¹¹ was used, according to the method of Rodriguez *et al*,¹² with minor modifications. Briefly, standards and

samples were incubated in microtitre plates (Dynatech) coated with anti-Ig class specific antibodies. After three washes with phosphate buffered saline containing 0.05% Tween 20 a class specific alkaline phosphatase conjugated antibody (Sigma) was added, and the plates were then incubated at 37°C for one hour. After three more washes the enzyme substrate (Sigma) was added, and the plates were read in a Titertek Multiskan MC photometer (Flow). The optical density values were converted to ng/ml by plotting against suitable reference curves.

Table 2 T and B cell membrane markers (percentage of positive cells), and T4/T8 ratio in the patients studied and in the control population

	Patients	Controls	p
OKT4	46.2±9.2*	49.4±7.9	—
OKT8	36.6±10.1	30.4±7.3	—
OKIa	12.3±7.8	3.7±2.1	<0.001
SmIg	13.1±7.2	7.7±3.0	<0.02
T4/T8	1.4±0.8	1.6±0.4	—

*Values are mean±SD.

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR IgM RF

Levels of IgM RF in the sera and supernatants of both unstimulated and PWM stimulated cultures were determined by an ELISA as previously described.⁸

Results

Table 2 shows the relative percentages of T cell subpopulations and B cells in patients and controls. Since the mean lymphocyte count did not differ significantly between the two groups the numbers shown also reflect changes in absolute values, the main finding being an increase in activated B lymphocytes (SmIg⁺, Ia⁺ cells).

No significant differences were noticed between patients and controls as far as Ig synthesis was concerned. This applied to both spontaneous and PWM induced production (Table 3). This finding was further supported by the observation that the serum Ig levels were not significantly different in patients and controls (see Table 1) even though three patients had IgM levels much higher than the normal range. In spite of this, patients' B lymphocytes produced large

Table 3 Spontaneous (PWM⁻) and PWM induced (PWM⁺) Ig synthesis by peripheral blood lymphocytes from patients and controls

	Patients		Controls	
	PWM ⁺	PWM ⁻	PWM ⁺	PWM ⁻
IgG (ng/ml)	7086±1692*	1099±300	5138±1351	603±104
IgA (ng/ml)	3483±1357	458±115	2682±643	650±215
IgM (ng/ml)	1987±971	384±199	1373±508	144±71

*Values are mean±SEM.
SI conversion: ng/ml×10⁻⁶=g/l.

Fig. 1 Spontaneous (a) and PWM induced (b) IgM RF synthesis by peripheral blood lymphocytes from patients and controls.

amounts of IgM RF, which was not the case with the cells of control subjects (PWM⁻: mean $\pm$ SEM 77.8 $\pm$ 34.6 v 1.2 $\pm$ 0.8 ng/ml, $p < 0.02$; PWM⁺: 286.1 $\pm$ 141.7 v 3.1 $\pm$ 1.8 ng/ml, $p < 0.05$) (Fig. 1). In particular, 10 out of 14 patients (71%) produced amounts of IgM RF greater than the normal range, calculated as the mean $\pm$ 2SD of the controls. Interestingly a good correlation existed between the amount of RF produced in vitro and serum RF levels ($p < 0.01$ for basal production; $p < 0.05$ for PWM induced production). On the other hand, no correlation was found with other clinical features.

Discussion

To our knowledge this is the first study of in vitro production of RF by peripheral blood lymphocytes from patients with type II EMC. This has been done extensively in RA, a closely related disorder, where an increased production has been described by many workers.^{7 12 13} Such an increased production has been found in our patients as well and, interestingly, it was not associated with other significant disturbances in Ig production. A good correlation existed between serum IgM RF and in vitro production of RF, as described for RA patients.^{12 13} Moreover, no major changes were found in peripheral blood lymphocytes except for a marked increase in activated B cells.

These data suggest an expansion of B cells committed to IgM RF production. Whether this is a primary event or, as suggested by others,^{5 14} a result of an impaired T cell regulation, cannot be decided on the basis of our findings. It is, however, interesting to notice that since T cell subsets appeared to be in the normal range an intrinsic B cell defect seems to be more likely, even if a functional T cell defect cannot be ruled out in the presence of normal T lymphocyte subsets.

Moreover, the coexistence of a B and T helper defect has been reported in type III EMC to explain a reduced in vitro Ig synthesis.¹⁵ On the basis of our results we can only speculate on the nature of the RF which is synthesised; it has been recently shown, however, that PWM induced blasts from the peripheral blood of EMC patients, and bone marrow B cells, express the same cross reactive idiotype of circulating RF.^{16 17} It should also be noted that, as described for RA,⁷ some of our patients did not produce IgM RF, the only distinguishing feature of these subjects being the low amount of circulating RF. When cryoglobulinaemic lymphocytes were stimulated with PWM they responded by secreting increased levels of RF (from 77.8 $\pm$ 34.6 to 286.1 $\pm$ 141.7 ng/ml), but the IgM RF/IgM ratio was lower than in unstimulated cultures, suggesting the presence of B lymphocytes committed to in vivo IgM

RF production. These could hardly be influenced by a polyclonal activator in vitro. Our results seem to indicate that type II EMC should be considered related in some way to lymphoproliferative disorders, as already suggested by the development of such diseases in patients with EMC^{3 18} and by the high incidence of secondary cryoglobulinaemias in such malignancies.^{2 12} Normal in vitro polyclonal Ig production appears to be maintained in EMC, as also suggested by normal Ig serum levels. This is frequently not the case in lymphoid neoplasms (unpublished data).^{19 20}

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