Phospholipids of *Mycobacterium intracellulare* inhibit T cell blastogenesis

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A crude lipid fraction obtained from *Mycobacterium intracellulare* (MI whole lipids) suppressed concanavalin A (Con A)-induced blastogenesis of murine spleen cells (SPCs). Among three lipid fractions, the phospholipid fraction possessed the highest inhibitory activity, followed by the polar mycoside fraction, but the apolar mycoside fraction showed no activity. Since MI whole lipid and phospholipid fractions inhibited the Con A-induced proliferative response of SPCs which had been treated with Con A before the addition of the lipid fractions, their action is not due to hindrance of Con A binding to T cells. These two MI lipid fractions reduced IL-2-producing ability, acquisition of IL-2 reactivity, and expression of IL-2 receptors in Con A-stimulated T cells. However, they did not affect IL-2-induced proliferation of an IL-2-dependent cytotoxic T cell line, CTLL-2. When SPCs were pretreated with either MI whole lipid or phospholipid fraction for 24 h, an irreversible reduction in Con A responsiveness was seen only in the phospholipid-treated SPCs. The action of these MI lipid fractions was phase-dependent and they exhibited considerably decreased but still significant inhibitory activity against Con A blastogenesis of SPCs, even when they were added at 24 h after the initiation of SPC culture with Con A. MI whole lipids and the three lipid fractions (polar mycoside, apolar mycoside, and phospholipid fractions) did not exhibit suppressor cell-inducing activity, while MI whole lipid fraction antagonized the Con A-mediated generation of suppressor cells. Silica gel thin layer chromatography of the phospholipid fraction showed four spots containing phosphate and one spot without. SPC Con A blastogenesis-inhibitory activity was shared by the two least polar phosphate-containing substances. One of those apparently contained amino groups and carbohydrate moieties. The second component contained no such moieties.

Keywords: *Mycobacterium avium*–*intracellulare* complex, T cells, concanavalin A blastogenesis, immunomodulation

INTRODUCTION

The *Mycobacterium avium*–*intracellulare* complex (MAC), which frequently causes disseminated infections in AIDS patients, is known to persist for long periods at the sites of infection (Collins, 1987, 1989). Previously, we found no obvious elimination of MAC organisms from the liver of mice during the first week after infection (Saito & Tomioka, 1990). This indicates a high resistance of the MAC to the microbicidal activity of liver macrophages

(Mφs). Thereafter, gradual bacterial elimination was seen in MAC-resistant mouse strains with the Bcg^r genotype (CBA, C3H/He, etc.), but continuous bacterial growth occurred in MAC-susceptible mouse strains with a Bcg^s genotype (BALB/c, C57BL/6, etc.) (Skamene, 1989; Saito & Tomioka, 1990). Bacterial growth in athymic nude mice (BALB/c strain) during the first 4 weeks was the same as in euthymic mice, but thereafter growth 1–2 orders of magnitude greater was seen in athymic nude mice, resulting in their death around week 16. Therefore, even in Bcg^s mice, T lymphocytes are important for prevention of the progression of MAC infection to a terminal state. In Bcg^r mice, T cells play an important role in the elimination of MAC organisms from the sites of infection in the immune phase (Gross *et al.*, 1983).

Abbreviations: Con A, concanavalin A; MAC, *Mycobacterium avium*–*intracellulare* complex; MI, *Mycobacterium intracellulare*; Mφs, macrophages; SPCs, spleen cells; TdR, thymidine.

Some bacterial components are known to modulate the host immune system, including T cell functions (Wadee *et al.*, 1980; Kleinhenz *et al.*, 1981; Edwards & Kirkpatrick, 1986; Pabst *et al.*, 1988; Rastogi & David, 1988; Tsuyuguchi *et al.*, 1990; Sussman & Wadee, 1991; Tomioka & Saito, 1992). For instance, polysaccharides of *M. tuberculosis*, including arabinomannan, enhance prostaglandin production of Mφs, thereby reducing T cell functions (Kleinhenz *et al.*, 1981). Similarly, phospholipids such as phosphatidylethanolamine of *M. tuberculosis* (Wadee *et al.*, 1980, 1983) and a lipid component with molecular mass of more than 200 kDa (Sussman & Wadee, 1991) act as suppressor T cell-activating factors. Furthermore, certain types of mycobacterial lipids, including glycopeptidolipids (Brownback & Barrow, 1988), phenolic glycolipid (Stanford, 1986) and sulfatide (Pabst *et al.*, 1988), possess an immunosuppressing activity against T cell functions. Therefore, it is possible that in MAC infections some lipid components of the pathogens modulate the T cell-mediated immune system of the host, thereby resulting in an increase in persistence of the organisms at the sites of infection and an enhanced progression of infection. Indeed, a recent study revealed that a crude lipid fraction from MAC organisms inhibited antigen-presenting cell function (Tsuyuguchi *et al.*, 1990). We therefore studied the effects of some lipid fractions obtained from *M. intracellulare* (MI) on the T cell proliferative response to concanavalin A (Con A).

METHODS

Organisms. *M. intracellulare* N-260 (serovar 16) isolated by our laboratory from a patient with MAC infection was grown on 7H11 agar (Difco) at 37 °C for 1 week, harvested into distilled water, washed twice with distilled water and lyophilized.

Mice. Eight- to ten-week-old female BALB/c mice were purchased from Japan Clea Co.

Medium. RPMI 1640 medium supplemented with 25 mM HEPES, 2 mM glutamine, 100 µg streptomycin ml⁻¹, 100 units penicillin G ml⁻¹, 0.05 mM 2-mercaptoethanol and 5% (v/v) foetal bovine serum (FBS) (M. A. Bioproducts) was used for cell culture.

Preparation of lipid fractions from *M. intracellulare*. The whole lipid fraction was extracted with chloroform-methanol (2:1 by vol.) from lyophilized *M. intracellulare*. After evaporation of the solvent, the lipids were dissolved in dimethylsulphoxide (DMSO).

Three lipid fractions were prepared by the method of Dhariwal *et al.* (1986) from *M. intracellulare* (1.6 g, 2.2×10^{12} c.f.u.). All procedures were done under the oxygen-free air conditions to reduce oxidation of lipids. Briefly, lyophilized organisms were extracted with acetone by sonication (Ultrasonic Cleaner B220H, Branson) for 10 min and subsequent incubation at 25 °C for 5 h. After centrifugation (700 g, 10 min), the extract was filtered through No. 50 filter paper and evaporated (apolar mycoside fraction; 131 mg). The remaining cell pellet was then extracted with hot (60 °C) methanol in a sonic water bath for 10 min followed by 1 h incubation at 60 °C. After centrifugation (20000 g, 15 min), the methanol extract was evaporated and extracted again with hexane by sonicating for 5 min. The hexane extract was evaporated (phospholipid fraction; 134 mg). The remaining lipid residue was then extracted with acetone by

sonication, centrifuged (20000 g, 15 min) and the resultant acetone extract evaporated (polar mycoside fraction; 4.3 mg). The above three lipid fractions were dissolved in DMSO.

Blastogenic response of splenic T cells. Con A-induced blastogenesis of spleen cells (SPCs) was measured as reported previously (Tomioka *et al.*, 1990). Briefly, 2.5×10^5 SPCs were cultured in 5% FBS-RPMI medium containing 2 µg Con A ml⁻¹ (Miles-Yeda) in the presence or absence of test lipid fractions in flat-bottomed microtitre wells (Corning) at 37 °C in a CO₂ incubator for 72 h, with pulsing with 0.25 µCi [³H]thymidine (TdR) (2 Ci mmol⁻¹; New England Nuclear) for the final 6–8 h, unless otherwise specified. Cells were harvested onto glass fibre filters and counted for radioactivity using a liquid scintillation counter (Packard).

IL-2 production of splenic T cells. IL-2 production of Con A-stimulated splenic T cells was measured as previously reported (Tomioka & Saito, 1992). Briefly, 2.5×10^5 SPCs were cultured in the medium containing 2 µg Con A ml⁻¹ in the presence or absence of test lipid fractions in microtitre wells at 37 °C for 24 h. Then, the culture fluid was withdrawn and measured for IL-2 activity, in terms of supporting proliferative response of an IL-2-dependent cytotoxic T cell line, CTLL-2. One unit of IL-2 bioactivity was defined as the amount of IL-2 required to support half-maximal [³H]TdR incorporation by CTLL-2 cells.

Generation of IL-2-reactive T cell subsets. Con A-induced generation of an IL-2-reactive T cell subpopulation was measured as previously reported (Tomioka *et al.*, 1990). Briefly, 2.5×10^5 SPCs were cultured in the medium containing 2 µg Con A ml⁻¹ in the presence or absence of test lipid fractions in microtitre wells at 37 °C for 48 h. Then, non-adherent cells were transferred to new wells, rinsed with Hanks' balanced salt solution (HBSS) containing 1% FBS (v/v) and 10 mg α-methyl mannoside ml⁻¹, and their proliferative response to 100 units mouse recombinant IL-2 (rIL-2) ml⁻¹ (Genzyme) was measured during a further 3 d cultivation.

Assay for expression of IL-2 receptors on Con A-activated T cells. SPCs (2.5×10^5 cells per well) were cultured in medium containing 2 µg Con A ml⁻¹ in the presence or absence of test lipid fractions at 37 °C for 48 h. Then non-adherent cells harvested from 12 wells for each regimen were pooled and washed twice with PBS containing 0.1% (w/v) bovine serum albumin (BSA-PBS). These cells were incubated in 0.1 ml BSA-PBS, with or without 25 µg fluorescein-conjugated anti-murine IL-2 receptor/CD25 (α chain) monoclonal rat antibody ml⁻¹ (Boehringer Mannheim). The cells were then washed with 3 ml BSA-PBS and resuspended in 0.5 ml PBS. Fluorescence of the resultant cells was analysed with FACSTAR (Becton Dickinson).

RESULTS

Suppressive action of MI lipid fractions on SPC blastogenesis

Fig. 1 shows the effects of MI whole lipid, apolar mycoside, polar mycoside, and phospholipid fractions upon Con A-induced SPC blastogenesis. As shown in Fig. 1(a), MI whole lipid fraction suppressed Con A-induced SPC blastogenesis in a dose-dependent fashion. Con A blastogenesis of SPCs of the control culture (no lipid added), was reduced by the addition of MI whole lipids at the following inhibition rates: $17.9 \pm 1.9\%$, $46.0 \pm 0.5\%$ and $59.4 \pm 3.8\%$ in the presence of 15.6, 62.5 and 250 µg lipid fraction ml⁻¹, respectively. However, the MI whole

Fig. 1. Effects of whole lipids (a) and the three lipid fractions (b) of *M. intracellulare* on the SPC blastogenic response to Con A. Lipid fractions dissolved in DMSO at 50 mg ml^{-1} , were added to the medium used for SPC Con A blastogenesis, to give the indicated final lipid concentrations. (a) SPC mitogenesis was measured in the presence (○) or absence (●) of $2 \mu\text{g ml}^{-1}$ Con A. (b) ○, Apolar mycoside fraction; ●, polar mycoside fraction; △, phospholipid fraction. Final concentrations of DMSO in the medium containing 31.3 – $250 \mu\text{g ml}^{-1}$ were 0.063 – 0.5% (v/v) (1600- to 200-fold dilution). SPC blastogenesis was not affected by DMSO at these concentrations. Each point indicates the mean $\pm$ SEM ($n = 3$).

lipid fraction itself exhibited some degree of mitogenic activity for SPCs. The MI whole lipid fraction caused 3.5-, 5.6- and 5.9-fold increases in spontaneous $[^3\text{H}]\text{TdR}$ incorporation by SPCs in the absence of Con A, when it was added at concentrations of 15.6, 62.5, and $250 \mu\text{g ml}^{-1}$, respectively.

Fig. 1(b) shows the effects of apolar mycoside, polar mycoside, and phospholipid fractions upon Con A-induced SPC blastogenesis. The phospholipid and polar mycoside fractions caused almost complete inhibition at $250 \mu\text{g ml}^{-1}$. The phospholipid fraction had about four times more activity than the polar mycoside fraction. The apolar mycoside fraction was inactive. In this experiment, DMSO alone at 0.5% (v/v), equivalent to its concentration added to the medium with the lipids, caused no reduction of SPC blastogenesis. However, in many of the other experiments, DMSO exhibited weak inhibitory activity. Therefore, in all experiments the percentage inhibition is related to the effects seen with cells treated with an equivalent concentration of DMSO. Since the yield of polar mycoside fraction was low, only MI whole lipid and phospholipid fractions were studied further.

The MI lipid-mediated reduction of Con A blastogenesis of SPCs is not due to cytotoxicity: when 3.75×10^6 SPCs were incubated in Con A-free medium at 37°C for 24 h in the presence of either MI whole lipid or phospholipid fractions, recovery of viable cells (measured by dye-exclusion) was 3.1×10^6 cells (82% of original inoculum) and 3.0×10^6 cells (78% of original inoculum), respectively. Therefore, these MI lipid fractions had little cytotoxicity. This conclusion is supported by the finding mentioned above that MI lipids increased $[^3\text{H}]\text{TdR}$ incorporation of SPCs during 72 h culture in the absence of Con A.

It seems possible that certain mannose-rich Con A-binding structures in the MI lipids reduced the active Con A concentration in the culture medium, causing an apparent reduction in the proliferative response of SPCs. However, this possibility can be excluded for the following reasons. Firstly, as shown in Table 1, MI lipid fractions exhibited an inhibitory activity even when the SPCs were treated with Con A for 60 min prior to the exposure to the MI lipids. The degree of inhibition was

Table 1. Effect of time of exposure of SPCs to MI lipids upon the expression of their Con A blastogenesis-inhibitory activity

SPCs were exposed to the indicated MI lipid fractions 10 min before or 60 min after the addition of $2 \mu\text{g ml}^{-1}$ Con A into the cell culture system.

Addition	Concn	SPC Con A mitogenesis ($10^{-3} \times \text{c.p.m.} \pm \text{SEM}$) [percentage inhibition]	
		Before Con A addition ($n = 3$)	After Con A addition ($n = 6$)
None		75.1 ± 2.2	54.4 ± 2.5
DMSO	$0.5\%*$	58.7 ± 5.2	46.4 ± 1.5
MI whole lipids	$250 \mu\text{g ml}^{-1}$	2.9 ± 0.4 [95 $\pm 1\%$]†	5.1 ± 0.1 [89 $\pm 0\%$]†
DMSO	$0.25\%*$	52.5 ± 5.6	55.5 ± 1.7
MI phospholipids	$125 \mu\text{g ml}^{-1}$	7.2 ± 0.2 [86 $\pm 2\%$]†	13.1 ± 0.1 [77 $\pm 1\%$]†

* Equivalent to the concentration of DMSO added to the medium with the lipids.

† Percentage inhibition was calculated from raw data and not from the round figures indicated in the Table.

Table 2. Phase-dependent inhibitory effects of MI lipid fractions on Con A blastogenesis of SPCs

SPCs (2.5×10^5 cells per well) were cultivated in the medium containing $2 \mu\text{g}$ Con A ml^{-1} with or without addition of MI lipid fractions at the indicated phase of cultivation (total 72 h).

Addition	Concn	Time of addition (h)	SPC mitogenesis ($10^{-3} \times \text{c.p.m.}$)*	Inhibition (%)
Medium (25 μl)	—	0	90.6 ± 1.6	—
		24	74.8 ± 1.6	—
		48	70.8 ± 4.3	—
DMSO	0.5 %†	0	70.4 ± 1.5	—
		24	64.3 ± 1.9	—
		48	55.6 ± 1.1	—
MI whole lipids	$250 \mu\text{g ml}^{-1}$	0	17.4 ± 0.4	75.3 ± 0.6
		24	41.9 ± 1.6	34.8 ± 2.5
		48	49.6 ± 0.9	10.8 ± 1.8
MI phospholipids	$125 \mu\text{g ml}^{-1}$	0	20.8 ± 1.0	70.5 ± 1.4
		24	44.1 ± 3.1	31.4 ± 4.8
		48	61.1 ± 2.5	-9.9 ± 4.5

* Mean value $\pm$ SEM ($n = 4$).

† Equivalent to the concentration of DMSO added to the medium with lipids.

Table 3. Effect of MI whole lipid and phospholipid fractions on the Con A-induced mitogenic response, IL-2 production and IL-2-reactive T cell generation of SPCs

Addition (concn)	Con A mitogenesis ($10^{-3} \times \text{c.p.m.} \pm \text{SEM}$)*	IL-2 production		IL-2-reactive T cell generation ($10^{-3} \times \text{c.p.m.} \pm \text{SEM}$)§
		Response of CTLL-2 cells ($10^{-3} \times \text{c.p.m.} \pm \text{SEM}$)†	IL-2 concn (units $\text{ml}^{-1} \pm \text{SEM}$)‡	
None	75.1 ± 2.2	48.5 ± 0.7	58 ± 4	22.1 ± 4.0
DMSO (0.5 %)¶	58.7 ± 5.1	47.1 ± 0.7	52 ± 2	20.7 ± 2.5
MI whole lipids ($250 \mu\text{g ml}^{-1}$)	2.9 ± 0.4	0.013 ± 0.002	< 0.02	0.067 ± 0.012
DMSO (0.25 %)¶	52.5 ± 5.6	42.3 ± 0.8	40 ± 2	28.4 ± 1.4
MI phospholipids ($125 \mu\text{g ml}^{-1}$)	7.2 ± 0.2	0.023 ± 0.001	< 0.02	5.0 ± 0.2

* The proliferative response of SPCs (2.5×10^5 cells per well) in the presence of $2 \mu\text{g}$ Con A ml^{-1} with or without the addition of the indicated lipid fractions during 72 h cultivation is indicated ($n = 3$).

† The proliferative response of CTLL-2 cells (1×10^4 cells) in the medium with the addition of the 24 h culture fluid of SPCs for Con A mitogenesis, as mentioned above, at a concentration of 50 % (v/v) during 40 h cultivation is indicated ($n = 6$).

‡ The concentration of IL-2 produced in the above 24 h culture fluid of SPCs for Con A response is indicated ($n = 6$).

§ The proliferative response of non-adherent cells, harvested from the 48 h culture of SPCs in response to Con A as above, to exogenous rIL-2 ($100 \text{ units ml}^{-1}$) is indicated ($n = 4$).

¶ Equivalent to the concentration of DMSO added to the medium with the lipids.

the same as when the MI lipids were added 10 min before the addition of Con A. It is known that Con A binding to T cells is completed within 30 min (Hadden, 1988).

Secondly, as shown in Table 2, these MI lipid fractions caused significant reduction in the proliferation of SPCs, even if they were added 24 h after the initiation of SPC

Table 4. Effect of MI whole lipid and phospholipid fractions upon Con A-induced blastogenesis and IL-2 receptor (IL-2R) expression of SPCs

Addition	Concn ($\mu\text{g ml}^{-1}$)	SPC mitogenesis		IL-2R expression	
		^3H TdR uptake ($10^{-3} \times \text{c.p.m.}$)*	Inhibition (%)	Percentage of IL-2R ⁺ cells†	Inhibition (%)
None	—	48.3 ± 2.6	—	51.6 ± 1.0	—
DMSO	0.25%‡	51.6 ± 1.6	—	52.7 ± 2.2	—
MI whole lipids	250	15.5 ± 0.3	69.9 ± 0.9	16.7 ± 0.3	68.3 ± 1.3
MI phospholipids	125	18.0 ± 0.3	65.1 ± 1.1	14.0 ± 0.1	73.4 ± 1.1

* Mean $\pm$ SEM ($n = 4$).† The percentage of IL-2R-positive cells measured by FACS analysis (FL1 > 15) is indicated. The mean $\pm$ SEM ($n = 3$) is given.

‡ Equivalent to the concentration of DMSO added to the medium with lipids.

culture in the presence of Con A. When the MI lipid fractions were added after 48 h, however, they did not cause any significant inhibition, if any (MI whole lipids caused 10.8% inhibition). Thirdly, the MI whole lipid fraction caused a similar reduction of SPC blastogenesis, whether the concentration of Con A was 2 or $10 \mu\text{g ml}^{-1}$. While the cell proliferation of SPCs induced with 2, 5 and $10 \mu\text{g Con A ml}^{-1}$ in the absence of MI whole lipids was ($10^{-3} \times \text{c.p.m.}$) 47.0 ± 2.0 , 50.4 ± 0.5 and 50.0 ± 1.4 (mean $\pm$ SEM; $n = 3$), respectively, the same values in the presence of $250 \mu\text{g MI whole lipids ml}^{-1}$ were ($10^{-3} \times \text{c.p.m.}$) 10.1 ± 0.3 , 9.8 ± 0.3 , and 12.3 ± 0.3 , respectively.

Mechanisms for SPC blastogenesis-inhibitory action of MI lipids

As shown in Table 3, both MI whole lipid and phospholipid fractions reduced the IL-2-producing ability of Con A-activated T cells and the generation of IL-2-reactive T cell populations in response to Con A signals (activation of T cell subsets to the IL-2-reactive state; Isakov *et al.*, 1986) in a manner almost parallel to the Con A-induced SPC blastogenic response. Table 4 shows the effect of lipid fractions on the IL-2 receptor expression in Con A-stimulated T cells. Both MI whole lipid and phospholipid fractions reduced IL-2 receptor expression in a fashion parallel to the observed reduction of Con A-induced SPC blastogenesis. Table 5 shows the effects of MI whole lipid and phospholipid fractions on the IL-2-induced proliferation of CTLL-2 cells. The whole lipid fraction only weakly inhibited it and the phospholipid fraction caused a slight increase. This result excludes the possibility that the observed reduction in the IL-2 production of T cells in the presence of the MI lipid fractions was due to carry over of test lipids to the IL-2 assay system using CTLL-2 cells as responder cells.

To determine whether the Con A blastogenesis-inhibitory actions of the MI whole lipid and phospholipid fractions are reversible, SPCs exposed to lipids at 37°C for 24 h in

Table 5. Effect of MI lipids on the IL-2-induced proliferative response of CTLL-2 cells

CTLL-2 cells (1×10^4 cells) were cultured in the medium containing 25 units rIL-2 ml^{-1} with or without the indicated lipids at 37°C for 64 h.

Addition	Concn	Proliferative response of CTLL-2 cells ($10^{-3} \times \text{c.p.m.}$)*	Inhibition (%)
None	—	23.2 ± 1.6	—
DMSO	0.5%	25.4 ± 1.5	—
MI whole lipids	$250 \mu\text{g ml}^{-1}$	16.6 ± 1.3	34.6 ± 5.1
DMSO	0.25%	32.6 ± 1.3	—
MI phospholipids	$125 \mu\text{g ml}^{-1}$	38.6 ± 1.6	-18.4 ± 4.9

* Mean $\pm$ SEM ($n = 4$).

the Con A-free medium and then washed with 2% FBS-HBSS were measured for their Con A mitogenic response. As shown in Table 6, the action of the whole lipid fraction on SPCs was reversible, that is SPCs preincubated with the MI lipids (the first culture) showed no decrease in their mitogenic responsiveness to Con A when they were cultivated in the absence of the lipid in the second culture for Con A-stimulation. In this case, a considerable increase in their Con A mitogenic responsiveness was noted, apparently due to mitogenic activity of the lipid fraction itself. In contrast, the action of MI phospholipids was irreversible, that is SPCs preincubated with the phospholipid fraction showed markedly reduced mitogenic responsiveness in the second culture for Con A-stimulation in the absence of the MI phospholipids. These SPCs showed a similarly reduced Con A mitogenic response as in the case where fresh SPCs without preincubation were measured for Con A blastogenesis in the presence of the MI phospholipid fraction.

Table 6. Reversibility of the inhibitory effects of MI lipid fractions on the Con A response of SPCs

Addition	Preincubation of SPCs*	Concn	SPC mitogenesis ($10^{-3} \times \text{c.p.m.}$)†	Inhibition (%)
None	—	—	89.2 ± 1.3	—
None	+	—	67.3 ± 2.8	—
DMSO	—	0.5%‡	69.8 ± 0.6	—
DMSO	+	0.5%§	76.4 ± 3.4	—
MI whole lipids	—	250 $\mu\text{g ml}^{-1}$ ‡	17.8 ± 0.1	74.5 ± 0.3
MI whole lipids	+	250 $\mu\text{g ml}^{-1}$ §	100.2 ± 2.6	-31.2 ± 1.0
MI phospholipids	—	125 $\mu\text{g ml}^{-1}$ ‡	19.9 ± 0.7	71.5 ± 5.9
MI phospholipids	+	125 $\mu\text{g ml}^{-1}$ §	26.3 ± 1.1	65.6 ± 1.5

* (—) Fresh SPCs without preincubation were cultivated in the medium containing 2 $\mu\text{g Con A ml}^{-1}$ in the presence or absence of MI lipids at a density of 2.5×10^5 cells per well. (+) SPCs were preincubated in Con A-free medium (3 ml) with or without the addition of MI lipids at 37 °C for 24 h (first culture). Preincubated SPCs were washed three times with 2% FBS-HBSS and resuspended in fresh FBS-RPMI 1640 medium. In the second culture, the resulting SPCs were cultivated in the MI lipid-free medium in the presence of 2 $\mu\text{g Con A ml}^{-1}$ at a density of 2.5×10^5 cells per well.

† Mean $\pm$ SEM ($n = 4$).

‡ The lipids were added in the medium for Con A blastogenesis of control SPCs (without preincubation).

§ The lipids were added in the medium for the 24 h preincubation of SPCs in Con A-free medium (first culture).

Effects of the MI lipids on Con A-induced generation of immunosuppressive cells

SPCs (2×10^5 cells per well) were cultured in the presence or absence of 2 $\mu\text{g Con A ml}^{-1}$ with or without the addition of the MI whole lipid fraction (0–250 $\mu\text{g ml}^{-1}$) for 72 h. The resulting cells were harvested, washed with 1% (v/v) FBS-HBSS, transferred to new wells, to which 2×10^5 fresh SPCs were also added, and their Con A (2 $\mu\text{g ml}^{-1}$) response was measured. As shown in Table 7, when SPCs were cultured in the absence of Con A in the primary culture, there was no generation of suppressor cells, irrespective of the addition of the MI whole lipid fraction. The Con A response of the fresh SPCs in the secondary culture was 78.9×10^3 c.p.m. and those of fresh SPCs co-cultured with the SPCs obtained from the primary culture in the absence or presence of 250 $\mu\text{g MI lipids ml}^{-1}$ were 75.6×10^3 and 83.7×10^3 c.p.m., respectively.

As shown in Table 7, when SPCs were cultivated in the presence of 2 $\mu\text{g Con A ml}^{-1}$ in the primary culture, potent suppressor cells were generated. Con A response of fresh SPCs in the secondary culture was 78.9×10^3 c.p.m. and this was reduced to 24.5×10^3 c.p.m., when the fresh SPCs were cultivated with the Con A-treated SPCs obtained from the primary culture. In this case, the addition of 250 $\mu\text{g MI whole lipid fraction ml}^{-1}$ into the primary culture medium decreased the activity of Con A-induced suppressor cells. That is the Con A response of fresh SPCs in the presence of Con A-stimulated SPCs was not so severely reduced (65.0×10^3 c.p.m.) when Con A-treatment of SPCs in the

primary culture was done in the presence of MI whole lipids.

In a separate experiment, no significant suppression of the Con A-response of fresh SPCs in the secondary culture (fresh SPCs alone; $50.8 \times 10^3 \pm 1.2 \times 10^3$ c.p.m.) was found even when they were co-cultivated with the SPCs preincubated in Con A-free medium with or without addition of 250 $\mu\text{g ml}^{-1}$ each of the three MI lipid fractions. In this case, the Con A response of fresh SPCs co-cultivated with the SPCs obtained from the primary culture in the presence of apolar mycosides, polar mycoside, or phospholipids was ($10^{-3} \times \text{c.p.m.}$) 48.7 ± 0.7 , 51.2 ± 1.9 and 63.8 ± 0.6 , respectively, and that in the absence of the lipids was 47.4 ± 1.0 . These results indicate that the inhibitory activity of the MI lipid fractions against Con A blastogenesis of SPCs is not due to their suppressor cell-inducing action.

Thin layer chromatography analysis of MI phospholipid fraction

Table 8 shows the result of thin layer chromatography of the MI phospholipid fraction on silica gel with a solvent system of CHCl_3 :methanol: H_2O (65:30:8 by vol.). There were five spots as follows: spot 1 ($R_F = 0.23$) containing a ninhydrin-reactive amino group, an α -naphthol- H_2SO_4 -reactive carbohydrate, and an anisaldehyde- H_2SO_4 -reactive carbohydrate; spot 2 ($R_F = 0.34$) containing an ammonium molybdate- HgCl_2 - H_2SO_4 -reactive phosphate, an amino group, and α -naphthol- and anisaldehyde-reactive carbohydrates; spot 3 ($R_F = 0.66$) containing phosphate only; spot 4 ($R_F = 0.81$) containing phosphate,

Table 7. Effects of MI whole lipid fraction on the Con A-induced generation of suppressor cells

Primary culture of SPCs (2×10^5 cells per well) was done in the medium with or without addition of either Con A or MI whole lipids for 72 h. Thereafter, the resulting cells were transferred to new wells and thoroughly washed. Fresh SPCs (2×10^5 cells) were further added to the wells and cultivated in the presence of $2 \mu\text{g}$ Con A ml^{-1} for a further 72 h and [^3H]TdR incorporation was measured during the final 8 h.

Addition to primary culture of SPCs		Con A response of SPCs in secondary culture ($10^{-3} \times \text{c.p.m.}$)*	Suppressor activity of pretreated SPCs†
Con A ($2 \mu\text{g ml}^{-1}$)	MI whole lipids ($\mu\text{g ml}^{-1}$)		
—	0	75.6 ± 2.3	4.1 ± 3.0
—	15.6	81.5 ± 1.6	-3.3 ± 2.0
—	62.5	78.0 ± 1.6	1.2 ± 2.0
—	250	83.7 ± 2.1	-6.1 ± 2.5
+	0	24.5 ± 3.9	69.0 ± 5.0
+	15.6	43.3 ± 1.7	45.1 ± 2.2
+	62.5	40.7 ± 2.5	48.5 ± 3.2
+	250	65.0 ± 3.1	17.6 ± 4.0

* [^3H]TdR incorporation into fresh SPCs in the secondary culture in the absence of pretreated SPCs from the primary cultivation was $78.9 \times 10^3 \pm 1.0 \times 10^3$ c.p.m. The mean $\pm$ SEM ($n = 3$) is indicated.

† Suppressor activity was calculated by the following equation from the values of Con A-induced SPC blastogenesis in the secondary culture: Suppressor activity = [(SPC blastogenesis in the absence of pretreated SPCs) - (SPC blastogenesis in the presence of pretreated SPCs)] / (SPC blastogenesis in the absence of pretreated SPCs) $\times 100$.

an amino group, and an anisaldehyde-reactive carbohydrate; and spot 5 ($R_F = 0.96$), from which only a phosphate group was detected. Among them, high R_F value spots 4 and 5 having phosphate groups possessed a potent inhibitory activity against Con A blastogenesis of SPCs. These spots caused 82.2 and 99.8% inhibition of SPC Con A blastogenesis, respectively, when added at the concentration equivalent to $125 \mu\text{g}$ MI phospholipids ml^{-1} .

DISCUSSION

This study revealed that whole lipid and phospholipid fractions obtained from *M. intracellulare* had a suppressive activity against the Con A response of T cells. Relatively high concentrations of the lipids were needed to inhibit blastogenesis. However, some host cells such as M ϕ s which phagocytose *M. intracellulare* may accumulate the lipids into their cell membrane and present them to the target T cells in a sufficiently condensed manner. Indeed, Stallcup *et al.* (1986) reported that M ϕ hybridoma cells inhibited the growth of B lymphocytes through cell to cell contact by presenting a lipid-like cytotoxic substance to target B cells.

The MI polar mycoside fraction also exhibited a measurable level of inhibitory activity against the Con A response of SPCs and this is consistent with the finding by Tassell *et al.* (1992) that lipopeptide derived from polar mycosides possesses a suppressing activity. However, such lipid components seem to have only minor importance in the MI lipid-mediated suppression of the SPC mitogenic response for the following reasons. Firstly, polar mycoside is a minor component of the MI whole lipids. Secondly, its activity is much lower than that of the phospholipid fraction. Therefore phospholipids are the most important inhibitory components of MI lipids. In relation to this, some lipids such as phenolic glycolipid (Stanford, 1986), glycopeptidolipids (Brownback & Barrow, 1988) and sulfolipids (Pabst *et al.*, 1988) are known to exhibit immunosuppressive activity. Thus, lipid components are thought to play some important role in the establishment of immuno-unresponsiveness frequently seen in hosts with disseminated mycobacterial infections. In relation, because free long chain fatty acids also inhibit SPC Con A blastogenesis (Tomioka & Saito, 1992), fatty acyl groups may play a certain role in the expression of the immunosuppressive activity of the MI lipid fractions.

The present findings indicate that MI whole lipid and phospholipid fractions inhibited Con A blastogenesis of SPCs largely by reducing IL-2-producing ability of Con A-stimulated T cells. The MI lipid fractions also inhibited Con A-induced IL-2-reactive T cell generation, that is the acquisition of IL-2-reactivity in Con A-stimulated T cell subpopulations (Isakov *et al.*, 1986). Furthermore, the expression of IL-2 receptors in Con A-stimulated T cell subsets was inhibited by MI lipids. It is noteworthy that the IL-2-producing function of T cells was somewhat more affected than was the step of T cell activation by the IL-2-reactive state. Impairment of IL-2-producing ability in host T cells is known in some cases of microbial infections (Hoffenbach *et al.*, 1983; Tarleton & Kuhn, 1984). In contrast, these MI lipid fractions did not reduce the IL-2-induced proliferation of CTLL-2 cells, growth of which is entirely dependent on IL-2. This indicates that MI whole lipid and phospholipid fractions do not affect the late phase event of Con A-induced SPC blastogenesis, that is IL-2-induced proliferation of Con A-stimulated T cell subsets which acquired IL-2 receptors. The finding in Table 2 that the MI lipids exerted no significant inhibitory action, when added at 48 h after the initiation of SPC culture for Con A blastogenesis, supports this concept.

In relation to this, it is noteworthy that certain phospholipids, such as phosphatidylinositol (PI) and phosphatidylethanolamine (PE) released from blood monocytes which had phagocytosed *M. tuberculosis* have been reported to exhibit a suppressor T cell-inducing ability (Wadee *et al.*, 1980, 1983). However, in our study, such a phenomenon was not observed. As shown in Table 7, none of the MI lipid fractions induced suppressor cells from normal SPCs during 72 h *in vitro* cultivation. Moreover, MI whole lipids antagonized Con A-mediated generation of suppressor T cells *in vitro*. Therefore, the possibility that the MI lipids exhibit their suppressive

Table 8. SPC blastogenesis-reducing activities of some components in the MI phospholipid fraction separated by silica gel thin layer chromatography

The MI phospholipid fraction (10 mg) was applied onto preparative silica gel for thin layer chromatography with the solvent system $\text{CHCl}_3:\text{MeOH}:\text{H}_2\text{O}$ (65:30:8, by vol.). The developed plates (3 plates) were roughly visualized for the major lipid components by spraying with H_2O_2 and the silica gels were divided into nine zones. After drying under vacuum, each zone of the silica gel was collected and extracted with CHCl_3 -methanol (2:1, v/v). The extract was evaporated and the lipid residue was finely emulsified into 5 ml 5% FBS-RPMI medium by sonication. The resultant specimens were added at 50% (v/v) into the culture medium for Con A blastogenesis of SPCs and measured for their inhibitory activity against SPC blastogenesis. Variations of inhibitory activity did not exceed 5.9% (mean 2.6%).

Spot*	R_F	Inhibitory activity (percentage inhibition)	Colour development with			
			Ammonium molybdate- HgCl_2 - H_2SO_4	Ninhydrin	α -Naphthol- H_2SO_4	Anisaldehyde- H_2SO_4
1	0.23	16.8	—	+++	++	+
2	0.34	4.5	+	+	++	++
3	0.66	9.3	+++	—	—	—
4	0.81	82.2	+	+	—	+
5	0.96	99.8	++	—	—	—

* On a separate thin layer chromatograph, the five indicated spots were detected by spraying with 90% H_2SO_4 followed by heating at 120 °C.

action against the Con A-induced blastogenic response of SPCs by enhancing the generation of suppressor cells may be excluded.

In separate experiments, we have found that of the various authentic phospholipids, phosphatidylserine (PS) and PI possessed a potent inhibitory activity against SPC blastogenesis, but phosphatidylcholine (PC) and PE lacked such activity (unpublished observations). Both PS and PI exhibited an inhibitory activity against IL-2 production of T cells and their activation by the IL-2-reactive state, as in the case of the MI phospholipid fractions. However, *M. avium* and *M. intracellulare* lack PS, PC, acylphosphatidylglycerol, and phosphatidylmethylethanolamine (Brennan, 1988). They possess only three kinds of phospholipids, PE, PI mannoside and diphosphatidylglycerol. Therefore, PS cannot serve as a mediator component of the MI phospholipid fractions tested here. The existence of PI mannoside supports the view that PI is widespread in these organisms (Goodfellow & Minnikin, 1964). Therefore, PI may be a candidate as the SPC blastogenesis-inhibitory phospholipid of *M. intracellulare*.

It is of interest to know the detailed nature of the lipid component having SPC blastogenesis-inhibitory activity contained in the MI phospholipid fraction. Thin layer chromatography analysis of the MI phospholipid fraction revealed that the fraction contained five separable components of which two showed inhibitory activity against SPC Con A blastogenesis. Spot 4 possessed phosphate groups, amino groups and anisaldehyde- H_2SO_4 -reactive carbohydrate moieties but was not re-

active to α -naphthol- H_2SO_4 . Spot 5 possessed phosphate groups but was not reactive to ninhydrin, α -naphthol- H_2SO_4 or anisaldehyde- H_2SO_4 . Large-scale purification of these two components and more detailed studies on their chemical structure is necessary to find a conclusive solution to this problem.

REFERENCES

- Brennan, P. J. (1988). *Mycobacterium* and other actinomycetes. In *Microbial Lipids*, pp. 203–298. Edited by C. Ratledge & S. G. Wilkinson. London: Academic Press.
- Brownback, P. E. & Barrow, W. W. (1988). Modified lymphocyte response to mitogens after intraperitoneal injection of glycopeptidolipid antigens from *Mycobacterium avium* complex. *Infect Immun* **56**, 1044–1050.
- Collins, F. M. (1987). *Mycobacterium avium* complex infections and immunodeficiency. In *Mycobacterium tuberculosis, Interactions with the Immune System*, pp. 389–414. Edited by M. Bendinelli & H. Friedman. New York: Plenum Press.
- Collins, F. M. (1989). Mycobacterial disease, immunosuppression, and acquired immunodeficiency syndrome. *Clin Microbiol Rev* **2**, 360–377.
- Dhariwal, K. R., Liav, A., Vatter, A. E., Dhariwal, G. & Goren, M. B. (1986). Haptenic oligosaccharides in antigenic variants of mycobacterial C mycosides antagonize lipid receptor activity for mycobacteriophage D4 by masking a methylated rhamnose. *J Bacteriol* **168**, 283–293.
- Edwards, D. & Kirkpatrick, C. H. (1986). The immunology of mycobacterial diseases. *Am Rev Respir Dis* **134**, 1062–1071.
- Goodfellow, M. & Minnikin, D. E. (1964). Circumstances of the genus. In *The Mycobacteria. A Source Book, Part A*, pp. 1–24. Edited by G. P. Kubica & L. G. Wayne. New York: Mercel Dekker Inc.

- Gross, P., Skamene, E. & Forget, A. (1983). Cellular mechanisms of genetically controlled host resistance to *Mycobacterium bovis* (BCG). *J Immunol* **131**, 1966–1972.
- Hadden, J. W. (1988). Transmembrane signals in the activation of T lymphocytes by mitogenic antigens. *Immunol Today* **9**, 235–239.
- Hoffenbach, A., Lagrange, P. H. & Bach, M. A. (1983). Deficit of interleukin 2 production associated with impaired T-cell proliferative response in *Mycobacterium lepraemurium* infection. *Infect Immun* **39**, 109–116.
- Isakov, N., Scholz, W. & Altman, A. (1986). Signal transduction and intracellular events in T-lymphocyte activation. *Immunol Today* **7**, 271–277.
- Kleinhenz, M. E., Ellner, J. J., Spagnuolo, P. J. & Daniel, T. M. (1981). Suppression of lymphocyte response by tuberculous plasma and mycobacterial arabinogalactan. Monocyte dependence and indomethacin reversibility. *J Clin Invest* **68**, 153–162.
- Pabst, M. J., Gross, J. M., Brozna, J. P. & Goren, M. B. (1988). Inhibition of macrophage priming by sulfatide from *Mycobacterium tuberculosis*. *J Immunol* **140**, 634–640.
- Rastogi, N. & David, H. L. (1988). Mechanisms of pathogenicity in mycobacteria. *Biochimie* **70**, 1101–1120.
- Saito, H. & Tomioka, H. (1990). The role of macrophage in host defense mechanisms against *Mycobacterium avium* complex infection induced in mice. *Res Microbiol* **141**, 206–212.
- Skamene, E. (1989). Genetic control of susceptibility to mycobacterial infections. *Rev Infect Dis* **11**, S394–S399.
- Stallcup, K. C., Martin, Y. L., Dorf, M. E. & Mescher, M. F. (1986). Inhibition of lymphoid cell growth by a lipid-like component of macrophage hybridoma cells. *J Immunol* **136**, 2723–2728.
- Stanford, J. L. (1986). Some immunological aspects of leprosy, and yet more hypothesis. In *Mycobacteria of Clinical Interest*, pp. 310–321. Edited by N. Casal. Amsterdam: Excerpta Medica.
- Sussman, G. & Wade, A. A. (1991). Production of a suppressor factor by CD8⁺ lymphocytes activated by mycobacterial components. *Infect Immun* **59**, 2828–2835.
- Tarleton, R. L. & Kuhn, R. E. (1984). Restoration of *in vitro* immune response of spleen cells from mice infected with *Trypanosoma cruzi* by supernatants containing interleukin 2. *J Immunol* **133**, 1570–1575.
- Tassell, S. A., Pourshafie, M., Wright, E. L., Richmond, M. G. & Barrow, W. W. (1992). Modified lymphocyte response to mitogens induced by the lipopeptide fragment derived from *Mycobacterium avium* serovar-specific glycopeptidolipids. *Infect Immun* **60**, 706–711.
- Tomioka, H. & Saito, H. (1992). Characterization of immunosuppressive functions of murine peritoneal macrophages induced with various agents. *J Leukocyte Biol* **51**, 24–31.
- Tomioka, H., Saito, H. & Yamada, Y. (1990). Characteristics of immunosuppressive macrophages induced in spleen cells by *Mycobacterium avium* complex infections induced in mice. *J Gen Microbiol* **136**, 965–973.
- Tsuyuguchi, L., Kawasumi, H., Takashima, T., Tsuyuguchi, T. & Kishimoto, S. (1990). *Mycobacterium avium*–*Mycobacterium intracellulare* complex-induced suppression of T-cell proliferation *in vitro* by regulation of monocyte accessory cell activity. *Infect Immun* **58**, 1369–1378.
- Wade, A. A., Sher, R. & Rabson, A. R. (1980). Production of a suppressor factor by human adherent cells treated with mycobacteria. *J Immunol* **125**, 1380–1386.
- Wade, A. A., Mendelsohn, D. & Rabson, A. R. (1983). Characterization of a suppressor cell activating factor (SCAF) released by adherent cells treated with *M. tuberculosis*. *J Immunol* **130**, 2266–2270.

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