THE IMMUNE RESPONSE OF SPLENIC LYMPHOCYTES AFTER CIMICIFUGOSIDE TREATMENT *IN VITRO* AND PRETREATMENT *IN VIVO*

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Pretreatment of mouse splenocytes with *Shigella* lipopolysaccharide and concanavalin A followed by 50 ng/ml of cemicifugoside resulted in a 69% and 31% inhibition of blastogenesis compared to controls.

The plaque forming colony assay using sheep erythrocytes (SRBC) showed a decreased number of plaque forming colonies after exposure of the splenic cells to 1 μ g/ml of cemicifugoside.

Cemicifugoside, 0.1 mg/mouse *i.p.* suppressed the anti-SRBC response in the plaque forming assay. The major inhibition of the antibody response occurred when cemicifugoside was administered 1 day before the primary immunization with SRBC. The delayed type hypersensitivity to picryl chloride was suppressed after *i.v.* administration of cemicifugoside, 1.0–2.0 mg/mouse.

The immunosuppressive activity of cemicifugoside is preferentially directed toward B-cell function with larger doses being required for suppression of T-cell function.

Keywords—cemicifugoside; triterpenoid xyloside; lymphocyte blastogenesis; Con A; LPS; anti-SRBC PFC; DTH response; immunosuppression

INTRODUCTION

As reported previously,¹⁾ 24 triterpenoids from *Cimicifuga* species, showed potent inhibition of thymidine-³H incorporation into human peripheral blood lymphocytes stimulated with phytohemagglutinin (PHA). Cemicifugoside, a triterpenoid xyloside from *C. simplex*,²⁾ is the most potent inhibitor of thymidine uptake among the rested compounds. In previous studies,^{1,3)} cemicifugoside was found to inhibit transport of nucleosides across the plasma membrane of the mouse lymphoblast L-5178Y cells without affecting their cellular growth and viability.

The uptake of nucleic acid bases, sugars and amino acid was not inhibited by cemicifugoside in L-5178Y cells.

The inhibition by cemicifugoside of the net uptake of L-leucine-1-¹⁴C after stimulation with lipopolysaccharide (LPS) and concanavalin A

(Con A) was studied using mouse spleen cells.

The response of the plaque forming cells (PFC) to sheep erythrocyte (SRBC) immunization after the mouse spleen cell culture was exposed to cemicifugoside given *in vitro* or *in vivo* was studied. Picryl chloride (PCI)-induced delayed-type hypersensitivity (DTH) response was tested after *i.v.* cemicifugoside administration.

MATERIALS AND METHODS

Animals — DBA/2 male, C3H/He male and Balb/C female mice, age 8–12 weeks old (18–23 g weight), were provided by the Institute for Experimental Animals, Tohoku University School of Medicine.

Materials — The L-leucine-1-¹⁴C (56 mCi/mmol) and thymidine-methyl-³H (50 Ci/mmol) were purchased from New England Nuclear (Boston, MA, USA). Cemicifugoside was donated by Dr. G. Kusano (Pharmaceutical Institute,

Tohoku University). The other materials were standard commercial products of reagent grade.

Preparation of Sample for Addition to Cultures and for Administration to Mice — Cimicifugoside was suspended (5 mg/ml) in sterile 0.9% NaCl saline containing 1% human serum albumin and the suspension was sonicated twice at 12 kc for 30 sec. Dilutions were then made with culture medium consisting of RPMI 1640 medium supplemented with 20% FCS and antibiotics as described previously,¹⁾ for additions to cultures or with saline for administration to animals.

Lymphocyte Preparation and Stimulation — Mouse spleen cells in single cell suspension from male DBA/2 mice were washed twice with RPMI 1640 medium and then treated with 0.75% NH₄OH-Tris-HCl buffer (10 mM, pH 7.2) to remove erythrocytes. The ghosts were removed by the bovine fetal calf serum (FCS) gradient procedure. The packed spleen cells were resuspended in the culture medium.

The mitogen stimulated splenic cell cultures were grown in triplicate in microplates (Falcon, 3042). The splenic lymphocytes (4×10^5 cells) were grown in 200 μ l of culture medium containing graduated doses of cimicifugoside and constant doses of Con A (0.4 μ g; Miles-Yeda Ltd., Israel) or *Shigella* LPS (4 μ g; Difco Lab.) in a 37° humidified-5% CO₂ incubator. After incubation for 14 hr, L-leucine-1-¹⁴C (0.5 μ Ci) was added to each culture well, and the cultures were incubated for an additional 4 hr. Then the lymphocytes were harvested and the incorporated radioactivity was counted by a liquid scintillation counter. In some experiments, blastogenesis after the addition of appropriate mitogens was determined by thymidine-³H (0.2 μ Ci/culture) incorporation into 60–72 hr cultures.¹⁾

Hemolytic Plaque Assay — The *in vitro* anti SRBC plaque-forming cells (anti-SRBC PFC) assay was performed using the method of Marbrook.⁶⁾ Spleen cells of DBA/2 male mice were cultured with 3×10^6 SRBC at 37° in a Marbrook chamber. After incubation for 4 days, the number of PFC to SRBC was determined in duplicate using the Cunningham chamber.

The *in vitro* assay for anti-SRBC PFC in C3H/He male mice was performed according to the method of Jerne and Nordine⁴⁾ and Cunningham and Svenberg.⁵⁾ Cimicifugoside was administered intraperitoneally.⁸⁾

Delayed-type of Hypersensitivity Response — The assay for PCI-induced DTH response was performed using the method of Zembra and Asherson.⁷⁾ Immunization with 0.1 ml of 7% PCI in alcohol was applied to the abdomen of Balb/C mice (8 weeks old). Five days later, the immune mice were challenged with 1% PCI in olive oil applied to both sides of both ears. After 2 days, ear thickness was measured by a dial thickness gauge (Peacock, type G, Osaki MFG. Co., Ltd.). Cimicifugoside was administered intravenously 1 day before and 5 days after the primary immunization.

RESULTS

Cimicifugoside Inhibition of Mitogen Stimulated Blastogenesis

L-Leucine-1-¹⁴C uptake by mitogen stimulated splenic lymphocytes after exposure to cimicifugoside was determined. Increasing dose

TABLE I. *Differential Suppression of Blastogenesis by Cimicifugoside on Lectin-Stimulated Lymphocyte^{a)}*

Cimicifugoside (ng/ml)	Percent inhibition			
	Leucine- ¹⁴ C		Thymidine- ³ H	
	LPS	Con A	LPS	ConA
None	0 ^{b)}	0 ^{b)}	0 ^{c)}	0 ^{c)}
5	8	10	76	79
20	36	22	97	96
50	69	31	99	96
100	77	42	>99	>99
500	86	61	>99	>99
1000	99	75	>99	>99

a) Spleen cells from DBA/2 mice at the age of 10 weeks old.

b) Incorporated dpm were: LPS, 2013 ± 96 ; Con A, 3114 ± 63 ; mitogen non, 511 ± 39 .

c) Incorporated dpm were: LPS, 3744 ± 156 ; Con A, 95238 ± 1253 ; mitogen non, 888 ± 52 .

of cimicifugoside inhibited L-leucine-1-¹⁴C uptake in mitogen stimulated lymphocytes. Suppression of L-leucine-1-¹⁴C uptake was greater in LPS stimulated lymphocytes. There was no difference in the uptake of thymidine by the mitogen stimulated lymphocytes exposure to cimicifugoside. However, the thymidine transport system is more sensitive to the inhibitory effects of cimicifugoside than the L-leucine transport system (Table I).

Differential suppression of mitogen stimulated blastogenesis as measured by L-leucine-1-¹⁴C and thymidine-³H uptake in the presence of cimicifugoside occurred. Leucine uptake into the lectin stimulated lymphocytes was less sensitive to cimicifugoside suppression compared to thymidine uptake. However, in the L-5178Y lymphoblast cell line, cimicifugoside did not suppress L-leucine uptake, but did suppress thymidine uptake,^{1,3)} similarly to the suppression of the mitogen stimulated spleen cells.

The inhibition of uptake of L-leucine-1-¹⁴C is not secondary to possible cytotoxic effects of cimicifugoside. Lymphocytes cultured for 4 days with cimicifugoside at 1000 ng/ml showed no cytotoxic effects (data not shown).

Suppression of Sheep RBC Plaque-Forming Cell by

Cimicifugoside

Cimicifugoside, 1 µg/ml, significantly reduced the anti-SRBC PFC number to a non-primed control number (Table II) without affecting the cell viability as measured by trypan blue dye exclusion.

The *in vivo* activity of cimicifugoside was examined. As described previously, cimicifugoside administered to mice at 25 and 50 µg/mouse

TABLE II. *Cimicifugoside Inhibition of the Anti-body-forming Cells to SRBC in Spleen Cell Culture*^{a)}

Condition	PFC ^{b)} /10 ⁶ Cells	%
Non-primed	54	0
SRBC ^{c)}	844	100
+0.01% HSA ^{d)}	735	86
+Cimicifugoside, 0.1 µg/ml	694	81
0.5 µg/ml	325	34
1.0 µg/ml	51	< 0

a) Spleen cells from DBA/2 mice at the age of 8 weeks old.

b) PFC: plaque forming cells.

c) SRBC: sheep erythrocytes.

d) HSA: human serum albumin.

TABLE III. *Decreased Ear Thickness after Cimicifugoside Suppression of the PCL-induced DTH Response in Mice*^{a)}

Primary ^{c)} Challenge	Secondary ^{d)} Challenge	Cimicifugoside mg/mouse	Thickness ^{b)} mm	% Max. Response
None	None	—	0.202 ± 0.0011	—
None	Yes ^{e)}	—	0.216 ± 0.0010	—
Yes	Yes	—	0.290 ± 0.0021	100
Yes	Yes	0.1 × 2	0.285 ± 0.0145	93
Yes	Yes	0.5 × 2	0.264 ± 0.0260	65
Yes	Yes	1.0 × 2	0.227 ± 0.0116	15

a) PCi: picryl chloride; mouse: Balb/C (8 weeks old).

b) Means ± S.D. < 10%; average of 10 ears.

c) Application onto abdomen.

d) Application onto ears, bilaterally.

e) control for local inflammation.

intraperitoneally 2 days before the primary immunization with SRBC, reduced the anti-SRBC PFC to 73% and 62% of the SRBC-primed control, respectively.⁸⁾ In this report, the effect of varying the time of administration of cimicifugoside on the anti-SRBC PFC response was examined. Suppression of anti-SRBC PFC response was found and was most significant when cimicifugoside was given on day 1, before the SRBC priming (Fig. 1). When cimicifugoside injection was delayed after the priming, suppression of anti-SRBC PFC response gradually decreased and approached control level. Therefore, cimicifugoside may be involved with the early stages of the immune response which occurs during antigen recognition in or antigen presentation to immunocompetent cells.

Delayed-type of Hypersensitivity Response

The *in vivo* effect of cimicifugoside on the T-

cell function in the mouse was studied by using the PCI-induced DTH response. The PCI-induced DTH response was found to be markedly suppressed when 1 mg of cimicifugoside was injected intravenously into the mice on days 1 and 5 (Table III). But, it was unclear whether the cimicifugoside suppression of the PCI-induced DTH response may be secondary to immunological specificity, or by high concentration of cimicifugoside, 1 mg/ml, which results in a suspension of fine particles. It is known that fine particles such as silica and latex can suppress macrophage function.⁹⁻¹¹⁾

DISCUSSION

In previous studies,^{1,3,8)} cimicifugoside showed potent inhibition of radioactive thymidine uptake in PHA-treated human peripheral blood lymphocytes and several established animal and human

FIG. 1. *Cimicifugoside Inhibition of the Antibody-producing Cells to SRBC in Immunized Mice*

SRBC (2×10^8 cells) were administered to C3H/He 10-week old male mice intravenously, and 4 days later, the PFC number of spleen cells was measured in a Cunningham chamber. Cimicifugoside (0.1 mg/mouse) was administered intraperitoneally on indicated days (see arrows) before or after the primary immunization with SRBC.

cell lines. *Cimicifugoside* was found to inhibit the uptake of thymidine into the mouse splenocytes stimulated with LPS and Con A at a low dose (5 ng/ml; Table I). The inhibition by *cimicifugoside* of thymidine uptake into mouse splenocytes did not result in the inhibition of blastformation as determined by leucine uptake. The inhibition by *cimicifugoside* on leucine- ^{14}C uptake into the splenocytes treated with LPS and Con A required higher doses of *cimicifugoside* (20–100 ng/ml) compared to inhibitory doses on nucleoside transport. Moreover, this inhibition on leucine- ^{14}C uptake was found to be directed to the splenocytes stimulated by the B-cell mitogen, LPS, rather than the T-cell mitogen, Con A. The difference in sensitivity to *cimicifugoside* of the splenocytes stimulated by B-cell mitogen was by a factor of ten when compared to the results obtained with the T-cell mitogen (Table I). The suppression of B-cell function by *cimicifugoside* was further demonstrated by the results obtained with the anti-SRBC PFC assay compared with DTH response in the mouse (Fig. 1 and Table III).

Many immunosuppressive agents with potent cytotoxicity are known to show a specific suppression of B-cell functions.^{9,10} *Cimicifugoside* shows no cytotoxicity toward lymphocytes, and cell lines that have been exposed to *cimicifugoside*.^{1,3} Although the mechanism of immune suppression by *cimicifugoside* is not known, *cimicifugoside* at low doses inhibits the expression of B-cell functions.

Since the anti-SRBC PFC was suppressed prior to the immunization with SRBC (Fig. 1), *cimicifugoside* exerts its action during the antigen recognition phase or during the antigen presentation process. It is possible that the immune suppression by *cimicifugoside* may have a direct effect on immunocompetent cells. It is thought that *cimicifugoside* did not undergo significant metabolic modifications, since potent suppression of splenocytes occurred after both *in vitro* and *in vivo* administration of *cimicifugoside* when performing the anti-SRBC PFC assay (Table II, Fig. 1).

The role of nucleosides in the immune

response is not known, but thymidine and thymidine-like molecules, are believed to be released by stimulated macrophages *in vitro*, and are thought to serve as immunoregulatory factors.¹¹ Further study of the mechanism of action of *cimicifugoside* in the expression of B-cell function during the immune response is warranted.

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