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Cell Walls, Peptidoglycans, and Teichoic Acids of Gram-Positive Bacteria as Polyclonal Inducers and Immunomodulators of Proliferative and Lymphokine Responses of Human B and T Lymphocytes

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In this study the mitogenic and immunomodulating effects of bacterial cell wall preparations were investigated. Cell walls, peptidoglycans, and teichoic acids from *Bacillus subtilis* and *Staphylococcus aureus* Wood 46 activated both human T cells (supplemented with 10% monocytes) and B cells to proliferate and to produce leukocyte-inhibitory factor. Similar results were obtained with adult and umbilical cord blood cells, suggesting that these bacterial preparations were acting as mitogens. Cell walls and peptidoglycans had a modulating effect on purified protein derivative- or protein A-induced proliferation. In the presence of suboptimal concentrations of these stimulants, bacterial components enhanced the proliferative response. However, at optimal concentrations of purified protein derivative or protein A, bacterial components suppressed lymphocyte proliferation. Peptidoglycans solubilized by lysozyme activated B lymphocytes but not T cells. Solubilization had no effect on the immunomodulating capacity.

Polyclonal activation of human lymphocytes by many bacteria (2, 18, 19) and other microbes is an intriguing phenomenon, the role of which has been discussed recently by several authors (6, 15, 19). Briefly, it has been suggested that polyclonal activation may be useful as a common mechanism of resistance in infectious diseases, on the one hand, but on the other hand, it may also have pathogenetic significance in many chronic inflammatory diseases.

We have tried to identify some of the components on the bacterial surface which might trigger such a response and have confirmed the mitogenicity of peptidoglycans and also found teichoic acids to be mitogenic (Räsänen, Mustikkamäk, and Arvilommi, in press). There are a number of publications on the mitogenicity and immunomodulating or adjuvant activity of peptidoglycans (5, 8, 10–12, 14, 16, 21), but most of these investigations have been made with laboratory animals, using unpurified mixtures of T and B lymphocytes. Further work is thus required to identify the target lymphocytes for bacterial preparations, especially in humans.

In this study, we investigated the capacity of cell walls, peptidoglycans, and teichoic acids from *Bacillus subtilis* and *Staphylococcus aureus* Wood 46 to activate both adult and umbilical cord blood T and B lymphocytes. In addition, the modulating effect of bacterial cell wall preparations on antigen- or mitogen-induced lymphocyte proliferation was studied.

MATERIALS AND METHODS

Preparation of bacterial cell walls. *B. subtilis* (NCTC 3610) and *S. aureus* Wood 46 (a gift from A. Forsgren, Malmö, Sweden) were cultured at 37°C for 20 h in a medium consisting of the following: beef extract, 5 g; glucose, 10 g; NaCl, 1 g; $Na_2HPO_4 \cdot 2H_2O$, 7.5 g; NaH_2PO_4, 3 g; peptone, 10 g; NH_4Cl, 1 g; $Na_2SO_4 \cdot 10H_2O$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 6.2 g; thiamine, 2 mg; and nicotinic acid, 4 mg, per liter of distilled water. The bacterial suspension was centrifuged at 1,500 × *g* for 15 min, and the cells were washed twice with distilled water.

To disintegrate the bacteria, they were subjected to ultrasonic vibration for 1 to 3 h in an MSE 150-W ultrasonic disintegrator. The suspension was centrifuged twice at 1,500 × *g* for 15 min, and the supernatant containing the crude cell walls was collected. The walls were sedimented by centrifuging at 15,000 × *g* for 30 min and then rinsed three times with distilled water. The washed cell walls were treated with 200 μg of trypsin (1:250; Difco Laboratories, Detroit, Mich.) per ml in phosphate-buffered saline for 2.5 h at 37°C, followed by a similar treatment with 100 μg of RNase B and 50 μg of DNase I (both purchased from Sigma Chemical Co., St. Louis, Mo.) per ml. The digested cell walls were then washed three times with distilled water and lyophilized.

Isolation of teichoic acid and peptidoglycan from bacterial cell walls. Teichoic acid was extracted from the digested bacterial cell walls according to the meth-

od of Armstrong et al. (1) with modifications. The walls (200 mg) were stirred with 10 ml of 10% trichloroacetic acid (TCA) at 4°C for 24 h, after which they were spun down and reextracted with TCA for 48 h. The supernatants were pooled and extracted three times with a double volume of ethyl ether. The aqueous layer was then mixed with ethanol (3 volumes) and kept at 4°C for 24 h. The precipitate of teichoic acid was dissolved in a minimum volume of 10% TCA and reprecipitated with ethanol (2 volumes), washed twice, and dried.

The residue remaining after extraction with TCA was further extracted with 10% TCA at 60°C for 2 h (20) and then centrifuged at 15,000 × *g* for 30 min. The sediment was washed three times with distilled water and lyophilized to obtain peptidoglycan. Before bacterial preparations were used in cell cultures, they were heated at 70°C for 30 min and plated for sterility checks.

Analysis of bacterial cell wall preparations. The phosphorus content of the digested cell walls and peptidoglycans was determined by the method of Chen et al. (4). Originally the enzyme-digested walls contained 5.02 and 2.54% phosphorus (*B. subtilis* and *S. aureus* Wood 46, respectively). After a 3-day extraction with 10% TCA, there was 0.46 and 0.11% phosphorus, respectively, left in the peptidoglycan fractions.

The purity of the bacterial preparations was checked by thin-layer chromatography. Cell walls, teichoic acids, and peptidoglycans from *B. subtilis* and *S. aureus* Wood 46 were hydrolyzed in 6 N HCl at 100°C for 6 h. After evaporation of the acid, the samples were examined with Silica Gel 60 aluminium sheets (E. Merck AG, Darmstadt, West Germany). The following solvent systems were used: *n*-propanol–ammonia–water (60:30:10, by volume) and *n*-butanol–acetic acid–water (60:15:15, by volume). Visualization of the spots was accomplished by the ninhydrin reagent for amino acids and amino sugars (7), the H_2SO_4-$KMnO_4$ spray reagent for polyols (13), and the aniline phthalate reagent for reducing sugars (17). The following amino acids and amino sugars were detected in the acid hydrolysates of *B. subtilis* and *S. aureus* Wood 46 walls: muramic acid, glucosamine, alanine, diaminopimelic acid, and glutamic acid (*B. subtilis* walls) and muramic acid, glucosamine, alanine, glutamic acid, glycine, and lysine (*S. aureus* Wood 46 walls). Alanine, ribitol, anhydroribitol, and glucose were identified in the acid hydrolysate of *B. subtilis* teichoic acid, whereas in *S. aureus* Wood 46 there was glucosamine in the place of glucose. Peptidoglycans contained the same amino acids and amino sugars as the cell walls and traces of glucose and anhydroribitol (*B. subtilis*) or anhydroribitol (*S. aureus* Wood 46) evidently originating from teichoic acids.

Treatment of peptidoglycans with lysozyme. The peptidoglycans from *B. subtilis* and *S. aureus* Wood 46 were first subjected to ultrasonic vibration for 2 h. Egg white lysozyme (final concentration, 0.01%; Sigma Chemical Co.) was then added to 2 mg of peptidoglycan in 2 ml of 0.1 M sodium phosphate buffer, pH 6.0. The suspension was incubated at 37°C for 4 h and then fractionated on a calibrated column of Sephadex G-25 M (Pharmacia Fine Chemicals, Uppsala, Sweden) to separate lysozyme from the peptidoglycan degradation products. The degradation products with a molecular weight below 5,000 were collected.

Separation and identification of the cells. Mononuclear cells were obtained by Ficoll-Isopaque centrifugation of heparinized or citrated adult venous or umbilical cord blood (3). To obtain T and B cells, the cell suspension was rosetted with sheep erythrocytes and centrifuged on Ficoll-Isopaque as described earlier (19). B lymphocytes were then incubated with 0.2 g of carbonyl iron in Hanks balanced salt solution supplemented with 10% AB serum at 37°C for 30 min, after which the phagocytic cells were removed with a magnet. T cells were not treated with carbonyl iron since their monocyte contamination was very small. Monocytes were purified on the basis of their adherence to plastic surfaces as described earlier (19).

The purity of the cell populations was studied, using sheep erythrocyte rosette formation to demonstrate T cells, staining of surface membrane immunoglobulin to demonstrate B cells, and staining of nonspecific esterase to demonstrate monocytes (18). T cells contained an average of 95.0% sheep erythrocyte rosette-forming cells, 2.5% immunofluorescence-positive cells, and <0.1% monocytes. B cells contained 71.6% immunofluorescence-positive cells, 2.7% sheep erythrocyte rosette-forming cells, and 0.5% monocytes. The purity of monocytes was 95.5%.

Cell cultures. The cells were suspended at a concentration of 10^6/ml in RPMI 1640 (Flow Laboratories, Irvine, Scotland) supplemented with 10% autologous plasma. Duplicate (for leukocyte-inhibitory factor [LIF] production) or triplicate (for transformation) cultures containing 10^5 cells per well were set up in flat-bottomed microplates. The cells were incubated in the presence of various concentrations of cell walls, peptidoglycans, or teichoic acids from *B. subtilis* and *S. aureus* Wood 46. The controls for LIF production contained corresponding amounts of bacterial preparations and 5 μg of the protein synthesis inhibitor puromycin (Sigma Chemical Co.) per ml. In a series of experiments, lymphocytes were cultured in the presence of bacterial components and purified protein derivative (PPD; State Serum Institute, Copenhagen, Denmark) or soluble protein A (Pharmacia Fine Chemicals, Uppsala, Sweden). Cultures for LIF and transformation were harvested after 4 and 5 days of incubation, respectively. At 16 h before harvesting, 0.125 μCi of 5-[^{125}I]iodo-2′-deoxyuridine containing 1 μM fluorodeoxyuridine was added per well of transformation cultures. The uptake of the isotope was measured with a gamma counter.

Leukocyte migration inhibitory factor assay. LIF activity was tested in the culture supernatants by the agarose migration method, as described earlier (18). The migration index was defined as follows: migration index = area of migration in the presence of test supernatant/area of migration in the presence of control supernatant. Generally, migration indexes smaller than 0.85 represented significant inhibition of migration.

Statistics. The significance of differences in lymphocyte responses was calculated by the Mann-Whitney U test.

RESULTS

Activation of adult and umbilical cord blood T and B cells by bacterial preparations. T cells responded to bacterial preparations only after

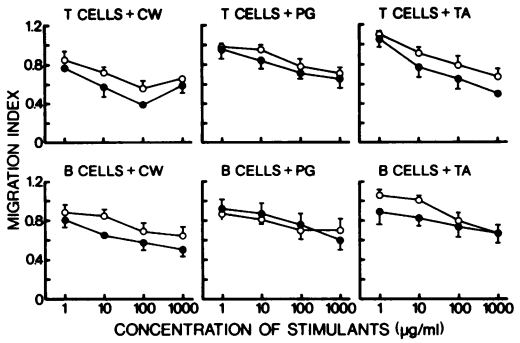

FIG. 1. Elaboration of LIF by human T and B lymphocytes stimulated by products from *S. aureus* Wood 46 (○) or *B. subtilis* (●). Circles represent mean and bars represent standard error of five experiments. CW, Bacterial cell walls; PG, peptidoglycan; TA, teichoic acid.

reconstitution with monocytes, whereas B cells did not require the help of additional monocytes. Monocytes by themselves did not produce LIF or proliferate in the presence of bacterial stimulants (data not shown). *B. subtilis* and *S. aureus* Wood 46 cell wall preparations activated both B and T cells (supplemented with 10% monocytes) to elaborate LIF (Fig. 1) and to proliferate (Fig. 2). The bacterial walls were slightly better stimulants than peptidoglycans or teichoic acids.

The possible mitogenicity of bacterial preparations was tested by using umbilical cord blood cells, which, lacking prior contact with antigens, should respond only to mitogens. The result obtained with cord blood cells was similar to that obtained with adult cells. All bacterial preparations used activated both umbilical cord T and B lymphocytes (Table 1).

Immunomodulating effect of cell walls and peptidoglycans on lymphocyte proliferation. T cells (supplemented with 10% monocytes) were stimulated with PPD and B cells were stimulated with protein A in the presence of various doses of peptidoglycans or bacterial walls. PPD and protein A were used in both optimal and suboptimal concentrations. Figure 3 shows the results obtained with *B. subtilis*. Experiments with *S. aureus* Wood 46 gave similar results (data not shown). Bacterial cell walls or their peptidoglycan components potentiated the lymphocyte response to suboptimal concentrations of PPD or protein A. The peak enhancement was most often seen in the presence of 0.1 μg of cell walls or peptidoglycans per ml. In contrast, however, with optimal concentrations of PPD or protein A, addition of bacterial preparations either diminished or did not significantly affect lymphocyte proliferation.

Comparison between particulate and lysozyme-digested peptidoglycans. We investigated whether the solubilization of peptidoglycan with lysozyme changes its capacity to stimulate lymphocytes or to modulate the lymphocyte response to other stimulants. Enzymatically digested peptidoglycan could activate B cells, but not T cells, to proliferate (Fig. 4). The B-cell response was only slightly lower with the soluble peptidoglycan than with the particulate preparation. The immunomodulating effects of soluble and particulate peptidoglycans were very similar (Fig. 5). Both preparations enhanced lymphocyte proliferation in the presence of suboptimal concentrations of PPD or protein A. Furthermore, in the presence of optimal concentrations of these stimulants, enzymatically treated and nontreated peptidoglycans diminished lymphocyte proliferation. The suppression was more clear-cut with the particulate peptidoglycan.

DISCUSSION

These results extend our previous work on the polyclonal activation capacity of whole bacteria to their surface components. It is demonstrated here that bacterial cell walls, peptidoglycans, and teichoic acids induce a proliferative and a lymphokine response in both T and B lymphocytes. As far as we know, this kind of analysis has not been made previously. The significance of the lymphokine response to bacterial stimulants has been discussed in our earlier paper (18).

It is noteworthy that lymphocytes respond polyclonally not only to whole bacteria but also to fractions which may be released from them in vivo. This makes it more plausible to think that polyclonal activation may occur in different phases of microbial infections, namely, before and after degradation of the microbes by host enzymes. The activation of lymphocytes and the consequences thereof become even more com-

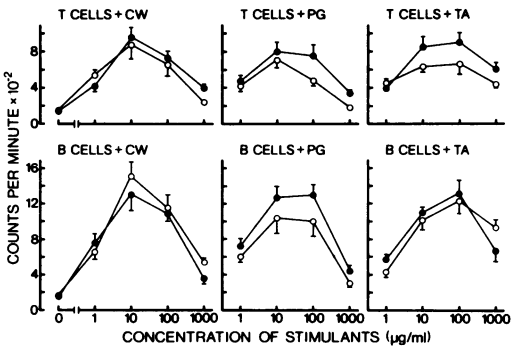

FIG. 2. Mitogenicity of bacterial cell walls, peptidoglycans, and teichoic acids to human T and B cells. For details and abbreviations, see legend to Fig. 1.

TABLE 1. Response of umbilical cord blood lymphocytes to bacterial cell wall preparations

Stimulant[a]	T cells		B cells	
	LIF (MI)[b]	Transformation (cpm)	LIF (MI)	Transformation (cpm)
None		123 ± 11		241 ± 21
B. subtilis walls	0.65 ± 0.08[c]	647 ± 57	0.67 ± 0.07	1,434 ± 138
B. subtilis peptidoglycan	0.66 ± 0.08	606 ± 67	0.68 ± 0.08	1,570 ± 33
B. subtilis teichoic acid	0.78 ± 0.07	575 ± 60	0.85 ± 0.06	1,377 ± 109
S. aureus walls	0.54 ± 0.04	834 ± 94	0.53 ± 0.03	1,319 ± 155
S. aureus peptidoglycan	0.65 ± 0.05	750 ± 92	0.61 ± 0.04	1,368 ± 176
S. aureus teichoic acid	0.68 ± 0.09	731 ± 80	0.67 ± 0.08	1,407 ± 135

[a] The dose of all bacterial preparations was 100 μg/ml.
[b] MI, Migration index (see text).
[c] Values represent the mean ± SEM of eight experiments.

plex when the immunomodulating properties of the bacterial products are taken into consideration. The presence of bacterial components may result in enhanced or diminished response to other antigens.

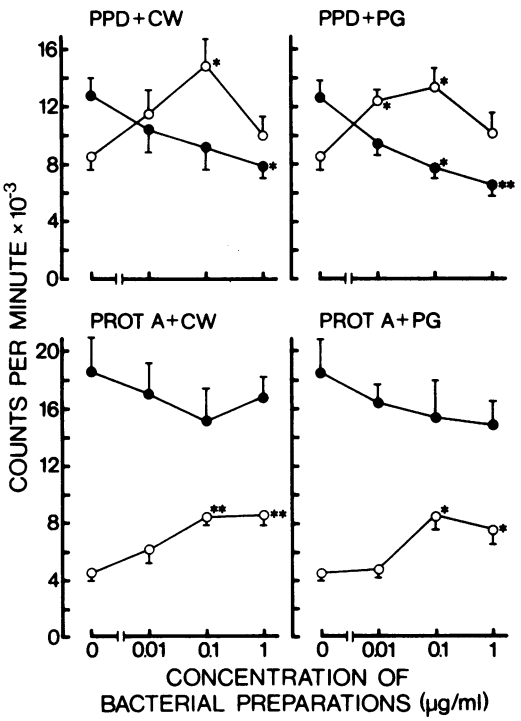

FIG. 3. Effect of *B. subtilis* walls (CW) and peptidoglycan (PG) on PPD-induced proliferation of T lymphocytes and protein A-induced proliferation of B lymphocytes. Symbols: ●, optimum concentrations of PPD (10 μg/ml) or protein A (20 μg/ml); ○, suboptimal concentrations of PPD (1 μg/ml) or protein A (2 μg/ml). The asterisks denote the significance of differences in responses between cultures with and without bacterial walls or peptidoglycan: **, $P < 0.01$; *, $P < 0.05$. The results represent the mean ± standard error of five experiments.

The physical properties of bacterial wall constituents may affect the response of lymphocytes. According to some investigators, soluble peptidoglycan components obtained by lysozyme digestion or ultrasonication were no longer mitogenic but possessed immunomodulating or adjuvant activities (5, 8, 10). Other investigators have found that enzymatically digested peptidoglycan or muramyl dipeptide is mitogenic (9, 21). In this study lysozyme-digested peptidoglycan could activate B lymphocytes but not T cells to proliferate. The results resemble those of Takada et al. (21). Their studies have shown, in guinea pigs, that after solubilization the bacterial walls or peptidoglycans were no longer mitogenic to thymocytes but retained their activity

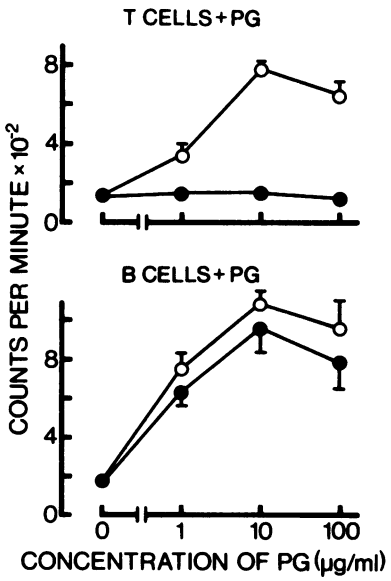

FIG. 4. Proliferation of T or B cells in the presence of intact (○) and lysozyme-digested (●) peptidoglycan (PG) from *S. aureus* Wood 46. Mean results ± standard error of five experiments are given.

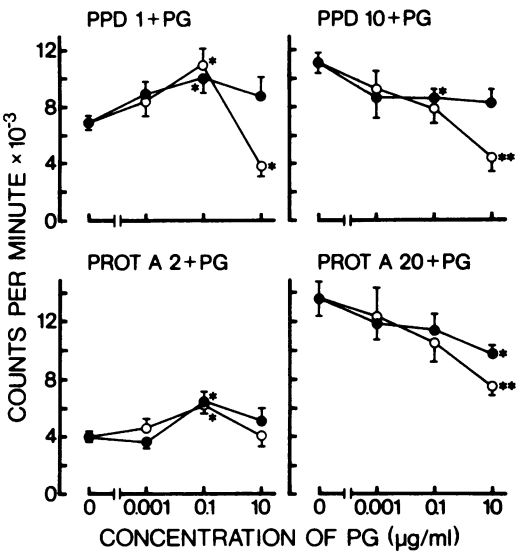

FIG. 5. Effects of particulate (○) and solubilized (●) peptidoglycan (PG) from *B. subtilis* on PPD- or protein A-induced T- or B-cell proliferation. Mean results ± standard error of five experiments are given. PPD 1 = PPD, 1 μg/ml.

against splenic lymphocytes. In the present study lysozyme-treated peptidoglycan did not lose its immunomodulating capacity. This result confirms the dissociation of mitogenicity and immunomodulating activities of bacterial wall constituents recently discussed by Dziarski (10).

In conclusion, bacteria and their products have various ways of affecting the host's T- and B-cell responses. Bacteria may activate lymphocytes in a specific or a polyclonal fashion and they may modulate lymphocyte responses to other stimulants. How this complex spectrum of events is regulated in vivo merits further study.

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