Phosphatidylserine Suppresses Antigen-Specific IgM Production by Mice Orally Administered Sheep Red Blood Cells (43469)

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Abstract. **Phosphatidylserine is an endogenous acidic phospholipid that has been shown to modulate nervous system function. In the immune system, phosphatidylserine has been shown to suppress T dependent and T independent immune responses after systemic administration of antigen and lipid. However, no studies on the possible regulation by phosphatidylserine on mucosal immunity have been undertaken. Therefore, we studied the action of phosphatidylserine on immunocompetence using orally immunized mice. Mice orally administered phosphatidylserine (25 mg/kg/day) and subsequently intubated intragastrically with sheep red blood cells showed a significant decrease in antigen-specific IgM production by splenic lymphocytes compared with controls. Furthermore, the response of splenic lymphocytes obtained from phosphatidylserine-treated, antigen-primed animals to antigen or pokeweed mitogen in proliferation assays was markedly suppressed, compared with splenic lymphocytes obtained from nontreated, antigen-primed mice. Similarly, splenic lymphocytes from phosphatidylserine-treated, antigen-primed animals cultured in the presence of antigen produced no measurable interleukin 4 and low levels of interleukin 2, whereas splenic lymphocytes from antigen-primed animals produced measurable levels of interleukin 4 and significantly higher levels of interleukin 2. By fluorescence-activated cell sorter analysis, brightly stained B lymphocytes (Ig+) take up a larger portion of phosphatidylserine than do brightly stained T lymphocytes (Thy 1.2+). Collectively, these results point to the immunosuppressive qualities of phosphatidylserine. Given that phosphatidylserine is released upon injury and destruction of eukaryotic cells, these results suggest that phosphatidylserine may be an endogenous anti-inflammatory molecule.**

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Phosphatidylserine (PS), an acidic phospholipid, is situated predominantly on the inner surface of the eukaryotic cell membrane and has been shown to affect both the immune and neuroendocrine systems. Within the neuroendocrine system, PS injected *in vivo* reduces serum prolactin levels in humans (1) and rats (2), enhances adenylate cyclase activity in brain synaptosomes (3), and stimulates growth hormone and prolactin release from perifused pituitary cells (4). Under inflammatory conditions, PS exposed to the surface of damaged cells potentially can modulate immune cells directly or following conversion to lysophosphatidylserine by phosphalipase A_2, which activates mast cells *in vitro* and *in vivo* (5, 6). Recently, the intravenous administration of PS vesicles in mice immunized intravenously against trinitrophenyl, nitrophenyl-conjugated sheep red blood cells (SRBC), and 2,4-dinitrophenyl-dextran significantly reduced the antigen-specific antibody response (7). This effect was apparent when the animals were exposed to the phospholipid prior to (4 hr) receiving the antigen, but not after antigen immunization. Moreover, the response was dose dependent, with maximum suppression observed at 25 mg/kg of PS. In addition, lymphocytes exposed *in vitro* to PS or lysophosphatidylserine produced substantially less interleukin (IL)-2 than untreated controls in response to concanavalin A (ConA) or phorbol myristate acetate.

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However, in aged rats (10–14 months), chronic treatment of PS (50 mg/kg, in drinking water) reverses the effects of the aging process on immunocompetence by increasing antigen-specific antibody titers to levels equivalent to those of young rats (2–3 months) (8). The immunologic boost in aged rats after PS exposure in the drinking water is reasoned to be linked to protein kinase C activity, since PS is a cofactor for protein kinase C (9) and protein kinase C has been linked to IL-2 receptor expression and IL-2 production (10). Moreover, cellular synthesis of PS has been shown to be associated with IL-1 signaling for IL-2 production (11).

Although PS has been shown to be an immunoregulatory molecule in the systemic immune system, very little, if any, data have been forthcoming concerning its effects on aspects of the mucosal immune system. Early work has shown that antigen-specific B lymphocytes from the gut associated lymphoid tissue (GALT) peak in the spleen of animals on Day 9 after oral immunization (12). Therefore, by sacrificing mice on the ninth day following exposure to antigen intragastrically, it would be possible to observe B lymphocytes from the GALT undergoing proliferation and maturation in the spleen (13). Moreover, recent studies using this model have identified that the majority of antigen-specific immunoglobulin-producing B lymphocytes consistently occurs in the spleen rather than Peyer's patches or mesenteric lymph nodes in mice on Day 9 after oral immunization (14, 15). Therefore, a study investigating the effects PS administration has on the immune response after intragastric incubation was undertaken (i) to determine the overall immunocompetence of the animal as measured by serum levels of antigen-specific antibody production; (ii) to determine changes in trafficking and/or processing of antigen by lymphocytes obtained from the spleen after oral immunization; and (iii) to identify mechanisms of immunosuppression or enhancement after PS exposure.

Materials and Methods

Mice. Male BALB/c mice (6–8 weeks old) were obtained from Charles River Breeding Laboratory (Wilmington, MA).

Immunization and Exposure to PS. Two groups of mice (n = 10/group) were orally administered PS (20 mg/kg) daily for 7 days in 100-μl volumes, while another two groups of animals (n = 10/group) received 100 μl of saline orally per day for 7 days. After the 7-day exposure, one group of the PS-treated mice and one group of saline-treated mice were intragastrically intubated with 5×10^7 SRBC in 100 μl of PS or saline daily for 4 days (hence, PS + antigen and antigen-only groups, respectively). The other two groups of mice were intragastrically intubated with 100 μl of PS or saline daily for 4 days (hence, PS-only and control groups). After an additional 5 days from the last oral exposure to antigen, mice were sacrificed and splenic lymphocytes were obtained from each individual mouse. Serum was obtained by cardiac puncture after CO_2 asphyxiation and used in the enzyme-linked immunosorbent assay.

Culture Conditions for Total and Antigen-Specific Ig Production. Spleens were surgically removed and lymphocytes were teased from the organ by mechanical dispersion. Red blood cells were lysed using 0.84% NH_4Cl. Lymphocytes (2×10^6 cells/2 ml) were cultured in 10% fetal bovine serum (Gibco, Grand Island, NY) in RPMI 1640 media containing 100 units of penicillin/ml, 100 μg of streptomycin/ml, and 40 units of nystatin/ml (designated culture media) for 7 days at 37°C in a 5% CO_2 atmosphere in the presence of SRBC (2×10^6 cells/well). After the incubation period, the supernates were harvested and assayed for (i) total and antigen-specific IgA, IgG, and IgM by enzyme-linked immunosorbent assay and (ii) IL-2 and IL-4 content using the IL-2/IL-4-sensitive CTLL-2 T cell line.

The enzyme-linked immunosorbent assay method used to detect total and antigen-specific Ig levels has been described previously (14, 15).

Lymphocyte Proliferation Assay. Lymphocyte proliferation assays were conducted in 96-well microtiter tissue culture plates. Splenic lymphocytes (2×10^5 cells/well) obtained from the various groups of mice were placed in each well in 200 μl of culture media. Cells were stimulated with either ConA (200 ng/well), pokeweed mitogen ([PWM] 200 ng/well), or SRBC (5×10^5 cells/well). After incubation at 37°C in 5% CO_2 and 95% air for 48 hr, 200 nCi of [^{3}H]thymidine (TdR) in culture media were added (10 μl/well) to each well and the cells were cultured an additional 16 hr. SRBC-stimulated cell cultures were incubated for 72 hr before the addition of [^{3}H]TdR. The cells were then harvested (Cambridge Technology Cell Harvester) on glass fiber filters, dried, and counted to determine cell-associated radioactivity.

CTLL-2 Proliferation Assay. Supernates obtained from the cultured (7 days in the presence of SRBC) lymphocytes of the various treated mice were assayed for IL-2/IL-4 content using the CTLL-2 T cell line. CTLL-2 cells (5×10^4/well) in 125 μl of culture media were placed in wells of 96-well microtiter plates. Lymphocyte culture supernate (75 μl) was added to each well, along with 200 nCi of [^{3}H]TdR (10 μl/well). In neutralization experiments, the lymphocyte supernates were preincubated (60 min) with 11B.11 anti-murine IL-4 monoclonal antibody (16) or anti-murine IL-2 monoclonal antibody (0.1–3.0 μg) prior to adding to the CTLL-2 cells. After a 16–18-hr incubation period in 5% CO_2 and 95% air at 37°C, the cells were harvested on glass fiber filters, dried, and counted to determine cell-associated radioactivity.

Fluorescence-Activated Cell Sorter Analysis of PS + B (Ig+) and T (Thy 1.2+) Lymphocytes. Lymphocytes obtained from the spleen of mice were collected and washed in 1.0 ml of phosphate-buffered saline (PBS) containing 01.% bovine serum albumin (BSA) and 0.05 *M* NaN_3. Lymphocytes (1×10^6 cells) were resuspended in 0.1 ml of ice-cold PBS-BSA plus azide containing 25 μM 1-oleic acid-2-[6-(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]caproyl phosphatidylserine ([PS-N-NDB] Avanti Polar Lipids, Inc., Albaster, AL) and allowed to incubated for 30 min on ice. Cells were subsequently centrifuged (500*g*, 5 min), washed with ice-cold PBS-BSA plus azide and resuspended in 0.1 ml of ice-cold PBS-BSA plus azide containing goat $(Fab)'_2$ anti-mouse Ig-conjugated with phycoerythrin (final dilution, 1/40) or rat $(Fab)'_2$ anti-mouse Thy 1.2 antigen conjugated with phycoerythrin (final dilution, 1/20) and allowed to incubate for 30 min on ice. Cells were washed twice with ice-cold PBS-BSA plus azide fixed in 1% paraformaldehyde solution, and analyzed by fluorescence-activated cell sorter (Becton-Dickinson, Mountainview, CA). Excitation for PS-N-NDB is 460 nm and emission is 534 nm. Excitation for phycoerythrin is 488 nm and emission is 580 nm. A total of 5000 events/sample were read and displayed by contour map.

Reagents. Goat anti-mouse IgA-, IgG-, and IgM-alkaline-phosphatase-conjugated antibodies, goat anti-mouse polyvalent Ig, *p*-nitrophenol phosphate, ConA, and PWM were purchased from Sigma Chemical Co. (St. Louis, MO). Mouse IgA, IgG, and IgM used as standards were obtained from Zymed Laboratories (San Francisco, CA). The phycoerythrin-conjugated goat $(Fab)'_2$ anti-mouse Ig (heavy and light chain specific) was purchased from Tago, Inc. (Burlingame, CA). The phycoerythrin-conjugated rat $(Fab)'_2$ anti-mouse Thy 1.2 monoclonal antibody was purchased from Boehringer Mannheim (Indianapolis, IN). [^{3}H]TdR (sp act 20 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Anti-mouse IL-2 monoclonal antibody was obtained from Collaborative Research, Inc. (Bedford, MA). Sheep red blood cells were obtained from Colorado Serum Co. (Denver, CO). Brain PS of bovine cortical origin was prepared and purified as described previously (17).

Statistics. Statistical significance ($P < 0.05$) comparing groups of treated animals was determined using the nonparametric Mann-Whitney *U* test.

Results

PS Suppresses Antigen-Specific IgM Production by Mice Intragastrically Intubated with SRBC. PS injected intravenously has been shown previously to suppress antigen-specific antibody production to T dependent and T independent antigens, with a maximum effect of PS obtained at 25 mg/kg (7). In order to determine whether PS modulates immunocompetence after oral administration, mice received PS intragastrically and were subsequently orally immunized with SRBC. Sera obtained from the mice show no differences in SRBC-specific antibody content or total (IgA, IgG, or IgM) antibody content of PS-treated antigen-primed mice compared with antigen-primed animals (data not shown). However, both antigen-primed and PS-treated antigen-primed groups had detectable serum levels of SRBC-specific IgM (920–930 ng/ml) compared with control, unprimed animals (<10 ng/ml of anti-IgM serum antibody). SRBC-specific IgA or IgG were not detectable in the sera of any of the animals. Splenic lymphocytes obtained from the mice were re-exposed to SRBC *in vitro* and assessed for total and antigen-specific Ig production after culture for 7 days. The results showed no differences in total immunoglobulin production by the cultured splenic lymphocytes from any of the groups (Table I). However, splenic lymphocytes obtained from PS-treated, antigen-primed mice

Table I. PS Suppresses Antigen-Specific IgM Production by Mice Orally Immunized with SRBC[a]

Treatment[b]	Total Ig			SRBC-specific Ig		
	IgA (ng/ml)	IgG (ng/ml)	IgM (ng/ml)	IgA (ng/ml)	IgG (ng/ml)	IgM (ng/ml)
Control (saline only)	95 ± 63[c]	1295 ± 1023	1703 ± 670	0	0	12 ± 15
PS only	64 ± 50	506 ± 454	1999 ± 1455	0	0	12 ± 15
Antigen only	92 ± 82	6478 ± 6247	2614 ± 961	0	0	76 ± 11[d]
Antigen + PS	118 ± 82	1810 ± 1474	1756 ± 458	0	0	23 ± 5[e]

[a] BALB/c mice (n = 10/group) were intragastrically intubated with PS and SRBC as described in Materials and Methods. Lymphocytes obtained from the spleens were cultured (2×10^6/cells/culture) for 7 days in culture media in the presence of 2×10^6 SRBC. After the 7-day culture period, supernates from the designated populations were collected (individually) and assayed for total and antigen-specific Ig content by enzyme-linked immunosorbent assay.
[b] Mice were intragastrically intubated with saline (control and antigen only) or PS (PS and antigen + PS groups) for 7 days in a 100-μl volume. After this treatment, mice were orally immunized with 100 μl of saline (control and PS only groups) or 100 μl of 5×10^7 SRBC (antigen only and antigen + PS groups) daily for 4 days. After an additional 5 days, the animals were sacrificed.
[c] Numbers ± SD, n = 10. Each individual supernate was measured in triplicate.
[d] $P < 0.01$, comparing antigen-primed to untreated mock (control) groups.
[e] $P < 0.001$, comparing antigen-primed to PS-treated, antigen-primed groups.

Table II. PS Suppresses Antigen-Specific and Mitogen-Stimulated Lymphocyte Proliferation *In Vivo*[a]

Treatment[b]	Mitogen or antigen		
	ConA	PWM	SRBC
Control (saline only)	50,727 ± 6,904[c]	37,392 ± 5,580	2,637 ± 1,154
PS only	50,139 ± 6,345	39,736 ± 2,914	2,514 ± 766
Antigen only	47,148 ± 9,533	38,101 ± 4,791	6,893 ± 1,444[d]
Antigen + PS	54,914 ± 7,506	30,547 ± 5,642[e]	3,405 ± 1,332

[a] Splenic lymphocytes (2×10^5 cells/well) obtained from the treated groups of mice were cultured in 200 μl of culture media in the presence of ConA (200 ng/well), pokeweed mitogen (200 ng/well), or SRBC (5×10^5 cells/well). After 48 hr (or 72 hr for SRBC-stimulated cultures), 200 nCi of [^{3}H]TdR/well were added to cultures, the cells were cultured an additional 16 hr and then harvested, and the cell-associated radioactivity was determined for each sample.
[b] For treatment details, see Table I.
[c] Numbers are in cpm ± SE. Lymphocytes obtained from each individual mouse (n = 10/group) were stimulated and counted in quadruplicate.
[d] $P < 0.05$, comparing untreated, antigen-primed group with any of the other groups.
[e] $P = 0.0865$, comparing untreated, antigen-primed group with PS-treated, antigen-primed group, or comparing PS-treated, antigen-primed group with each of the other groups (pokeweed mitogen).

had a significantly reduced level of SRBC-specific IgM detected in culture supernates compared with cultured splenic lymphocyte supernates from untreated, antigen-primed animals (Table I). Both splenic lymphocyte from PS-treated and untreated, antigen-primed mice produced more SRBC-specific IgM compared with unprimed animals (Table I) as detected by measuring antigen-specific antibody in lymphocyte culture supernates.

Intragastric Intubation with PS Suppresses Splenic Lymphocyte Proliferation to Antigen and PWM. Splenic lymphocytes obtained from the animals represented in Table I were also investigated for their response to mitogens and SRBC (antigen) in proliferation assays as measured by uptake of [^{3}H]TdR. The blastogenic response by lymphocytes obtained from the antigen-primed animals treated with PS and exposed to SRBC was significantly suppressed compared with the antigen-primed mice (Table II). However, no differences in lymphocyte proliferation were observed between treated groups in response to ConA or PWM (Table II).

PS Suppresses IL-2 and IL-4 Production in Vitro. Supernates obtained from the lymphocyte cultures used to measure total and antigen-specific antibody production as shown in Table I were also investigated for IL-2/IL-4 content using the T cell line CTLL-2. The results (Fig. 1) indicated that lymphocytes obtained from antigen-primed animals produced more IL-2/IL-4 compared with lymphocyte from PS-treated antigen-primed mice. However, lymphocyte obtained from PS-treated, antigen-primed or antigen-primed animals produced significantly more IL-2/IL-4 compared with lymphocytes obtained from saline-treated (control) animals (Fig. 1). In order to determine whether a difference in IL-2 and/or IL-4 production exists between lymphocytes taken from the antigen-primed animals versus the PS-treated, antigen-primed mice, neutralization assays

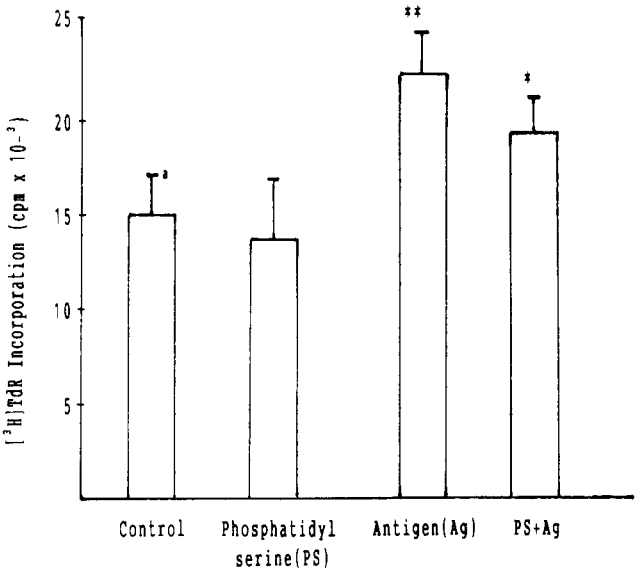

Figure 1. CTLL-2 proliferation after culturing with supernates from lymphocyte obtained from PS- and/or antigen-treated mice. Splenic lymphocytes obtained from the treated groups of mice were cultured (2×10^6 cells/culture) in culture media in the presence of 2×10^6 SRBC/culture for 7 days in a 5% CO_2 and 95% air incubator at 37°C. After the incubation period, supernates were harvested independently and assayed for IL-2/IL-4 content using the CTLL-2 T cell line. Unstimulated lymphocyte supernates were used to determine background counts. Cells were incubated for 16 to 18 hr in a 5% CO_2 and 95% air incubator at 37°C in the presence of 200 nCi of [^{3}H]TdR. Background counts were subtracted out in order to display specific [^{3}H]TdR uptake. a, Bars represent SE. Each mouse splenic lymphocyte supernate (n = 5/group) was determined in quadruplicate. *$P < 0.05$, comparing PS-treated, antigen-primed mice with unprimed (control) supernates. **$P < 0.01$, comparing antigen-primed with unprimed (control) supernates.

were conducted using anti-mouse IL-2 and IL-4 monoclonal antibodies. In a dose-dependent fashion, anti-IL-2 monoclonal antibody blocked as much as 64% of the stimulatory activity in the supernates from the lymphocyte cultures of the antigen-primed mice (Fig. 2) and 100% of the stimulatory activity in the supernates from

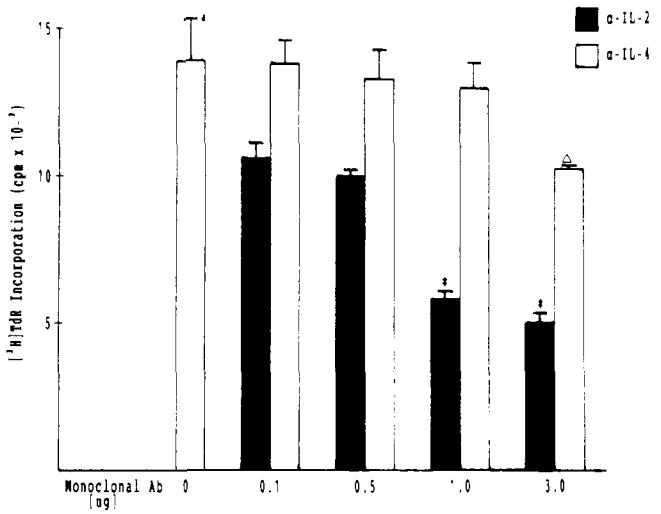

Figure 2. Lymphocyte cultures from antigen-primed mice contain IL-2 and IL-4. Supernates were obtained from lymphocyte cultures of antigen-primed mice and preincubated with or without anti-mouse IL-2 (0.1–3.0 μg) or anti-mouse IL-4 (0.1–3.0 μg) monoclonal antibodies for 60 min at 37°C before the addition of CTLL-2 cells (5×10^4 cells/well). Cells were incubated for 16 hr in a 5% CO_2 and 95% air incubator at 37°C in the presence of 200 nCi of [^{3}H]TdR, then harvested and counted for [^{3}H]TdR uptake. Unstimulated lymphocyte supernates were used to determine background counts. Background counts were subtracted out in order to display specific [^{3}H]TdR uptake. αIL-4 monoclonal antibody (3.0 μg) will block 100% of the stimulatory action of 10 units of recombinant IL-4 and 50% of the stimulatory action of 50 units of recombinant IL-4 in CTLL-2 proliferation assays (data not shown). a, Bars represent SE ($n = 4$). *$P <$ 0.05, comparing antibody-treated with untreated supernates. Δ, $P =$ 0.1573, comparing antibody-treated with untreated supernates.

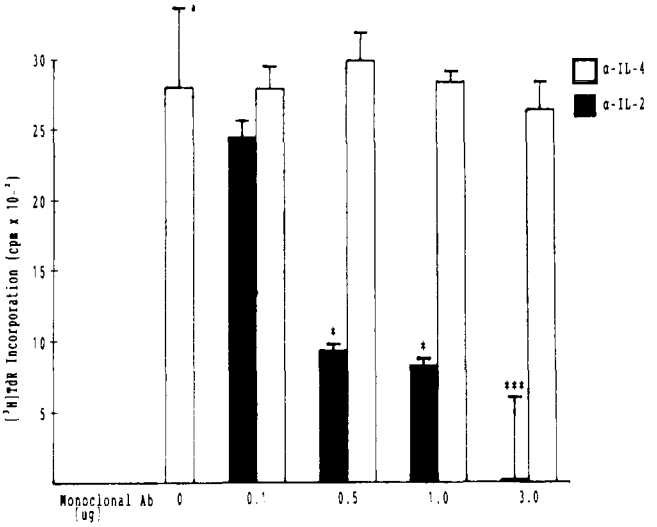

Figure 3. Lymphocyte cultures from PS-treated, antigen-primed mice contain IL-2 only. Supernates were obtained from lymphocyte cultures of PS-treated, antigen-primed mice and preincubated with or without anti-mouse IL-2 or anti-mouse IL-4 monoclonal antibodies, as described in the legend of Figure 2. After preincubation, CTLL-2 cells (5×10^4/well) were added along with 200 nCi of [^{3}H]TdR and allowed to incubate in a 5% CO_2 and 95% air incubator at 37°C for 16 hr. Cells were then harvested and counted to determine specific [^{3}H]TdR uptake. Unstimulated lymphocyte supernates were used to determine background counts. Background counts were subtracted out in order to display specific [^{3}H]TdR uptake. a, Bars represent SE ($n = 4$). *$P < 0.05$, comparing antibody-treated with untreated supernates. ***$P < 0.001$, comparing antibody-treated with untreated supernates.

the lymphocyte cultures of the PS-treated, antigen-primed mice (Fig. 3). Likewise, in a dose-dependent fashion, anti-IL-4 monoclonal antibody blocked as much as 26% of the stimulatory activity in the supernates from the lymphocyte cultures of the antigen-primed mice (Fig. 2). However, using the same concentration of anti-IL-4 monoclonal antibody to neutralize IL-4 activity in the supernates from the lymphocyte cultures of the PS-treated, antigen-primed mice, no effect was observed, which indicates the absence of IL-4 in these supernates (Fig. 3).

B Lymphocytes Are the Initial Targets of PS. To define which lymphocyte population is the primary target in incorporating PS, double staining on splenic lymphocytes was carried out using PS-N-NDB and phycoerythrin-conjugated anti-Thy 1.2 and anti-Ig antibodies. The results (Fig. 4) showed that 15 ± 4% ($n =$ 2) of the relatively bright Ig+-stained (B lymphocytes) population of cells incorporated PS within 30 min of exposure, whereas 3 ± 2% of the relatively bright Thy 1.2+-stained (T lymphocytes) cells incorporated PS-N-NDB into their membranes in the same time period.

Discussion

In the present report, intragastric intubation of mice with PS followed by oral immunization with SRBC resulted in suppression of immunocompetence. It is currently unknown whether this effect is mediated principally by PS or PS metabolites, including phosphatidylethanolamine, phosphatidylcholine, lysophosphatidylserine, or neutral lipid. PS or PS metabolites may be able to block antigen processing and/or the trafficking of stimulated lymphocytes from the GALT to the spleen and mesenteric lymph nodes. The data (Table I) showing no differences in total immunoglobulin production by cultured lymphocytes of the various treated groups suggest that absolute numbers of T and B lymphocytes may not be affected by PS at the level of the spleen. However, antigen-stimulated B and/or T lymphocyte migration to the spleen from the GALT may be hindered or altered after PS treatment. The fact that less SRBC-specific antibody is produced by splenic lymphocytes from antigen-primed, PS-treated animals compared with untreated, antigen-primed animals (Table I) after culture and re-exposure to antigen indicates that (i) there are fewer numbers of presensitized B lymphocytes in the spleen of mice treated with PS at Day 9 compared with untreated mice; (ii) there are fewer numbers of activated or functional T lymphocytes in the spleen at Day 9 after PS exposure orally; or (iii) there are effects on the cellular machinery with regard to the production of cytokines necessary to mount an optimal immune response resulting in a diminution in immune activity in these mice exposed to PS.

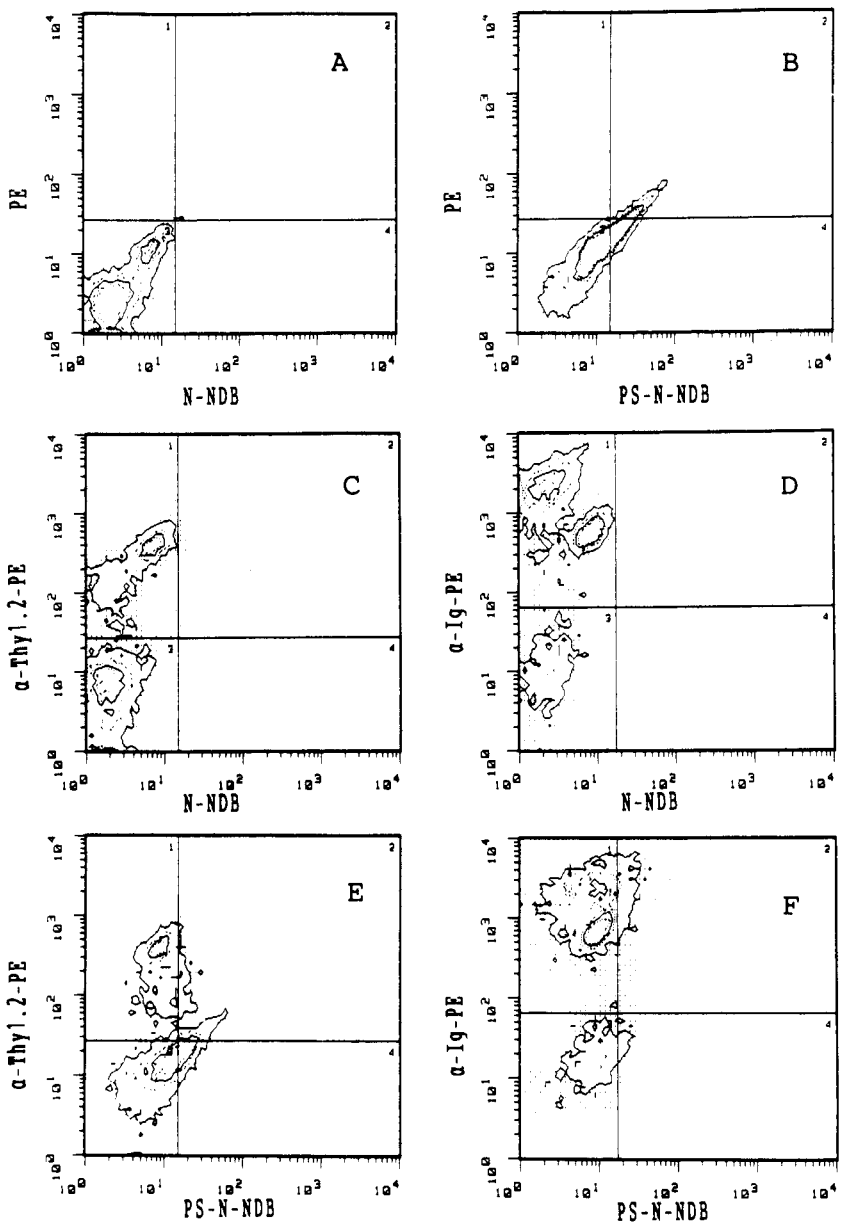

Figure 4. Contour rnap display of PS$^+$ T (Thy 1.2)- and B (Ig$^+$)-lymphocytes. See Materials and Methods section for details. (A) Double negative. (B) PS-N-NDB$^+$ splenic lymphocytes. (C) Thy 1.2$^+$ splenic lymphocytes. (D) Ig$^+$ splenic lymphocytes. (E) PS-N-NDB$^+$/Thy 1.2$^+$ splenic lymphocytes (3%). (F) PS-N-NDB$^+$/Ig$^+$ splenic lymphocytes (15%).

Certainly, B lymphocytes are directly or indirectly affected by PS. As shown in Figure 4, B lymphocytes are early targets of PS compared with T lymphocytes. Although macrophages could be a third cell source for targeting of PS, previous studies indicate that this is unlikely (18, 19). Likewise, splenic lymphocytes obtained from PS-treated, antigen-primed mice respond less to the T dependent, B cell mitogen, pokeweed mitogen, compared with lymphocytes from the other groups (Table II). This effect is only seen using lymphocytes from the PS-treated, antigen-primed animals, because splenic lymphocytes from the unprimed, PS-treated group respond at the same level as control and antigen-primed groups (Table II). These results seem to suggest that lymphocyte activation may be a prerequisite for PS-induced immunosuppression or that lymphocytes which have migrated from the GALT to the spleen are immunosuppressed, contributing to the diminution in proliferation. The data indicating no differences in proliferation by splenic lymphocytes taken from any of the groups in response to the T cell mitogen concanavalin A also indicate that B lymphocytes are the primary target of PS action.

Interestingly, the measurement of IL-2/IL-4 content in the cultured lymphocyte supernates suggests that T cells are also a target of PS. Using the IL-2/IL-4-sensitive T cell line CTLL-2 and anti-IL-2 and anti-IL-4 monoclonal antibodies in neutralization assays, PS treatment *in vivo* inhibits IL-4 production by lymphocytes re-exposed to antigen *in vitro* (Fig. 3). Since

IL-4 is synthesized by T_H2 lymphocytes (20), PS appears to target this subpopulation of T lymphocytes. Whether T_H1 lymphocyte production of IL-2 is affected by PS cannot be determined with the present data.

The suppression in B lymphocyte proliferation in response to pokeweed mitogen and the absence of IL-4 production by lymphocytes obtained from PS-treated, antigen-primed animals correlate with the reduction in SRBC-specific antibody production by the cultured lymphocytes (Table I). However, no differences in the serum level of SRBC-specific antibody between the antigen-primed, PS-treated, and antigen-primed, untreated animals were found. It should be noted the serum samples represent the primary immune response, whereas supernatant levels represent the secondary immune response. The exposure of PS-treated and untreated animals to antigen followed by measuring serum levels of antigen-specific antibody would address the immunosuppressive qualities of PS administered orally. The present model was developed to investigate the effects PS might have on the trafficking or processing of antigen by GALT lymphocytes after a primary immunization orally. Previous studies have only investigated serum or splenic lymphocyte production of antibodies after PS administration peripherally (7, 8).

From a pathophysiologic point of view, the release of PS and lysophosphatidylserine after the destruction of cells may be an internal check (independent of the immune system) to control and limit inflammation. Specifically, the greater number of cells destroyed locally during an immune response would lead to an increase in PS and metabolic products that could reach levels ultimately resulting in immunosuppression. Thus, external to the immune system, there may be control mechanisms eukaryotic cells have engineered in regulating the degree and intensity of an immune reaction. However, autoimmune episodes would tend not to support this idea unless these immune cells are not as sensitive to PS as normal cells. It is hoped that future work will address this question.

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1. Falaschi P, Rocco A, D'Urso R, Jellamo R, Toffano G, Frajese G. Phospholipid liposomes and prolactin secretion: Effect on spontaneous drug-induced hyperprolactinemia. Life Sci **31**:17–23, 1982.
2. Canonico PL, Annumziato L, Toffano G, Bernardini R, Stanzani S, Foti M, Clementi G, Drago F, Scapagnini U. In vivo and in vitro interference of phosphatidylserine liposomes on prolactin secretion in the rat. Neuroendocrinimmunology **33**:358–362, 1981.
3. Floreani M, Carpenedo F. Phosphatidylserine vesicles increase rat brain synaptosomal adenylate cyclase activity. Biochem Biophys Res Commun **145**:631–636, 1987.
4. Spangelo BL, Ross PC, Harvis WD, MacLeod RM. Brain cortex phosphatidylserine stimulates anterior pituitary hormone release in vitro. Progress in NeuroEndocrinImmunol **3**:35–42, 1990.
5. Bruni A, Bigon E, Battistella A, Boarato E, Mietto L, Toffano G. Lysophosphatidylserine as histamine releaser in mice and rats. Agents Actions **14**:619–625, 1984.
6. Chang HW, Inove K, Bruni A, Boarato E, Toffano G. Stereoselective effects of lysophosphatidylserine in rodents. Br J Pharmacol **93**:647–653, 1988.
7. Ponzin D, Mancini D, Toffano G, Bruni A, Doria G. Phosphatidylserine-induced modulation of the immune response in mice: Effect of intravenous administration. Immunopharmacology **17**:167–176, 1989.
8. Guarcello V, Triolo G, Cioni M, Morale MC, Farinella Z, Scapagnini U, Marchetti B. Phosphatidylserine counteracts physiological and pharmacological suppression of humoral immune response. Immunopharmacology **19**:185–195, 1990.
9. Nishizuka Y. Turnover of inositol-phospholipids and signal transduction. Science **225**:1365–1370, 1984.
10. Isakov N, Malley MI, Scholz W, Altman A. T-lymphocyte activation: The role of protein kinase C and the inositol phospholipid signal transduction pathway. Immunol Rev **95**:89–111, 1987.
11. Didier M, Aussel C, Pelassy C, Fehlmann M. IL-1 signaling for IL-2 production in T cells involves a rise in phosphatidylserine synthesis. J Immunol **141**:3078–3080, 1988.
12. Andre C, Bazxin H, Herzmans F. Influence of repeated administration of antigen by the oral route on specific antibody-producing cells in the mouse spleen. Digestion **9**:166–175, 1973.
13. Anderson AO. Structure and organization of the lymphatic system. In: Oppenheim JJ, Shevach EM, Eds. *Immunophysiology.* New York: Oxford University Press, p14, 1990.
14. Carr DJJ, Radulescu RT, Blalock JE. Phentolamine augmentation of total and antigen-specific immunoglobulin production by splenic and Peyer's patch lymphocytes from mice orally immunized with SRBCs. Progress in NeuroEndocrinImmunol **3**:125–135, 1990.
15. Carr DJJ, Woolley TW, Blalock JE. Phentolamine but not prooanolol blocks the immunopotentiating effect of cold stress on antigen-specific IgM production in mice orally immunized with SRBCs. Brain Behav Immun **6**:50–63, 1992.
16. Ohara J, Paul WE. B-cell stimulating factor-1/interleukin-4. Annu Rev Immunol **5**:429–459, 1987.
17. Lees WG. Preparation and analysis of phosphatides. Methods Enzymol **3**:328–345, 1957.
18. Mietto L, Boarato E, Toffano G, Bruni A. Lysophosphatidylserine-dependent interaction between rat leukocytes and mast cells. Biochim Biophys Acta **930**:145–153, 1987.
19. Gilbreath MJ, Hoover DL, Aluing CR, Swartz GM, Meltzer MS. Inhibition of lymphokine-induced macrophage microbiocidal activity against Leishmania major by liposomes: Characterization of the physicochemical requirements for liposome inhibition. J Immunol **137**:1681–1687, 1986.
20. Arai K, Lee F, Miyajima A, Miyatake S, Arai N, Yokota T. Cytokines: Coordinators of immune and inflammatory responses. Annu Rev Biochem **59**:783–836, 1990.