Staphylococcal Endo-β-*N*-Acetylglucosaminidase Inhibits Response of Human Lymphocytes to Mitogens and Interferes with Production of Antibodies in Mice

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Abstract

The effect of a bacteriolytic enzyme, the endo-β-*N*-acetylglucosaminidase excreted by *Staphylococcus aureus* (SaG) on the response of human lymphocytes to mitogens and on the immune response in mice has been studied. SaG inhibited incorporation of [^{3}H]thymidine into TCA-precipitable material by human peripheral lymphocytes stimulated either by phytohemagglutinin or by concanavalin A, as well as formation of cytoplasmic immunoglobulin-containing cells by B lymphocytes treated with pokeweed mitogen. In all cases the level of inhibition first increased with the SaG concentrations reaching values of over 80% at an enzyme concentration of 100 μg/ml, and then decreased. Heat-inactivated SaG as well as SaG treated with both polyclonal and monoclonal specific antibodies or enzyme inhibitors such as chitotriose or hydrolyzed peptidoglycan had no effect on lymphocyte response to mitogens. In mice, SaG at a dose of 300 μg per mouse was found to cause a fourfold decrease in the anti-BSA antibody titer and an ~ 70–75% reduction in the immunoglobulin-containing cells in the spleens of mice injected with sheep red blood cells. SaG also completely abolished the enhancing effect of adjuvants such as muramyldipeptide, Freund's complete adjuvant, and *Escherichia coli* lipopolysaccharide. When SaG was injected into mice together with *S. aureus* peptidoglycan hydrolyzed either by SaG or by human lysozyme, the inhibitory effect on both production of anti-BSA circulating antibodies and appearance of Igc cells in the spleens of mice injected with sheep red blood cells was enhanced. As we know that (*a*) human tissues contain endo-β-*N*-acetylglucosaminidases; (*b*) other human hexosaminidases (lysozymes) have previously been shown to interfere with the functions of immunocompetent cells; and (*c*) products of hexosaminidase hydrolysis of peptidoglycan (muropeptides) known to modulate immune response are ordinarily found in the urine of healthy persons, the possibility that hexosaminidases play a major role in the regulation of the immune response is raised and discussed. (*J. Clin. Invest.* 1991. 87:1969–1976.)

Key words: bacteriolytic enzyme • lysozyme • immune response • *Staphylococcus aureus* • pathogenicity

Introduction

It has long been known that bacteria can release enzymes that hydrolyze peptidoglycan and cause bacteriolysis (bacteriolytic enzymes) in the growth medium (1). More recently, it has been shown that extracellular release of bacteriolytic enzymes is a property common to all strains of some genera of great importance for human pathology, such as the genus *Staphylococcus* (2) and the genus *Enterococcus* (3), or a property of all isolates of that species which is the most important human pathogen in a given genus, such as *Pseudomonas aeruginosa* among the *Pseudomonadaceae* (3).

Several bacteriolytic enzymes excreted by bacteria have been isolated, purified, and characterized. Among these, those isolated from pathogenic bacteria, namely, *Staphylococci* and *Enterococci,* were found to act as hexosaminidases; in particular the enzymes from *Staphylococci* were found to act as *N*-acetylglucosaminidases (4) and those from *Enterococci* as muramidases (5).

Bacteriolytically active muramidases and glucosaminidases are present in vertebrates, where they are produced by cells of the immune system such as macrophages, monocytes, and granulocytes (6, 7). Although the role these enzymes play in human physiology is largely unknown, some observations such as the findings that human lysozyme (the muramidase produced by the vertebrate cells mentioned above) regulates the activated state of human polymorphonuclears (8) and that both hen egg white and human lysozyme drastically inhibit the in vitro response of human lymphocytes to mitogens (9) indicate a possible role of these enzymes in regulating the activity of nonspecific and specific host defenses. Despite this, the possibility that bacteriolytic enzymes produced by bacteria could interfere with immunocompetent cell functions and with immune response has not yet been considered.

We have previously made the novel and, at the time, unexpected observation that the endo-β-*N*-acetylglucosaminidase produced by *Staphylococcus aureus* (SaG),[1] an enzyme with substrate specificity very close to that of lysozyme since it hydrolyzes peptidoglycan by splitting the 1-4 β-glucosidic bonds in the same way as lysozyme, but yielding reducing *N*-acetylglucosamine instead of muramic acid (10), is capable of interfering with several aspects of vertebrate cell physiology (11, 12). Others have shown that endo-β-*N*-acetylglucosaminidases are produced by cells of some mammals including humans (13, 14). These facts, together with the well-established knowledge

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1. *Abbreviations used in this paper:* cIg+ cells, cytoplasmic immunoglobulin-containing cells; HL, human lysozyme; MDP, muramyldipeptide; PFC, plaque-forming cells; SaG, *Staphylococcus aureus* endo-β-*N*-acetylglucosaminidase; SRBC, sheep red blood cells.

that glucosaminidases of the exo type share the property of being produced by cells of the immune system with lysozyme (15), prompted us to evaluate the possibility that SaG might interfere with human lymphocyte functions and, perhaps, with the immune response in mammals.

In this work we show that SaG prevents in vitro response of human lymphocytes to mitogens and strongly interferes with the immune response of mice, significantly reducing production of circulating antibodies and completely abolishing the enhancing effect of muramyldipeptide, Freund's complete adjuvant, and *Escherichia coli* lipopolysaccharide.

Methods

Purification of S. aureus glucosaminidase, bacteriolytic activity assay, and purity criteria. SaG was purified and its lytic activity measured turbidimetrically as described previously (4). Human lysozyme (HL) was purified from urine of a patient with monocytic leukemia as described previously (12).

The purity of each different SaG preparation was checked by SDS-PAGE (16) by loading up to 1 mg of proteins on wells in 3-mm thick gel. In addition to this, protease, lipase, phosphatase, heat-stable DNase, coagulase, and hemolytic and leucocidin activity were each tested using samples containing at least 1 mg of proteins. Coagulase activity was assayed as described elsewhere (17) by adding 0.2 ml of pure SaG preparation to 2 ml of rabbit plasma. Protease, lipase, phosphatase, and heat-stable DNase were assayed as described (18–21), respectively, by depositing 0.1 ml of pure SaG preparations (sterilized by filtration) in adequate wells prepared in the specific solid media. Positive controls were represented by protease type I from bovine pancreas, lipase type VI-S from porcine pancreas, phosphatase type I from bovine intestine, and deoxyribonuclease I type II from bovine pancreas (Sigma Chemical Co., St. Louis, MO). Plates were incubated for 12 h at 37°C and then observed. Hemolysis was assayed on blood agar plates prepared according to current criteria (17) with rabbit or sheep erythrocytes by depositing 0.1 ml of the pure and filtration sterilized SaG preparation onto the plates, which were incubated for 12 h at 37°C. Leucocidin activity was assayed following the procedure described by Noda et al. (22). Only preparations that gave only one band in the SDS-PAGE (Fig. 1) and did not contain any of the biological activities mentioned above in 1 mg protein, were used in all the experiments. All experiments described throughout this work were carried out using at least two different enzyme preparations from the same staphylococcal strain (AT12). Some of the most crucial experiments were further repeated using SaG purified from a different *S. aureus* strain (A128).

Experiments on the lymphocyte response to mitogenic lectins. Total lymphocytes from human peripheral blood were used in the experiments with PHA and Con A, whereas for the experiments with PWM, B and T cells were separated. The lymphocyte preparation and both the experiments on lymphocyte stimulation induced by PHA or Con A and those on B cell differentiation induced by PWM were performed according to the methods detailed elsewhere (9). In particular, the effects of SaG (or other test substances) were studied in the same experiments by adding the enzyme together with the mitogens to the culture medium, and in others by adding the mitogens in the absence of SaG to lymphocytes that had been pretreated with the enzyme for 1 h. The mitogenic lectins were generally added immediately, but in some experiments the microplates were left at 37°C for definite time intervals before the mitogens were added.

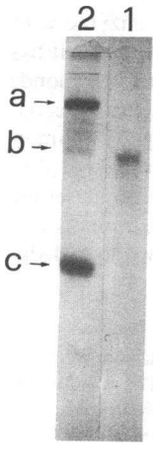

Figure 1. SDS-PAGE of pure preparation of SaG (lane *1*) and marker proteins (lane *2*) of known molecular weight, namely β-galactosidase (*a*, mol wt 130,000) and hen egg albumin (*b*, dimeric form, mol wt 86,000; *c*, monomeric form, mol wt 43,000). 5 μg of SaG was loaded on lane 1 of the gel. The gel was stained with Coomassie blue.

Effect of SaG on antibody-producing cells in mouse spleens. Male Swiss albino mice weighing ~ 20 g were used. Sheep red blood cells (SRBC), washed three times with saline to make an appropriate concentration, were used as antigens. Both SRBC (10^8 cells/mouse) and SaG or other substances were injected intraperitoneally simultaneously but separately. The animals were killed 5 d after immunization and the number of plaque-forming cells (PFC) in the spleens was counted by the technique of localized hemolysis in agar (23). Five mice were used for each experimental condition and the inhibitory effect of SaG was estimated by comparison between the mean of the SaG-treated mice and that in nontreated controls.

Effect of SaG on production of circulating antibodies in mice. Male Swiss albino mice were also used in these experiments. BSA (Sigma) was used as test antigen (25 μg/mouse) and SaG or other substances were inoculated by subcutaneous injection, at the same time but separately. On the contrary, when adjuvants were employed, they were given together with the antigen. The animals were killed 30 d after immunization and anti-BSA titers were evaluated by passive hemagglutination on the serum of each individual animal in each different experimental group (15 mice each). A passive hemagglutination test was performed according to the Hirata and Brandiss procedure (24), using sheep erythrocytes stabilized by formaldehyde and coated with antigen. For titration, 0.1 ml of the 1% suspension of sensitized erythrocytes was added to 0.1 ml of twofold serial dilutions of test serum. Antibody titers were expressed as the reciprocal of the final dilution of serum that indicated positive agglutination.

Other test substances and chemicals. Tri-*N*-acetylglucosamine (chitotriose) and anti-SaG antisera were prepared in our laboratory. Chitotriose was obtained by partial acid hydrolysis of chitin (ICN Pharmaceuticals, Plainview, NY) followed by charcoal column fractionation, as described by Rupley (25). Specific anti-SaG antisera were obtained from rabbits, using SaG preparations that met the above-described criteria for purity. The initial antigen dose was given intramuscularly as a water-in-oil emulsion with CFA (Difco Laboratories Inc., Detroit, MI). Two booster doses were given subcutaneously as emulsions with incomplete Freund's adjuvant (Difco) 10 and 30 d later. Blood for serum was collected 5–7 d after the second booster.

To determine the effect of SaG on the enhancing effect of immunoadjuvants, muramylpeptide (MDP; Protein Research Foundation, Tokyo) and lipopolysaccharide of *E. coli* 055:B5 (Difco) were also employed. Lysostaphin, bovine pancreatic RNase, and poly-L-lysine (mol wt 4,000–15,000) were purchased from Sigma Chemical Co.

Other methods. To estimate the effect of SaG on lymphocyte viability, 0.9 ml of cell suspensions was mixed with 0.1 ml of 1% trypan blue saline solution. The suspensions were examined microscopically after 30 min exposure of the cells to the dye, and the numbers of stained and unstained cells were analyzed. The results were compared to controls without SaG and expressed as percentage of viable cells.

S. aureus peptidoglycan was purified using the same procedure used for purifying the *M. luteus* peptidoglycan (4). Purity of the polymer was checked by determining the amino acid composition after hydrolysis in 6N HCl for 18 h at 100°C with an automatic amino acid analyzer (119; Beckman Instruments, Inc., Fullerton, CA). The peptidoglycan preparation was considered pure when at least 95% of amino acids present were those of the *S. aureus* peptidoglycan in their typical molar ratio. For enzymatic digestion of peptidoglycan, SaG or HL was added to a suspension of 1 g pure peptidoglycan of *S. aureus* in 99 ml of

0.15 M sodium acetate buffer at a final concentration of 100 μg/ml and incubated at 37°C for 6 h. The insoluble material was removed by centrifugation (150,000 *g* for 3 h) and the supernatant was lyophilized.

To obtain trypsin-inactivated SaG, a solution of 200 μg/ml of SaG was added with 20 μg/ml of trypsin and incubated at room temperature up to complete inactivation of SaG bacteriolytic activity. Ovomucoid was then added to the mixture to inactivate trypsin. Boiled SaG and heat-inactivated SaG were prepared by 15 min heating at 100°C and 20 min heating at 120°C, respectively. Determination of possible residual lytic activity after heating and enzymatic digestion, and in the presence of both monoclonal and polyclonal antibodies and chitotriose, was performed according to the standard procedures referred to above for assay of lytic activity (4).

MAbs to pure SaG (and to lysostaphin) were prepared following standard protocols (26). A total of five different MAbs capable of binding SaG was obtained. The one that gave the highest inhibition of SaG bacteriolytic activity was used in this work. A monoclonal antibody capable of inhibiting lysostaphin bacteriolytic activity was also obtained.

Results

SaG prevents human lymphocyte response to Con A and PHA. SaG inhibited incorporation of radioactive thymidine by mitogen-treated lymphocytes both when the enzyme was present in the cultures and when it was used to pretreat the cells before mitogenic stimulation (Fig. 2). The level of inhibition depended on the enzyme concentration, first increasing and then decreasing as the enzyme concentration increased. When added together with the mitogen, SaG caused inhibitions of up to ~ 75 and 85% for Con A and PHA, respectively (Fig. 2 *A*). When the enzyme was removed immediately before mitogen treatment, inhibition was also substantial, being ~ 65% for Con A and > 85% for PHA (Fig. 2 *B*). Under the latter conditions the inhibitory effect caused by SaG was reversible. It was maintained for 20–30 min after removal of the enzyme (in both PHA and Con A) and then began to drop, being completely absent after 50–60 min (Fig. 3).

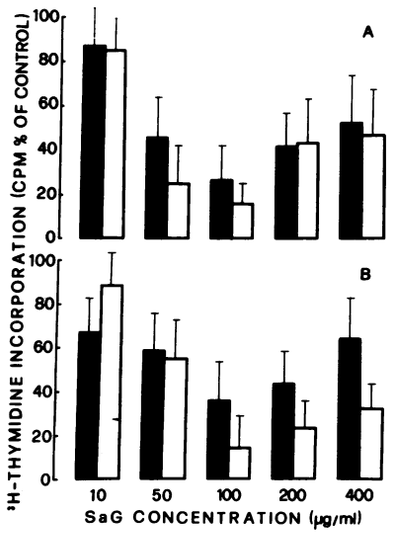

Figure 2. Effect of SaG on lymphocyte response to Con A (■) and PHA (□). (*A*) Lymphocytes were cultured in the presence of SaG and Con A or PHA. (*B*) Lymphocytes were pretreated with SaG for 1 h, washed, and then cultured in the presence of the mitogens. Each figure represents the mean±SD from five experiments. Values are expressed as percentage of stimulated control cultures without SaG. In the latter, the absolute values yielded by the five experiments ranged from 39,000 to 57,000 cpm for Con A, and from 56,000 to 77,000 cpm for PHA. The cpm values were consistently below 5,000 in the unstimulated control cultures (i.e., not exposed to Con A or PHA).

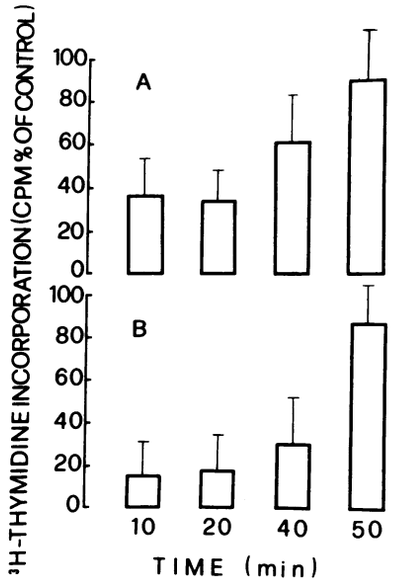

Figure 3. SaG-pretreated lymphocyte response to Con A (*A*) and PHA (*B*). Lymphocytes were pretreated with SaG (100 μg/ml), washed, and subdivided into samples that were added with Con A or PHA after various time intervals. Each figure represents the mean±SD from five experiments. Values are expressed as percentage of stimulated control cultures without SaG. In the latter, the absolute values yielded by the five experiments ranged from 44,000 to 57,000 cpm for Con A, and from 65,000 to 80,000 cpm for PHA. The cpm values were consistently below 5,000 in the unstimulated control cultures (i.e., not exposed to Con A or PHA).

SaG inhibits human B cell differentiation induced by PWM. PWM, when added to B lymphocytes in the presence of T cells, induces differentiation of the B lymphocytes into plasmoblasts or plasmacells that are identified as cells with detectable amounts of cytoplasmic immunoglobulins (cIg+ cells; see ref. 27). Fig. 4 shows that SaG caused a decrease of as much as 80% in the number of cIg+ cells recovered after stimulation. Such inhibition demonstrated the same characteristics as for PHA- and Con A-treated lymphocytes. In fact, it increased with enzyme concentration up to a dose of 100 μg/ml and diminished at higher concentrations. When the lymphocyte mixture (B cells plus T cells) was treated with SaG for 60 min, washed free of SaG, and added with PWM at various intervals, blast formation was again inhibited. This inhibition, however, as observed with Con A and PHA under similar conditions, was reversible and completely disappeared 90 min after treatment with SaG (Fig. 5).

SaG does not interfere with lymphocyte viability and its inhibitory effects are linked to enzyme activity. The effect of SaG on viability of human lymphocytes was assayed at concentrations varying from 10 to 400 μg/ml. After 5 d contact with the

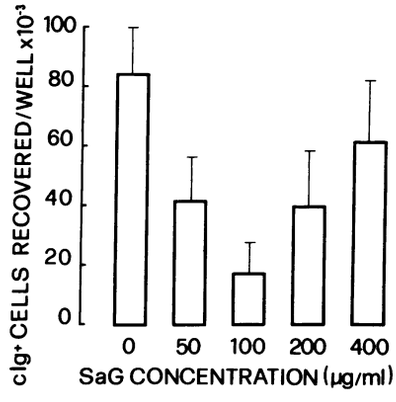

Figure 4. Effect of SaG on PWM-induced B lymphocyte differentiation into plasmablasts or plasma cells (cIg+ cells) in the presence of T cells. Each figure represents the mean±SD from five experiments. The number of cIg+ cells recovered per well was consistently below 1×10^3 in the unstimulated control cultures (i.e., not exposed to PWM).

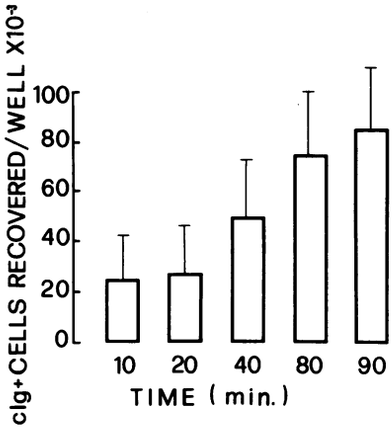

Figure 5. SaG-pretreated lymphocyte response to PWM. Lymphocytes were pretreated with SaG (100 μg/ml), washed, and subdivided into samples that were added with PWM after various time intervals. The number of cIg+ cells recovered per well was consistently below 1×10^3 in the unstimulated control cultures (i.e., not exposed to PWM).

enzyme at the concentrations mentioned above, the percentage of cells unable to exclude the vital dye was similar to that of the controls treated with the mitogen and of those not treated and incubated in the absence of SaG.

Figs. 6 and 7 show that the inhibitory effect of the purified SaG was unlikely to be caused by possible contaminants of the enzyme preparation. In fact, (*a*) proteolytic digestion (up to complete loss of enzyme activity), (*b*) inactivation with polyclonal specific antibodies, (*c*) treatment with an anti-SaG monoclonal antibody that inhibited 80% of the bacteriolytic activity of the enzyme, and (*d*) denaturation by autoclaving, all caused SaG to completely lose its inhibitory activity on human lymphocyte response to mitogenic stimulation by PHA, Con A (Fig. 6), and PWM (Fig. 7). When the SaG was incubated at 100°C for 10 min (a treatment that denatures most protein, but has little effect on enzyme activity of SaG [4, 28]), the protein conserved almost all its inhibitory effect on lymphocyte response to mitogens (Figs. 6 and 7).

The data presented in Figs. 6 and 7 also clearly indicate that the inhibitory property of SaG is linked to its enzyme activity.

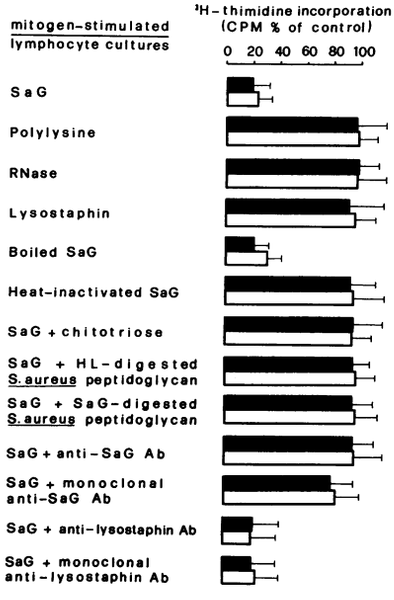

Figure 6. Effect of SaG, untreated or submitted to various treatments, and other basic proteins on lymphocyte response to PHA (■) and Con A (□). SaG was consistently used at the concentration of 100 μg/ml. The other test substances were used at the following concentrations: chitotriose, 1 mg/ml; lysostaphin up to 200 μg/ml; RNase up to 100 μg/ml; polylysine up to the highest nontoxic concentration (50 μg/ml). Each figure represents the mean±SD from five experiments. Values are expressed as percentage of control cultures stimulated with the mitogen only. With such control cultures, the absolute values ranged from 39,000 to 64,000 cpm for Con A, and from 53,000 to 86,000 cpm for PHA.

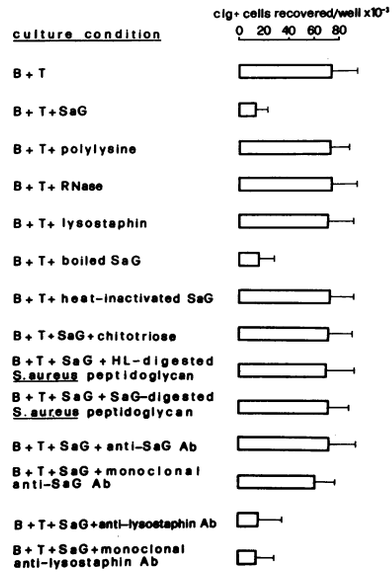

Figure 7. Effect of SaG, both untreated and submitted to various treatments, and other basic proteins on PWM-induced B lymphocyte differentiation into plasmablasts or plasma-cells (*cIg+ cells*) in the presence of T cells. SaG was consistently used at the concentration of 100 μg/ml. The other test substances were used at the following concentrations: chitotriose, 1 mg/ml; lysostaphin up to 200 μg/ml; RNase up to 100 μg/ml; polylysine up to the highest nontoxic concentration (50 μg/ml). Each figure represents the mean±SD from five experiments.

Table I. Inhibition of SaG (100 μg/ml) by Chitotriose, Peptidoglycan, and Anti-SaG Antibodies

Inhibitor	Inhibitor concentration*	Relative SaG activity‡
None	—	1.00
Chitotriose	0.01	0.78
	0.02	0.62
	0.04	0.43
	0.08	0.26
S. aureus peptidoglycan	0.01	0.93
	0.02	0.84
	0.04	0.63
	0.08	0.48
S. aureus peptidoglycan digested by HL	0.01	0.82
	0.02	0.61
	0.04	0.54
	0.08	0.42
S. aureus peptidoglycan digested by SaG	0.01	0.75
	0.02	0.64
	0.04	0.58
	0.08	0.38
Anti-SaG antibodies	1:10	0.10
Anti-SaG MAbs	5:1	0.20

* Inhibitor concentrations are expressed as milligrams per milliliter, except for anti-SaG antibodies. In this case either 0.1 ml of polyclonal anti-SaG antiserum was added to 0.9 ml of SaG solution or 0.1 ml of anti-SaG MAbs was added to 0.9 ml of SaG to give a final Ab/SaG molar ratio of 5/1. ‡ SaG activity was evaluated as described in Methods. SaG residual activity after inhibitor treatment is expressed relatively to the activity of untreated sample taken equal to 1.00.

Table II. Effect of Enzymatically Active and Inactivated SaG on the Recovery of PFC from Spleens of SRBC-Injected Mice and on Anti-BSA Antibody Production in Mice

Substances injected* (μg/mouse with SRBC or BSA)	No. PFC (×10³)‡ per spleen ±SD	P§	Anti-BSA titer‡ (reciprocal) ±SD	P§
None	69.8±12.5		240±89.44	
SaG (50)	69.2±7.69	NS	220±109.54	NS
SaG (100)	56.6±10.45	NS	ND	
SaG (200)	ND		110±54.77	<0.05
SaG (300)	19.6±4.33	<0.01	55±27.38	<0.01
SaG (500)	25.8±5.97	<0.01	55±27.38	<0.01
Boiled SaG (300)	22.4±4.72	<0.01	55±27.38	<0.01
Heat-inactivated SaG (300)	71.2±10.59	NS	220±109.54	NS
Trypsin-inactivated SaG (300)	68.4±7.09	NS	220±109.54	NS
Lysostaphin (300)	124.2±17.28	<0.01	240±89.44	NS
Polylysin (300)	111.6±14.92	<0.01	220±109.54	NS

* SaG and other materials were given as described in Methods. ‡ Means of five experiments are shown. § *P* values were calculated by Student's *t* test in comparison to controls (BSA- or SRBC-treated mice). NS, $P > 0.05$.

In fact, substances such as chitotriose and solubilized *S. aureus* peptidoglycan, that specifically inhibited the enzyme activity of SaG (Table I), almost completely abolished its inhibitory effect on lymphocyte response to mitogenic stimulation. In addition to this, other polypeptides which, like SaG, are positively charged, such as polylysine, RNase, and lysostaphin (which is a bacteriolytic enzyme produced by some staphylococcal strains, but acts mainly as an endopeptidase [29]), did not demonstrate any effect on lymphocyte response to mitogens. It is also important that the monoclonal antibody that prevented the inhibitory effect of SaG on the human lymphocyte response to mitogenic stimulation also abolished the bacteriolytic activity of the enzyme. In contrast, both monoclonal and polyclonal antibodies specific for another staphylococcal protein (lysostaphin) had no effect on the inhibitory activity of SaG.

SaG interferes with anti-BSA antibody production in mice. To test whether SaG in vitro inhibition of lymphocyte response to mitogenic stimuli might be a manifestation of a possible modulating activity of the enzyme on immunocompetent cells, we analyzed the effect of SaG on antibody production in mice. Table II shows that SaG demonstrated a clear inhibitory effect, as evaluated by determination both of anti-SRBC antibody-containing cells in spleens of immunized mice and of anti-BSA circulating antibodies (again in mice). In both systems the inhibitory effect increased with enzyme concentration, reaching a maximum at the concentration of 300 μg/mouse. This SaG dose caused a 3.6 and a 4-fold reduction in antibody production in the former and in the latter system, respectively.

Boiled SaG maintained its inhibitory activity which, in contrast, was completely lost after autoclaving. Moreover, trypsin-digested SaG, injected together with the antigens, had no effect on the mouse immune response in either of the two experimental systems. Two other cationic peptides devoid of glucosaminidase activity, lysostaphin (see above) and polylysine, did not influence production of anti-BSA circulating antibodies, but caused a slight increase in the number of anti-SRBC immunoglobulin-containing cells detectable in the spleens of immunized mice.

When the experiments described in Table II were repeated using SaG purified from another *S. aureus* strain (A128), antibody production was again inhibited in both experimental systems at levels virtually identical to those observed with SaG purified from strain AT12.

SaG completely abolishes the enhancing effect of immunoadjuvants. In mice where BSA was injected together with any one of the immunoadjuvants MDP, LPS, or CFA, the anti-BSA antibody titer was four to eight times higher than that found in mice injected with BSA only (Table III). However, when 300 μg of SaG was injected together with the antigen and one of the three adjuvants, the anti-BSA antibody titer was in

Table III. Effect of Enzymatically Active and Inactivated SaG on the Enhancing Activity of Muramyldipeptide, E. coli Lipopolysaccharide, and Freund's Complete Adjuvant

Substances injected with BSA		Anti-BSA titer‡ (reciprocal) ±SD	P§
Adjuvant	Protein*		
None	None	240±89.44	
None	SaG	70±27.38	<0.01
MDP	None	1600±879.79	<0.01
MDP	SaG	240±89.44	NS
MDP	Autoclaved SaG	1760±876.35	<0.01
MDP	Lysostaphin	1600±879.79	<0.01
MDP	Polylysine	1600±979.79	<0.01
LPS	None	880±438.17	<0.05
LPS	SaG	200±0.00	NS
LPS	Autoclaved SaG	880±438.17	<0.05
LPS	Lysostaphin	960±357.77	<0.01
LPS	Polylysine	880±438.17	<0.05
FCA	None	960±357.77	<0.01
FCA	SaG	220±109.54	NS
FCA	Autoclaved SaG	880±438.17	<0.05
FCA	Lysostaphin	960±357.17	<0.01
FCA	Polylysine	880±438.17	<0.05

* Injected doses of SaG, heat-inactivated SaG, lysostaphin, and polylysine were 300 μg/mouse. Injected doses of MDP, LPS, and FCA were 100 μg/mouse, 50 μg/mouse, and 0.1 μg/mouse, respectively. ‡ Means of five experiments are shown. § *P* values were calculated by Student's *t* test in comparison to controls (BSA-treated mice). NS, $P > 0.05$.

all cases lower than that of the controls injected with the antigen only. It is interesting that identical results were obtained when SaG purified from a different *S. aureus* strain was used. In contrast to this, neither SaG inactivated by autoclaving, nor polylysine or lysostaphin, interfered with the enhancing effect of any of the three adjuvants.

S. aureus peptidoglycan solubilized either with human lysozyme or with SaG enhances the inhibitory effect of SaG on antibody production in mice. Insoluble peptidoglycans have been shown to enhance antibody production (30). In addition, both insoluble and hydrolyzed peptidoglycans are competitive inhibitors of bacteriolytic enzymes (see also Table I). In infections caused by *S. aureus* SaG must always be present together with staphylococcal wall peptidoglycan that is likely to be partly in a soluble form due to the hydrolytic activity of the lysozyme of human tissues and of bacterial autolysin(s). This fact raises the possibility that the inhibitory effect of SaG in infections could be prevented by peptidoglycan (in the soluble or insoluble form). Table IV shows that *S. aureus* peptidoglycan, whether insoluble or hydrolyzed by HL or SaG, when injected into mice together with antigens, slightly enhances both production of anti-SRBC immunoglobulin-containing cells in the mouse spleens and production of anti-BSA circulating antibodies. On the contrary, when SaG was injected together with the antigens and the hydrolyzed peptidoglycan, not only was the enhancing effect of the peptidoglycan completely abolished, but the inhibitory effect of SaG on immune response was slightly, though unquestionably, enhanced. In fact, under these conditions the number of anti-SRBC immunoglobulin-containing cells in the spleens and the titer of the anti-BSA circulating antibodies were more than 8 and 12 times lower, respectively, than in controls injected with the antigens only.

Insoluble peptidoglycan did not appear to influence the inhibitory effect of SaG, but its enhancing effect was abolished. In addition, neither autoclaving-inactivated SaG, nor polylysine or lysostaphin interfered with the enhancing effect on the immune response caused by both insoluble and solubilized peptidoglycan in the two different systems.

Discussion

This work presents a number of novel observations that were rather unexpected, based on our current knowledge of the possible biological effects of peptidoglycan hydrolytic enzymes produced by bacteria. We have, in fact, shown here that an endo-β-*N*-acetylglucosaminidase excreted by one of the most important human pathogens almost completely inhibits formation of blasts and immunoglobulin-containing cells after treatment with mitogens, strongly depresses the immune response in mice, and completely abolishes the enhancing effect of the most powerful adjuvants. The possibility that peptidoglycan hydrolytic enzymes may interfere with host defenses has never been considered before. This is the first description of a hexosaminidase (namely an endo-β-*N*-acetylglucosaminidase) interfering with antibody production in mice and the first description of a microbial bacteriolytic enzyme preventing in vitro response of human lymphocytes to mitogens and impairing immune response in mice. Such findings are important for a better understanding of the mechanisms by which bacteria express their pathogenicity and for further clarifying the mechanism of regulation of the immune response.

As far as the former problem is concerned, we have already stated that bacteriolytic enzymes, many of which act as endo-β-*N*-acetylglucosaminidases (4, 5), are secreted by various microbial species that are important in human pathology (2, 3). In most of these cases, the virulence determinants of the microorganism are as yet unknown. Our findings suggest the possibility that, in *S. aureus* and in other species that produce enzymes of the SaG type, the bacteriolytic enzyme is one of the pathogenicity determinants. This is strongly supported by the fact, as

Table IV. Effect of Insoluble and Solubilized S. aureus Peptidoglycan on SaG-Induced Inhibition of Production of PFC and Anti-BSA Antibodies in Mice

Substances injected with SRBC or BSA*	No. PFC (×10³)‡ per spleen ±SD	P§	Anti-BSA titer‡ (reciprocal) ±SD	P§
None	73.6±10.96		240±89.44	
Insoluble *S. aureus* peptidoglycan	91.4±13.44	NS	480±178.88	<0.05
HL-digested *S. aureus* peptidoglycan	107.4±17.57	<0.01	480±178.88	<0.05
SaG-digested *S. aureus* peptidoglycan	101.6±15.75	NS	480±178.88	<0.05
SaG	23.2±10.82	<0.01	70±27.38	<0.01
Autoclaved SaG	75.0±5.70	NS	240±89.44	NS
Insoluble *S. aureus* peptidoglycan + SaG	21.2±3.83	<0.01	60±22.36	<0.01
HL-digested *S. aureus* peptidoglican + SaG	9.2±2.16	<0.01	17±6.84	<0.01
SaG-digested *S. aureus* peptidoglycan + SaG	7.4±3.36	<0.01	15±5.59	<0.01
HL-digested *S. aureus* peptidoglycan + autoclaved SaG	102.2±18.67	<0.05	240±89.44	NS
SaG-digested *S. aureus* peptidoglycan + autoclaved SaG	94.2±21.14	NS	240±89.44	NS
HL-digested *S. aureus* peptidoglycan + polylysine	105.8±17.71	<0.01	280±109.54	NS
SaG-digested *S. aureus* peptidoglycan + polylysine	108.8±20.58	<0.01	280±109.54	NS
HL-digested *S. aureus* peptidoglycan + lysostaphin	111.0±19.14	<0.01	280±109.54	NS
SaG-digested *S. aureus* peptidoglycan + lysostaphin	107.4±12.40	<0.01	280±109.54	NS

* Injected doses of SaG, heat-inactivated SaG, lysostaphin, and polylysine were 300 μg/mouse. Injected doses of insoluble and digested peptidoglycan were 800 μg/mouse. ‡ Means of five experiments are shown. § *P* values were calculated by Student's *t* test in comparison to controls (BSA- or SRBC-treated mice). NS, $P > 0.05$.

shown elsewhere (31), that SaG greatly enhances intraperitoneal pathogenicity of *S. aureus* for mice, whereas anti-SaG antisera strongly inhibit such pathogenicity; furthermore *S. aureus* mutants that do not excrete SaG are much less pathogenic for mice than parental strains (31). Further support also comes from the studies of other investigators, who have shown that mutants lacking an endo-β-*N*-acetylglucosaminidase isolated from a *S. pneumoniae* strain are much less pathogenic for mice than parental strains (32).

It is quite possible that SaG may exert effects similar to those observed both in mice and in vitro also in natural staphylococcal infections in humans. The two *S. aureus* strains from which we have purified SaG are capable of producing up to 50 μg SaG per 10^9 bacteria, i.e., an amount consistent with the possibility that, in the infected tissues and adjacent body zones, concentrations of SaG not much lower than those present in mice after the injection of 300 μg could reasonably be reached. It has been, in fact, calculated that in serious staphylococcal infections such as pneumonia, bronchopneumonia, empyema, or lung abscesses, bacteria can reach concentrations that may exceed 10^9 per milliliter (or gram) of infected tissue (33). We should also consider that proteins are rapidly metabolized by living animals and that the metabolism of mice is much faster than that of humans. In infections SaG is continuously produced by the infecting staphylococci thus replacing the enzyme metabolized (and destroyed) at the host organism. On the other hand the amount of SaG which, after administration of 300 μg to mice, arrives at immunopoietic organs such as lymph nodes distant from the site of injection, is so low that it is likely that an equivalent amount can easily be produced by staphylococci that invade regional lymph nodes in natural infections in humans. It is therefore likely that, as a result of the SaG released, staphylococci that multiply in the lymph nodes may cause impairment of the immune response in the invaded organ, even when they are not present as large populations.

Lastly it is interesting to recall that, during phagocytosis of staphylococci and other bacteria, human granulocytes release lysozyme and other hexosaminidases (exo-β-*N*-acetylglucosaminidase) at high concentrations. Since we have found that hen egg white lysozyme also inhibits antibody production in mice, it is possible that such enzymes may exert an effect of the type observed with SaG thus determining further impairment of host defenses.

We have previously shown that, in some Gram-positive and Gram-negative pathogens, the different species can be identified, for both taxonomic and clinical purposes, on the basis of the peculiar properties of the bacteriolytic enzymes the strains secrete (2, 3, 34). The finding that such enzymes may contribute to microbial pathogenicity relates this identification system to a pathogenicity determinant and makes it particularly suitable for separating the different staphylococcal species that may be clinically important.

The relevance of the findings described here for a better understanding of the mechanism of immunocompetent cell regulation appears evident when such findings are taken together with other observations. We have recently shown that both HL and hen egg white lysozyme (i.e., endo-hexosaminidases that hydrolyze peptidoglycan with a mechanism very similar to that of SaG) inhibit the response of human lymphocytes to mitogens (9). Others have demonstrated that HL regulates the activated state of human granulocytes (8) and contributes to regulation of lymphocyte proliferation in mixed lymphocyte cultures (35). In addition to this, exo-β-*N*-acetylglucosaminidases have long been known to be produced by macrophages, monocytes, and granulocytes (15, 36) while an endo-β-*N*-acetylglucosaminidase has been purified from hen oviduct (37) and has been described in tissues of humans and other mammals (13). More recently, an exo-β-*N*-acetylglucosaminidase that hydrolyzes peptidoglycan has been shown to be carried by human granulocytes (38). On the other hand, it is well established that both polymeric peptidoglycan and peptidoglycan glycopeptides (which are substrates and competitive inhibitors of these enzymes) have an immunomodulating effect (30, 39). It is also known that bacterial peptidoglycan undergoes extensive turnover (up to 50% per generation) during which a variety of glycopeptides (muropeptides) are released (40). Probably as a consequence of this, muropeptides are ordinarily found in urine of people who do not suffer from infections (41). All the above-mentioned observations together with the findings of this study make it very likely that hexosaminidases that hydrolyze peptidoglycan play a role in the regulation of immune response and that the alterations which peptidoglycan and peptidoglycan derivatives, such as MDP, cause in immune response may be due to their interaction with these enzymes. Knowledge of these previously unknown facts may be the starting point for a novel approach to the study of regulation of immune response.

All the information mentioned above also provides a probable explanation of the mechanism by which SaG interferes with the response of human lymphocytes to mitogenic stimuli and with antibody production in mice. Hexosaminidases may perform their possible regulatory function by operating on a double pathway, where, on the one hand, they interact directly with immunocompetent cells by binding with specific receptors and, on the other, they generate (from bacterial envelopes) muropeptides that modulate hexosaminidase interaction with the aforementioned receptors, and also directly interact with effector cells. In this context, SaG may cause inhibition of the in vitro response of human lymphocytes to mitogens by binding to specific receptors through which it triggers, in the specific cells, nonresponse to mitogenic stimuli. Some of the data in our possession actually indicate that SaG binds specific receptors of immunocompetent cells. However, the mechanism by which SaG exerts its effect is probably rather complex, as indicated by the fact that hydrolyzed peptidoglycan of *S. aureus,* which is a competitive inhibitor of SaG (and other bacteriolytic hexosaminidases), induces slight though distinct enhancement of depression of antibody production caused by SaG in mice. It is possible that immunocompetent cells carry two components that act as receptors for SaG (and probably for other hexosaminidases including the endogenous ones), of which one has high and one low affinity for the enzyme. The first receptor, which is bound at low concentrations of SaG, triggers the nonresponse effect, while the latter, bound at higher concentrations only, antagonizes the effects of the former. At the concentrations needed for saturating the high affinity receptor, SaG may partially bind the low affinity receptor which to a certain extent moderates depression of response. The solubilized peptidoglycan might have an affinity for SaG that is lower than that of the high-affinity receptor, but higher than that of the low-affinity receptor, and may thus prevent binding of SaG (and other hexosaminidases) to the receptor potentially responsible for antagonizing response depression. Alternatively, muropeptides present in the body might normally stimulate im-

munocompetent cells. SaG and other hexosaminidases may contribute to regulation of immune response both by generating such muropeptides and by modulating their interaction with the target cells.

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