INFECTION AND IMMUNITY, Oct. 1984, p. 279–281
0019-9567/84/100279-03$02.00/0

Vol. 46, No. 1

Interaction of Anti-kojibiose Antibody with the Lipoteichoic Acids from *Streptococcus faecalis* and *Streptococcus faecium*

ROBERT E. KESSLER,[1]†* JODIE DUKE,[2] AND IRWIN J. GOLDSTEIN[2]

The Dental Research Institute, School of Dentistry, Departments of Microbiology and Immunology[1] and Biological Chemistry,[2] School of Medicine, University of Michigan, Ann Arbor, Michigan 48109-2007

Received 30 April 1984/Accepted 2 July 1984

Antisera prepared in rabbits by immunization with *p*-aminophenyl β-kojibioside conjugated to bovine serum albumin (antikojibiose sera), readily agglutinated whole cells of *Streptococcus faecalis* or *Streptococcus faecium*, and showed specific reactions with the lipoteichoic acids (LTAs) of these streptococci by passive hemagglutination, microscale enzyme-linked immunosorbent assay, and crossed immunoelectrophoresis. The interaction of the antikojibiose sera with the LTAs was inhibited best by kojibiose [α-D-glucopyranosyl-(1→2)-D-glucose], somewhat less by the dextran from which the kojibiose was prepared, and not measurably by maltose [α-D-glucopyranosyl-(1→4)-D-glucose]. The sera reacted only minimally in only the most sensitive assay (microscale enzyme-linked immunosorbent assay) with LTA from group A streptococci (this LTA contains a single kojibiosyl residue as part of the glycolipid moiety of the molecule and failed to react with the *Lactobacillus fermentum* LTA which is substituted with α-D-galactopyranosyl-(1→2)-D-glucosyl units.

Antisera raised in rabbits by immunization with *p*-aminophenyl β-kojibioside conjugated to bovine serum albumin (kojibiose-BSA) react with other substances bearing kojibiosyl residues such as a large number of dextrans containing α-(1→2) glucosidic bonds (J. L. Duke and I. J. Goldstein, Abstr. 172nd Annual Meeting Am. Chem. Soc. 1976, CARB 005). There is chemical evidence for the occurrence of kojibiose or kojitriose (23) or mixtures of both (6) on the lipoteichoic acids (LTAs) from *Streptococcus faecalis* and *Streptococcus faecium*. Structural studies of these LTAs indicate that the C-2 position of glycerol of the poly(glycerol phosphate) chains are substituted by these sugars (23). The degree of substitution varies from strain to strain and appears to be influenced by inhibition of protein synthesis (11, 13). As expected, these LTAs readily react with concanavalin A (16). The LTA from *S. faecium* NCIB 8191 cross-reacts with antipneumococcal type XII sera, and the reaction is inhibited by kojibiose (7). In this report, we show the specific interaction of antisera prepared against the kojibiose-BSA conjugate with the LTAs from *S. faecalis* and *S. faecium*.

The dextran from *Leuconostoc mesenteroides* NRRL B-1299-S, kojibiose, and antisera to kojibiose was prepared as previously described (3). The LTA from a group A streptococcus was available from a previous study (12). The LTA from *Lactobacillus fermentum* NCTC 6991 was the generous gift of A. Wicken. LTAs were prepared from *S. faecalis* JH2-2 (obtained from D. Clewell, The University of Michigan), *S. faecium* ATCC 9790 (obtained from G. Shockman, Temple University), and *S. faecium* NCIB 8191 (obtained from A. Wicken, University of New South Wales). *S. faecalis* JH2-2 was grown in Oxoid nutrient broth no. 2 as previously described (4). *S. faecium* ATCC 9790 and NCIB 8191 were grown in chemically defined media (18, 20). LTA was extracted with 45% phenol in water at 65°C (25). The aqueous phases were dialyzed, lyophilized, suspended in 0.2 M ammonium acetate, and chromatographed on AcA 22 (LKB Instruments, Inc., Rockville, Md.) (10). LTA-containing fractions, determined by monitoring at 206 nm and phosphorus content, were combined, dialyzed, and lyophilized. Phosphorus was estimated by the method of Lowry et al. (15). Glucose was determined by enzymatic assay (1) after hydrolysis with 2 N H_2SO_4 at 100°C for 2 h under N_2 in a sealed tube (22) and by phenol-sulfuric acid assay (2). The glucose-to-phosphorus ratios of the LTAs from *S. faecalis* JH2-2 and *S. faecium* ATCC 9790 and NCIB 8191 were 0.38:1, 1.04:1, and 2.47:1, respectively.

Whole cells of *S. faecium* ATCC 9790 and NCIB 8191 were agglutinated with a 1:10 dilution of antisera raised against the kojibiose-BSA conjugate. These strains did not agglutinate spontaneously or in the presence of preimmune sera. Crossed immunoelectrophoresis (performed essentially as described previously [16]) of LTA from *S. faecium* ATCC 9790 yielded a diffuse but distinguishable immunoprecipitate that could be shown to contain the LTA by the transposition of the immunoprecipitate formed in a main gel containing anti-poly(glycerol phosphate) (PGP) antibody into an intermediate gel containing the anti-kojibiose serum (data not shown). The anti-kojibiose sera also reacted with the LTA from *S. faecium* ATCC 9790 in microscale enzyme-linked immunosorbent assay (micro-ELISA) (Table 1). As expected, the titer was higher when an LTA with a greater degree of substitution was used to sensitize the microtiter plate wells (Table 2). The numbers in parentheses in Table 2 indicate the approximate percentage substitution of the PGP backbone, based on the glucose-to-phosphorus ratios of these LTAs and the assumption that all of the glucose was present as kojibiose side groups, or kojitriose on *S. faecium* NCIB 8191 LTA, except for two glucosyl residues in the glycolipid moiety. The antibody titer to *S. faecium* ATCC 9790 LTA is higher in this experiment than in the one shown in Table 1 due to longer development time and possibly a higher ambient temperature during plate development. There was little or no reaction of anti-kojibiose serum with the LTA from a group A streptococcus (Table 2). This result was expected since the group A streptococcal LTA contains a single kojibiosyl residue as part of the glycolipid moiety to which the PGP chain is linked (19). There was no reaction

* Corresponding author.
† Present address: Bristol-Meyers Co., Pharmaceutical Research and Development Division, Syracuse, NY 13221-4755.

TABLE 1. Micro-ELISA titers of antikojibiose sera against *S. faecium* LTA

Serum sample, rabbit no./bleed no.	Titer[a]
33/1	1,024
34/1	1,024
34/2	256
35/1	2,048
36/1	2,048
36/2	2,048

[a] Titer is the reciprocal highest two-fold serial dilution yielding an absorbance of 0.2 (2× background) at 10 min of incubation. Assay components, buffers, and procedures were basically as described by Voller et al. (21). Microtiter plates were sensitized with 5 μg of LTA per ml for 90 min at 37°C. Swine anti-rabbit immunoglobulin (Dako; Accurate Scientific, Waterbury, N.Y.) conjugated to alkaline phosphatase was used (1:5,000 dilution) as the second antibody. Plates were read after development with *p*-nitrophenylphosphate with a Titertek-Multiskan (Flow Laboratories, Inc.) at 405 nm.

with the LTA from *L. fermentum* which is substituted with α-D-galactopyranosyl-(1→2)-D-glucose (14). However, extensive reactivity of the group A streptococcal and lactobacillus LTAs was found with sera from certain rabbits (unpublished data). This appeared to be due to PGP-specific antibodies which presumably resulted from environmental sensitization (17) since preimmune sera from these animals also exhibited significant titers of antibody to PGP. Careful screening of preimmune sera of animals to be immunized and use of a diet free of teichoic acid and gram-positive bacteria (17) may be required for consistent production of sera free from PGP reactivity.

The reaction of anti-kojibiose sera with *S. faecium* ATCC 9790 LTA in the micro-ELISA was inhibited by both kojibiose and dextran NRRL B-1299-S (Fig. 1). The dextran was not as potent an inhibitor as the kojibiose. Although a high percentage of kojibiosyl residues are found within the dextran, the polymer contains other linkages as well (3). Comparison of inhibition with maltose (1-4 linkage) to kojibiose (1-2 linkage) was also done. There appeared to be little or no inhibition of serum sample 36/1 (rabbit number/bleed number) by maltose (Fig. 2B), but the results with serum sample 35/1 were not as cleanly interpretable (Fig. 2B). Maltose appeared to inhibit this serum sample 30 to 45% over a broad concentration range (0.03 to 300 nmol). Inhibition analysis in another system, passive hemagglutination, did not show significant inhibition with maltose (Table 3). Passive hemagglutination is a semiquantitative assay in which a single well or twofold dilution difference, as is the difference between titers of 128 and 256, is not significant. Thus, the only significant reduction in hemagglutination titers were at 0.15 and 0.44 μmol per ml of kojibiose, although a trend was apparent at lower concentrations of

TABLE 2. Comparison of reactivity of serum sample 35/1 with LTAs from different sources

LTA source	Carbohydrate substitution (%)[a]	Titers[b]
S. faecium NCIB 8191	Glcα-(1→2)Glc(α-1→2)Glc (60)	40,960
S. faecium ATCC 9790	Glcα-(1→2)Glc (50)	40,960
S. faecalis JH2-2	Glcα-(1→2)Glc (15)	10,240
Group A streptococcus	None	10
L. fermentum	Galα-(1→2)Glc	<10

[a] Glc, Glucose; Gal, galactose.

[b] ELISA titer is the reciprocal of the highest fourfold dilution giving an absorbance of 0.2 at 30 min of incubation. Initial dilution was 1:10.

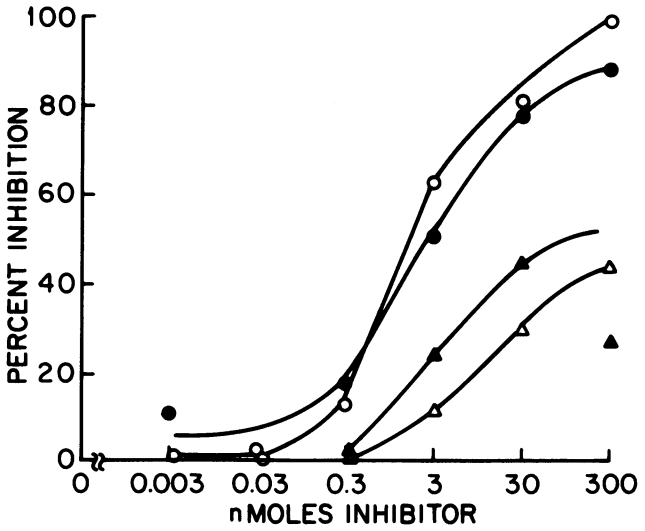

FIG. 1. Inhibition by kojibiose and dextran B-1299-S of the binding of antikojibiose serum samples 35/1 and 36/1 with the LTA from *S. faecium* ATCC 9790. Symbols: ● and ○, kojibiose additions; ▲ and △, dextran additions; closed symbols, serum sample 35/1; open symbols, serum sample 36/1.

kojibiose. The data from Table 3 and Fig. 2 were interpreted to indicate that maltose was at least 100- to 1,000-fold less potent an inhibitor than kojibiose.

The reaction of anti-kojibiose serum sample 36/1 with the *S. faecium* ATCC 9790 LTA in micro-ELISA was also inhibited by a lipopolysaccharide isolated from *Salmonella tel-aviv* and its derivative lipid-free polysaccharide. These polymers appear to carry kojibiose constituents, (Peter Z. Allen, personal communication). Approximately 50 and 30 μg/ml, respectively, were required for 50% inhibition under the same assay conditions used for the hapten inhibition studies.

Taken in sum, these results show the specific interaction of antikojibiose sera with the LTAs from *S. faecalis* and *S. faecium*. The specificity appears to be dependent upon both the α(1→2) linkage and the diglucosyl combination since maltose was not inhibitory and there was no reaction with a closely related LTA which bears galactosyl-glucose moieties

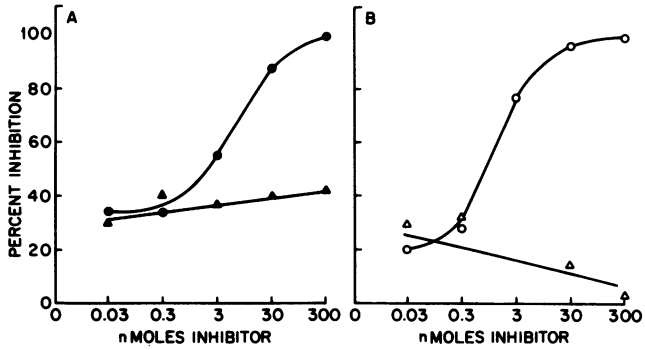

FIG. 2. Comparison of the ability of kojibiose and maltose to inhibit the binding of serum samples 35/1 and 36/1 to the LTA from strain ATCC 9790. Symbols: ● and ○, kojibiose additions; ▲ and △, maltose additions. (A) Serum sample 35/1. (B) Serum sample 36/1.

TABLE 3. Inhibition analysis with kojibiose and maltose of serum sample 36/1 agglutination of LTA sensitized erythrocytes[a]

Inhibitor	Concn (μmol/ml)	Titer
None		256
Kojibiose	0.004	128
	0.015	128
	0.044	128
	0.15	64
	0.44	32
Maltose	0.004	256
	0.015	256
	0.044	256
	0.15	256
	0.44	256
	1.46	128
	4.38	128

[a] Passive hemagglutination was carried out as described by Hewett et al. (8) with strain JH2-2 LTA.

with the same linkage. These results confirm the chemical evidence in support of the presence of kojibiose or kojitriose side chains or both on these LTAs (6, 23) and suggest that a specific reagent is now available for the distinction of LTAs from these organisms in natural samples, including those which might contain LTAs from other organisms as well. In addition, considering that the LTA of the group D streptococcus is the group antigen (5, 24), preparation of Lancefield group D sera for diagnostic purposes may be much simpler and more efficient by using a kojibiose-BSA conjugate, provided that the kojibiose moiety alone is the group immunodeterminant. This would presumably be done best via production of monoclonal antibody which appears to have been successful by using LTA bearing kojibiose (9). Lastly, the cross-reactivity of a group D streptococcal lipoteichoic acid and *S. tel-aviv* lipopolysaccharide by virtue of bearing a common immunodeterminant, kojibiose, must also be taken as an important caveat in the development and usage of immunodiagnostic reagents for direct detection of bacteria in clinical specimens. Reliance on a direct detection immunoassay alone, without confirmation by other means, could result in a choice of inappropriate therapy.

This work was supported by Public Health Service grants DE02731, DE05916, and GM29470 from the National Institutes of Health.

We thank D. Mangan and D. Lopatin for help in application of the micro-ELISA to our project, B. Thivierge for technical assistance, and P. Z. Allen for providing the lipopolysaccharide and lipid-free derivative polysaccharide from *Salmonella tel-aviv*.

LITERATURE CITED

1. **Dahlqvist, A.** 1961. Determination of maltase and isomaltase activities with a glucose oxidase reagent. Biochem. J. **80**:547–555.
2. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
3. **Duke, J., N. Little, and I. Goldstein.** 1973. Preparation of crystalline α-kojibiose octaacetate from dextran B-1299-S: its conversion into *p*-nitrophenyl and *p*-isothiocyanatophenyl-kojibioside. Carbohydr. Res. **27**:193–198.
4. **Dunny, G., B. Brown, and D. B. Clewell.** 1978. Induced cell aggregation and mating in *Streptococcus faecalis*: evidence for a bacterial sex pheromone. Proc. Natl. Acad. Sci. U.S.A. **75**:3479–3483.
5. **Elliott, S. D.** 1962. Teichoic acid and the group antigen of group D streptococci. Nature (London) **193**:1105–1106.
6. **Fischer, W., P. Rösel, and H. U. Koch.** 1981. Effect of alanine ester substitution and other structural features of lipoteichoic acids on their inhibitory activity against autolysins of *Staphylococcus aureus*. J. Bacteriol. **146**:467–475.
7. **Heidelberger, M., and J. Baddiley.** 1974. Cross-reactivity of the membrane teichoic acid of *Streptococcus faecalis* NCIB 8191 in antipneumococcal type XII and type XVI sera. Carbohydr. Res. **37**:5–7.
8. **Hewett, M. J., K. W. Knox, and A. J. Wicken.** 1970. Studies on the group F antigen of lactobacilli: detection of antibodies by haemagglutination. J. Gen. Micro. **60**:315–322.
9. **Jackson, D. E., W. Wong, M. T. Largen, and G. D. Shockman.** 1984. Monoclonal antibodies to immunodeterminants of lipoteichoic acids. Infect. Immun. **43**:800–803.
10. **Jacques, N. A., L. Hardy, K. W. Knox, and A. J. Wicken.** 1979. Effect of growth conditions on the formation of extracellular lipoteichoic acid by *Streptococcus mutans* BHT. Infect. Immun. **25**:75–84.
11. **Kessler, R. E., and B. H. Thivierge.** 1983. Effects of substitution on polyglycerol phosphate-specific antibody binding to lipoteichoic acids. Infect. Immun. **41**:549–555.
12. **Kessler, R. E., I. van de Rijn, and M. McCarty.** 1979. Characterization and localization of the enzymatic deacylation of lipoteichoic acid in group A streptococci. J. Exp. Med. **150**:1498–1509.
13. **Kessler, R. E., A. J. Wicken, and G. D. Shockman.** 1983. Increased carbohydrate substitution of lipoteichoic acid during inhibition of protein synthesis. J. Bacteriol. **155**:138–144.
14. **Knox, K. W., and A. J. Wicken.** 1973. Immunological properties of teichoic acids. Bacteriol. Rev. **37**:215–257.
15. **Lowry, O. H., N. R. Roberts, K. Y. Leiner, M. Wu, and A. L. Farr.** 1954. The quantitative histochemistry of brain. 1. Chemical methods. J. Biol. Chem. **207**:1–17.
16. **Owen, P., J. D. Oppenheim, M. S. Nachbar, and R. E. Kessler.** 1977. The use of lectins in the quantitation and analysis of macromolecules by affinoelectrophoresis. Anal. Biochem. **80**:446–457.
17. **Rozmiarek, H., R. W. Bolton, and F. W. Chorpenning.** 1977. Environmental origin of natural antibodies to teichoic acid. Infect. Immun. **16**:505–509.
18. **Shockman, G. D.** 1963. Amino acids, p. 567–673. *In* F. Kavanagh (ed.), Analytical microbiology. Academic Press, Inc., New York.
19. **Slabyj, B. M., and C. Panos.** 1973. Teichoic acid of a stabilized L-form of *Streptococcus pyogenes*. J. Bacteriol. **114**:934–942.
20. **Terleckyj, B., N. P. Willett, and G. D. Shockman.** 1975. Growth of several cariogenic strains of oral streptococci in a chemically defined medium. Infect. Immun. **11**:649–655.
21. **Voller, A., D. Bidwell, and A. Bartlett.** 1976. Microplate enzyme immunoassays for the immunodiagnosis of virus infections, p. 506–512. *In* N. E. Rose and H. Friedman (ed.), Manual of clinical immunology. American Society for Microbiology, Washington, D.C.
22. **Wicken, A. J.** 1966. The glycerol teichoic acid from the cell wall of *Bacillus stearothermophilus* B65. Biochem. J. **99**:108–116.
23. **Wicken, A. J., and J. Baddiley.** 1963. Structure of intracellular teichoic acids from group D streptococci. Biochem. J. **87**:54–62.
24. **Wicken, A. J., S. D. Elliott, and J. Baddiley.** 1963. The identity of streptococcal group D antigen with teichoic acid. J. Gen. Microbiol. **31**:231–239.
25. **Wicken, A. J., J. W. Gibbens, and K. W. Knox.** 1973. Comparative studies on the isolation of membrane lipoteichoic acid from *Lactobacillus fermenti*. J. Bacteriol. **113**:365–372.