SCIENTIFIC PAPERS

Production of IgM rheumatoid factor by normal lymphocytes after stimulation with preparations containing IgM rheumatoid factor from patients with juvenile rheumatoid arthritis

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Abstract

Preparations containing IgM rheumatoid factor (RF) and hidden IgM RF were isolated from the serum samples of nine patients with juvenile rheumatoid arthritis. Six of these preparations stimulated lymphocytes from normal donors to produce IgG and IgM, of which up to 11% had IgM RF activity. In contrast, the polyclonal activator pokeweed mitogen also stimulated IgM production, but only 1% had IgM RF activity.

A relation between the activator and IgM RF or hidden IgM RF is suggested. This is based on the positive correlation between IgM RF concentration in these preparations and their ability to stimulate lymphocytes to produce IgG, IgM, and IgM RF.

These data indicate that preparations from patients with juvenile rheumatoid arthritis containing IgM RF and hidden IgM RF are potent stimulants of lymphocytes from normal donors, with specific production of IgM RF.

Rheumatoid factors (RFs) are immunoglobulins of the IgM, IgG, and IgA classes directed toward the Fc fragment of autologous, homologous, or heterologous IgG.¹ They are an important and integral component of the inflammatory process, yet their role in the disease process is not well understood.¹ Several infections and inflammatory disorders cause reversible increased synthesis of IgM RF.¹ In rheumatoid arthritis and juvenile rheumatoid arthritis, however, this increased synthesis of IgM RF is chronic, usually irreversible, and related to disease activity in juvenile rheumatoid arthritis.¹ The cause of this prolonged synthesis of IgM RF is not clear. Several explanations have been suggested, such as chronic antigenic stimulus, lymphocyte dysfunction, expansion of a B cell population committed to IgM RF synthesis, or alteration of the idiotype-anti-idiotype interactions.²

Polyclonal B cell activators, Epstein-Barr virus and pokeweed mitogen, have been shown to increase the synthesis of RF in patients with rheumatoid arthritis.²⁻⁶ Low concentrations of human aggregated IgG have been shown to stimulate production of IgM RF in peripheral blood lymphocytes from patients with rheuma-

toid arthritis but not in lymphocytes from normal donors.⁷

Evidence for the role of IgM RF itself in B cell activation came from studies showing stimulation of lymphocyte transformation induced by preparations containing RF from patients with rheumatoid arthritis.⁸ Hobbs *et al* reported that preparations containing IgM RF from two adults (one with rheumatoid arthritis and the other with primary Sjögren's syndrome) induced polyclonal activation of B lymphocytes from normal subjects.⁹ They suggested that RFs contribute to the formation of B cell stimulating immune complexes.⁹ This has been supported by a recent study showing stimulation of IgM RF synthesis by immune complexes from patients with rheumatoid arthritis, but not by immune complexes from patients with Hodgkin's disease.¹⁰ We undertook this study to determine whether such stimulation may be induced with preparations containing RF and hidden RF from patients with juvenile rheumatoid arthritis.

Materials and methods

SOURCE OF SERUM SAMPLES

Blood was drawn from nine female patients with juvenile rheumatoid arthritis—seven had polyarticular onset disease and two pauciarticular onset disease. All were outpatients at the St Louis University Children's Arthritis Clinic at Cardinal Glennon Children's Hospital or the St Louis University Doctors' Office Building. The study was previously approved by the St Louis University institutional review board. All patients met the criteria for the diagnosis of juvenile rheumatoid arthritis established by the committee on juvenile rheumatoid arthritis of the American Rheumatism Association.¹¹ Mean age of onset of disease was 10 years. Mean disease duration before the study was 8.2 years. Four of nine patients were seropositive for 19S IgM RF and five were positive for hidden IgM RF—that is, 19S IgM RF which can be detected in the fraction containing IgM after separation of serum by acid-gel filtration.¹²

ISOLATION OF IgM RICH FRACTIONS

Serum samples (3 ml) were subjected to acid-gel filtration.¹² IgM rich fractions were pooled,

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concentrated, and dialysed against phosphate buffered saline (PBS).

ISOLATION OF RF RICH PREPARATIONS

Heat denatured human IgG was coupled to Reacti-Gel ($\times 6$) beads (Pierce Chemical Co, Rockford, IL). IgM rich preparations were applied to the column and incubated for 72 hours at 4°C. The fractions were then eluted sequentially with PBS and 0.1 M glycine-HCl (pH 2.9) and monitored for absorbance at 280 nm. Fractions containing protein from each eluant were pooled. The acid fractions were neutralised with NaOH to pH 7.0.

SEPARATION OF PERIPHERAL BLOOD

LYMPHOCYTES

Heparinised blood (20 ml) from healthy adults was separated on Ficoll-Hypaque cushions. Peripheral blood lymphocytes were collected and washed with RPMI 1640 (Gibco Laboratories Life Technologies, Chagrin Falls, OH), then further separated by the E rosetting technique. Rosette forming cells (E^+) and E^- cells were resuspended separately in 2 ml RPMI 1640 with 10% fetal calf serum, 100 μ g penicillin G, and 10 μ g gentamicin.

CULTURE OF PERIPHERAL BLOOD LYMPHOCYTES WITH RF RICH PREPARATIONS

Phosphate buffered saline and acid-glycine preparations were added to microtitre plate wells (Dynatech Laboratories, Alexandria, VA) in triplicate. E^+ and E^- cells were added in 2:1 ratio, at a final cell concentration of 10^6 cells/well (E^+) and 5×10^5 cells/well (E^-). As controls were used peripheral blood lymphocytes stimulated with pokeweed mitogen (50 μ g/ml) or phytohaemagglutinin (100 μ g/ml), lymphocytes cultured with RPMI 1640 and fetal calf serum without stimulants, and also PBS and acid-glycine preparations containing RF without added lymphocytes. Samples were incubated at 37°C for five days in duplicate, the third well harvested immediately, and supernatants stored at -70°C until assayed (day 0 sample). Cultures were fed with 100 μ l/well RPMI 1640 on the second day. Duplicate wells were harvested after five days and the supernatants stored at -70°C until assayed.

DETERMINATION OF ANTIBODY PRODUCTION

Antibody production was determined by an enzyme linked immunosorbent assay (ELISA) for IgM RF measurement.¹³ Briefly, microtitre plate wells were coated with human IgG. Serially diluted samples and WHO standard were added and incubated overnight, allowed to react with alkaline phosphatase conjugated anti-human IgM (Sigma Chemical Co, St Louis, MO), developed with Sigma 104 phosphatase substrate, and the absorbance at 405 nm monitored.

For IgM determination the wells were coated with goat antihuman IgM (Pel Freez, Rogers, AR) and the procedure continued as outlined above.

For the IgG determination the wells were coated with goat antihuman IgG (Pel-Freez, Rogers, AR). Samples were added, allowed to react with alkaline phosphatase conjugated goat antihuman IgG ($F(ab')_2$) (Sigma), and the procedure continued as outlined above.

STATISTICAL ANALYSIS

The significance of the difference between means was evaluated by Student's *t* test and coefficients of correlation for relationship of variables by Pearson's method.

Results

PREPARATIONS CONTAINING IgM RF

IgM enriched fractions obtained by gel filtration on Sephacryl S-300 were separated to obtain IgM RF enriched fractions and IgM RF depleted fractions by affinity column chromatography specific for human IgG. The RF depleted fractions (eluted with PBS, pH 7.2) contained IgG (474 (SD 450) μ g/ml), IgM (790 (1126) μ g/ml), and a small amount of IgM RF (1.2 (1.9) μ g/ml), representing 0.3 (SD 0.3)% of the total IgM. Rheumatoid factor enriched fractions (eluted with glycine buffer, pH 2.9) contained small amounts of IgG (110 (140) μ g/ml) and IgM (90 (106) μ g/ml), but the IgM RF content was increased (6.6 (7.8) μ g/ml) and it contributed a larger percentage of the total IgM (10 (8.3)%).

The four preparations from patients with the 'classic' IgM RF (Nos 1-4) had the largest concentration of IgM RF in the fractions eluted with both PBS and acid-glycine (mean (SD) 2.6 (2.3) μ g/ml and 12.8 (8.3) μ g/ml respectively). The five preparations from patients with 'hidden' RF (Nos 5-9) had smaller concentrations of IgM RF in their two fractions (0.2 (0.3) μ g/ml and 1.6 (1.7) μ g/ml respectively).

SYNTHESIS OF IgM RF

The preparations containing IgM RF were used to stimulate immunoglobulin synthesis by peripheral blood lymphocytes from normal donors. Significantly increased synthesis of IgM RF was noted after incubation with six of the nine acid-glycine fractions and two of the nine PBS fractions as measured by comparing stimulated IgM RF synthesis and synthesis in cells without stimulation (table 1). Figure 1 shows the strong positive correlation between the concentration of IgM RF (the stimulant) from nine serum samples and the synthesis of IgM RF by the peripheral blood lymphocytes ($r=0.91$, $p<0.001$ for the acid-glycine fractions; $r=0.96$, $p<0.001$ for the PBS fractions). Figure 1 also shows that the acid-glycine fractions were more stimulatory than the PBS fractions, the slopes being $y=275x$ and $y=42x$ respectively.

The RF percentage of the synthesised IgM was increased significantly after incubation with the acid-glycine fractions (mean (SD) 5 (1)% *v* 1.1 (0.1)% from the pokeweed mitogen stimulated cells, $p<0.02$) (table 1). This suggests a specific increase in IgM RF synthesis by the acid-glycine fractions.

IgM SYNTHESIS

Increased synthesis of IgM was noted in six samples stimulated with acid-glycine fractions containing RF and in three samples stimulated

with PBS fractions containing RF. These were significant when compared with IgM production of unstimulated peripheral blood lymphocytes (table 1). Here, too, there was a strong

Table 1 Synthesis of IgM rheumatoid factor, IgM, and IgG by peripheral blood lymphocytes after stimulation with phosphate buffered saline and acid-glycine fractions, pokeweed mitogen, and phytohaemagglutinin†

Case No	Stimulant†	IgM RF† ($\mu\text{g}/10^6$ PBL†)	IgM ($\mu\text{g}/10^6$ PBL)	IgM RF/ IgM (%)	IgG ($\mu\text{g}/10^6$ PBL)	IgM RF (stimulant) ($\mu\text{g}/\text{ml}$)
1	G	4.6 (0.3)*	77 (2.6)*	6.0	126 (11)*	22
	P	0.02 (0.002)	34 (6)*	0.06	13.7 (1.8)*	1.9
2	G	6.5 (0.26)*	169 (42)*	3.8	150 (5.8)*	17.5
	P	0.3 (0.08)*	114 (10)*	0.3	20 (2.9)*	6.0
3	G	3.66 (0.45)*	33 (7)*	11.1	79 (0.4)*	7.2
	P	0.03 (0.02)	0.8 (0.6)	3.7	19.3 (2)*	1.5
4	G	0.63 (0.18)*	7.5 (0.8)*	8.4	43.7 (4)*	4.5
	P	0.05 (0.003)*	2.1 (1)	2.3	3.0 (1)	0.9
5	G	0.46 (0.04)*	14.4 (4.5)*	3.2	30.1 (3.6)*	4.2
	P	0.01 (0.002)	9.2 (2)*	0.1	12.6 (0.9)*	0.8
6	G	0.32 (0.1)*	4.5 (1.5)*	7.0	1.4 (0.5)	2.5
	P	0	4.4 (3)	0	4.0 (3.5)	0
7	G	0.04 (0.04)	1 (0.5)	4.0	17.8 (0.3)*	0.9
	P	0	0.1 (0.1)	0	0.4 (0.3)	0
8	G	0.04 (0.03)	3 (2)	1.3	9.8 (7.0)	0.4
	P	0.01 (0.01)	2.3 (1.3)	0.6	3.5 (1.6)	0.1
9	G	0.01 (0.01)	2.1 (1.0)	0.5	7.0 (3.7)	0.1
	P	0	1.1 (1.0)	0	0.7 (0.6)	0
	Unstimulated PBL	0	0.16 (0.01)	0	0.42 (0.02)	0
	PWM†	0.013 (0.002)*	1.2 (0.04)*	1.1	5.8 (0.8)*	
	PHA†	0	0.1 (0.04)	0	7.8 (6.8)	

* $p < 0.05$ v unstimulated peripheral blood lymphocytes.

†G=acid-glycine fraction; P=phosphate buffered saline fraction; RF=rheumatoid factor; PBL=peripheral blood lymphocytes; PWM=pokeweed mitogen; PHA=phytohaemagglutinin.

‡Serum samples from nine patients with juvenile rheumatoid arthritis were subjected to chromatography on Sephacryl S-300 to obtain IgM fractions, which were subjected to IgG affinity chromatography and elution with phosphate buffered saline (P) and acid-glycine (G). The data represent the difference in content between cultures on days 0 and 5, per 10^6 peripheral blood lymphocytes. Results are mean (SEM) of four to eight assays. The right column shows the corresponding concentration of IgM RF used as the stimulant.

Figure 1 Correlation between stimulation of peripheral blood lymphocytes (PBL) by IgM rheumatoid factor (RF) and synthesis of IgM RF. ●=synthesis after stimulation with acid-glycine fractions containing RF; ○=synthesis after stimulation with phosphate buffered saline fractions containing RF.

Figure 3 Correlation between stimulation of peripheral blood lymphocytes (PBL) by IgM rheumatoid factor (RF) and synthesis of IgG. ●=synthesis after stimulation with acid-glycine fractions containing RF; ○=synthesis after stimulation with phosphate buffered saline fractions containing RF.

Figure 2 Correlation between stimulation of peripheral blood lymphocytes (PBL) by IgM rheumatoid factor (RF) and synthesis of IgM. ●=synthesis after stimulation with acid-glycine fractions containing RF; ○=synthesis after stimulation with phosphate buffered saline fractions containing RF.

Table 2 Dose dependency of the response of peripheral blood lymphocytes (PBL) to fractions containing IgM rheumatoid factor (RF)*

IgM RF (stimulant) ($\mu\text{g}/\text{ml}$)	Immunoglobulin synthesis ($\mu\text{g}/10^6$ PBL)		
	IgM RF	IgM	IgG
22 (0.7)	5.5 (2)	127 (30)	166 (15)
16.6 (0.5)	6.0 (1.1)	139 (32)	156 (8)
11 (0.1)	4.0 (0.5)	101 (11)	111 (31)
7.2 (0.1)	3.7 (0.1)	33 (2)	79 (5)
3.6 (0.01)	0.4 (0.001)	7 (1)	21 (0.4)
2.2 (0.01)	0.4 (0.001)	5 (1)	2 (0.2)

*IgM RF enriched fractions from serum samples of four patients with juvenile rheumatoid arthritis were further concentrated or serially diluted to equalise their IgM RF concentration. Peripheral blood lymphocytes were incubated with these fractions for five days, and the de novo IgG, IgM, and IgM RF concentration determined by an enzyme linked immunosorbent assay (ELISA). Results are mean (SEM) of three to six assays with each of the four serially diluted fractions. Controls were PBL incubated without stimulants and IgM RF enriched fractions incubated without PBL.

positive correlation between the concentration of IgM RF used as a stimulant and the stimulatory effect on IgM production (fig 2). In contrast with the IgM RF synthesis, here the PBS fractions were more stimulatory than acid-glycine fractions ($y=16.7x$ and $y=4.8x$) respectively. There was no correlation between the initial IgM concentration and IgM synthesis.

IgG SYNTHESIS

IgG synthesis was significantly increased in six samples after stimulation with acid-glycine fractions and in four samples after stimulation with the PBS fractions, when compared with spontaneous IgG synthesis by unstimulated peripheral blood lymphocytes (table 1). This also correlated positively with the presence and concentration of IgM RF in the stimulating preparation (either PBS or acid-glycine fraction) (fig 3). There was no correlation between the initial IgG concentration and IgG synthesis.

IgG RF SYNTHESIS

Trace amounts of IgG RF were detected in only three samples (Nos 4, 7, and 8) (data not shown).

DOSE DEPENDENCY OF IMMUNOGLOBULIN SYNTHESIS

As most fractions had very low concentrations of IgM RF (figs 1–3), making the dose-response relations somewhat difficult to analyse, we diluted or further concentrated four acid-glycine fractions (Nos 1–4) to obtain equal IgM RF concentrations, then incubated the lymphocytes with these samples after serial dilutions. The results (table 2) show again the IgM RF dose dependency of immunoglobulin synthesis by peripheral blood lymphocytes. There was no correlation between the initial IgG and IgM concentrations of these fractions and their stimulatory properties.

Discussion

Stimulation of peripheral blood lymphocytes from normal donors to produce IgM RF was induced by six of nine IgM RF rich samples isolated from patients with juvenile rheumatoid arthritis. There was a strongly positive correlation between IgM RF content in the preparations and the ability to induce lymphocyte response. This suggests that IgM RF isolated from patients with juvenile rheumatoid arthritis has a role in producing this activation.

A major concern was the possibility that the IgM RF, IgM, and IgG which were measured in the culture supernatants might be largely composed of the stimulant fraction added at the start of the culture and might not indicate actual synthesis. This is most unlikely as there was a significant difference in IgM RF, IgM, and IgG concentrations in wells with or without lymphocytes after five days of incubation, while day 0 samples that were assayed for IgM RF, IgM, and IgG concentration showed no difference between wells with or without lymphocytes. Furthermore, if a 'carry over' existed, one

would expect a significant correlation between initial and final concentrations of IgG, IgM, and IgM RF. Although there was a good correlation between IgM RF concentration in the stimulant and the final IgM RF at the end of incubation, no such correlation could be found for initial and final content of IgM and IgG. This suggests that *de novo* synthesis of IgM RF, IgM, and IgG is related to the IgM RF content in the stimulant.

IgM RF synthesis induced by the fractions containing IgM RF was quite specific. Pokeweed mitogen, a polyclonal B cell activator, stimulated the peripheral blood lymphocytes to synthesise IgM with a 1.1% content of IgM RF (table 1), which is comparable with previous studies.¹⁴ Yet, these peripheral blood lymphocytes from normal donors synthesised IgM with significantly higher content of IgM RF (5%) upon incubation with preparations containing IgM RF. Nevertheless, the percentage of IgM RF produced was less than reported with lymphocytes from adult patients with rheumatoid arthritis.^{14 15}

It was of interest to note the marked difference between the stimulatory properties of the PBS and acid-glycine fractions. The stimulatory effect of both was dependent on concentration (table 1, figs 1–3), but the PBS fractions (presumably containing low affinity IgM RF) were more stimulatory for total IgM production and less for IgM RF production ('non-specific' stimulation), whereas the acid-glycine fractions (presumably containing high affinity IgM RF) showed the opposite pattern, being more 'specific' stimulants for IgM RF synthesis (figs 1 and 2).

Whether IgM RF itself or IgM RF-IgG immune complexes are responsible for this activation is unclear. Most patients with juvenile rheumatoid arthritis with classic or hidden IgM RF have immune complexes containing IgM RF and IgG in their sera.^{13 16} Hobbs *et al* suggested that the immune complexes were responsible for peripheral blood lymphocyte activation.⁹ This observation was based on their inability to induce such a response using purified monoclonal IgM RF, and their demonstration that a high degree of large complexes containing RF were characteristic of their stimulatory preparations. Other studies showed that immune complexes from patients with rheumatoid arthritis,¹⁰ antigen-antibody complexes,¹⁷ or aggregated IgG^{18 19} were able to activate B cells. Our observation is in agreement with these studies, particularly as the activation seems to be specific, but we cannot rule out the possibility that IgM RF itself may activate peripheral blood lymphocytes. IgM RF has the ability to bind complement.¹ *De novo* complement fixation during the incubation period was unlikely, however, as the fetal calf serum in the incubation medium was prewarmed to 56°C. Therefore, it is improbable that the noted immunoglobulin response is related to complement activation.

The mechanism by which preparations containing RF stimulate B cells is unknown. We cultured the peripheral blood lymphocytes using a mixture of B and T cells. Fractions

containing RF from adults' serum samples do activate B cells cultured without T cells, but the B cell activation was optimal in the presence of T cells,⁹ suggesting a T cell dependence of the RF associated antibody response. Still, it is not clear whether the RF activated the B cells through primary activation of T cells, or directly, with some non-specific permissive role of the T cells.

A recent observation may suggest another explanation. Okawa-Takatsui *et al* demonstrated a suppressive role for normal monocytes in the production of RF.²⁰ We suggest that RF, or immune complexes containing RF, might have been processed by the monocytes, causing inhibition of this suppressive function, and consequently increased synthesis of IgM RF.

Our study confirms other reports showing that cells able to synthesise IgM RF are present in peripheral blood lymphocytes from healthy subjects and that such cells can be activated by various stimuli.^{8-10 21 22} In this study we showed that fractions containing IgM RF and hidden IgM RF from patients with juvenile rheumatoid arthritis also have this property. Thus in this respect RF and hidden RF from patients with juvenile rheumatoid arthritis were shown to be comparable with RF from adult patients with rheumatoid arthritis. Our observation might explain the self perpetuating and chronic nature of juvenile rheumatoid arthritis; yet, the initiating mechanism remains to be identified.

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