Detection of Immunoglobulin G Antibodies to Cholesterol in Antisera to Mycoplasmas

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Antisera were produced by inoculation of mycoplasma cells grown in PPLO broth supplemented with rabbit serum alone or rabbit serum plus Freund complete or incomplete adjuvant. By using an enzyme-linked immunosorbent assay, immunoglobulin G antibodies to cholesterol were detected in antisera to mycoplasmas (Mycoplasma salivarium ATCC 23064, M. orale ATCC 15539, M. buccae IID 802, M. faucau IID 996, and M. hominis IID 801) and rabbit serum.

The microorganisms included within the class Mollicutes are procaryotyes devoid of cell walls. Mycoplasma species, members of this class, require a source of cholesterol for growth, because they cannot synthesize cholesterol and it is an essential constituent of their cell membranes (8). Therefore, they are normally grown in medium supplemented with animal sera as a cholesterol source. They incorporate cholesterol without any modifications into their cell membranes (8). Medium components are easily adsorbed onto the surfaces of mycoplasma cells and cannot be removed easily by repeated washing (1, 9). Mycoplasma salivarium cells grown in medium supplemented with rabbit serum (RaSe) (10% (vol/vol)) contain rabbit immunoglobulin G (IgG) (approximately 4 to 10% [wt/wt] of total cell proteins), and IgM autoantibodies are produced when the cells are inoculated into rabbits (12).

Cholesterol had previously been considered to be nonimmunogenic, because it is widely distributed and plays important biological roles. However, it has recently been shown that cholesterol is an excellent immunogen (10). Therefore, we investigated whether mycoplasmas induce antibodies to cholesterol. In this report, we describe the detection of IgG antibodies to cholesterol in antisera to mycoplasmas.

Test sera included antisera to M. salivarium ATCC 23064 (MS, MS+FIA, and MS+FCA), M. orale ATCC 15539 (MO+FCA), M. buccae IID 802 (MB+FCA), M. faucau IID 996 (MF+FCA), M. hominis IID 801 (MH+FCA), and RaSe (RS, RS+FIA, and RS+FCA). Sera (PS) from rabbits inoculated with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 9.6 mM phosphate; pH 7.2) were also tested for comparative purposes. Preimmune serum was drawn from rabbits immediately before the first immunization. All test sera had been prepared previously and were stored at −100°C. Antisera had been produced as described previously elsewhere (12). MS, RS, and PS sera were from rabbits inoculated with 1 ml of M. salivarium cell suspension (1 mg of protein per ml), RaSe (diluted 1:200 in PBS), and PBS, respectively, intravenously for 5 consecutive days each week for 4 weeks. The other antisera were sera from rabbits immunized as follows. On day 0, rabbits were inoculated with 1:1 (vol/vol) mixtures (1 ml per rabbit) of immunogens plus Freund complete or incomplete adjuvant (FCA or FIA, respectively; Difco Laboratories, Detroit, Mich.) subcutaneously and intramuscularly; on days 4 and 8, they were inoculated with the mixtures (2 ml per rabbit); and on days 22, 26, and 30, they were inoculated intravenously with 1, 2, and 3 ml of the immunogens, respectively. The immunogens were mycoplasma cell suspensions (4 mg of protein per ml) or RaSe (diluted 1:20 in PBS; GIBCO Laboratories, Grand Island, N.Y.). Mycoplasma cell suspensions were prepared by harvesting mycoplasma cells grown in PPLO broth (90% [vol/vol]; Difco) supplemented with RaSe (10% [vol/vol]) by centrifugation at 15,000 × g for 15 min, three washes in PBS, and suspension in the same buffer.

IgG antibodies to cholesterol were measured by enzyme-linked immunosorbent assays (ELISA) performed by the method of Loizou et al. (6), which was modified, as described below, on the basis of the results obtained in preliminary experiments.

Cholesterol was coated onto the surfaces of 8 wells (each) in rows 2, 4, 6, 8, 10, and 12 of Linbro EIA microtitration plates (Flow Laboratories, Inc., McLean, Va.) by the addition of 30 μl (each) of an ethanol solution (45 μg/ml; Daiichi Chemical Co., Tokyo, Japan) to wells, which were then dried under vacuum for 30 min. The other wells were treated with 30 μl (each) of ethanol. To prevent nonspecific binding of immunoglobulins, the wells were treated with 110 μl of PBS supplemented with fetal bovine serum (10% [vol/vol]) (PBS-FBS) for 3 h at room temperature. PBS-FBS was discarded, and the wells were washed. Each test serum sample (100 μl) diluted 1:25 with PBS-FBS and PBS-FBS controls (100 μl) were added to four wells coated with cholesterol (C wells) and four wells treated with ethanol (E wells). The plates were allowed to stand at 4°C for 18 h. Serum and PBS-FBS were discarded, and the wells were washed. The wells were incubated at 37°C for 2 h with 100 μl of goat anti-rabbit IgG-alkaline phosphatase (AP) conjugate (lot 1401; Tago, Inc., Burlingame, Calif.) diluted 1:2,000 with PBS-FBS and then incubated for 1 h with 100 μl of AP substrate solution (1 mg of p-nitrophenyl phosphate [Sigma Chemical Co., St. Louis, Mo.] per ml of DEA buffer [pH 9.8], which consists of 45 ml of distilled water, 5 ml of ethanolamine [Kanto Chemical Co., Tokyo, Japan], and 100 μl of 0.25 mM MgCl2 · 6H2O). The plates were scanned and A405 values were read with a Microplate Reader (Bio-Rad Laboratories, Richmond, Calif.). Antibody titers were calculated as follows: ([mean A405 of 4 C wells incubated with test serum − mean A405 of 4 E wells incubated with test serum])
The method of Loizou et al. (6) is a double-antibody ELISA, while a single-antibody method was employed in this study. Therefore, to augment the sensitivity of the method, the duration of incubation with test sera and enzyme conjugate was increased and incubation with enzyme conjugate and substrate was carried out at 37°C, instead of at room temperature. The antigen concentration used for coating the wells was 45 μg/ml (Table 1). MS-FIA was diluted with PBS-FBS in doubling dilutions ranging from 1:25 to 1:400 and titers of anticholesterol antibodies were measured (Table 2). On the basis of the results obtained (Table 2), the 1:25 serum dilution was used for the assay. Antibody titers are expressed as A405 values after 1-h incubation with substrate.

Anticholesterol antibodies were detected in all test sera except for sera from rabbits inoculated with PBS (Table 3). However, antibody titers of immune sera were more than 8 times those of preimmune sera (Table 3). Therefore, it was concluded that inoculation with mycoplasma cells or RaSe caused production of anticholesterol antibodies. We had hypothesized that mycoplasma cells would induce anticholesterol antibodies, since the antibodies are induced by inoculating liposomes consisting of dimyristoyl phosphatidylcholine, cholesterol, and lipid A into mice (10). However, the finding that RaSe induced antibodies was unexpected. Incidentally, attempts were made to isolate mycoplasmas from RaSe by using liquid medium consisting of PPLO broth (70% [vol/vol]; Dißco) supplemented with horse serum (20% [vol/vol]; Gibco), yeast extract (10% [vol/vol]; Flow), penicillin G (1,000 U/ml) and thallium acetate (0.5% [wt/vol]) and by using solid medium prepared by the addition of Agar 1 (1% [wt/vol]; Oxoid Ltd., Basingstoke, Hampshire, England) to liquid medium, but mycoplasmas were not detected. Other mycoplasmas may also be considered to cause similar antibody responses, although only antisera to four Mycoplasma species were tested in the present study.

**M. buccale** and **M. fauca** are rare members of the normal flora of the human oropharynx, making up less than 2% of the total number of mycoplasmas recovered (3). However, **M. salivarium** and **M. orale** are very common inhabitants of the human oral cavity (11), and **M. hominis** is common in the lower urogenital tract (7). Antibodies to **M. salivarium** are detected in 60% of patients with oral infections and 40% of control subjects, and a fourfold or greater rise or fall in antibody titer is detected in paired serum samples from some patients (11). A greater percentage of antibody responses to **M. hominis** is detected in paired serum samples from patients with acute pelvic inflammatory disease than in a selected control group (2). Furthermore, antibody responses to **M. hominis** are detected in 50% of sera from women having febrile abortions but in only 14% of sera from women having afebrile abortions (5). The antibodies presumably include those directed to cholesterol.

It is suggested that anticholesterol antibodies participate in the pathogenesis of atherosclerosis through activation of the classical complement pathway (4). It may not be unreasonable to speculate that mycoplasmas play a role in the mechanisms involved in production of the antibodies, but this role has not been entirely elucidated yet.

**REFERENCES**


buccale nom. nov. and *Mycoplasma faucium* nom. nov. for *Mycoplasma orale* "types" 2 and 3, respectively. Int. J. Syst. Bacteriol. 24:252–255.


