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Production and Epitope Analysis of Monoclonal Antibodies against a *Rhizobium leguminosarum* Biovar Trifolii Strain

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Heat-treated cells of *Rhizobium leguminosarum* biovar trifolii strain 162X95 were used to produce monoclonal antibodies (MAbs). The fusion produced three cross-reactive MAbs and eight MAbs specific for the immunizing strain and a group of five other *R. trifolii* strains from the same geographic region where 162X95 was isolated (California). Seven MAbs were analyzed by competitive enzyme-linked immunosorbent assay to determine the number of different epitopes detectable on strain 162X95. The results indicated that six MAbs reacted with the same or overlapping epitopes, and the seventh MAb gave inconclusive results.

Monoclonal antibodies (MAbs) are used, or are projected for use, for identification of bacteria (4, 9, 11, 22). Ecological studies of bacteria, such as rhizobia, depend upon identification of strains which are exposed to harsh conditions in mixed cultures in soils and have undergone physiological changes in nodules. Discrete antigenic sites recognized by an antibody (13) might be altered under such conditions and render an MAb against a single determinant an ineffective probe. It is suggested, therefore, that several MAbs be mixed or used in sequence to produce a defined polyclonal antiserum for identification of a bacterial strain (4).

Antibodies against O antigen lipopolysaccharide (LPS) are generally the most strain- or serogroup-specific antibodies obtainable (3, 7, 15). Typical analysis of the reactive site (epitope) of an MAb is done by using Western immunoblot analysis (2) and by detection of competition between pairs of antibodies for an epitope (1, 5, 11, 18). Western immunoblot analysis determines the position of the antigen as a band on an electrophoretic profile and provides qualitative information for comparison of reactive sites of antibodies. Because LPS may elicit antibodies to different determinants, more specific analysis is required to determine whether MAbs react with the same epitope. The most commonly used procedures for epitope analysis are competitive enzyme-linked immunosorbent assay (ELISA) or competitive radioimmunoassay (17, 18), which indicate whether an unlabeled MAb inhibits binding of a labeled (detectable) MAb. The objective of this study was to determine whether a competitive ELISA could be used to characterize MAbs for use in a defined polyclonal antiserum.

Rhizobium trifolii 162X95 (Nitragin Co., Milwaukee, Wis.) was grown to the late log phase in defined mannitol broth (14), centrifuged at 25,000 × *g*, and washed three times in physiological saline. Cells were steamed for 30 min, centrifuged, and suspended in physiological saline. Two female BALB/c mice were injected with cell suspensions (0.5 ml intraperitoneally) on day 0 (optical density at 450 nm [OD_{450}], 0.283), day 12 (OD_{450}, 0.539), and day 37 (OD_{450}, 0.539). Spleen cells were harvested 3 days after the last injection and fused with myeloma cell line P3-NS1/1-Ag4-1 by the procedure of Oi and Herzenberg (16). At 10 to 15 days postfusion, supernatants were tested by ELISA for antibodies to whole cells (22). Cell lines that tested positive were screened for cross-reactivity to a strain of *R. trifolii* (WV22) isolated from a different geographic region and having a different electrophoretic profile (22). Cell lines that produced antibodies reactive only with the immunizing strain were cloned. Tissue culture supernatants from cloned cell lines were tested against a panel of antigens (22).

The fusion produced 150 hybridomas. Three cell lines produced MAbs which were cross-reactive with WV22, and eight MAbs were specific for the six strains of *R. trifolii* previously reported to have a common antigen: 162X95, 162X84, 162X68, 162X47, and 162X92 (22). Six cell lines (C5, C9, D7, E6, E8, and G9) survived all stages of cloning, freezing, and regrowth and were used along with one MAb (E10) from a previous fusion (22) for epitope analysis.

The first step in epitope analysis of the seven MAbs was Western immunoblot analysis as previously described (2, 22). A cell lysate of strain 162X95 was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10 to 20% T), and profiles were transferred to nitrocellulose membrane. An ELISA was performed to detect the antigen on the nitrocellulose. Profiles electrophoresed simultaneously with those used for Western blots were subjected to silver staining to reveal LPS bands (8).

Results of electrophoresis and Western immunoblot analysis are presented in Fig. 1. Antibody C5 reacted with the slower-migrating band (Fig. 1C), which was also stained by the LPS silver stain (Fig. 1B). All MAbs except the controls reacted with the slower-migrating band and were, therefore, presumed to react with the O antigen of LPS (data not shown).

The second stage of epitope analysis was by competition between unlabeled and biotinylated MAbs as described by Shively et al. (17). The seven MAbs were produced in tissue culture supernatant and were concentrated by ammonium sulfate precipitation (6). Concentrated antibodies were stored at −20°C in 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, pH 8.0. Biotinylation of MAbs was by the method of Updyke and Nicholson (20). Avidin-phosphatase (Sigma Chemical Co., St. Louis, Mo.) was used as the enzyme-linked probe, and the substrate was *para*-nitrophenylphosphate (Sigma) dissolved in diethanolamine buffer (19). The antigen was LPS obtained by phenol-water extraction (21). Briefly, 100 ml of a late-log-phase culture was centrifuged, suspended in 3.5 ml of water, and extracted with 3.5 ml of phenol at 65°C. The emulsion was centrifuged, and the water layer was removed. The residue was extracted again in the same manner. Water extracts

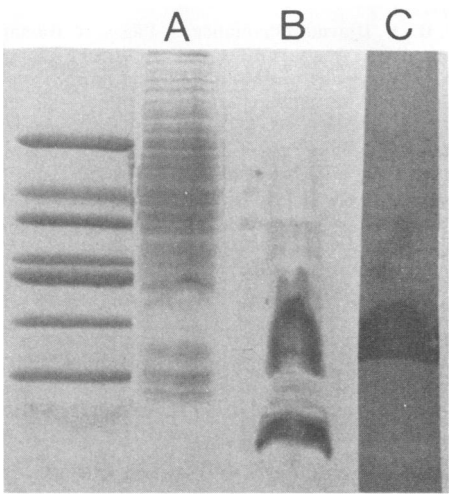

FIG. 1. Electrophoretic profiles and Western immunoblot of a cell extract of *R. trifolii* 162X95. Lanes: A, protein profile stained with Coomassie blue; B, profile A stained with silver stain for LPS; C, Western blot of profiles A reacted with MAb C5. Molecular mass indicators, from the top, are 66, 45, 36, 29, 24, 20, and 14.2 kilodaltons.

were combined and dialyzed for 3 to 4 days against distilled water. The LPS was diluted 1:125 in 0.02 M $MgCl_2$ (10) and attached to 96-well polyvinylchloride microtiter plates (50 μl per well) by incubation overnight at 37°C with the plates covered. Unlabeled MAbs, beginning with 50 μl of 1 mg/ml in HEPES (1.0 M, pH 8.0), were serially diluted to 1:512 in HEPES, and 50 μl of the biotinylated antibody (diluted in HEPES to give an ELISA OD_{405} reading of 0.7 to 1.5) was added. Twenty microliters of 0.1 M $MgCl_2$ was added to each well, and the plates were placed on a rotating shaker at 100 rpm for 2 h. The ELISA was performed as previously described (22), except that Tris-buffered saline (20 mM Tris, 250 mM NaCl, pH 7.4) in 0.02 M $MgCl_2$ was used to dilute all reagents and was used as the washing solution with the addition of 0.2 ml of Tween 20 per liter. Incubation with avidin-phosphatase and color development incubation were for 1 h each. Absorbance was read at 405 nm. Controls for the competition experiment were an MAb which was cross-reactive against many other rhizobia (2FE11) and an MAb which was not reactive against rhizobia (32DB10). The results shown are means of duplicate tests.

Competitive ELISA results are shown in Table 1. Absorbance readings for the lowest dilution of unlabeled antibody in competition with the biotinylated antibody are presented to illustrate these results. The dilution curves of these experiments were similar to those reported by Shively et al. (17) for competition studies using biotinylated MAbs. Lack of reciprocal inhibition of biotinylated MAbs by unlabeled MAbs is shown by comparing the absorbance readings of pairs of antibodies. For example, biotinylated E6 (B-E6) was not prevented from binding to the epitope by E10 (A_{405}, 1.07), but E6 inhibited binding of B-E10 (A_{405}, 0.01). Also, C9 only partially inhibited binding of B-E6 (A_{405}, 0.55), but E6 completely inhibited binding of B-C9 (A_{405}, 0.01). MAb E8 gave results which were difficult to interpret and is discussed below. The controls, 2FE11 and 32DB10, did not prevent the biotinylated MAbs from binding.

Nonreciprocal inhibition of paired MAbs shown in Table 1 may be due to steric hindrance, which interferes with the specificity of the competition test in the case of polymeric

TABLE 1. Competition ELISA with MAbs to the O antigen of *R. trifolii* 162X95

MAb 1	A_{405} with the following MAb 2 (biotinylated)[a]:						
	C5	C9	D7	E6	E8	E10	G9
C5	0.02	0.01	0.03	0.21	0.00	0.01	0.00
C9	0.15	0.06	0.42	0.55	0.00	0.04	0.00
D7	0.05	0.01	0.00	0.03	0.00	0.04	0.00
E6	0.04	0.01	0.00	0.03	0.00	0.01	0.00
E8	0.66	0.37	0.58	0.99	0.38	1.02	0.28
E10	0.29	0.32	0.72	1.07	0.11	0.11	0.09
G9	0.14	0.06	0.23	0.57	0.00	0.06	0.01
2FE11[b]	0.84	0.79	0.83	1.11	0.33	1.03	0.46
32DB10[b]	0.83	0.88	0.88	1.15	0.30	0.96	0.43

[a] The A_{405}s of the substrate reacted with avidin phosphatase are shown.
[b] Unlabeled antibody which was not reactive with the O antigen.

antigen formed of repeating epitopes (11). Steric hindrance is a factor (11, 12) in nonreciprocal or partial inhibition of binding between two MAbs which react with distinct polysaccharide epitopes as determined by different reactions with two strains of the target bacterium. For the seven MAbs to strain 162X95, there were no differential reactions with the panel of organisms tested. That is, all seven MAbs reacted with the same six *Rhizobium* isolates (22), providing no evidence that nonreciprocal binding was due to a factor other than overlap of epitopes.

Biotinylated E8 was prevented from binding by all MAbs except the controls and the homologous antibody. Lack of inhibition of the biotinylated antibody by its homologous antibody is not consistent with expected results from a competitive ELISA. Therefore, further investigation of the reactions of E8 was necessary. For the competitive ELISA, all MAbs were adjusted to a concentration of 1 mg/ml and serial dilutions were made. The concentration of immunoglobulin G (IgG) used in these experiments was based upon the total IgG present in the concentrate from culture supernatant which contained fetal bovine serum (i.e., bovine immunoglobulins) in addition to mouse IgG. Therefore, 1 mg of IgG of E8 per ml may have been weak in the MAb. However, increasing the concentration of unlabeled E8 to 2 or 3 mg/ml did not prevent binding of the biotinylated MAb (data not shown).

As an indication of the relative affinity of E8 for its antigen, ELISA readings were ranked by IgG subclass. The IgG subclass of MAbs was determined by the double-diffusion method on microslides (6). The relative affinities of MAbs were determined by the peroxidase ELISA reaction of an excess of antibody reacting with whole cells of strain 162X95 (OD_{450}, 0.010). Hybridomas were allowed to overgrow in growth medium, and the supernatant was reacted undiluted and at a 1:2 dilution in eight replicate ELISA wells. No different between absorbance readings (OD_{414}) of the ELISA for undiluted and diluted supernatant indicated that the antibody was in excess, and MAbs were ranked by mean absorbance readings in each subclass. Subclasses of MAbs were as follows: C5 and G9 were IgG1, and D7, E6, C9, E8, and E10 were IgG3. The relative affinity ranking of IgG1 antibodies was C5>G9 (OD_{414}s of 1.21 and 1.02, respectively), and that of IgG3 antibodies was D7>E6>B10>C9>E8 (OD_{414}s of 1.05 to 0.66). Affinities were compared within subclasses because of the possibility of differences in subclass reactions with the enzyme-conjugated anti-mouse IgG probe. Antibody E8 was the weakest one in subclass IgG3, and this is possibly why unlabeled E8 did not inhibit binding of the biotinylated antibodies and gave a low

absorbance reading of the biotinylated antibody, even in the presence of control MAbs (Table 1). These results are inconsistent with the placement of MAb E8 in the same group with the other six MAbs which apparently detect the same or overlapping epitopes or with consideration of MAb E8 as an antibody which recognizes a separate epitope.

These results indicated that there will be difficulty in establishing by standard procedures that MAbs to the O antigen of at least some strains of *R. trifolii* react with different epitopes. Carlin and Lindberg (3) used structurally well-defined LPS from different serotypes to select and characterize MAbs to the O antigens of *Shigella flexneri* and *S. sonnei*. They stated that selection and characterization of MAbs would have been next to impossible without access to the structurally defined LPS. While they were able to establish specificity for many of the clones, they were still unable to give an exact definition of an MAb in terms of its combining-site specificity.

Rabbit polyclonal antiserum has been recommended as a better probe than a single MAb for tracing bacteria in environmental samples because of diversity of epitope recognition. The inability to isolate individual antibodies from rabbit antiserum makes it impossible to determine whether there are differences in animal responses to LPS which allow rabbits to produce antibodies to distinctly different epitopes on LPS, and highly specific rabbit polyclonal antiserum may or may not recognize distinctly different epitopes. While the MAbs to the O antigen of *R. trifolii* 162X95 have been shown to be specific for six strains of *R. trifolii* from California, the results presented here also make it difficult to recommend a combination of MAbs to use in a defined polyclonal antiserum to detect this group of organisms.

The work presented here showed that MAbs to the O antigen of an *R. trifolii* strain were easily obtained by injecting BALB/c mice with heat-treated cells. However, the antibodies may react with the same or overlapping epitopes on the LPS. Further work using an organism mutated to express structurally different LPS and a series of MAbs which show differential reactions to these organisms may make epitope mapping of LPS possible. This will provide information for a practical approach to the use of MAbs against LPS for identification of environmental microorganisms.

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