INFECTION AND IMMUNITY, Jan. 1984, p. 139–142
0019-9567/84/010139-04$02.00/0

Vol. 43, No. 1

Detection of Soluble Peptidoglycan in Urine After Penicillin Administration

HYDOW PARK,[1] ALLEN R. ZEIGER,[2]* AND H. RALPH SCHUMACHER[3]

Department of Biochemistry, Thomas Jefferson University, Philadelphia, Pennsylvania 19107,[2] and Rheumatology-Immunology Center, Veterans Administration Medical Center,[1] and Department of Medicine, University of Pennsylvania Medical School,[3] Philadelphia, Pennsylvania 19104

Received 1 July 1983/Accepted 27 September 1983

A unique enzyme-linked immunosorbent assay was developed to detect soluble peptidoglycans in biological fluids. It makes use of the similar affinities of vancomycin and purified rabbit antibodies to peptidoglycan precursor sequences found in soluble peptidoglycans. This assay has been used to detect as little as 500 pg of soluble peptidoglycan per ml of serum and 5 pg/ml of urine. Studies of normal individuals and *Staphylococcus aureus*-infected patients revealed only a few sera with detectable levels of soluble peptidoglycans. Studies of normal volunteers who were given a single oral dose of 250 mg of penicillin VK showed that about half had detectable levels of soluble peptidoglycans in their urine up to 6 h after ingestion. This suggests that soluble peptidoglycans can be released by indigenous bacteria in detectable amounts. In one volunteer, a detectable level of soluble peptidolgycan in the urine at 6 h decreased to an undetectable level at 12 h. Such an ephemeral appearance of soluble peptidoglycan in the urine could account for the small number of human sera that had detectable levels of soluble peptidoglycan.

Cell wall-derived peptidoglycans (PG) are known to have a variety of biological properties, including the ability to activate the complement system and to stimulate macrophages and B lymphocytes (4, 13). In addition, PG are under consideration as possible factors in the pathogenesis of some diseases of unknown causes, such as the systemic rheumatic diseases (1). Recently, soluble forms of peptidoglycans (SPG) have been described which are secreted in vitro by several species of gram-positive bacteria in response to penicillin treatment but not to vancomycin treatment (7, 9, 11, 14, 18). Preliminary studies indicate that SPG from *Staphylococcus aureus* are murine B-lymphocyte activators (U. M. Babu and A. R. Zeiger, submitted for publication).

Examination of the peptide portion of SPG indicated that the untranspeptidated C-terminal sequence, Lys-D-Ala-D-Ala, was present (9, 18). Antibodies that are predominantly directed to this sequence have been found in a variety of normal animal populations, including humans (5, 6, 17). Recently, we reported that elevated antibody levels against the D-Ala-D-Ala sequences were correlatable with *S. aureus*-caused endocarditis among patients treated with penicillin-like (β-lactam) antibiotics but not among those treated with vancomycin (17). The antibody specificity and the time course of the rise in antibody titers suggested that SPG was the responsible immunogen.

The development of a sensitive, reliable, and specific method to detect SPG in biological fluids is essential for further in vivo studies. In this paper, we describe a novel enzyme-linked immunosorbent assay (ELISA) for SPG which makes use of the specificities of both vancomycin (2, 10) and rabbit antibodies against a synthetic peptide immunogen (15, 16) for binding D-Ala-D-Ala sequences.

MATERIALS AND METHODS

Reagents. Vancomycin was purchased from U.S. Biochemical Corp., Cleveland, Ohio. Biotinylated goat anti-rabbit immunoglobulin G (IgG), avidin DH, and biotinylated horseradish peroxidase H were purchased from Vector Laboratories, Inc., Burlington, Calif. The avidin-biotin-horseradish peroxidase complex (ABC) was freshly prepared according to the manufacturer's instructions by adding 50 μl of avidin DH and 50 μl of biotinylated horseradish peroxidase H to 8.0 ml of phosphate-buffered saline (PBS) (pH 7.4) containing 0.5% Tween 20 (Sigma Chemical Co., St. Louis, Mo.) and 1% bovine serum albumin (Sigma). *O*-Phenylenediamine was obtained from Eastman Chemical Co., Rochester, N.Y.

SPG. SPG was obtained from a clinical strain of *S. aureus* which was incubated for 1 h at 37°C in a minimal cell wall growth medium containing PG precursors and penicillin G (50 μg/ml) as described previously (18). The SPG preparation had a high molecular weight (eluted at V_0 of Sephadex G-100) and contained PG precursor peptide (bound by vancomycin-Sepharose 4B gel [2]). The concentration of SPG was determined by amino acid hydrolysis (6 N HCl, 110°C, 16 h). Calculation of the SPG concentration was based on the assumption that all of the muramic acid residues in the glycan were linked to peptide (3).

Rabbit anti-SPG antibodies. The rabbit anti-SPG antibodies were prepared against a synthetic immunogen containing PG precursor pentapeptide (Ala-γ-D-Glu-Lys-D-Ala-D-Ala) multiply linked to a random polypeptide carrier (16). The antibodies were affinity purified by chromatography on a PG precursor pentapeptide-Sepharose 4B gel (15). The concentration of protein was estimated spectrophotometrically using an $\varepsilon_{280}^{1\%}$ of 14 (8).

Tripeptide antibody inhibitor. The tripeptide α-t-butoxycarbonyllysyl-D-alanyl-D-alanine (Boc-Lys-D-Ala-D-Ala) was synthesized stepwise by classical chemical coupling procedures as described previously (16).

ELISA for SPG. A 96-well polyvinyl microtiter plate (Dynatech Laboratories, Alexandria, Va.) was used as the solid-phase carrier. The volume of test materials or reagents added to each well was 0.1 ml. Each washing step consisted of four to five washings with PBS (pH 7.4) containing 0.05% Tween 20. Enzyme substrate solutions were freshly prepared by mixing 0.1 ml of 5% H_2O_2 and 0.5 ml of *o*-phenylenediamine (10 mg/ml in methanol) in 50 ml of 0.1 M

* Corresponding author.

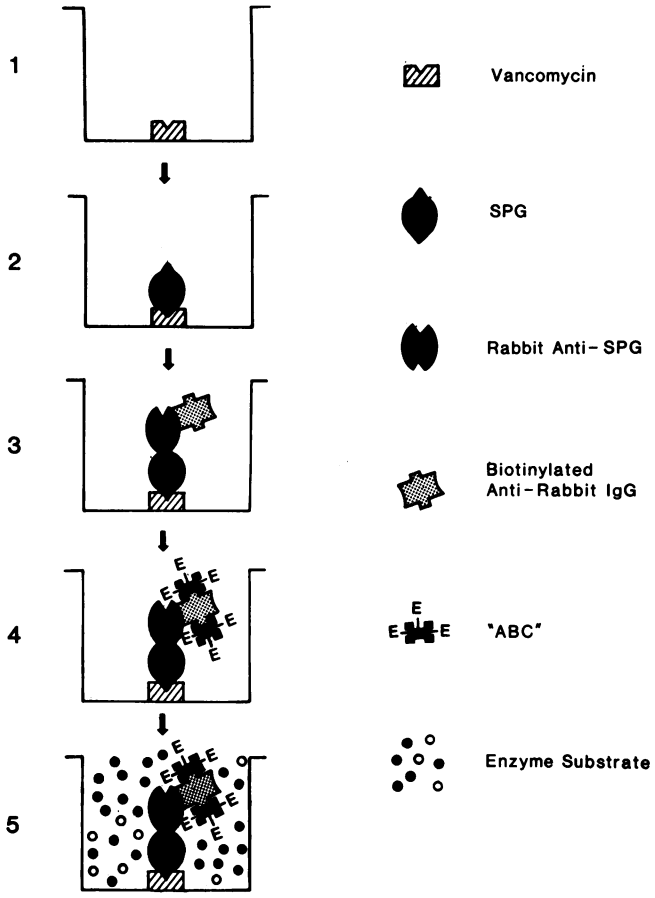

FIG. 1. Sequential steps in the ELISA to detect SPG.

sodium phosphate buffer (pH 8.0). All other reagents were diluted in PBS-Tween-bovine serum albumin just before the assay. The sequential steps of the ELISA are shown in Fig. 1. The polyvinyl wells were incubated with vancomycin (100 μg/ml) in carbonate buffer (1.59 g of Na_2CO_2, 2.93 g of $NaHCO_3$, 0.2 g of NaN_3 in 1 liter of water [pH 9.6]) for at least 18 h at 4°C before use. After washing, each of the samples and standard SPG solutions was placed into four wells, two test wells and two control wells. The plate was incubated in a moist chamber at room temperature for 18 h. After another washing, the wells received reagent mixture containing affinity-purified rabbit anti-SPG (5 μg/ml) and biotinylated anti-rabbit IgG (1:660 dilution), either with the tripeptide inhibitor Boc-Lys-D-Ala-D-Ala (5 μg/ml) for the control wells or without the tripeptide for the test wells. This tripeptide has been shown to be capable of inhibiting antibodies with SPG specificity (16, 17). The plate was incubated at 37°C for 2 h. After washing, the ABC complex solution was added to each well. After 1 h of incubation at room temperature, the plate was washed again, and the substrate solution was added. The plate was incubated at room temperature for 1 h in the dark, and the enzyme reaction was stopped by addition of one drop of concentrated H_2SO_4 to each well. The entire contents of the wells were transferred to test tubes containing 1.5 ml of deionized water. After vortexing, absorbances were read at 490 nm in a Beckman model 34 spectrophotometer. The mean absorbance values of test and control wells were compared for interpretation.

TABLE 1. Detectability of SPG in PBS-Tween buffer

Concn of SPG (pg/ml)	Absorbance at 490 nm[a]	
	Test	Control
5,000	0.248	0.021
500	0.145	0.013
50	0.026	0.011
5	0.012	0.011
0	0.011	0.014

[a] Average of two determinations.

Ratios of test to control values greater than 2 were considered positive.

Experiments on the detectability of SPG added to PBS-Tween and serum. Serial 10-fold dilutions (5 to 5,000 pg/ml) of SPG were made in PBS-Tween and a normal human serum containing no detectable antibodies to peptidoglycan. Initial experiments showed a 100-fold difference in detectability of SPG between the serum samples and PBS-Tween solutions (data not shown), suggesting the presence of an interfering substance(s) in serum. The following method was devised to remove the interfering substance(s) from serum. One volume of serum was mixed with nine volumes of 5% trichloroacetic acid by vortexing. The mixture was incubated at room temperature for 20 min and then centrifuged at 1,000 × *g* for 10 min. The supernatant fluid was dialyzed against PBS (pH 7.4) overnight at 4°C. Serial dilutions of SPG in the serum were treated as described above and then assayed for SPG.

Detection of SPG in human sera. Eighty sera from 30 patients with bacterial endocarditis caused by *S. aureus*, 52 sera from patients with various bacterial infections, and 24 sera from healthy blood donors were assayed for SPG by our ELISA after trichloroacetic acid precipitation. Sera had been collected over several years and stored at −20°C. No urine specimens were available from these patients.

Experiments on urinary excretion of SPG after penicillin ingestion in healthy volunteers. Urine specimens were collected at various times of the day from 13 healthy individuals (7 males and 6 females) before and 6 h after oral administration of penicillin VK (250 mg). One individual was given 250 mg of penicillin VK every 6 h for 2 days, and eight sequential posttreatment urine specimens were collected. Both pre- and posttreatment urine specimens were concentrated 20-fold and kept at −70°C. For the SPG assay, the concentrated urine specimens were thawed and mixed with the same volume of PBS-Tween.

RESULTS

Detectability of SPG added to PBS-Tween and serum. The results of the experiments are shown in Tables 1 and 2. The detection limit in PBS-Tween was 50 pg/ml, whereas the detection limit in serum was 500 pg/ml. The 10-fold differ-

TABLE 2. Detectability of SPG in human serum

Concn of SPG (pg/ml)	Absorbance at 490 nm[a]	
	Test	Control
50,000	0.263	0.030
5,000	0.113	0.019
500	0.032	0.015
50	0.019	0.015
0	0.016	0.015

[a] Average of two determinations.

TABLE 3. Study of SPG in urine samples before and after penicillin treatment

Subject sex and age (yr)	Absorbance at 490 nm[a]			
	Pretreatment		Postreatment	
	Test	Control	Test	Control
M 41	0.010	0.009	0.043[b]	0.011
F 36	0.014	0.013	0.167[b]	0.015
F 13	0.010	0.010	0.110[b]	0.012
M 40	0.009	0.010	0.013	0.012
F 37	0.010	0.007	0.157[b]	0.013
F 50	0.015	0.017	0.042[b]	0.011
M 36	0.012	0.012	0.009	0.011
M 27	0.010	0.013	0.012	0.016
F 47	0.011	0.014	0.233[b]	0.021
M 34	0.013	0.012	0.013	0.012
M 36	0.015	0.014	0.013	0.015
F 58	0.010	0.011	0.028[b]	0.010
M 26	0.007	0.009	0.008	0.009

[a] Average of two determinations.
[b] Considered positive.

ence was as expected from the ten-fold dilution used in the trichloroacetic acid precipitation.

Detection of SPG in patient sera. From the detection limits above, only 2 of 80 sera from patients with staphylococcal endocarditis and/or bacteremia were positive. The concentrations of the positive sera were estimated to be in the range of 1 ng/ml. The 52 sera from patients with various bacterial infections and 25 sera from healthy blood donors were all negative.

Detection of SPG in pre- and post-penicillin-treated urine samples. Six of six female posttreatment urine samples and one of seven male posttreatment samples were positive, whereas all pretreatment samples were negative for SPG (Table 3). Only the first urine sample from the individual given 250-mg doses of penicillin VK every 6 h for 2 days contained detectable SPG. Of the positive urine samples, three had SPG concentrations of greater than 500 pg/ml (or >50 pg/ml in the original specimens if one takes into account the 10-fold concentration of the specimens before the assay).

DISCUSSION

The ELISA technique has become a very popular method for measuring antigens and antibodies in biological fluids. Normally, the sandwich ELISA to detect antigen utilizes antibodies both for coating the wells (step 1 in Fig. 1) and reacting with the antigen (step 3 in Fig. 1). The ELISA described in this paper is unique in coating the wells with an antibiotic which has a specificity (10) toward the Lys-D-Ala-D-Ala sequence similar to that of the antibody (16). This feature makes it highly unlikely that any other peptide sequences would be detected in the ELISA. This appears to have been confirmed by the extremely effective inhibition by the tripeptide Boc-Lys-D-Ala-D-Ala.

The obvious source of the SPG found in the two patients with positive sera would be the bacteria from the site of infection. Yet the data with urine samples from normal volunteers suggest an additional or alternative source of SPG, gram-positive indigenous bacteria. If the SPG is from intestinal bacteria, it would appear that SPG is capable of crossing the mucosal barrier in detectable quantities. Among the intestinal bacteria, *Streptococcus faecium* (11) and *S. aureus* (18) have been shown capable of secreting SPG in vitro in response to β-lactam antibiotics. On the other hand, the apparent preponderance of SPG in urine from women may indicate that the major source of SPG is vaginal bacteria.

Our studies indicate that whatever the source of SPG, its appearance in biological fluids appears to be ephemeral. The observation that about half of the population can be induced by penicillin to secrete detectable levels of SPG in the urine coincides with observations that about half of the human population develops anti-SPG (6, 17). It may be significant that only about 30 to 50% of the population has *S. aureus* colonizing the large intestine (12). In view of the recent observation that SPG, like PG, is a murine B-cell activator (Babu and Zeiger, submitted), further studies on the physiology of SPG would seem to be in order.

ACKNOWLEDGMENTS

This work was supported by Public Health Service research grant AI 13525 from the National Institute of Allergy and Infectious Diseases (to A.R.Z.) and a grant from the Arthritis Foundation, Eastern Pennsylvania Chapter (to H.P.).

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