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Soluble Peptidoglycan from *Staphylococcus aureus* Is a Murine B-Lymphocyte Mitogen

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Soluble peptidoglycan from *Staphylococcus aureus* has been shown to be capable of causing murine B lymphocytes from the spleen to proliferate and to secrete immunoglobulins in both an in vitro and an in vivo assay. The optimal concentration in vitro was between 33 and 100 μg/ml. A 3-day incubation with soluble peptidoglycan was more stimulatory than was a 1- or 2-day incubation. Removal of most of the T lymphocytes with anti-theta serum did not result in any significant change in the mitogenic activity of soluble peptidoglycan on the remaining B cells.

Soluble peptidoglycans (SPG) are secretory by-products of penicillin action on some gram-positive bacteria (8, 11, 12, 15, 18). The composition of SPG is similar to that of cell-wall-derived peptidoglycans (PG), although SPG are non-cross-linked (11, 18). PG have been shown to have a wide variety of biological properties, many of which are shared with lipopolysaccharide (LPS) (6, 14). Among these is the ability to cause B lymphocytes to proliferate and to secrete immunoglobulins in vitro and in vivo (1–3). Heretofore, no such properties have been shown for high-molecular-weight SPG. The monomer from lysozyme-treated SPG from *Brevibacterium divaricatum* has been shown to be adjuvant active (7). In this report, we show that SPG from *Staphylococcus aureus*, like PG, can cause proliferation and immunoglobulin secretion by B lymphocytes both in vitro and in vivo.

MATERIALS AND METHODS

Mice. Female BALB/c and Swiss Webster mice weighing 18 to 20 g were obtained from the National Institutes of Health, Bethesda, Md. Female B10.M mice of the same weight were purchased from the Jackson Laboratory, Bar Harbor, Maine.

Mitogens. SPG were obtained from a clinical strain of *S. aureus* (19) and from *Micrococcus luteus* (11), as previously described. Amino acid analyses of these SPG that had been previously prepared under identical conditions in a minimal cell wall growth medium showed no evidence of protein contamination (11, 18). PG from *S. aureus*, kindly provided by R. Dziarski, was prepared free of protein and teichoic acid, as previously described (2). LPS and concanavalin A (con A) were purchased from Sigma Chemical Co., St. Louis, Mo.

Mitogenic assay. Spleen cells were cultured as published previously (10). Briefly, mice were killed by cervical dislocation, spleens were removed aseptically, single-cell suspensions were obtained, and erythrocytes were lysed with 0.83% ammonium chloride solution. The cells were cultured in RPMI 1640 supplemented with 5% fetal calf serum at a concentration of 2×10^5/0.2 ml for 24, 48, or 72 h. One microcurie of tritiated thymidine was added 4 h before harvesting. Cells were harvested on glass-fiber filters and lysed with distilled water. Radioactivity on the filters was counted in Formula 949 (New England Nuclear Corp., Boston, Mass.).

Coupling of protein A to SRBC. Protein A (Pharmacia Fine Chemicals, Piscataway, N.J.) was coupled to sheep erythrocytes (SRBC) with reagent-grade $CrCl_3$ (Fisher Scientific Co., King of Prussia, Pa.) by the method of Gronowicz et al. (5) with minor modifications. Briefly, SRBC were washed four times with 0.9% saline. Equal volumes of protein A (1 mg/ml in saline) and packed SRBC were mixed well. To this suspension, 10 volumes of freshly prepared aqueous $CrCl_3$ (60 μg/ml) were added while this suspension was gently stirred. After 1 h at room temperature, excess phosphate-buffered saline (50 volumes) was added. After centrifugation, the protein A-coupled SRBC were washed three times with Hanks balanced salt solution.

PFC assay. The polyclonal response determined by the number of plaque-forming cells (PFC) was measured by a modified Jerne technique (16). Polyvalent rabbit anti-mouse immunoglobulin (Cappel Laboratories, Cochranville, Pa.) was used as a developing serum, and lyophilized guinea pig serum (GIBCO Laboratories, Grand Island, N.Y.) was used as a source of complement. This polyvalent serum appears to be mainly of immunoglobulin G (IgG) specificity (unpublished data). Plaques scored on petri dishes with protein A-coupled SRBC alone were subtracted from all of the experimental values. This control number of PFC generally was about 10 PFC per 10^6 cells. The determination of IgM and IgG plaques was performed by R. Dziarski, using monospecific anti-

TABLE 1. Mitogenicity of SPG from *S. aureus* in murine splenocyte cultures

Addition	Concn (μg/ml)	BALB/c		Swiss Webster		B10.M	
		cpm	SI[a]	cpm	SI	cpm	SI
None		4,569		6,142		4,888	
Con A	1	183,152	40.1	ND[b]		ND	
LPS	30	111,301	24.4	134,037	21.8	19,103	3.9
SPG	100	127,518	27.9	131,978	21.5	45,419	9.3
SPG	33	177,823	38.9	119,439	19.5	25,010	5.2
SPG	11	32,488	7.1	19,254	3.1	24,003	4.9
SPG	3.8	24,447	5.4	ND		ND	

[a] SI, Stimulation index.
[b] ND, Not determined.

mouse μ and γ sera, instead of the polyvalent anti-mouse immunoglobulin (2).

In vivo studies. Mice were injected in the retroorbital plexus with 100 μl (1 mg/ml) of mitogen in sterile saline. After 4 days, the animals were sacrificed by cervical dislocation, and single-cell suspensions were made from their spleens. For the mitogenic assay, these cells were plated at 2×10^5 cells per well for 68 h in RPMI 1640 and then incubated with 1 μCi of tritiated thymidine for 4 h more, as described above. For the PFC assay, the cells were plated immediately by the modified Jerne technique used for the in vitro studies.

T-cell depletion assay. Anti-Thy 1.2 obtained from HO-13-4 cells and fresh guinea pig serum were the kind gifts of T. T. MacDonald. Splenocytes (10^7 cells) from a Swiss Webster mouse were incubated in 1 ml of the anti-Thy 1.2 supernatant at 4°C for 30 min. The cells were then centrifuged and suspended in a 1:10 dilution of guinea pig serum in Hanks balanced salt solution. After 30 min at 37°C, the cells were centrifuged, washed twice, and then incubated at 2×10^5 viable cells per well, according to the conditions for the proliferation assay.

RESULTS

In vitro proliferation of splenocytes. Splenocytes from outbred (Swiss Webster), inbred (BALB/c), and congenic (B10.M) mice were incubated with different amounts of SPG from *S. aureus* and with previously determined optimal concentrations of LPS for 3 days (Table 1). In each case, SPG and LPS were significantly mitogenic for the splenocytes. The optimal concentration of SPG varied to some extent and was maximal at 33 or 100 μg/ml. In one experiment, con A also caused significant mitogenic stimulation. SPG from *M. luteus*, which has a glycine residue on the α-carboxyl moiety of D-glutamic acid, was not mitogenic (data not shown).

Incubation of BALB/c splenocytes for only 1 or 2 days with SPG, as with LPS and con A, was less mitogenic than was incubation for 3 days (Table 2). Consequently, all further proliferation experiments were done with a 3-day incubation.

Treatment of Swiss Webster splenocytes with anti-theta serum and fresh guinea pig complement resulted in the loss of about 45% of the splenocytes. The treated splenocyte population was much less stimulated by con A than was the population treated only with complement (Table 3). SPG, on the other hand, was equally as good a mitogen in both populations (taking into account that the wells with the anti-theta serum-treated cells had approximately 80% more B lymphocytes). LPS appeared to be a better mitogen among the anti-theta serum-treated population (even taking into account the greater number of B lymphocytes).

In vitro maturation of splenocytes. BALB/c splenocytes were incubated for 3 days with about 30 μg of SPG or LPS per ml (Table 4). Both LPS and SPG caused significant increases in the numbers of PFC. Under the same condi-

TABLE 2. Kinetics of the mitogenic stimulation of BALB/c splenocytes by SPG from *S. aureus*

Addition	Concn (μg/ml)	1[a]		2		3	
		cpm	SI[b]	cpm	SI	cpm	SI
None		3,169		10,586		15,760	
Con A	1	5,851	1.9	58,109	5.5	150,371	9.6
LPS	30	8,549	2.7	36,239	3.4	126,886	8.1
SPG	100	3,532	1.1	22,049	2.1	85,782	5.5
SPG	50	ND[c]		ND		101,125	6.5
SPG	25	2,319	0.7	24,097	2.3	33,635	2.2

[a] Day in culture.
[b] SI, Stimulation index.
[c] ND, Not determined.

TABLE 3. Mitogenicity of SPG from *S. aureus* in T-cell-depleted Swiss Webster splenocyte cultures

Addition	Concn (μg/ml)	Complement only[a]		Complement + anti-θ[b]	
		cpm	SI[c]	cpm	SI
None		1,478		2,069	
Con A	1	98,000	66.3	5,522	2.7
LPS	34	12,500	8.5	75,963	36.7
SPG	25	14,674	9.9	24,108	11.7

[a] Complement only resulted in a 10% loss of cellular viability.
[b] Complement + anti-θ (anti-theta serum) resulted in a 45% loss of cellular viability.
[c] SI, Stimulation index.

tions, no immunoglobulin-secreting cells were detected by plaques among the cells incubated in medium alone.

Since the polyvalent anti-mouse immunoglobulin serum was predominantly anti-IgG, another experiment was performed with BALB/c splenocytes, using monospecific anti-mouse μ and γ sera. The number of IgM-secreting cells stimulated by SPG was about 10-fold that of IgG-secreting cells (Table 4). For the comparison with PG from *S. aureus*, a concentration of PG previously shown to be optimal for splenic stimulation (400 μg/ml) was used (2). Despite the relatively high concentration of PG, the stimulation of immunoglobulin-secreting cells by PG was not that much greater than that by SPG.

In vivo studies. Swiss Webster mice were injected intravenously with 100 μg of SPG, LPS, or saline. After 4 days, they were sacrificed, and their spleens were grated into single-cell suspensions. The LPS- and SPG-treated animals showed a splenomegaly of two- to threefold (Table 5). For the proliferation assay, these splenocytes were incubated for 3 days in vitro in RPMI- 1640. Both LPS and SPG showed three- to fourfold stimulation in terms of the [^{3}H]thymidine incorporation (Table 5). In an experiment in which splenocytes were assayed immediately for proliferation, similar results were obtained (data not shown). For the plaque-forming assay, the mitogenic effects of LPS and SPG were even more marked (Table 5) than was the increase in cell numbers or the results from the proliferation assay.

DISCUSSION

SPG from *S. aureus* has been shown to be capable of stimulating murine B lymphocytes to proliferate and to secrete immunoglobulins. The degree of stimulation by SPG and the optimal concentration range are similar to those by LPS. The kinetics of stimulation of murine B-cell proliferation by SPG show that a 3-day incubation is superior to a 2-day incubation. This is in contrast to the kinetics of stimulation by PG in which a 2-day incubation is optimal (1). Apparently, the actions of SPG and PG are not identical.

There are some similarities between the SPG and PG from *S. aureus* in their ability to activate B lymphocytes. For both, T cells have little or no effect on B-cell activation (4). For both, the activation of B lymphocytes appears to be polyclonal (2, 3). Neither the SPG nor the PG from *M. luteus* was an activator of B lymphocytes (9).

The physiological significance of SPG, however, may be different from that of PG. Its secretion from organisms such as *Streptococcus faecium* and *S. aureus* in vitro is triggered by β-lactam antibiotics which are commonly used to treat infections. Recently, we showed that SPG-like molecules can be detected in human urine after an oral dose of penicillin VK (H. Park, A. R. Zeiger, and H. R. Schumacher, submitted for publication). This indicates that SPG can be derived from indigenous bacteria.

A more indirect line of evidence for the secretion of SPG in vivo comes from serological studies of patients with *S. aureus*-caused endocarditis (17). Antibodies to determinants in SPG were found in higher titers among patients treated with β-lactam antibiotics than among patients treated solely with vancomycin. Vancomycin does not trigger SPG secretion in vitro. The time course of anti-SPG titers among these patients showed an increase in titer associated with β-lactam antibiotic administration rather than with infection. Thus, the potency of SPG during

TABLE 4. Effect of SPG from *S. aureus* on the PFC response of BALB/c splenocyte cultures

Addition	Concn (μg/ml)	PFC/10^6 cells					
		Expt 1	Expt 2	IgM (expt 1)	IgG (expt 1)	IgM (expt 2)	IgG (expt 2)
None		0	0	1,603	52	1,081	17
LPS	30	5,250	4,000	ND[a]	ND	ND	ND
SPG	33	7,750	5,250	49,725	4,492	38,076[b]	6,263[b]
PG	400	ND	ND	50,808	7,814	73,747	17,956

[a] ND, Not determined.
[b] The concentration of SPG for this experiment was 75 μg/ml.

TABLE 5. In vivo proliferation and activation of murine B lymphocytes[a]

Treatment	Animal	Cells/spleen $\times 10^6$	[^{3}H]thymidine incorporation (cpm/2 $\times 10^5$ cells)	PFC/2 $\times 10^5$ cells
Control	1	84	1,989	105
	2	77	1,147	150
	3	81	—[b]	35
SPG	1	226	3,696	750
	2	120	6,956	825
	3	154	5,745	715
LPS	1	190	4,652	660
	2	210	4,672	600
	3	200	7,322	775

[a] The methodologies used here are presented in the text.

[b] —, <200 cpm.

antibiotic therapy of human infectious diseases and the possible countereffects of anti-SPG may be of considerable practical interest. Antibodies to the immunodominant determinant in SPG have been shown to be capable of inhibiting murine B-cell stimulation by PG in vitro (4) and of inhibiting PG-induced pyrogenicity (13).

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