Relationships Between Adjuvant, Immunosuppressive, and Mitogenic Activities of Staphylococcal Peptidoglycan

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Received for publication 1 June 1979

Staphylococcal peptidoglycan (PG) possesses in vivo immunodulating activity and is a B-cell mitogen in mice. The effect of PG on in vitro immune response of mouse splenocytes to sheep erythrocytes (SRBC) was studied, as well as the relationships between in vivo and in vitro adjuvant, immuno-suppressive, and mitogenic activities of PG in terms of dose response, time kinetics, and physical state. Particulate PG suppressed in vivo anti-SRBC response when injected in a large dose before or simultaneously with SRBC. A small dose of particulate PG given before or along with the antigen was immunostimulatory. Soluble PG was adjuvant active in both high and low doses when injected before or along with the antigen. Both PG preparations were adjuvant active for mouse splenocytes in vitro immunized with SRBC, but particulate PG was more active. Even high doses of particulate PG were not directly suppressive for the in vitro immune response. Particulate PG was also mitogenic for mouse splenocytes, and the maximum increase in [3H]thymidine incorporation was observed after 2 days of culture. Soluble PG was not mitogenic during the 5-day incubation period. These results indicate that the physical state of PG, its dose, and its time of application are important factors determining its immunomodulating and mitogenic activities, and that by changing them it is possible to dissociate the adjuvant, immunosuppressive, and mitogenic properties of PG.

In vivo immunopotentiating activity of peptidoglycans (PG), basic cell wall heteropolymers of bacteria and actinomycetes, is a well-established phenomenon (11, 13, 15, 21). Studies on the relationships between PG structure and activity associated its adjuvant properties with the soluble monomeric subunits composed of sugar-peptide complexes (1, 21). Subsequent experiments on the synthetic analogs of naturally occurring PG defined N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) as the smallest adjuvant-active compound (2, 15, 19). More recent studies have shown that MDP also enhances in vitro immune responses, and T lymphocytes and macrophages have been identified as mediators of its adjuvant activity (8, 18, 24). Since the work on MDP started, however, less attention has been paid to the immunodulating activity of naturally occurring PG.

The majority of adjuvants act, in fact, as immunomodulators and can either enhance or depress the immune response, depending on the timing in relation to the antigenic stimulation and the dosage of both antigen and immunomodulator (22, 26). This is also true for PG, and an immunosuppressive effect of staphylococcal PG on the primary in vivo antibody response of mouse spleen cells to sheep erythrocytes (SRBC) was reported (5). The in vitro antibody response of spleen cells from PG-treated mice was also markedly depressed. It wasnext established that PG induced in the spleen suppressor cells capable of inhibiting the in vivo and in vitro immune responses of normal splenocytes to SRBC. The suppressor cells had properties of macrophages and could be induced by PG both in vivo and in vitro (5a).

In addition to its adjuvant and immunosuppressive activity, staphylococcal PG is also a potent B-cell mitogen in mice (6). Previous studies of Damais et al. (4) suggested a correlation and even a causal relationship between mitogenic and adjuvant activities of PG. More recent work on the adjuvant properties of MDP indicated, however, that mitogenic activity is not required for adjuvanticity of this compound (23, 25). This is also in agreement with the studies on the mechanism of adjuvant activity of MDP (8, 18, 24). MDP is a synthetic analog of basic PG structure and, as mentioned above, it is adjuvant active while having no mitogenic effect on mouse lymphocytes. This opened the possi-
bility of dissociating adjuvant from mitogenic properties of PG.

The objective of this study was to evaluate the effect of PG on in vitro immune response and then to explore the relationships between in vivo and in vitro adjuvant and immunosuppressive activities of PG, as well as its mitogenic properties. These activities of PG were studied in terms of dose-response, time kinetics, and the physical state of the PG preparation. Two PG preparations were used: insoluble, particulate PG naturally present in the cell wall, and soluble PG obtained by extensive ultrasonic disintegration. Striking differences in immunomodulating and mitogenic properties of these two preparations were observed.

MATERIALS AND METHODS

Mice. Female BALB/c mice, 6 to 8 weeks old, were obtained from Ace Animals, Inc., Boyertown, Pa., and fed a standard diet ad libitum.

PG. PG was isolated from Staphylococcus aureus 3528 cell walls by trichloroacetic acid extraction (7) and stored as an acetone-dried powder. Analysis of the chemical composition of PG revealed the presence of lysine, glutamic acid, glycine, alanine, glucosamine, and muramic acid (7), indicating no significant contamination with teichoic acid, proteins, nucleic acids, or other cell components. Accidental contamination with exogenous endotoxins was ruled out by toxicity tests in adrenalec tomized mice (6). Before use, PG was suspended in sterile phosphate-buffered saline solution and treated with ultrasonication for 1 h at 20 kHz (8-W output) in a W/185 Sonifier (Branson Ultrasonics Co., Plainview, N.Y.), to yield a particulate PG preparation. To obtain a soluble preparation, PG was suspended in sterile distilled water and treated with ultrasonication as above. Supernatants were pooled after several 1-h ultrasonic disintegrations and centrifuged at 30,000 x g. Both PG preparations were heated at 70°C for 1 h and tested for sterility.

In vivo immunomodulation experiments. Mice were immunized intravenously with 10^6 SRBC as previously described (6). PG preparations were appropriately diluted in PBS and injected intravenously in 0.2-ml volumes. SRBC-specific plaque-forming cells (PFC) were assayed by the method of Cunningham and Zsengere (3) 4 days after SRBC challenge. The spleen of each mouse was assayed in triplicate cultures.

In vitro immunomodulation experiments. In each experiment, spleen cells from at least three mice were pooled. In vitro cultures of dissociated spleen cells were established in 24-well tissue culture plates (Linbro Scientific, Inc., Hamden, Conn.) in Eagle basal medium with Hanks balanced salt solution supplemented with 10% fetal bovine serum, amino acids, and vitamins as described by Kamo et al. (14). Spleen cells (2-ml cultures, 5 x 10^6 cells/ml) were immunized with 5 x 10^5 SRBC and incubated at 37°C in a humid atmosphere containing 5% CO_2 and 95% air. PG preparations were appropriately diluted in the tissue culture medium and added (0.2 ml/well) at the initiation of the cultures. Cultures were always assayed individually for SRBC-specific PFC by the method of Jerne et al. (12). Numbers of viable cells (excluding trypan blue dye) were also counted.

Significance of differences in PFC numbers in both in vivo and in vitro experiments was evaluated according to Student's t test, P ≤ 0.05 being taken as a significant difference.

Mitogenicity studies. Mitogenicity was studied as previously described (6). Briefly, cultures of 10^6 mouse spleen cells (1 ml each) were established in 24-well tissue culture plates in RPMI 1640 medium supplemented with 5% fetal bovine serum and antibiotics. Appropriate concentrations of mitogens were added at the initiation of the cultures, and each culture was pulsed with 1 μCi of [3H]thymidine for the final 18 h of incubation. The cells were collected on glass microfiber filters, and radioactivity was determined in a liquid scintillation counter. Phytohemagglutinin-P (Difco Laboratories, Detroit, Mich.) and Escherichia coli O:127:B8 lipopolysaccharide (LPS), prepared by a phenol-water extraction method (Sigma Chemical Co., St. Louis, Mo.), were used as control mitogens.

RESULTS

In vivo immunomodulating activity of PG. Groups of mice were injected with different doses of either particulate or soluble PG along with SRBC antigen or 1, 2, or 3 days before SRBC challenge. Control mice received SRBC only. SRBC-specific PFC were assayed 4 days after SRBC injection (Table 1). As in our previous experiments (5), 400 μg of particulate PG, when injected 1, 2, or 3 days before or simultaneously with SRBC antigen, caused significant suppression of anti-SRBC PFC response. A small dose (10 μg/mouse) of particulate PG stimulated in vivo anti-SRBC PFC response when injected 1 day before or simultaneously with the antigen. Injection of intermediate doses of particulate PG (50 and 100 μg/mouse; data not shown) did not give clear-cut results; i.e., there was a great variability of the response, with suppression observed in some mice and stimulation observed in others. Consequently, construction of a dose-response curve in these in vivo studies was not possible. Some strain differences in susceptibility of mice to PG-induced immunosuppression were also observed when the above results, obtained from BALB/c mice, were compared with results of previous studies (5) performed on Swiss mice.

Soluble PG, when injected before or simultaneously with the antigen, markedly stimulated the anti-SRBC PFC response (Table 1). Both large (400 μg) and small (10 μg) doses were stimulatory, the higher dose being more effective. Intermediate doses (50 and 100 μg/mouse; data not shown) caused an intermediate increase
in PFC. Immunosuppression was not observed with any time regimens or doses of soluble PG tested.

In vitro immunomodulating activity of PG. Cultures of normal splenocytes were immunized in vitro with SRBC, and graded amounts of either particulate or soluble PG were added at the initiation of the cultures to obtain final PG concentrations ranging from 0.5 to 500 μg/ml. Control cultures received appropriate volumes of tissue culture medium instead of PG solution. After 4 days of incubation, SRBC-specific PFC were assayed (Fig. 1). A marked dose-dependent adjuvant activity of particulate PG on the in vitro immune response was observed. Optimal concentration for the adjuvant effect was 25 μg/ml, and none of the concentrations tested was immunosuppressive. Some decrease in the viability of spleen cells incubated with high concentrations of particulate PG was observed, but the surviving cells were still able to mount a good antibody response. This resulted in higher adjuvant effect of particulate PG when the data were expressed as PFC/10^6 viable cells rather than as PFC/culture.

Soluble PG also exhibited a dose-dependent adjuvant activity on the in vitro immune response (Fig. 1); the maximum effect was obtained with 200 μg/ml. It was less active than particulate PG, but it did not decrease viability of the cells.

The influence of PG on the time kinetics of the in vitro immune response was also studied (Fig. 2, Table 2). Particulate PG caused an acceleration of the immune response during the first 3 days of culture and an overall increase in the response throughout the entire culture period. On each of the days studied, the differences between treated and control cultures were statistically significant. Soluble PG caused an increase in the magnitude of the immune response during the first 4 days of culture, and on each of these days the differences between treated and control cultures were statistically significant. No acceleration of the response between days 1 and 4 of incubation was observed.

Mitogenic activity of PG. The above experiments revealed that both particulate and soluble PG preparations were adjuvant active. It was of interest to determine whether the adjuvant activity of PG could be dissociated from its mitogenic activity. To answer this question, the mitogenicity of particulate and soluble PG preparations was compared.

Graded concentrations of either particulate or soluble PG were incubated with mouse spleno-

### TABLE 1. Effect of particulate and soluble PG on in vivo anti-SRBC PFC response

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>PFC/spleen</th>
<th>% of control</th>
<th>Significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>151,880 (144,544–159,588)</td>
<td>100%</td>
<td>0.05</td>
</tr>
<tr>
<td>Particulate PG, 400 μg Day 0</td>
<td>100,485 (82,433–122,490)</td>
<td>66%</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Day 1</td>
<td>40,256 (34,088–47,534)</td>
<td>27%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Day 2</td>
<td>42,901 (32,976–55,821)</td>
<td>28%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Day 3</td>
<td>112,402 (104,328–121,116)</td>
<td>74%</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Particulate PG, 10 μg Day 0</td>
<td>201,280 (188,148–215,328)</td>
<td>133%</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Day 1</td>
<td>190,327 (179,515–201,790)</td>
<td>125%</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Day 2</td>
<td>156,964 (138,612–177,746)</td>
<td>103%</td>
<td>NS</td>
</tr>
<tr>
<td>Soluble PG, 400 μg Day 0</td>
<td>279,898 (238,616–286,322)</td>
<td>184%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Day 1</td>
<td>208,785 (176,279–247,286)</td>
<td>137%</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Day 2</td>
<td>237,875 (195,119–289,268)</td>
<td>156%</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Day 3</td>
<td>283,661 (278,227–296,483)</td>
<td>187%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Soluble PG, 10 μg Day 0</td>
<td>250,842 (230,622–272,835)</td>
<td>165%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Day 1</td>
<td>228,402 (208,882–249,747)</td>
<td>150%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Day 2</td>
<td>160,103 (144,178–177,878)</td>
<td>105%</td>
<td>NS</td>
</tr>
</tbody>
</table>

*10^6 SRBC were injected intravenously on day 0 (control). Each mouse in the experimental groups received an intravenous injection of SRBC on day 0 and the indicated dose of either particulate or soluble PG on one of the indicated days.

* Geometric means of SRBC-specific 19S PFC (mean ± standard error in parentheses) from 6 to 12 mice assayed on day 4.

* Significance (P value) of differences between the control and experimental groups; NS, not significant (P > 0.05).
spleen cells, soluble PG incubation.

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FIG. 1. Effect of particulate and soluble PG on in vitro anti-SRBC PFC response. Cultures of 10^7 mouse spleen cells were immunized with 5 x 10^6 SRBC, and indicated concentrations of either particulate PG (●) or soluble PG (○) were added at the initiation of the cultures. SRBC-specific 19S PFC were assayed after 4 days of incubation. The results are means obtained from 12 cultures in three experiments (particulate PG) or 8 to 10 cultures in two experiments (soluble PG). Standard error was usually lower than 10% and is not indicated.

cytes in vitro for 2 days, and the increase in [3H]thymidine incorporation was used to evaluate the proliferation of the cells (Fig. 3). Particulate PG caused a marked dose-dependent stimulation of mouse lymphocytes, with 400 μg/ml being the optimal mitogenic concentration, confirming our previous findings (6). Soluble PG did not stimulate mouse spleen cells.

Time kinetics studies were also performed to test whether the lack of mitogenic activity of soluble PG observed in the previous experiments was due to the early termination of the cultures. However, soluble PG was not stimulatory during 5-day culture period (Fig. 4). Particulate PG induced a maximal [3H]thymidine incorporation after 2 days of incubation with mouse spleno-}

19S PFC/CULTURE

FIG. 2. Kinetics of in vitro anti-SRBC PFC response of 10^7 mouse spleen cells immunized with 5 x 10^6 SRBC and cultured with an addition of 25 μg of particulate PG (●) or soluble PG (○) per ml or in medium alone (○). The results are means of eight cultures obtained in two experiments. Standard error was usually lower than 10% and is not indicated.

cytes, whereas the stimulation index peaked after 3 days of culture due to a natural decrease in background [3H]thymidine incorporation by control (unstimulated) cultures.

DISCUSSION

This study confirms the in vivo immunomodulating activity of PG and correlates this activity with the physical state of the PG preparation (particulate versus soluble), its dosage, and its time of injection. Particulate PG was immuno-suppressive when injected in a large dose before or along with a thymus-dependent SRBC antigen (Table 1). Previous studies have shown that PG does not suppress the response to a thymus-independent antigen (5). A small dose of partic-
TABLE 2. Kinetics of adjuvant effect of particulate and soluble PG on in vitro anti-SRBC PFC response*

<table>
<thead>
<tr>
<th>Prepn added and days in culture</th>
<th>% of control PFC/culture</th>
<th>% of control PFC/10^6 viable cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particulate PG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>181</td>
<td>154</td>
</tr>
<tr>
<td>2</td>
<td>278</td>
<td>270</td>
</tr>
<tr>
<td>3</td>
<td>410</td>
<td>399</td>
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<tr>
<td>4</td>
<td>240</td>
<td>262</td>
</tr>
<tr>
<td>5</td>
<td>190</td>
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</tr>
<tr>
<td>6</td>
<td>188</td>
<td>212</td>
</tr>
<tr>
<td>7</td>
<td>360</td>
<td>410</td>
</tr>
<tr>
<td>Soluble PG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>198</td>
<td>146</td>
</tr>
<tr>
<td>2</td>
<td>152</td>
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<td>3</td>
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<td>149</td>
<td>158</td>
</tr>
<tr>
<td>5</td>
<td>96</td>
<td>88</td>
</tr>
<tr>
<td>6</td>
<td>98</td>
<td>80</td>
</tr>
<tr>
<td>7</td>
<td>130</td>
<td>140</td>
</tr>
</tbody>
</table>

*Results presented in Fig. 2 are expressed as percentage of control response on each of the indicated days after in vitro immunization; details as in the legend to Fig. 2.

Fig. 3. Mitogenic activity of particulate (○) and soluble (○) PG. Cultures of 10^6 mouse spleen cells were incubated with the indicated concentrations of either particulate or soluble PG for 24 h and pulsed with [3H]thymidine for an additional 18 h. Stimulation with optimal mitogenic concentrations of LPS (100 µg/ml) and phytohemagglutinin (PHA) (38 µg/ml) is also shown. The results are means obtained from eight cultures in two experiments.

Fig. 4. Kinetics of blastogenic response of 10^6 mouse spleen cells cultured with 400 µg of particulate PG (○) or 100 µg of soluble PG (○) per ml. Cultures were pulsed with [3H]thymidine for the final 18 h of incubation. Incorporation of radioactivity in untreated control cultures is also shown (□). The results are means obtained from four cultures; stimulation index = mean counts per minute for treated cultures/mean counts per minute for control cultures.

Solubilate PG given before or along with the antigen was immunostimulatory. Soluble PG was adjuvant active in both high and low doses when injected before or along with the antigen. Its in vitro immunostimulatory effect was greater than that obtained with low doses of particulate PG (Table 1). Immunosuppression could not be induced by soluble PG with any doses or time regimens tested. It was recently shown, however, that multiple injections of high doses of MDP given before antigenic stimulation could suppress the anti-SRBC PFC response (16).

In the in vitro immunization system, both PG preparations displayed a dose-dependent adjuvant activity. In this system, however, particulate PG was more active than soluble PG (Fig. 1 and 2). Even high doses of particulate PG were not directly suppressive for the in vitro immune response when PG was added at the initiation of the cultures. Previous studies have shown, however, that the in vitro immune response of splenocytes from mice injected a day earlier with PG is suppressed to the same extent as the in vivo response (5a). This stresses the importance of time sequence in PG-induced immunosuppression; i.e., for the most effective suppression, PG must be injected before the antigenic stimula-
tion. In the same study, it was also found that the suppressive effect is mediated by macrophages and the suppression can be (at least to a certain extent) reproduced in vitro by treating macrophages with PG and then adding them to normal cell cultures.

The third important factor in PG-induced immunosuppression, as shown in this paper, is the physical state of PG. Only particulate PG was immunosuppressive; soluble PG was not. Both preparations were, however, adjuvant active, which indicates that different mechanisms are responsible for these two immunomodulatory events.

The adjuvant activity of PG could be dissociated from its mitogenic activity. As already discussed, both PG preparations were adjuvant active. Mitogenicity was associated only with particulate PG and was lost after its solubilization (Fig. 3 and 4). PG is a B-lymphocyte mitogen (6). B-cell mitogenicity and polyclonal activation was suggested as the mechanism of adjuvant activity of a number of adjuvants (20), including PG (4). From our studies, however, this seems not to be the case, at least for the adjuvant activity of PG. Soluble PG was adjuvant active but was not mitogenic, and even though particulate PG was both adjuvant active and mitogenic, the optimal concentration for its adjuvant effect was 25 μg/ml, whereas maximal mitogenic activity was obtained with 400 μg/ml (Fig. 1 and 3). Moreover, the adjuvant activity of PG was comparable when the results were calculated either per culture or per 10^6 viable cells, indicating that immunostimulatory effect did not depend on an increase in total cell number, but was due to increased numbers of SRBC-specific antibody-forming cells only.

Similar results were obtained with LPS, another potent B-cell mitogen and adjuvant. Studies on LPS-responder and -nonresponder mice indicated that adjuvanticity and mitogenicity represented distinct pathways of B-cell activation and were subject to different regulatory mechanisms (10). It was also possible to chemically dissociate adjuvant from mitogenic activity of LPS (9). A low-molecular-weight polysaccharide-rich preparation, extracted from Serratia marcescens LPS, stimulated the in vitro antibody response of mouse splenocytes and had no mitogenic activity.

However, some contribution of B-cell mitogenicity to the adjuvant effect of PG cannot be excluded, especially in the in vitro system, in which particulate PG exhibited higher activity than soluble PG. It appears from these studies that the overall effect of PG on the immune system is the result of interactions of different immunomodulating activities. The final outcome probably depends on a number of factors, including culture conditions, since it was recently reported that MDP had an adjuvant effect on in vitro immune response in low-density cultures (comparable to those described in this paper), whereas it was immunosuppressive for cells cultured at high densities (17). Further studies are required to define more precisely the mechanisms of immunomodulating activities of PG and its derivatives.

ACKNOWLEDGMENTS

I am grateful to Gail Higenell and Frank Leonardo for their technical assistance.

This work was supported by grants from the National Foundation for Infectious Diseases and from the Pennsylvania College of Podiatric Medicine.

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