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Phospholipids and reactive nitrogen intermediates collaborate in expression of the T cell mitogenesis-inhibitory activity of immunosuppressive macrophages induced in mycobacterial infection

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SUMMARY

We studied the role of phospholipids and nitric oxide in expression of the suppressor activity of splenic macrophages induced by *Mycobacterium avium-intracellulare* complex infection (MAIC-induced macrophages) in mice against mitogenic response of concanavalin A (Con A)-stimulated splenocytes (SPC) as follows. First, phosphatidylserine (PS) and phosphatidylinositol were found to suppress Con A-induced mitogenesis of SPC via inhibition of IL-2 production and acquisition of IL-2 reactivity in Con A-stimulated T cells. The mitogenesis-inhibitory activity of PS was increased when SPC were cultured under mildly acidic condition (pH 6·3). When SPC were pretreated with PS for 24 h prior to Con A blastogenesis, their mitogenic response was irreversibly abrogated. Second, N^G-monomethyl-L-arginine, an inhibitor of nitric oxide (NO) synthase, was found to attenuate in part the expression of the suppressor activity of MAIC-induced macrophages. Third, reactive nitrogen intermediates (RNI) including NO generated from acidified NO_2^- exerted potent inhibitory activity against SPC mitogenic response, and the suppressive activity of RNI was significantly augmented by the combination with PS. These findings indicate that phospholipids and RNI play an important role in the expression of suppressor activity of MAIC-induced macrophages as the effector molecules.

Keywords phospholipids NO reactive nitrogen intermediates immunosuppressive macrophages T cell mitogenesis *Mycobacterium avium-intracellulare* complex

INTRODUCTION

During the course of mytobacterioses including *Mycobacterium avium-intracellulare* complex (MAIC) infections in humans and experimental animals, generation of immunosuppressive macrophages is frequently encountered [1–4]. Previously, we found that immunosuppressive macrophages were induced in the spleens of host mice around 2 weeks after i.v. infection with MAIC, and that they possessed potent suppressive activity against concanavalin A (Con A)-induced mitogenesis of splenocytes (SPCs) [5,6]. Concerning the mediator molecules of the splenic macrophages, the following has been elucidated [5–7].

First, although MAIC-induced macrophages showed increased ability for active oxygen release, scavengers for oxygen radicals (superoxide dismutase and catalase) could not attenuate the suppressive activity [6]. Therefore, active oxygen intermediates are not involved. Prostaglandins (PG), especially PGE, are important for macrophage function, since indomethacin (inhibitor of PG synthesis) could partially overcome the suppressor activity of MAIC-induced macrophages, and moreover, PGE potently suppressed Con A-induced SPC blastogenesis [5]. In the course of screening for other mediators, we found SPC mitogenesis-inhibitory activity in some unsaturated free fatty acids [7]. In addition, preliminary experiments showed the possibility that reactive nitrogen intermediates (RNI), including nitric oxide (NO), act as the effector molecules of MAIC-induced macrophages, since ferrous myoglobin, scavenger of NO, attenuated their suppressor activity [7]. During the course of our investigation of the virulence factor of MAIC organisms, we found that some bacterial phospholipid-like components possessed potent inhibitory activity against SPC mitogenesis [8]. Since this phenomenon strongly suggests the possibility that phospholipids may play an important role in the expression of the suppressor activity of MAIC-induced macrophages, we carried out experiments to elucidate detailed profiles of phospholipid-mediated suppression of SPC mitogenesis. In addition, we studied the role of RNI in the suppressor activity of MAIC-induced macrophages in experiments using N^G-monomethyl-L- arginine (NMMA), a specific inhibitor of NO synthase [9], and examined combined effects of RNI

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with phospholipid in exerting suppressive activity against SPC mitogenesis.

MATERIALS AND METHODS

Microorganisms

MAIC N-260 strain isolated from an MAIC patient was used. This organism was identified as *Myco. intracellulare* belonging to serovar 16.

Mice

Female BALB/c mice, 8–10 weeks old, purchased from Japan Clea Co. (Osala, Japan), were used.

Special agents

Recombinant mouse IL-2 was purchased from Genzyme Co. (Cambridge, MA). Fluorescein-conjugated anti-murine IL-2 receptor/CD25 (α-chain) rat MoAb was obtained from Boehringer (Mannheim, Germany). Phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE) were purchased from Sigma (St Louis, MO), and phosphatidyl-choline (PC) was donated by Kewpie Co.(Tokyo, Japan).

Medium

RPMI 1640 medium (RPMI) supplemented with 25 mM HEPES, 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin G, 5×10^{-5} M 2-mercaptoethanol (2-ME) and 5% (v/v) heat-inactivated fetal bovine serum (FBS) (M. A. Bioproducts, Walkersville, MSD) was used for cell culture.

Mitogenic response of SPC

Con A-induced mitogenesis of SPC was measured as reported previously [5]. Briefly, $2{\cdot}5 \times 10^5$ SPC were cultured in 0·2 ml 5% FBS–RPMI containing 2 μg/ml Con A (Miles-Yeda Ltd., Israel) in the presence or absence of test agents in flat-bottomed 96-well microculture plates (Corning Glass Works Co., Corning, NY) at 37°C in a CO_2 incubator for 72 h, with pulsing with 0·25 μCi or in some experiments 0·5 μCi of ^{3}H-TdR (2 Ci/mmol; New England Nuclear, Boston, MA) for the final 6–8 h. Cells were harvested onto glassfibre filters and counted for radioactivity using Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, IL). Usually, 5×10^4–9×10^4 and 12×10^4–19×10^4 ct/min of ^{3}H-TdR incorporation were observed in this experimental system, when radioisotopic pulsing was done by using 0·25 and 0·5 μCi of ^{3}H-TdR, respectively.

Suppressor activity of MAIC-induced macrophages

Suppressor activity of MAIC-induced splenic macrophages was measured as described previously [5]. Briefly, SPC were harvested from mice infected intravenously with 1×10^8 colony-forming units (CFU) of MAIC 2 weeks after infection. The SPC (1×10^6–4×10^6) were cultured in 0·2 ml of 10% FBS–RPMI in four wells each of microculture plate in a CO_2 incubator for 2 h. The wells were then vigorously rinsed with Hank's balanced salt solution (HBSS) containing 2% FBS and then 0·1 ml of 5% FBS–RPMI was poured onto the resulting wells. This procedure usually gave >90% pure macrophage monolayer cultures, with active pinocytic ability of neutral red and with phagocytic ability against latex particles, containing about 5×10^4 cells per culture well from 2×10^6 of MAIC-induced SPC. Into the macrophage culture wells, 0·05 ml of 5% FBS–RPMI with or without 0·5 mM NMMA was added, and $2{\cdot}5 \times 10^5$ of normal SPC in 0·05 ml of the medium containing 8 μg/ml (final 2 μg/ml) of Con A were then poured onto the resultant macrophage cultures. Con A-induced mitogenesis of SPC was then measured as described above.

IL-2 production of splenic T cells

IL-2 producing activity of Con A-activated SPC was assayed as previously described [7]. Briefly, the 24-h culture fluid harvested from the above SPC culture of Con A mitogenesis was measured for IL-2 activity in terms of the proliferative response of 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 ^{3}H-TdR incorporation by CTLL-2 cells.

Generation of IL-2 reactive T cell population

Con A-induced generation of IL-2 reactive T cell population was measured as previously reported [7]. Briefly, $2{\cdot}5 \times 10^5$ SPC were cultured in the above medium for Con A mitogenesis in microculture wells at 37°C for 48 h. The non-adherent cells were transferred to new wells and rinsed with 1% FBS–HBSS containing 10 mg/ml of α-methyl mannoside. The proliferative response of these cells to 100 U/ml of recombinant IL-2 was measured during a further 3-day cultivation.

Assay for expression of IL-2 receptors on Con A-activated T cells

Normal SPC ($2{\cdot}5 \times 10^5$ cells/well) were cultured in 5% FBS–RPMI containing 2 μg/ml Con A at 37°C for 48 h. Then non-adherent cells harvested from 12 wells were pooled and washed twice with PBS containing 0·1% bovine serum albumin (BSA) (Sigma). The resultant cells were incubated in 0·1 ml of the BSA–PBS, with or without 25 μg/ml of fluorescein-conjugated anti-murine IL-2 receptor rat MoAb. The cells were washed with 3 ml of BSA–PBS and suspended into 0·5 ml of PBS. Fluorescence of these cells was then analysed with FACSTAR (Becton Dickinson, Mountain View, CA).

RESULTS

Suppressive activity of phospholipids against Con A mitogenesis of SPC

As shown in Table 1, PS and PI displayed a potent inhibitory action against Con A-induced SPC mitogenesis. They inhibited IL-2 production of Con A-stimulated T cells and Con A-induced generation of IL-2-reactive T cell populations (acquisition of IL-2 responsiveness in Con A-stimulated T cell subsets). The other phospholipids, such as PC and PE, did not exhibit such obvious inhibitory action, although a weak inhibition of IL-2 production was seen at the high concentration (125 μg/ml) of PC. In a separate experiment, PS at a concentration of 62·5 μg/ml was found to cause a significant decrease in expression of IL-2 receptors (α-chain) in Con A-stimulated T cells. The ratio of IL-2 receptor-positive T cells in the non-adherent cells harvested from the 48-h culture ($51{\cdot}6 \pm 1{\cdot}0\%$) was reduced to $33{\cdot}5 \pm 0{\cdot}7\%$ by the addition of 62·5 μg/ml of PS to culture medium (36% inhibition). In addition, Fig. 1 shows the effects of these phospholipids of IL-2-induced proliferation of CTLL-2 cells. PS exerted potent inhibitory action, whereas PC and PE lacked such an inhibitory activity, but caused a

Table 1. Effects of some phospholipids on concanavalin A (Con A)-induced mitogenic response, IL-2 production, and IL-2 reactive T cell generation of splenocytes (SPC)

Addition	Concentration (μg/ml)	SPC mitogenesis (10^3 ct/min ± s.e.m.)*	IL-2 production (U/ml ± s.e.m.)†	IL-2-reactive T cell generation (10^3 ct/min ± s.e.m.)‡
None	–	50·6 ± 1·9	52 ± 4	38·4 ± 1·0
DMSO-ethanol§	0·5%	45·5 ± 3·8	62 ± 3	38·2 ± 1·4
PS	15·6	98·1 ± 3·6	15 ± 1	51·2 ± 1·1
	31·3	0·35 ± 0·02	0·06	0·84 ± 0·47
	62·5	0·13 ± 0·02	<0·01	0·19 ± 0·04
	125	0·045 ± 0·003	<0·01	0·33 ± 0·12
PI	15·6	62·0 ± 0·9	38 ± 1	47·8 ± 3·8
	31·3	37·5 ± 0·8	18 ± 2	56·8 ± 1·7
	62·5	2·5 ± 0·3	9·9 ± 0·2	10·3 ± 3·0
	125	0·041 ± 0·008	<0·01	0·11 ± 0·02
PC	15·6	43·6 ± 3·3	66 ± 6	35·3 ± 1·9
	31·3	37·9 ± 1·1	56 ± 5	31·9 ± 0·4
	62·5	51·8 ± 3·8	48 ± 2	37·9 ± 1·8
	125	66·9 ± 1·2	18 ± 5	45·8 ± 2·5
PE	15·6	58·2 ± 3·2	39 ± 2	36·7 ± 1·2
	31·3	58·0 ± 0·6	38 ± 2	33·8 ± 0·9
	62·5	69·1 ± 3·6	36 ± 2	38·6 ± 1·6
	125	60·3 ± 0·7	54 ± 7	44·0 ± 1·7

Each phospholipid was dissolved or finely emulsified into dimethylsulfoxide (DMSO)–ethanol (1:1) by gentle sonication using Handy sonic at the concentration of 25 mg/ml, and then diluted 200-fold or more with 5% fetal bovine serum (FBS)–RPMI. Phospholipids were finely emulsified into the medium by gently sonication as above.

* Proliferative response of SPC in the presence of 2 μg/ml Con A with or without addition of test phospholipid is indicated ($n = 4$).

† Concentration of IL-2 produced in the 24-h culture fluid of the above SPC culture is indicated ($n = 4$).

‡ Non-adherent cells were harvested from the 48-h culture of SPC in the medium containing Con A with or without the addition of test phospholipid. Proliferative response of the resultant non-adherent cells to exogenous recombinant IL-2 (100 U/ml) is indicated ($n = 4$).

§ Solute control for phospholipid addition.

PS, Phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

considerable increase in CTLL-2 cell proliferation. PI showed a weak inhibitory action when added at 62·5 μg/ml. As shown in Fig. 2, PS exhibited greater inhibitory activity against SPC mitogenesis in the mildly acidified medium (pH 6·3) than in the regular medium at pH 7·2.

It is noteworhy that there was an apparent enhancement of SPC mitogenesis ($P < 0·005$; Student's *t*-test) with the lowest dose (15·6 μg/ml) of PS. Although the precise reason is unknown, this may be attributable to the saturated PS (such as stearoyl PS) contained in the present PS preparation obtained from bovine brain, since the saturated PS possesses a DNA synthesis-stimulating activity [10].

The profile of PS-mediated suppression of SPC mitogenic response is on the whole consistent with the feature of suppressor activity of MAIC-induced macrophages, as follows. MAIC-induced macrophages suppressed proliferation of SPC in response to Con A, primarily by inhibiting Con A-induced activation of T cells to an IL-2-reactive state [5]. In fact, MAIC-induced macrophages strongly reduced IL-2 receptor expression of Con A-stimulated T cells [6].

The mode of suppressive action of PS against Con A-induced SPC mitogenesis

PS was investigated for the mode of its suppressor function against SPC blastogenic response. First, we determined whether or not the suppressor activity of PS was due to its cytotoxic activity. When $2·5 \times 10^5$ of SPC were cultured in 0·2 ml of Con A-free medium in the presence or absence of 50 or 100 μg/ml PS, the recovery of viable cells (10^5 cells/well; determined by nigrosin staining) after 24-h culture was as follows: control (none added), 2·20 ± 0·08 ($n = 6$); solute control (0·2% DMSO), 1·91 ± 0·08 ($n = 4$); 50 μg/ml PS, 1·92 ± 0·13 ($n = 4$); 100 μg/ml PS, 1·86 ± 0·08 ($n = 4$). Here, the ratio of viable cell recovery compared with the control (none added) value was as follows: 0·2% DMSO (solute control), 87·2 ± 3·4%; 50 μg/ml PS, 87·5 ± 5·7%; 100 μg/ml PS, 84·5 ± 3·8%. Therefore, the recovery of viable cells was not significantly reduced by the addition of 0·2% DMSO, or 50 or 100 μg/ml of PS. Moreover, the relative recovery of viable cells compared with the solute control (DMSO) was 100·3 ± 6·5% for the case of 50 μg/ml PS addition, and 96·9 ± 4·3% for the case of 100 μg/ml PS addition. Therefore, it is thought that PS given at the doses of 50 and 100 μg/ml did not exert a significant level of cytotoxicity against SPC. In this experiment, Con A-induced SPC mitogenesis in the presence or absence of PS was as follows: control (none added), $129·5 \pm 3·8 \times 10^3$ ct/min ($n = 6$); 50 μg/ml PS, $1·02 \pm 0·21 \times 10^3$ ct/min ($n = 6$); 100 μg/ml PS, $0·16 \pm 0·04 \times 10^3$ ct/min ($n = 6$). Thus, PS at concentrations of 50–100 μg/ml caused about 99% inhibition of SPC mitogenesis.

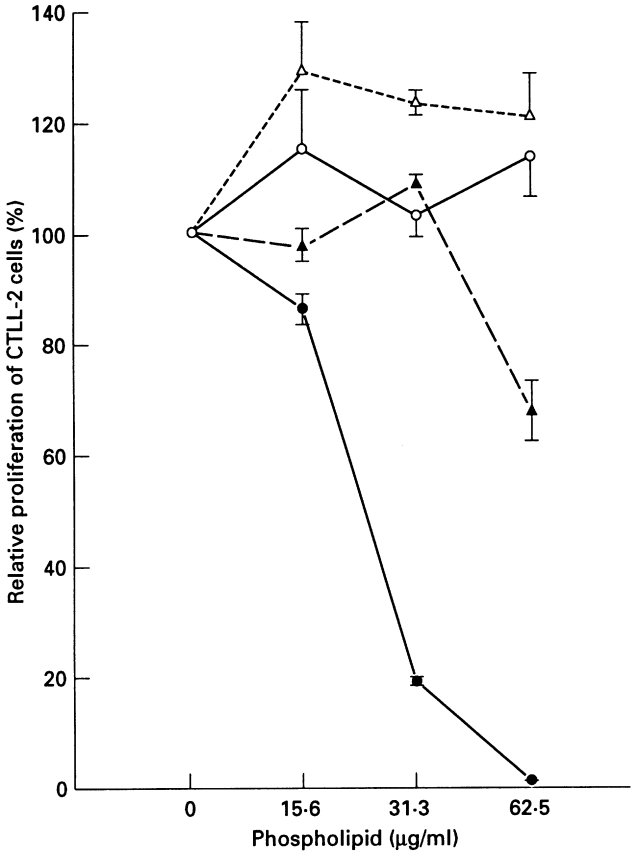

Fig. 1. Effects of some phospholipids on IL-2-induced proliferation of CTLL-2 cells. CTLL-2 cells (1×10^4 cells/well) were cultured in 5% fetal bovine serum (FBS)–RPMI containing 25 U/ml of recombinant IL-2 in the presence or absence of phosphatidylcholine (PC) (○), phosphatidylethanolamine (PE) (△), phosphatidylserine (PS) (●), or phosphatidylinositol (PI) (▲) at 37°C for 64 h. The extent of cell proliferation in the presence of 0·5% DMSO–ethanol (1:1) (solute control for phospholipid addition), $23{\cdot}2 \pm 1{\cdot}6 \times 10^3$ ct/min, was fixed as 100%. Each plot indicates the mean ± s.e.m. ($n = 4$).

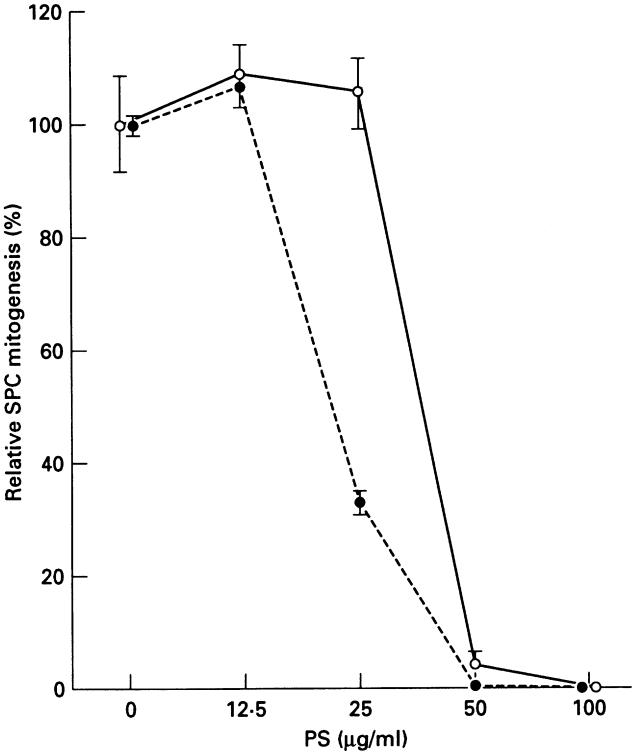

Fig. 2. The pH-dependency of the splenocyte (SPC) mitogenesis-inhibitory activity of phosphatidylserine (PS). SPC ($2{\cdot}5 \times 10^5$ cells/well) were cultured in 5% fetal bovine serum (FBS)–RPMI at pH 7·2 (○) or 6·3 (●) containing 2 μg/ml concanavalin A (Con A) with or without the addition of indicated concentrations of PS. SPC mitogenesis of the control cultures was as follows: none added (pH 6·3), 123 784 ± 11 228 ct/min; solute control (0·4% DMSO) (pH 6·3), 127 475 ± 4963 ct/min; none added (pH 7·2), 123 435 ± 1672 ct/min; solute control (0·4% DMSO) (pH 7·2), 106 561 ± 3630 ct/min. Each plot indicates the mean ± s.e.m. ($n = 3$).

These findings indicate that the suppressor activity of PS is largely attributable to mechanisms other than cytotoxicity.

Next, in order to know whether Con A mitogenesis-inhibitory action of PS is reversible or not, SPC exposed to PS (125 μg/ml) at 37°C for 24 h were measured for the Con A mitogenic response. As shown in Table 2, the PS-pretreated SPC showed almost completely diminished blastogenic response to Con A in PS-free medium, as in the case where Con A mitogenesis of untreated SPC was measured in the presence of the same concentration of PS. This indicates irreversibility of PS action on SPC.

Participation of NO in the suppressor activity of MAIC-induced macrophages

RNI including NO are reported to be responsible for the expression of the T cell mitogenesis-inhibitory effect by some kinds of immunosuppressive macrophages [11,12]. It has been also reported by Rockett *et al*. [13] that the reduction of Con A mitogenic response observed in the case of SPC from *Plasmodium vinickei*-infected mice was restored by the addition of NMMA (a specific inhibitor of NO synthase) [9], thereby indicating the role of NO in the suppression of T cell proliferation induced by malaria parasites. In addition, we previously found that ferrous myoglobin, a scavenger of NO [14], partly overcame the suppressive activity of MAIC-induced macrophages [7], thereby suggesting participation of NO in the immunosuppressive activity of MAIC-induced macrophages. As shown in Table 3, NMMA attenuated in part the suppressive activity of MAIC-induced macrophages ($P < 0{\cdot}01$; Student's *t*-test). This indicates that RNI, including NO, play an important role as effector molecules of MAIC-induced macrophages.

Collaboration of RNI with phospholipids in the suppression of SPC mitogenesis

It is of interest to know whether or not NO collaborate with phospholipids in the manifestation of their SPC mitogenesis-inhibitory action. As indicated in Table 4, RNI including nitrous acid (HNO_2), NO, and NO_2 which are generated from mildly acidified NO_2^- (added as $NaNO_2$) [14] could inhibit SPC mitogenic response in a dose-dependent fashion. Approximately half and nearly complete inhibitions were given by 2 and 8 mM $NaNO_2$ alone at pH 6·3, respectively. Combinations of $NaNO_2$ (1 mM) + PS (25 μg/ml), $NaNO_2$ (2 mM) + PS (12·5 or 25 μg/ml), and $NaNO_2$ (4 mM) + PS (25 μg/ml) displayed significant combined effects in suppressing SPC mitogenesis. These results imply that RNI in collaboration with phospholipids, in particular

Table 2. Irreversible inhibitory effect of phosphatidylserine (PS) against concanavalin A (Con A)-induced mitogenic response of splenocytes (SPC)

Addition	Concentration	Preincubation of SPC	SPC mitogenesis (10^3 ct/min ± s.e.m.; $n = 4$)
None	–	−*	89·2 ± 1·3
None	–	+†	67·3 ± 2·8
DMSO‡	0·5%	−*	69·8 ± 0·6
DMSO‡	0·5%	+†	76·4 ± 3·4
PS	125 μg/ml	−*	0·07 ± 0·01
PS	125 μg/ml	+†	0·06 ± 0·01

* Fresh SPC without preincubation were measured for their mitogenic response to 2 μg/ml Con A in the presence or absence of indicated concentrations of PS or DMSO.

† SPC were preincubated in the Con A-free medium (3 ml) with or without the addition of indicated concentrations of PS or DMSO at 37°C for 24 h (the first culture). Preincubated SPC were thoroughly washed with 2% fetal bovine serum (FBS)–Hank's balanced salt solution (HBSS) and thereafter measured for their Con A response in the absence of PS and DMSO (the second culture).

‡ Solute control.

PS, mediate the manifestation of the suppressor activity of MAIC-induced macrophages.

DISCUSSION

Some lipid components of microbial origin, such as glycopeptidolipids, phenolic glycolipid-I, and phospholipid-like substances, are known to exhibit suppressive activity against T cell functions, including mitogenic response [8,15,16]. Previously, we found suppressive activity of free fatty acids including oleic, linoleic, linolenic, and arachidonic acids against SPC blastogenesis [7]. The present study also showed that PS and PI displayed a potent inhibitory activity against Con A-induced SPC blastogenesis. These findings strongly suggest the important role of lipid components as an immunomodulatory molecule acting on T cells.

Recently, Caselli *et al*. [10] reported the PS-mediated inhibition of phytohaemagglutinin (PHA)-induced proliferation of human peripheral blood mononuclear cells (PBMC). In their experiments PS caused half-maximal inhibition of PHA-induced mitogenesis of human PBMC at a concentration of about 12 μg/ml, which is comparable to the dose of PS required for half-maximal suppression of Con A mitogenesis of murine SPC, i.e. about 25 μg/ml. Their observations that PS reduces the IL-2 expression of mitogen-stimulated PBMC and that the T cell mitogenesis-inhibitory action of PS is expressed in an irreversible manner are consistent with our findings obtained here. Although they observed the minor inhibition of IL-2-induced proliferation of PBMC by PS, we found that PS potently suppressed the IL-2-dependent growth of CTLL-2 cells.

Previously, Stallcup *et al*. [17] demonstrated that macrophage hybridoma cells established by them inhibited lymphoid cell growth via cell-to-cell contact with target cells, and that such cytostatic effect was mediated by a lipid-like substance of the macrophage hybridoma cells. In relation to this, Rich and her

Table 3. Participation of nitric oxide (NO) as effector molecules in the suppressive activity of *Mycobacterium avium-intracellulare* complex (MAIC)-induced macrophages against splenocyte (SPC) mitogenesis*

Addition	Concentration (mM)	MAIC-induced macrophages (10^6 SPC eq.)	SPC mitogenesis (10^3 ct/min ± s.e.m.; $n = 4$)	Macrophage-mediated suppression of SPC mitogenesis (%)†
None	–	–	66·9 ± 3·6	–
None	–	1·0	1·15 ± 0·19	99·3
None	–	2·0	0·30 ± 0·05	99·5
None	–	4·0	0·33 ± 0·02	99·5
NMMA	0·5	–	49·0 ± 1·1	–
NMMA	0·5	1·0	22·3 ± 1·0‡	54·5
NMMA	0·5	2·0	23·7 ± 1·1‡	51·7
NMMA	0·5	4·0	23·4 ± 1·7‡	52·7

* Normal SPC ($2·5 \times 10^5$) were cultured in the presence of concanavalin A (Con A) (2 μg/ml) with or without macrophage monolayer culture prepared by seeding indicated number of MAIC-induced SPC.

† Per cent suppression was calculated for each regimen of the N^G-monomethyl-L-arginine (NMMA) addition.

‡ Significantly higher than the control mitogenesis ($P < 0·01$; Student's *t*-test).

Table 4. Inhibitory effect of reactive nitrogen intermediates (RNI) in combination with phosphatidylserine (PS) against splenocyte (SPC) mitogenesis

Addition of $NaNO_2$ (mM)	Addition of PS (μg/ml)	SPC mitogenesis (10^3 ct/min $\pm$ s.e.m.; $n = 3$)	Per cent suppression of mitogenesis
–	–	123·8 ± 11·2	
–	12·5	131·9 ± 4·3	−6·5
–	25	40·5 ± 2·0	67·3
1·0	–	141·9 ± 2·5	−14·6
1·0	12·5	110·0 ± 3·7	11·1
1·0	25	17·4 ± 5·4*	85·9
2·0	–	62·2 ± 2·9	49·8
2·0	12·5	39·1 ± 1·8†	68·4
2·0	25	2·18 ± 0·31†	98·2
4·0	–	14·9 ± 0·91	88·0
4·0	12·5	8·47 ± 1·05	93·2
4·0	25	0·27 ± 0·01†	99·8
8·0	–	1·53 ± 0·03	98·8
8·0	12·5	0·62 ± 0·01	99·5
8·0	25	0·09 ± 0·00	99·9

SPC ($2{\cdot}5 \times 10^5$ cells/well) were cultured in 5% fetal bovine serum (FBS)–RPMI pH 6·3 containing 2 μg/ml concanavalin A (Con A) with or without the addition of indicated concentrations of $NaNO_2$ (as NO source) and PS alone or their combinations. SPC mitogenesis of solute control (0·4% DMSO) was $127 \pm 5{\cdot}0 \times 10^3$ ct/min.

† Significant combined effect was observed ($P < 0{\cdot}05$; †$P < 0{\cdot}01$; Student's *t*-test).

coworkers [18,19] recently reported that human alveolar macrophages exerted a suppressor activity against PHA-induced T cell mitogenesis through cell-to-cell contact with target lymphocytes. This inhibitory activity was partly attributable to a hydrophobic substance which contained phosphatidylglycerol. Previously we found that the suppressor activity of MAIC-induced macrophages was abrogated by cytochalasin B [5], thereby indicating the essential role of membrane function of these macrophages in the expression of their immunosuppressive activity. This suggests that cell-to-cell interaction is also important in the case of MAIC-induced macrophages. In our experiments, PS and PI caused 50% inhibition of SPC mitogenesis when added at concentrations of 25–40 μg/ml. Thus, PS and PI are regarded to have about three times or more stromger activity than phosphatidylglycerol, which required a concentration of 100–400 μg/ml to yield a similar degree of suppressor activity [19]. Therefore, PS and PI rather than phosphatidylglycerol may act as the principal effector molecules of immunosuppressive macrophages.

In addition to PG and free fatty acids which were previously evidenced as the possible effectors of MAIC-induced macrophages [5–7], RNI molecules in combination with phospholipids are considered to be important effectors for the suppressor activity of MAIC-induced macrophages, for the following reasons. First, specific NO synthase inhibitor, NMMA [9], strongly attenuated the suppressive activity of MAIC-induced macrophages. Second, monolayer culture of MAIC-induced macrophages prepared by seeding 2×10^6 MAIC-induced SPC accumulated a significant amount (i.e. about 10–25 μM concentrations) of nitrite ion, a sequential metabolic product derived from NO (unpublished observation). Third, RNI generated from acidified NO_2^- potently suppressed SPC mitogenesis in a dose-dependent manner. In addition, mitogenesis-inhibitory activity of RNI was strongly augmented in combination with PS. The potent combined effects of RNI with PS supports the concept that PS also acts as one of the major effector molecules of the MAIC-induced suppressor macrophages, although further studies are needed to find direct evidence for this speculation. Recently, we also found that PGE exhibited combined effects with free fatty acids in the inhitibion of SPC mitogenesis (unpublished observation). Therefore, it is of interest to know the precise feature of synergism among various effector molecules of MAIC-induced macrophages. With special reference to these points, further studies are currently under way.

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