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Defect of Suppressor Cell Induction in Patients with Juvenile Rheumatoid Arthritis

Hideo Murata and Junichi Yata

SUMMARY **We studied the suppressor cell activity induced by concanavalin A (Con A) in 9 patients with acute febrile juvenile rheumatoid arthritis (JRA). The suppressor activity of JRA patients was higher than that of normal controls. However, the activity was significantly reduced by treating Con A-activated cells with mitomycin C (MMC) ($P < 0.05$). On the other hand, the suppressor activity of normal controls and systemic lupus erythematosus (SLE) patients was not affected by MMC treatment. Two of 5 SLE patients showed low activity even before MMC treatment. The addition of the culture supernatant of Con A-stimulated peripheral blood mononuclear cells from a normal donor restored the induction of suppressor activity of JRA which was decreased by MMC treatment. The results indicated that patients with acute febrile type of JRA had reduced MMC resistant suppressor cell activity and that this was due to a defect in the ability of the cells to produce soluble factors needed to induce MMC resistant suppressor cells.**

Juvenile rheumatoid arthritis (JRA) is a well known collagen disease in childhood characterized by arthritis, fever, skin rash and frequently accompanied by pericarditis and/or iridocyclitis. JRA patients have hypergammaglobulinemia, and sometimes antinuclear antibody (ANA) and/or rheumatoid factor (RF). These suggest hyperreactivity of humoral immunity in JRA. Strelkauskas *et al*[1] reported that patients with active JRA had significantly higher numbers of circulating B cells secreting immunoglobulin. In SLE patients who also have hypergammaglobulinemia and autoantibodies, evidence suggesting a decreased suppressor T cell activity has accumulated.[2-6] Astrup *et al*[7] showed that JRA patients had decreased concanavalin A (Con A) induced suppressor cell activity as demonstrated by their reduced ability to suppress the proliferation in response to autologous cells. We studied the suppressor cell activity of JRA patients on immunoglobulin production from pokeweed mitogen (PWM) driven B cells. This report showed that Con A activated suppressor cells in JRA were more potent than normal, but in contrast to normal individuals their activity was significantly reduced by mitomycin C (MMC) treatment after induction culture. This reduced suppressor activity could be restored by addition of culture supernatant of Con A activated peripheral blood mononuclear cells (PBMC) from a normal subject.

MATERIALS AND METHODS

Patients

Subjects with acute febrile JRA were studied. Control blood samples were obtained from normal healthy volunteers and from patients with SLE. Only those for whom a positive diagnoses of JRA or SLE was made were included in this study. The diagnosis of JRA were made according to the ARA criteria.[8] There were 9 patients with JRA, 5 boys and 4 girls, whose mean age at the time of study was 7.2 years (range, 3-11). All of them had active disease at the time when blood samples were taken. The criteria for classification as severe or moderate disease were based on objective findings, *i.e.* pericarditis, serositis, iridocyclitis, and fever resistant to nonsteroidal anti-

From the Department of Pediatrics, Tokyo Medical and Dental University School of Medicine, Tokyo, Japan

inflammatory drugs (NSAIDs). Thus, children with one or more of the above findings were considered to have severe disease. Patients with moderate disease had arthritis, fever and/or rash which responded well to NSAIDs. Patients with SLE had active disease at the time of study. They had at least two or more of the following signs and symptoms : fever, butterfly rash, arthritis, active renal disease, serositis or hematological disorders. They were 1 boy and 5 girls whose mean age at the blood sampling was 12.3 years (range, 9-16).

Lymphocyte preparation

PBMC were isolated from heparinized venous blood by Ficoll-Conray density gradient centrifugation.[9] The cells were washed three times and resuspended in culture medium : RPMI 1640 (Grand Island Biological Co., N.Y., NY, USA.) supplemented with 10% FCS (Grand Island Biological Co., N.Y., NY, USA.), 20 mM HEPES buffer (Flow Laboratories, North Ryde, Australia), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cell concentration was adjusted to 2×10^6/ml.

Induction of suppressor cells

PBMC were incubated with 10 μg/ml of Con A (Pharmacia Fine Chemicals, Uppsala, Sweden) at a concentration of 10^6 cells/ml for 48 or 72 hr at 37°C in a humid atmosphere of 5% CO_2 and 95% air. After incubation, cells were divided into two groups, one of which was treated with 25 μg/ml of MMC (Sigma Chemical Co., St. Louis, MO, USA.) for 20 min. After treatment, both groups of cells were washed three times, and resuspended in culture medium to a concentration of 2×10^6 viable cells/ml.

Suppressor cell assay.

Freshly isolated PBMC from donor were cultured in round bottom microtiter culture plates (Nunc, Roskilde, Denmark; Cat. No. 16330) at 37°C in a humid atmosphere of 5% CO_2 and 95% air for 7 days in RPMI 1640 supplemented with 10% FCS, 20 mM HEPES buffer, 100 U/ml penicillin, 100 μg/ml streptomycin. The total well volume in all experiments was 0.2 ml. To this PWM (Grand Island Biological Co., N.Y., NY, USA.) was added to the final concentration of 250x because the maximum production of IgG took place at that concentration. To determine the suppressor activity of Con A-activated cells on the secretion of IgG, 10^5 Con A-activated cells with or without prior MMC treatment were added to 10^5 freshly isolated allogeneic PBMC at the initiation of cultures. Control cultures contained normal PBMC and PWM. PBMC used for IgG secretion were always taken from the same healthy donor. All cultures were performed in triplicate. On day 7, cultures were terminated and supernatants were harvested. IgG secreted into the supernatant was quantitiated by a solid phase radioimmunoassay using affinity purified γ-chain specific goat anti-human IgG (Cappel Laboratories Inc. USA; Cat. No. 06010121).[10] The percent suppression of IgG secretion was calculated according to the following formula :

$$\%\text{suppression} = \left[1 - \frac{\text{IgG (suppressor cell+PBMC)}}{\text{IgG (PBMC)}}\right] \times 100$$

Schwartz *et al.*[11] suggested that even the cultured PBMC without Con A could suppress IgG production although the suppressor activity was much lower than that of cells activated by Con A. We also had the same experience. Therefore we used wells containing only fresh PBMC and PWM as a control in order to escape from false positive results for reduced suppressor cell activity.

Preparation of Con A supernatant

To obtain a supernatant, 10^6 PBMC/ml were cultured for 12 hr in culture tubes (Falcon, Oxnard, CA; Cat. No. 2054) in 1 ml of RPMI 1640 supplemented with 10% FCS, 20 mM HEPES, and 10 μg/ml of Con A. The supernatant was membrane-filtered (pore size 0.45 μm, Millipore Co., Bedford, MA, USA.).

Detection of antilymphocyte antibody

The modified Terasaki method was used.[12] Briefly, 10^5 PBMC in 25 μl were incubated with heat inactivated serum at a concentration of 1:5 at 4°C for 1 hr. After washing twice, the cells were resuspended in 25 μl of RPMI 1640 containing baby rabbit complement (Pelfreeze, Rogers, AR.) at a dilution of 1:3, and incubated at 15°C for 3 hr. The viable cells were counted by the trypan blue dye exclusion method. Percent cytotoxity more than mean

$$+\sqrt{u^2 \times (n+1) \div n \times F^1_{n-1}\ (0.05)}$$

of normal sera were considered positive, where n is the number of normal sera used as control, and u^2 is the unbiased variance. Cytotoxic tests were performed using PBMC derived from at least 3 different donors.

Statistical analysis

Data were analysed with the Student's *t* test.

RESULTS

Fig. 1 shows the percent suppression of IgG production by Con A induced suppressor cells from JRA, SLE and control subjects. With regard to suppressor cell activity without MMC treatment, that of JRA patients was more potent than normal subjects ($P < 0.05$, Table 1). Two of 5 SLE patients showed reduced suppressor activity ($P < 0.01$, Fig. 1). On the other hand, when Con A activated

cells were treated by MMC, suppressor cell activity from JRA pa--tients was significantly reduced ($P < 0.05$, Table 1). Especially 6 of 9 JRA patients showed dramatic reduction of suppressor activity. In normal and SLE subjects, the difference between MMC-treated and non-treated Con A activated cells was not significant. A representative example is shown in Fig. 2. PBMC from a normal subject secreted 6400 ng/ml of IgG. When Con A activated cells from one JRA patient were added to this, the IgG production was suppressed to 700 ng/ml (% suppression = 89%). However, when Con A stimulated cells from the patient were treated by MMC, the suppressor activity was reduced to the level of 33%. The cells activated by Con A in the presence of the Con A supernatant from a normal subject showed enough suppressor activity (% suppression = 83%) even when treated by MMC. Two other JRA patients that could be tested showed similar results. Table 2 shows the relation between disease activity and suppressor function or anti-lymphocyte antibody. Severely ill patients seemed to have profound reduction of suppressor activity after MMC treatment and/or higher rate of presence of anti-lymphocyte antibody.

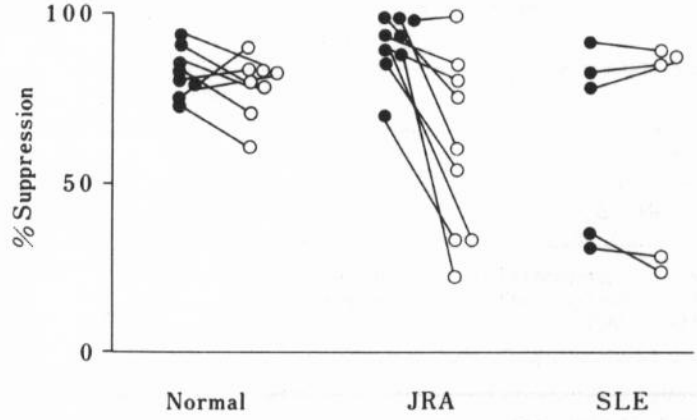

Fig. 1 PBMC (10^6/ml) were stimulated by Con A (10 μg/ml) and incubated for 48 hr. Then half of them were treated by MMC (25 μg/ml) for 20 min. Con A activated cells with (○), or without (●) MMC treatment were added to PWM driven Ig production system.

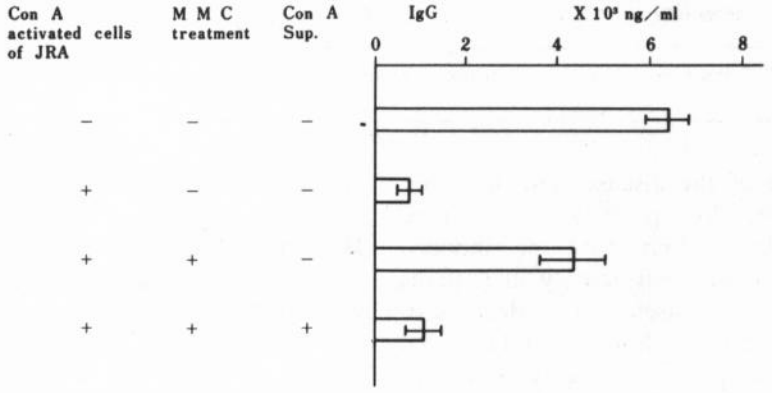

Fig. 2 Restoration of the suppressor cell activity lost by MMC treatment by addition of supernatant of Con A stimulated normal cells. PBMC from a healthy individual were incubated with Con A (10 μg/ml) for 12 hrs, then supernatant was harvested and used as Con A-sup. Patient's PBMC were stimulated by Con A (10 μg/ml) and incubated for 12 hr, then Con A-sup. was added to the culture (final concentration 80%). After more than 36 hr cultured cells were treated by MMC and were added to the allogeneic PWM system. All three JRA patients that could be tested showed similar patterns.

DISCUSSION

In the present study, we have investigated Con A induced suppressor cell activity on IgG production driven by PWM in 9 patients with acute febrile JRA. Though suppressor activity of Con A stimulated cells was somewhat higher than that of normal controls, the activity was significantly reduced when they were treated by MMC. Suppressor activity from normal individuals and patients with SLE was not affected by MMC treatment. Kuritani *et al*[13] reported that Con A induced suppressor cells were still sensitive to MMC treatment even after activation to show their activity. However, according to Ozer *et al*,[14] Con A induced suppressor cells were resistant to MMC treatment in concordance with our results. This discrepancy could be due to the difference in the length of the MMC incubation periods; the times used by Ozer *et al* and ourselves were significanly shorter than used by Kuritani *et al.*[13] Dobloug *et al.*[15] reported that Con A induced suppressor cell activity in patients with JRA was normal, and in agreement with the present study, they also found increased suppressor activity in acute febrile

Table 1 Sensitivity to MMC treatment of suppressor cells induced by Con A

Groups of patients	% Suppression		
	A*	B**	A-B
JRA (N = 9)	91±2.9***	60±8.3	30±7.3
SLE (N = 5)	64±11	63±13	1.0±2.8
Normal (N = 8)	82±2.4	78±2.9	4.4±3.1

* A = % suppression of MMC-non-treated suppressor cells
** B = % suppression of MMC-treated suppressor cells
*** Mean ± SEM

Table 2 The relationship between disease activity and immunological parameters of JRA

Disease activity	Number of patients	
	Profound reduction of suppressor activity *	Anti-lymphocyte antibody positive
Severe	4 / 5	4 / 5
Moderate	2 / 4	1 / 4

* Profound reduction of Con-A induced suppressor cell activity after MMC treatment

type of the disease. On the other hand, Astrup *et al*[7] presented evidence that Con A induced suppressor cell activity in patients with the disease was decreased. The latter authors pointed out that Dobloug *et al*[15] worked in an allogeneic system whereas they used an autologous system. It could be assumed that Con A activated PBMC from a JRA patient could generate suppressor activity using the factor released from normal allogeneic PBMC in the co-culture system. This hypothesis could explain the reduced suppressor cell activity using the autologous system presented by Astrup *et al.*[7] Using a similar allogeneic system, we confirmed the results of Dobloug *et al*,[15] in terms of suppressor activity of cells tested without MMC treatment. The present observation that the suppressor cell activity was reduced significantly by MMC treatment could indicate that either, Con A activated suppressor cells from JRA are MMC sensitive or that in the particular case of JRA, the time for MMC resistant suppressor cell induction required more than 48 hr. In order to differentiate between these two possibilities, we cultured patients' PBMC with Con A for 72 hr, but the suppressor activity was decreased even without MMC treatment (data not shown). This negated the latter possibility. Moreover, supernatants of Con A stimulated PBMC from normal individuals restored MMC resistant suppressor cell activity induced by Con A. The results indicated that PBMC in patients with JRA lacked the ability to produce the humoral factor needed to induce MMC resistant suppressor cells. Morimoto *et al*[16] investigated patients with JRA in an active state. They found that JRA sera were reactive with helper/inducer subsets of T cells and that T cells reacted with JRA sera worked as suppressor inducer cells on IgG secretion stimulated by PWM. Our results resembled that of Morimoto *et al.*[16] However, in our system, it seemed that the impaired function of Con A induced suppressor cells was not due to anti-lymphocyte antibody in patients' sera; treatment of PBMC from normal subjects with antilymphocyte antibody positive patient sera and complement did not affect the sensitivity of Con A induced suppressor cells to MMC (data not shown). One explanation could be that anti-lymphocyte antibodies in the sera we used might react not only with suppressor inducer cells but also with other types of T cells or non-T cells. Anyway, our results seemed to indicate an abnormality in the suppressor circuit in JRA. The patients may lack the T-cell subset which induces suppressor effector T cells via the production of soluble factor in the Con A induced regulatory system. Characterization of the cells which secrete soluble suppressor cell-inducing factor is under investigation.

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