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Bacterial peptidoglycan induces *in vitro* rheumatoid factor production by lymphocytes of healthy subjects

R. J. LEVY, M. HAIDAR, H. PARK, L. TAR & A. I. LEVINSON *Allergy-Immunology Section, University of Pennsylvania School of Medicine and Rheumatology-Immunology Center, Veterans Administration Medical Center, Philadelphia, USA*

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SUMMMARY

The present studies were carried out to further characterize the polyclonal B cell activating properties of bacterial peptidoglycan (PG) and to determine if this ubiquitous agent induces *in vitro* IgM rheumatoid factor (RF) production by lymphocytes from healthy volunteers. Peripheral blood mononuclear cells (PBMC) were cultured in the presence of peptidoglycan, pokeweed mitogen (PWM), a standard polyclonal B cell activator, or additional culture medium. Supernatants were harvested on days 7–8 for determination of total IgM, total IgG, and IgM RF by an enzyme-linked immunosorbent assay (ELISA). PG and PWM induced comparable amounts of total IgM production but PG was a less potent stimulant of total IgG production. PG induced *in vitro* IgM-RF production in 9/33 experiments, a frequency of response of less than that observed in corresponding PWM stimulated cultures (22/33 experiments). PG-induced IgM-RF production depended upon active protein synthesis and did not correlate with the magnitude of PG-induced total IgM production. The latter finding suggests that PG-induced IgM-RF may not merely reflect polyclonal B cell activation. These results add to a growing list of PG's functional properties and provide further impetus for considering this ubiquitous agent as a potential stimulant for *in vivo* RF production.

Keywords peptidoglycan rheumatoid factor polyclonal B cell pokeweed mitogen

INTRODUCTION

Stimulation of lymphocytes obtained from experimental animals or healthy human donors with polyclonal B cell activators may elicit the production of multiple antibody specificities, some of which are directed against self determinants. Thus, pokeweed mitogen (PWM), lipopolysaccharide, and Epstein-Barr virus have all been shown to stimulate the production of rheumatoid factor (RF) (Slaughter *et al.*, 1977; Koopman & Schrohenloher, 1980; Primi *et al.*, 1977; Izui, Eisenberg & Dixon, 1979), an autoantibody directed against the Fc fragment of IgG. Furthermore, chronic polyclonal B cell activation induces autoimmune disease in the mouse (Hang, 1983). These observations have suggested that exaggerated responses to ubiquitous environmental polyclonal B cell activators could lead to the appearance of autoantibodies in certain clinical disorders.

Bacterial peptidoglycan (PG) is a major cell wall constituent of bacteria (particularly Gram positive), actinomycetes, and blue green algae (Schleifer & Kandler, 1972, review). We have reported that this ubiquitous agent is a mitogenic for murine and human lymphocytes (Dziarski, Dziarski & Levinson, 1980) and is a polyclonal B cell activator in these species (Dziarski *et al.*, 1980;

Correspondence: Dr A. I. Levinson, Allergy-Immunology Section, University of Pennsylvania School of Medicine, 515 Johnson Pavilion/G2, 36th & Hamilton Walk, Philadelphia, PA 19104, USA.

Levinson *et al.*, 1983). In addition, PG has arthritogenic properties; PG-polysaccharide complexes induce a chronic relapsing form of arthritis in experimental animals (Cromartie *et al.*, 1977). These findings prompted us to explore the PG-induced polyclonal B cell differentiation responses of blood cells from patients with rheumatoid arthritis, a disorder associated with *in vivo* polyclonal B cell activation (Pardo & Levinson, 1983). We found that cells from a large subset of patients with rheumatoid arthritis had markedly reduced *in vitro* polyclonal B cell responses to PG although their responses to a standard polyclonal activator (PWM) were intact (Pardo *et al.*, 1984).

The studies reported herein provide further clarification of the polyclonal B cell activating properties of peptidoglycan and demonstrate for the first time its capacity to induce RF production by normal human lymphocytes.

MATERIALS AND METHODS

Blood donors. Healthy adult volunteers served as lymphocyte donors. They ranged in age from 19–55 years and were without a history of arthritis, recent illness or drug use.

Polyclonal activators. PWM was purchased from GIBCO (Grand Island, NY, USA), PG (a gift from R. Dziarski, Indiana University School of Medicine) was isolated as previously described (Dziarski & Kwarecki, 1976) from *Staphylococcus aureus* 845 cell walls by trichloracetic acid extraction. Quantitative analysis of the chemical composition and purity of PG was established by paper chromatography. In addition, as previously reported (Dziarski, 1984) quantitative analysis of the chemical composition of PG on an amino acid analyser revealed that 97.5% of cell amino acids were those typical of *S. aureus* (Schleifer & Kandler, 1972). Accidental contamination with exogenous endotoxins was ruled out by toxicity tests in adrenalectomized mice (Dziarski & Dziarski, 1979). Before use, PG was suspended in sterile phosphate-buffered saline, treated with ultrasonication for 1 h, and then heated to 70°C for 1 h, and tested for sterility (Dziarski, 1979).

Cell cultures. Peripheral blood mononuclear cells (PBMC) were obtained from the blood of healthy volunteers by Ficoll-Hypaque density gradient separation. PBMC were prepared in RPMI-1640 medium (Microbiological Assoc., Walkersville, MD, USA) supplemented with 10% fetal bovine serum (Microbiological Assoc.), penicillin-streptomycin 1% v/v, and glutamine 2% v/v. In some experiments, cycloheximide 25 μg/ml was incorporated into the medium. Replicate cultures of 2×10^5 cells in 0·2 ml medium were established in 96-well flat bottom tissue culture plates (Linbro, Flow Laboratories, Hamden, CT, USA). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. After 7–8 days, cultures were centrifuged and supernatants were removed and stored at −20°C until analysed for total IgM, total IgG and IgM RF secretion.

Assay for supernatant IgM and IgG. Supernatant IgM and IgG was determined by an enzyme-linked immunosorbent assay (ELISA). To wells of a 96 well polystyrene microtiter plate designed for ELISA (Costar, Cambridge, MA, USA) were added 0·1 ml of a 10 μg/ml solution of affinity purified goat anti human IgG or anti IgM (Tago, Burlingame, CA, USA) in pH 9·6 carbonate buffer. After incubation overnight at 4°C, the wells were drained and 'blocked' with 0·1 ml of 1% bovine serum albumin/phosphate buffered saline (BSA/PBS) solution for 1 h. Next, dilutions of (a) a purified IgG or IgM standard (Tago, Burlingame, CA, USA), (b) neat, 1:10, and 1:100 dilutions of unknown supernatants; or (c) medium alone were added to replicate wells. All dilutions were made in complete culture medium. After 3 h at room temperature, wells were drained and washed four times with 0·05% Tween/PBS. This was followed by the addition of 0·1 ml peroxidase conjugated goat anti-human Ig (Sigma Chemical Co., St Louis, MO, USA) diluted 1:1000 and further incubation overnight at 4°C. Following three washes the next day 0·4% *o*-phenylenediamine and 0·012% H_2O_2 were added and the plates incubated in the dark for 10 min. Colour development was read at 450 nm on a multichannel spectrophotrometer (Titertek). A standard curve was constructed by plotting log optical density (o.b.) *v* log concentration of input IgG or IgM. A linear regression line was developed and values for unknowns determined from the standard curve with a computer program.

Assay for IgM-rheumatoid factor (IgM-RF). Levels of IgM-RF in culture supernatants were

quantitated by a sensitive ELISA. Duplicate wells of polystyrene test cup trays (SPIA, R & D Lab Products, Newtown Square, PA, USA) were coated with 10 μg/ml Fc fragment of human IgG (Cappel Cochranville, PA, USA) in pH 9·4 carbonate buffer. Duplicate control wells received carbonate buffer alone. After incubation for 18–72 h at 4°C, wells were coated with 1% BSA (Sigma Chemical Co., St Louis, MO, USA), in PBS. After 3–6 h at room temperature, monoclonal IgM-RF (kindly provided by Dr R. Heimer, Jefferson Medical College, Philadelphia) diluted to 100, 50, 25, 10, 5, 2·5, and 1·0 ng/ml in complete medium was added to duplicate Fc coated and uncoated wells. Similarly, PBMC culture supernatants were added in parallel to duplicate Fc (IgG) coated and uncoated wells. After 18 h at room temperature, wells were incubated with 250 ng/ml of affinity purified goat antibody to human IgM (Kirkegaard & Perry Labs, Gaithersberg, MD, USA) in BSA/PBS for 2 h at room temperature. Wells were then incubated for 2 h at room temperature with 250 ng/ml of biotinylated affinity purified rabbit antibody to goat-IgG (Vector Labs, Burlingame, CA, USA) in PBS-tween. Avidin biotinylated horseradish peroxidase complex (Vector Labs, Burlingame, CA, USA) was diluted 1:80 in 0·05% Tween/PBS and added to all wells for a 1 h room temperature incubation. This was followed by the additon of enzyme substrate indicator, consisting of 0·01% hydrogen peroxide with *o*-phenylenediamine, to each well for 1 h at room temperature. Well contents were then mixed with 1·5 ml of deionized H_2O and transferred via the flow-through passages of the wells to test tubes containing 0·05 ml of 20 M sulphuric acid. Tubes were vortexed and colour development was read at 490 nm on a spectrophotometer. All reagents were added in 0·1 ml volumes. Excess reagents were removed between all steps by washing with 0·05% Tween/PBS as previously described (Park, 1981).

The absorbance for each dilution of monoclonal RF was corrected for nonspecific binding by subtraction of the absorbance in corresponding uncoated wells. A standard curve was constructed by plotting log O.D. *v* log concentration of input monoclonal RF. Absorbance from wells containing unknowns were similarly corrected and the concentration of RF determined from the standard curve. Values of RF were linear over a range of 2·5–100 ng/ml.

RESULTS

Quantitation of IgM and IgG in supernatants of PG stimulated cultures

In preliminary experiments we established that peak IgM and IgG responses were observed in cultures stimulated with 50–200 μg of PG. At optimal stimulatory concentrations for a given individual's cells, a wide range of secreted IgM and IgG responses was observed for PG (Fig. 1). For comparison, the distribution of PWM responses is also shown. The responses of each individual's cells are expressed as delta IgM and delta IgG, i.e. they have been corrected for amounts of Ig secreted in corresponding unstimulated cultures. The geometric means of IgM and IgG in such unstimulated cultures were 57 ng/ml and 302 ng/ml, respectively. The amounts of IgM elicited by PG and PWM were similar ($P > 0{\cdot}05$, Wilcoxin signed rank test) whereas PG elicited less IgG secretion than did PWM ($P < 0{\cdot}05$, Wilcoxin signed rank test).

Induction of rheumatoid factor by PG

Next we sought to determine if PG induced IgM rheumatoid factor production by PBMC from healthy donors. In these experiments, cells were cultured alone or in the presence of PG, PWM or additional culture medium. In no case was IgM-RF produced (>2·5 ng/ml) in unstimulated cultures (data not shown). By contrast PG induced IgM-RF production in 9/33 experiments with a mean response ± s.e.m. of 6·5 ± 1·5 ng/ml (Fig. 2). This frequency of response was lower than that observed for PWM (22/33, $P < 0{\cdot}05$, chi square). On the other hand, the magnitude of response was similar to that observed in PWM stimulated cultures (11 ± 4·5 ng/ml, mean ± s.e.m.; $P > 0{\cdot}05$, student's *t*-test). PG induced IgMRF production reflected active protein secretion, since it was abrogated when cells were cultured in the presence of cycloheximide (Fig. 3). No correlation was observed between the magnitude of IgM-RF produced in response to PG and the total IgM response induced by PG ($r = 0{\cdot}03$, linear regression analysis).

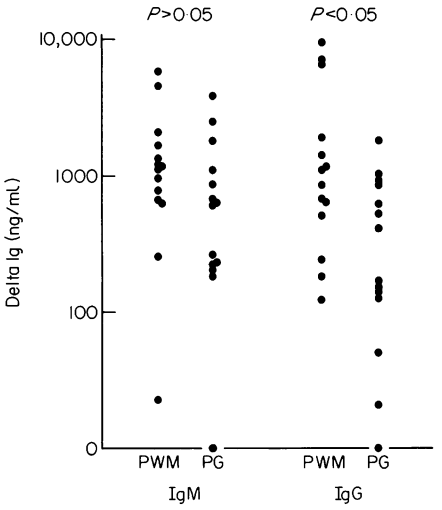

Fig. 1. *In vitro* total IgM and IgG production by lymphocytes of healthy volunteers. PBMC were incubated with varying concentrations of PWM and PG for 7–8 days and culture supernatants were assayed for total IgM and total IgG. Data are expressed as delta IgM and delta IgG responses where delta = Ig in supernatants of stimulated cultures minus Ig in supernatants of corresponding unstimulated cultures. Data points shown reflect responses at optimally stimulating doses of mitogen.

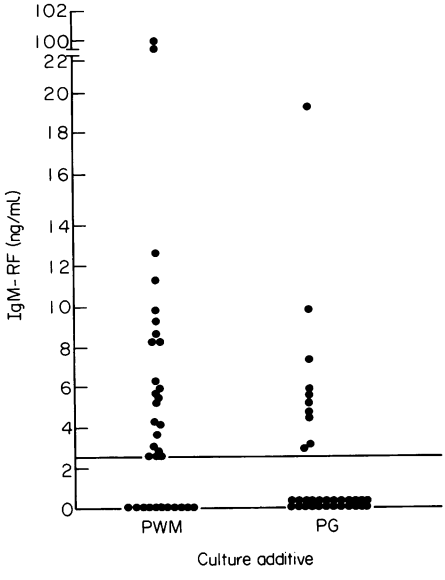

Fig. 2. *In vitro* IgM rheumatoid factor (IgM-RF) production by lymphocytes of healthy volunteers. PBMC were incubated with varying concentrations of PWM and PG for 7–8 days and culture supernatants were assayed for IgM-RF. Data reflect results of optimally stimulating doses of mitogen.

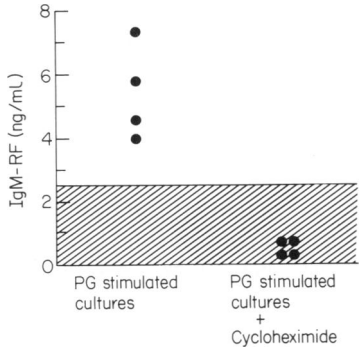

Fig. 3. *In vitro* IgM rheumatoid factor (IgM-RF) production by lymphocytes of healthy volunteers: Effect of cycloheximide. PBMC from four donors were incubated for 7–8 days with PG in the presence and absence of cycloheximide 25 μg/ml and culture supernatants were assayed for IgM-RF.

DISCUSSION

These studies extend our previously reported findings on the polyclonal B cell activating properties of PG. We had reported that PG was a relatively T cell independent polyclonal B cell activator, albeit a less potent one than PWM (Levinson *et al.*, 1983). However, quantitation of PG induced polyclonal B cell activation in the earlier studies relied solely on enumeration of total immunoglobulin secreting cells in stimulated cultures, irrespective of the isotype specificity of the immunoglobulins produced. In the current studies, it is apparent that PG induces both total IgM and IgG production in levels that are consistent with polyclonal B cell activation. The PG-induced IgM response is similar in magnitude to the IgM response elicited by PWM. By contrast, PG appears to be a less potent stimulant of IgG production.

The present experiments are highlighted by the finding that PG induces IgM-RF by cells of some healthy donors. Values determined are not merely indicative of nonspecific binding of the polyclonal IgM secreted. IgM-RF determinations were routinely corrected for nonspecific binding to uncoated wells. Furthermore, addition of as much as 200 μg of an IgM preparation, which lacked antiglobulin activity, to Fc (IgG) coated wells was not associated with detectable binding in the ELISA (Levinson, unpublished observations). This amount of IgM greatly exceeds the amounts of IgM secreted in our cultures. Since wells were coated with Fc fragments of IgG rather than the intact IgG molecule, only classical RF, i.e. Fc (IgG) reactive molecules, were detected. RF production required protein synthesis since it was abrogated when cells were cultured in the presence of cycloheximide.

Not all donors' PBMC produced RF in response to PG and the frequency of response was less than that observed in PWM stimulated cultures. In this regard, our finding that 70% of PWM stimulated cultures produced RF is somewhat higher than previously reported estimates (Koopman & Schrohenloher, 1980). This may reflect greater sensitivity of our double antibody ELISA which can detect RF responses as low as 2·5 ng/ml. The failure of several of our subjects cells to produce RF in response to PG could reflect an absence from the peripheral blood of clones committed to this autoantibody specificity. However, the results of the PWM stimulated cultures clearly indicated that a large number of subjects whose cells did not produce RF when stimulated with PG had B cells in their PBMC capable of differentiating into RF secreting cells. This observation suggests that PG and PWM may activate distinct subsets of RF secreting cells and that the PG reactive subset was missing from the peripheral blood of PG nonresponders. That two different polyclonal B cell activators can stimulate distinct subsets of RF secreting cell precursors is indicated by the studies of Pasquali *et al.* (1981) and Fong, Vaughan and Carson (1983). They showed that PWM and Epstein-Barr virus appear to stimulate distinct subsets of B cells to secrete RF.

Alternatively, the inability of normal PBMC to produce RF in response to PG could also reflect

potent downregulating mechanisms. Although PG is a relatively T cell independent (with regard to help) polyclonal B cell activator, PG may induce prominent suppressor T cell activity (Pardo *et al.*, 1984). Such suppressor T cell activity could be the dominant regulatory force in nonresponder cultures.

We have assumed that PG-induced IgM-RF production is caused by the polyclonal B cell activating properties of this agent. This property has likewise been assumed to account for the production of anti-DNA and anti-erythrocyte antibodies by PG-stimulated mouse splenocytes (Dziarski, 1984a). However, production of IgMRF in PG-stimulated cultures did not merely reflect the magnitude of the overall PG-induced polyclonal IgM response. RF responders could not be distinguished from nonresponders on the basis of the total IgM response (data not shown) and there was no correlation observed between total IgM and IgM-RF responses in the responding group of subjects. These observations suggest that other possibilities be considered as mechanisms by which PG induces RF production. In this regard, Johnson *et al.* (1985) have preliminarily reported that immunization of mice with affinity purified polyclonal RF of human origin elicits the production of anti-PG antibodies. One interpretation of these provocative data is that RF carries the internal image of a PG epitope and thus could function as an autoanti-idiotype against anti-PG antibodies. These results suggest a more specific link between PG and RF than previously entertained.

The data reported herein add to a growing list of PG's functional properties. They provide a basis for considering if this ubiquitous bacterial cell wall component contributes to the *in vivo* production of RF and perhaps other autoantibodies in man.

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REFERENCES

CROMARTIE, W.J., CRADDOCK, J.G., SCHWAB, J.H., ANDERLE, S.K. & YANG, C.H. (1977) Arthritis in rats after systemic injection of streptococcal cells or cell walls. *J. exp. Med.* **146,** 1585.

DZIARSKI, R. (1980) Polyclonal activation of immunoglobulin secretion in B lymphocytes induced by staphylococcal peptidoglycan. *J. Immunol.* **125,** 2478.

DZIARSKI, R. (1984) Opposing effects of Xid and nu mutations on proliferative and polyclonal antibody and autoantibody responses to peptidoglycan, LPS, Protein A and PWM. *Immunology*, **53,** 563.

DZIARSKI, R. (1984a) Anti-immunoglobulin autoantibodies are not preferentially induced in polyclonal activation of human and mouse lymphocytes, and more anti-DNA and anti-erythrocyte autoantibodies are induced in polyclonal activation of mouse than human lymphocytes. *J. Immunol.* **133,** 2537.

DZIARSKI, R. & DZIARSKI, A. (1979) Mitogenic activity of staphylococcus peptidoglycan. *Infect. Immun.* **23,** 706.

DZIARSKI, R., DZIARSKI A. & LEVINSON, A.I. (1980) Mitogenic responsiveness of mouse, rat and human lymphocytes to *Staphylococcas aureus* cell wall, teichoic acid, and peptidoglycan. *Int. Arch. Allergy Appl. Immunol.* **63,** 383.

DZIARSKI, R. & KWARECKI, K. (1976) Biological effects of staphylococcal cell wall and its component in mice. *Zentrahl. Bakteriol. Parastienkd. Infektionsk. Hyg. Abl.* **1 (Suppl. 5),** 393.

FONG, S., VAUGHAN, J.H. & CARSON, D.A. (1983) Two different rheumatoid factor-producing cell populations distinguished by the mouse erythrocyte receptor and responsiveness to polyclonal B cell activators. *J. Immunol.* **130,** 162.

HANG, L.M. (1983) Induction of murine autoimmune disease by chronic polyclonal B cell activation. *J. exp. Med.* **157,** 874.

IZUI, S., EISENBERG, A. & DIXON, F. (1979) IgM rheumatoid factors in mice injected with bacterial lipopolysaccharides. *J. Immunol.* **122,** 2096.

JOHNSON, P.M., PHUA, K. & EVANS, H.B. (1985) An idiotypic interaction between rheumatoid factor and anti peptidoglycan antibodies? *Arthritis Rheum.* **28 (Suppl.),** S33.

KOOPMAN, W.J. & SCHROHENLOHER, R.E. (1980) *In vitro* synthesis of IgM rheumatoid factor by lymphocytes from healthy adults. *J. Immunol.* **125,** 934.

LEVINSON, A.I., DZIARSKI, A., ZWEIMAN, B. & DZIARSKI, R. (1983) Staphylococcal peptidoglycan, T cell dependent mitogen and relatively T cell independent polyclonal B cell activator. *Infect. Immun.* **39,** 290.

PARDO, I., CARAFA, C., DZIARSKI, R. & LEVINSON, A.I. (1984) Analysis of *in vitro* polyclonal B cell differentiation responses to bacterial peptidoglycan and pokeweed mitogen in rheumatoid arthritis. *Clin. exp. Immunol.* **56,** 253.

PARDO, I. & LEVINSON, A.I. (1983) Circulating immunoglobulin secreting cells in rheumatoid arthritis. *Clin. Immunol. Immunopathol.* **29,** 29.

PARK, H. (1981) A simple washing technique for solid-phase radioimmunoassay and enzyme-linked immunosorbent assays. *J. clin. Pathol.* **34,** 1309.

PASQUALI, J.L., FONG, S., TSOUKAS, C.D., SLOVIN, S.F., VAUGHAN, J.H. & CARSON, D.A. (1981) Different populations of rheumatoid factor idiotypes induced by two polyclonal B cell activators, pokeweed mitogen and Epstein-Barr virus. *Clin. Immunol. Immunopathol.* **21,** 184.

PRIMI, D., HAMMERSTROM, L., SMITH, C.I.E. & MOLLER, G. (1977) Characterization of self reactive B cells by polyclonal activators. *J. exp. Med.* **145,** 21.

SCHLEIFER, K.M. & KANDLER, D. (1972) Peptidoglycan types of the bacterial cell walls and their toxonomic implications. *Bacteriol. Rev.* **36,** 407.

SLAUGHTER, L., CARSON, D.A., JENSEN, F.C., HOLBROOK, T.L. & VAUGHAN, J.H. (1978) *J. exp. Med.* **148,** 1429.