INFECTION AND IMMUNITY, Dec. 1981, p. 712-717
0019-9567/81/120712-06$02.00/0

Vol. 34, No. 3

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

LIISA RÄSÄNEN[1]* AND HEIKKI ARVILOMMI[2]

Institute of Biomedical Sciences, University of Tampere, 33101 Tampere 10,[1] and Public Health Laboratory, 40620 Jyväskylä 62,[2] Finland

Received 1 June 1981/Accepted 10 August 1981

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 migration inhibitory factor. Similar results were obtained with adult and umbilical cord blood cells, suggesting that these bacterial preparations acted as mitogens. Cell walls and peptidoglycans had a modulating effect on purified protein derivative-induced and 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 immunomodulating capacity.

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

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

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-induced and 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 5 g of beef extract, 10 g of glucose, 1 g of NaCl, 7.5 g of $Na_2HPO_4 \cdot 2H_2O$, 3 g of NaH_2PO_4, 10 g of peptone, 1 g of NH_4Cl, 0.5 g of $Na_2SO_4 \cdot 10H_2O$, 6.2 g of $MgSO_4 \cdot 7H_2O$, 2 mg of thiamine, and 4 mg of nicotinic acid 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, we subjected the cells 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 ribonuclease-B and 50 μg of deoxyribonuclease I per ml (both purchased from Sigma Chemical Co., St. Louis, Mo.). The digested cell walls were then washed three times with distilled water and were lyophilized.

Isolation of teichoic acid and peptidoglycan from bacterial cell walls. Teichoic acid was ex-

tracted from the digested bacterial cell walls by the method 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 time 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 (three volumes) and kept at 4°C for 24 h. The precipitate of teichoic acid was dissolved in a minimum volume of 10% TCA, reprecipitated with ethanol (two 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 the 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 contents of the digested cell walls and peptidoglycans were determined by the method of Chen et al. (4). Originally, the enzyme-digested walls contained 5.02 and 2.54% phosphorus (for *B. subtilis* and *S. aureus* Wood 46, respectively). After a 3-day extraction with 10% TCA, there was 0.46 and 0.11% phosphorus left in the peptidoglycan fractions (for *B. subtilis* and *S. aureus* Wood 46, respectively).

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, Germany). The following solvent systems were used: *n*-propanol–ammonia–water (60:30:10, vol/vol/vol), and *n*-butanol–acetic acid–water (60:15:15, vol/vol/vol). Visualization of the spots was accomplished with ninhydrin reagent for amino acids and amino sugars (7), H_2SO_4-$KMnO_4$ spray reagent for polyols (13), and 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 place of glucose. Peptidoglycans contained the same amino acids and amino sugars as the cell walls contained, as well as traces of glucose and anhydroribitol (*B. subtilis*) or anhydroribitol evidently originating from teichoic acids (*S. aureus* Wood 46).

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 peptidoglycan degradation products. Degradation products with molecular weights below 5,000 were collected.

Separation and identification of cells. Mononuclear cells were obtained by Ficoll-Isopaque centrifugation of heparinized or citrated adult venous or umbilical cord blood (3). For obtaining T and B cells, the cell suspension was rosetted with sheep erythrocytes and centrifuged on Ficoll-Isopaque as described previously (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 time the phagocytic cells were removed with a magnet. T cells were not treated with carbonyl iron because 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 by using sheep erythrocyte rosette formation to demonstrate T cells, by staining surface membrane immunoglobulin to demonstrate B cells, and by staining nonspecific esterase to demonstrate monocytes (18). T cells contained on the average 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 the monocytes was 95.5%.

Cell cultures. The cells were suspended at a concentration of 10^6 cells per ml in RPMI 1640 medium (Flow Laboratories, Irvine, Scotland) supplemented with 10% autologous plasma. Duplicate (for leukocyte migration 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 per ml (Sigma Chemical Co.). 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). Cultures for LIF and transformation were harvested after 4 and 5 days of incubation, respectively. Sixteen hours before harvesting took place, 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.

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

Statistics. The significance of differences among 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 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 were 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 have responded only to mitogens. The results obtained for umbilical cord blood cells was similar to that obtained for adult cells. All bacterial preparations used activated both umbilical cord T and B lymphocytes (Table 1).

Immunomodulating effect of cell walls

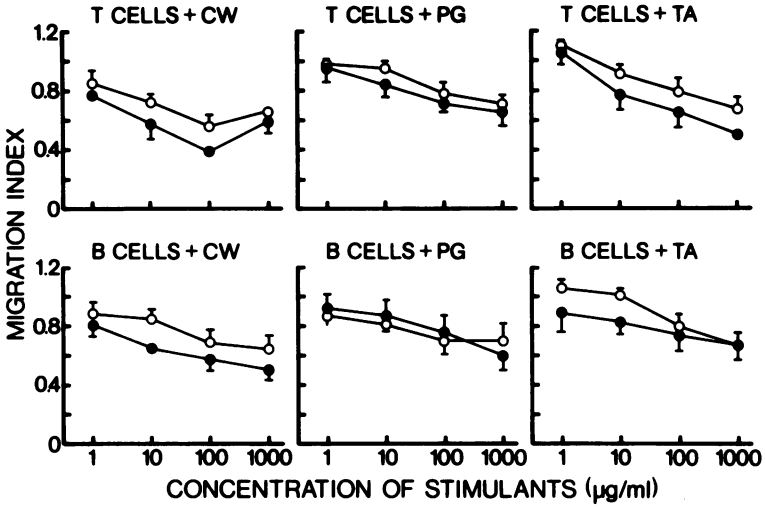

FIG. 1. *Elaboration of LIF by human T and B lymphocytes stimulated by products from S. aureus Wood 46 (○) or B. subtilis (●). Circles represent the means, and bars represent standard errors of the means of five experiments. CW, Bacterial cell walls; PG, peptidoglycan; TA, teichoic acid.*

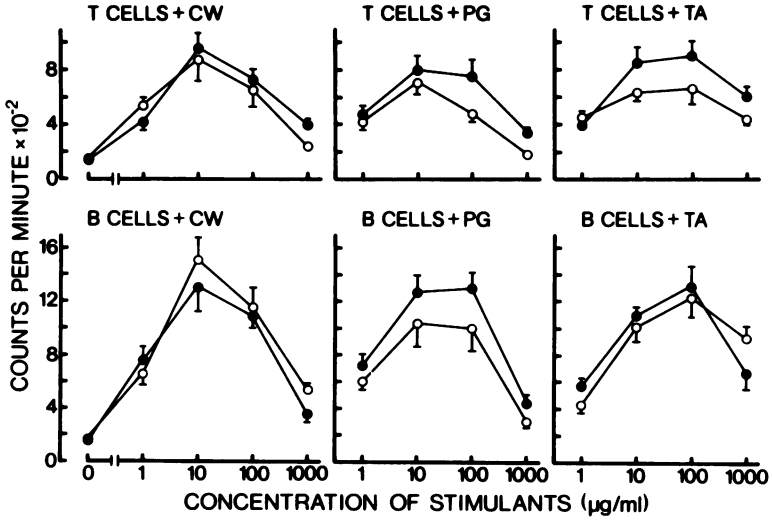

FIG. 2. *Mitogenicity of bacterial cell walls, peptidoglycans, and teichoic acids to human T and B cells. For explanations, see legend to Fig. 1.*

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

Stimulant[a]	T cells		B cells	
	LIF (MI)	Transformation (cpm)	LIF (MI)	Transformation (cpm)
None		123 ± 11[b]		241 ± 21
B. subtilis walls	0.65 ± 0.08	647 ± 57	0.67 ± 0.07	1434 ± 138
B. subtilis peptidoglycan	0.66 ± 0.08	606 ± 67	0.68 ± 0.08	1570 ± 33
B. subtilis teichoic acid	0.78 ± 0.07	575 ± 60	0.85 ± 0.06	1377 ± 109
S. aureus walls	0.54 ± 0.04	834 ± 94	0.53 ± 0.03	1319 ± 155
S. aureus peptidoglycan	0.65 ± 0.05	750 ± 92	0.61 ± 0.04	1368 ± 176
S. aureus teichoic acid	0.68 ± 0.09	731 ± 80	0.67 ± 0.08	1407 ± 135

[a] The dose of each bacterial preparation was 100 μg/ml.
[b] Values represent the means ± standard errors of the means of eight experiments.

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 from *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. 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 the capacity of peptidoglycan to stimulate lymphocytes or modulate the lymphocyte response to other stimulants. Enzymatically digested peptidoglycan could activate proliferation of B but not T cells (Fig. 4). 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 particulate peptidoglycan.

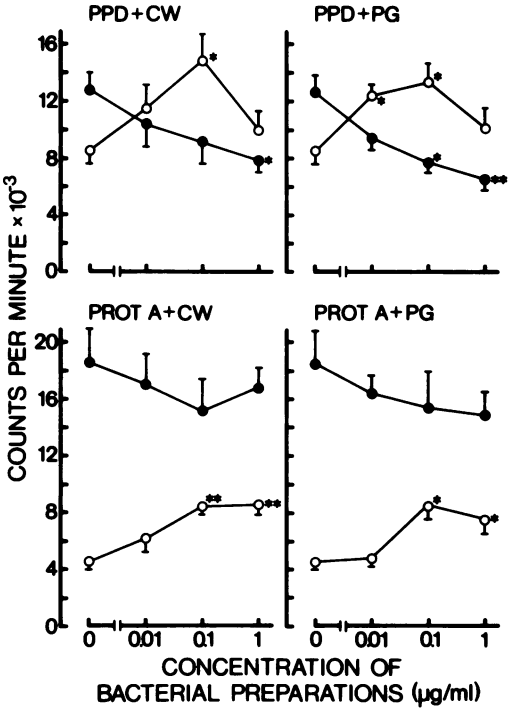

FIG. 3. *Effect of B. subtilis walls or peptidoglycan on PPD-induced proliferation of T lymphocytes and protein A-induced proliferation of B lymphocytes. Symbols: ●, optimum concentrations of PPD (10 μg/ml) or protein A (prot A) (20 μg/ml); ○, suboptimal concentrations of PPD (1 μg/ml) or protein A (2 μg/ml). Asterisks denote the significances of differences in responses between cultures with and without bacterial walls or peptidoglycan. **, $P < 0.01$; *, $P < 0.05$. Circles represent the means, and bars represent standard errors of the means of five experiments. Abbreviations are explained in the legend to Fig. 1.*

DISCUSSION

These results extend our previous work on the polyclonal activation capacity of whole bacteria to that of their surface components. We demonstrate 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 previously been made. The significance of the lymphokine response to bacterial stimulants has been discussed in an earlier paper (18).

It is noteworthy that lymphocytes reponded 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, viz., before and after degradation of the microbes by host enzymes. The activation of lymphocytes and the consequences thereof become even more complex 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.

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 are no longer mitogenic but possess immunomodulating or adjuvant activities (5, 8, 10). Other investigators found that enzymatically digested peptidoglycan or muramyl dipeptide is mitogenic (9, 21). In the present study, lysozyme-digested peptidoglycan was able to activate B lymphocytes but not T cells to proliferate. The results resemble those of Takada et al. (21). Their results showed that, in guinea pigs, the bacterial walls or peptidoglycans after solubilization were no longer mitogenic to thymocytes, but both retained activity against splenic lymphocytes. In our 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).

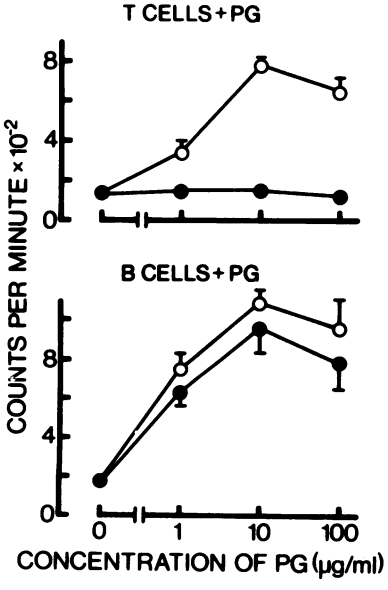

FIG. 4. *Proliferation of T and B cells in the presence of intact* (○) *and lysozyme-digested peptidoglycan (PG)* (●) *from S. aureus Wood 46. Circles represent the means, and bars represent standard errors of the means of five experiments.*

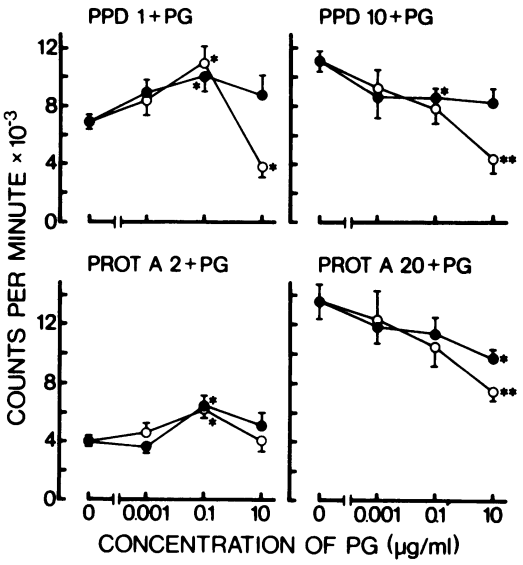

FIG. 5. *Effects of particulate* (○) *and solubilized* (●) *peptidoglycan (PG) from B. subtilis on PPD- or protein A (prot A)-induced proliferation. Circles represent means, and bars represent standard errors of the means of five experiments. Numbers after abbreviations indicate concentrations (micrograms per milliliter). *, $P < 0.05$; ** $P < 0.01$ (significances of differences in responses between cultures with particulate or solubilized peptidoglycan compared with control cultures without peptidoglycan).*

In conclusion, bacteria and their products have various ways of affecting the T and B cell responses of the host. Bacteria may activate lymphocytes in a specific or polyclonal fashion and may modulate lymphocyte responses to other stimulants. How this complex spectrum of events is regulated in vivo merits further study.

LITERATURE CITED

1. **Armstrong, J. J., J. Baddiley, and J. G. Buchanan.** 1970. Structure of the ribitol teichoic acid from the walls of Bacillus subtilis. Biochem. J. **76**:610-621.
2. **Banck, G., and A. Forsgren.** 1978. Many bacterial species are mitogenic for human blood B lymphocytes. Scand. J. Immunol. **8**:347-354.
3. **Böyum, A.** 1968. Isolation of mononuclear cells and granulocytes from human blood. Scand. J. Clin. Lab. Invest. **21**(Suppl.):77-89.
4. **Chen, P. S., T. Y. Toribara, and H. Warner.** 1956.

Microdetermination of phosphorus. Anal. Chem. **28**: 1756–1758.

5. **Ciorbaru, R., J.-P. Petit, E. Lederer, E. Zissman, C. Bona, and L. Chedid.** 1976. Presence and subcellular localization of two distinct mitogenic fractions in the cells of *Nocardia rubra* and *Nocardia opaca*: preparation of soluble mitogenic peptidoglycan fractions. Infect. Immun. **13**:1084–1090.
6. **Clagett, J. A., and D. Engle.** 1978. Polyclonal activation: a form of primitive immunity and its possible role in the pathogenesis of inflammatory diseases. Dev. Comp. Immunol. **2**:235–242.
7. **Consden, R., and A. H. Gordon.** 1948. Effect of salt on partition chromatograms. Nature (London) **162**:180–181.
8. **Damais, C., C. Bona, L. Chedid, J. Fleck, C. Nauciel, and J. P. Martin.** 1975. Mitogenic effect of bacterial peptidoglycans possessing adjuvant activity. J. Immunol. **115**:268–271.
9. **Damais, C., M. Parant, and L. Chedid.** 1977. Nonspecific activation of murine spleen cells in vitro by a synthetic immunoadjuvant (N-acetyl muramyl-L-alanyl-D-isoglutamine). Cell. Immunol. **34**:49–56.
10. **Dziarski, R.** 1979. Relationships between adjuvant, immunosuppressive, and mitogenic activities of staphylococcal peptidoglycan. Infect. Immun. **26**:508–514.
11. **Dziarski, R., and A. Dziarski.** 1979. Mitogenic activity of staphylococcal peptidoglycan. Infect. Immun. **23**: 706–710.
12. **Dziarski, R., A. Dziarski, and A. I. Levinson.** 1980. Mitogenic responsiveness of mouse, rat and human lymphocytes to Staphylococcus aureus cell wall, teichoic acid and peptidoglycan. Int. Arch. Allergy Appl. Immunol. **63**:383–395.
13. **Ertel, H., and L. Horner.** 1962. Zwei Universalreagentien zur Dünnschichtchromatographie. J. Chromatogr. **7**: 268–269.
14. **Kotani, S., Y. Watanabe, T. Shimono, T. Narita, K. Kato, D. E. S. Stewart-Tull, F. Kinoshita, K. Yokogawa, S. Kawata, T. Shiba, S. Kusumoto, and Y. Tarumi.** 1975. Immunoadjuvant activities of cell walls, their water-soluble fractions and peptidoglycan subunits prepared from various gram-positive bacteria, and of synthetic N-acetylmuramyl peptides. Z. Immun. Allergieforsch. **149**:302–319.
15. **Möller, E., H. Ström, and S. Al-Balaghi.** 1980. Role of polyclonal activation in specific immune responses. Scand. J. Immunol. **12**:177–182.
16. **Nauciel, C., and J. Fleck.** 1975. Adjuvant activity of bacterial peptidoglycans. Z. Immun. Allergieforsch. **149**:349–353.
17. **Partridge, S. M.** 1949. Aniline hydrogen phthalate as a spraying reagent for chromatography of sugars. Nature (London) **164**:443.
18. **Räsänen, L., E. Karhumäki, and H. Arvilommi.** 1978. Bacteria induce lymphokine synthesis polyclonally in human B lymphocytes. J. Immunol. **121**:418–420.
19. **Räsänen, L., E. Karhumäki, R. Majuri, and H. Arvilommi.** 1980. Polyclonal activation of human lymphocytes by bacteria. Infect. Immun. **28**:368–372.
20. **Shayegani, M., K. Hisatsune, and S. Mudd.** 1970. Cell wall component which affects the ability of serum to promote phagocytosis and killing of *Staphylococcus aureus*. Infect. Immun. **2**:750–756.
21. **Takada, H., M. Tsujimoto, S. Kotani, S. Kusumoto, M. Inage, T. Shiba, S. Nagao, I. Yano, S. Kawata, and K. Yokogawa.** 1979. Mitogenic effects of bacterial cell walls, their fragments, and related synthetic compounds on thymocytes and splenocytes of guinea pigs. Infect. Immun. **25**:645–652.