Immunization for Generation of Hybridoma Antibodies Specifically Reacting with Plants Infected with a Mycoplasmalike Organism (MLO) and Their Use in Detection of MLO Antigens

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ABSTRACT

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Employing a procedure by using mice neonatally injected with nontarget antigens present in immunogen preparation before immunization, mouse hybridomas that secreted antibodies to a mycoplasmalike organism (MLO) isolated from tomato plants with symptoms of big bud disease were produced. Neonatal mice were injected with extracts from normal host plant tissue on days 1 and 7. When mice were 8 wk old, they were immunized with a crude antigen preparation consisting of about 10% MLO as determined by dot-blot immunoassays. Based on indirect enzyme-linked immunosorbent assay 20 hybridomas that secreted specific antibodies to the plant pathogenic MLO were selected. Two hybridoma lines were used for development of a dot-blot immunoassay technique on nitrocellulose membranes for detection of the MLO. Grinding liquid nitrogen-frozen MLO-infected midrib tissues with a pestle in a mortar was an efficient method for preparation of antigen extracts for dot-blot immunoassays.

Additional keywords: immunological assay, immunological tolerance, monoclonal antibody.

Mycoplasmalike organisms (MLOs) are cell wall-free prokaryotes associated with yellows type plant disease. Most affected plants exhibit stunting, proliferation, phyllody, and virescence. More than 300 mycoplasma diseases have been reported in plants (18). Diagnosis of diseases caused by MLOs and detection and identification of the causal organisms are difficult and time consuming. Serology offers several reliable and rapid methods for pathogen detection and disease diagnosis, but high quality serological reagents for serodiagnosis of plant diseases caused by MLOs are currently unavailable.

Preparation of specific antisera requires pure immunogens for immunization. MLOs are restricted to phloem tissues in infected plants, and attempts to culture plant pathogenic MLOs in vitro have been unsuccessful. A fraction enriched in MLOs can be obtained from infected plants, but host contaminants in the MLO immunogen preparation still limit the production of MLO-specific antisera (3,5,11,19,20,21).

Antibodies from hybridomas are specific (13). Although hybridoma-secreting antibodies have been produced using crude immunogen preparations, selection of desirable hybridomas for MLOs is a problem when antigens of particular interest are difficult to purify in sufficient quantities for screening. Monoclonal antibodies, however, have been produced to a limited number of plant pathogenic MLOs, including the aster yellows agent, the maize bushy stunt agent, the primula yellows agent, and an agent inducing peach eastern X-disease (2,3,12,15). Previously using crude antigens, monoclonal antibodies have been made to MLO only after tedious and labor-intensive work (2,15). In addition, a genius approach in which an immunogen preparation obtained from one source (viruliferous leafhoppers) and screening antigen from another source (extracts of diseased lettuce plants) was employed (15).

Antigen presented neonatally can suppress the ability of the immune system to recognize that antigen in later life and enhance the generation of desirable hybridomas (6,10,22). In this communication, we report the utilization of mice neonatally injected with normal plant antigens prior to immunization with partially purified MLO preparation in the production of hybridomas secreting monoclonal antibodies specific to a plant pathogenic MLO. The use of these monoclonal antibodies in the development of an assay for the MLO associated with tomato big bud disease is also presented.

MATERIALS AND METHODS

Preparation of MLO-enriched antigens. A periwinkle (*Catharanthus roseus* (L.) G. Don) infected with an isolate of MLO from plants of tomato (*Lycopersicum esculentum* Mill.) with symptoms of big bud disease was kindly provided by James L. Dale of the University of Arkansas, Fayetteville, AR (4). The MLO was maintained in periwinkle by grafting. A 2-ml MLO-enriched suspension in PBS (0.02 M phosphate-buffered saline solution, pH 7.2) was prepared by enzyme, cellulase, and macerozyme (Yukult Pharmaceutical Industry Co., Nishinomiya, Japan) treatments from 6 g of midrib tissue excised from infected periwinkle leaves by a previously described method (14). A threefold concentrated (2 ml of suspension prepared from 6 g of midribs) MLO-enriched antigen stock solution was stored at −25 C and used for both immunization of mice and for antibody detection during selection of hybridomas. Dilutions of various MLO antigen and plant antigen preparations used throughout the entire study were normalized on the basis of fresh weight of starting materials (midribs).

Sample preparation for detection of MLO infection. Three methods of preparing samples for detection of MLO infection were evaluated by dot-blot immunoassays. Method I employed the procedure of preparing MLO-enriched antigens described above in which 0.2 g of MLO-infected midrib tissue was digested

by enzyme treatments. Sieve elements were transferred to a test tube containing 3.0 ml of PBS and sonicated with Sonicator Cell Disruptor, Model W-375 (Heat Systems–Ultrasonic, Inc., Plainview, NY). Following clarification by low-speed centrifugation at 500 *g* for 10 min, the MLO was pelleted from suspension at 17,000 *g* for 40 min. In method II, 0.2 g of MLO-infected tissue was treated only by enzyme digestion and sonication before testing. Method III of sample preparation consisted of grinding 0.2 g of liquid nitrogen frozen MLO-infected tissue in 3.0 ml of buffer in a mortar with a pestle. All extracted MLO-suspensions were adjusted by adding PBS to give a 1:15 dilution and transferred into test tubes. They were left undisturbed for 5 min at room temperature before spotting on nitrocellulose membranes. Leaving a sample suspension undisturbed for a few min allowed particulates which might otherwise interfere with sample application onto membranes to settle by gravity. Healthy plant tissues treated similarly were used as controls.

Preparation of normal plant antigens. A threefold concentrated stock solution of plant extract from midrib tissues of healthy periwinkle was prepared using the same procedure used to prepare MLO antigens. The extract of healthy plant preparation was used as immunogens to produce normal plant antigen-specific mouse antisera and as antigens in later immunoassays. It was also used as tolerogens to inject newborn mice prior to immunizations with MLO-enriched preparation.

Mouse anti-plant antigen serum. Antiserum to normal plant constituents was produced in female adult BALB/c mice (Dominion Laboratories, Dublin, VA). Adult 25 g BALB/c mice were injected intraperitoneally four times with normal plant antigens (200 μl of antigen preparation plus 200 μl of complete Freund's adjuvant) over a period of 6 wk. Two weeks after the last injection, blood was collected from arteries while mice were under ether-induced anesthesia. Serum was separated by centrifugation and stored in a −25 C freezer for use.

Injection schedules for hybridoma formation. Pregnant BALB/c mice were obtained from Dominion Laboratories. Six newborn mice were each injected intraperitoneally within 24 hr after birth with 15–20 μl of normal healthy plant antigens. A second injection of 20 μl of the same extract was made 7 days later through the same route. When the mice were 8 wk old, one mouse was injected intraperitoneally with 200 μl of the MLO-enriched suspension, and the other mouse was injected intraperitoneally with 200 μl of MLO preparation that had been cross-absorbed with 200 μl of undiluted mouse antiserum prepared against healthy host plant extracts. Single spleen cell suspensions were prepared separately from the two mice 3 days after injection with MLO antigens.

Cell cultures and fusions. Procedures and reagents for myeloma cell line P3/NS1/1-Ag4-1 cultures and hybridization were essentially similar to those reported for barley yellow dwarf and carnation etched ring viruses (7,9). A modified growth medium used in current studies contained 360 ml of RPMI-1640 (GIBCO Laboratories, Grand Island, NY), 60 ml of Nu-serum (Collaborative Research, Inc., Bedford, MA), 25 ml of heat-inactivated (56 C, 30 min) CPSR-3 serum (Sigma Chemical Co., St. Louis, MO), 2 mM glutamine, 2×10^{-2} mM 2-Mercaptoethanol, and 10 mM HEPES. Two fusions were made individually 10 min apart. Cells from each fusion were seeded into a total of ten 96-well culture plates. Twice weekly, starting 4 days after fusion, one-half of the medium was replenished with fresh medium. For the first 2 wk after fusion, 10^{-1} mM hypoxanthine, 4×10^{-4} mM aminopterin, and 1.6×10^{-2} mM thymidine (17) were added to growth media. Thereafter, aminopterin was omitted from the media for about 4–5 wk before regular growth media were used.

Enzyme-linked immunosorbent assay. Indirect ELISA, using antigen-coated polyvinyl chloride (PVC) microtiter plates (Becton-Dickinson and Co., Oxnard, CA) and alkaline phosphatase-labeled goat anti-mouse IgA, IgG, and IgM immunoglobulins (Kirkegaard and Perry Laboratory, Gaithersburg, MD) were employed for detection of antibodies reacting with MLO-infected plant extracts during the selection of hybridomas (8,9). Both MLO-infected and healthy plant preparations, 50 μl at 1:3 dilutions in 0.05 M carbonate, pH 9.6, were used to sensitize PVC ELISA plates. MLO-positive antibody activities from culture fluids were tested and confirmed three times, once each from 96-well plates, 24-well plates, and T-25 flasks for each culture.

Hybridomas. Methods used in cloning and stabilization of desirable monoclonal antibody-secreting hybridomas, determination of immunoglobulin isotypes, and in vivo production of monoclonal antibodies were previously described (7,9).

Dot-blot immunoassay. Unless otherwise stated, the following protocols were used throughout the entire study. Nitrocellulose membranes, 0.2 μm pore size (Bio-Rad Laboratories, Richmond, CA), were immersed into TBS buffer (0.02 M Tris-base, 0.15 M NaCl pH 7.5) before being assembled onto a Minifold I apparatus (Schleicher & Schuell, Inc., Keene, NH). Antigen samples diluted in TBS were added 50 μl to each well while the manifold was attached to a vacuum line. Each well was then rinsed twice with 100 μl of TBS under vacuum. The manifold was disassembled, and the nitrocellulose membrane was removed before the vacuum was disconnected. The membranes were placed in TBS containing 1% natural nonfat dry milk (Carnation Company, Los Angeles, CA), 0.5% bovine serum albumin (BSA), at room temperature for 60 min to block unbound sites and incubated overnight at 4 C with monoclonal antibodies diluted in TBS-BSA (TBS containing 0.1% natural nonfat dry milk and 0.05% BSA). Following three successive 5 min washes in TBS-BSA, the blots were incubated for 2 hr in a solution of goat anti-mouse immunoglobulin-alkaline phosphatase conjugate prepared in TBS-BSA. The blots were then washed five times in TBS-BSA and incubated 2–10 min in a substrate solution containing 14 mg of nitroblue tetrazolium and 7 mg of 5-bromo-4-chloro-3-indolyl phosphate in 40 ml of substrate buffer containing 0.1 M Tris, 0.1 M NaCl, 5 mM $MgCl_2$, pH 9.5. Reactions were stopped with 10 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5.

Blind tests of MLO infection. A blind test was conducted to determine the applicability of dot-blot immunoassays for diagnosis of MLO infections. Ten MLO-infected and 10 healthy leaves were collected by one of us (I.M.L.), coded by a second person (R.E.D.), and tested by a third one (Y.C.W.). Therefore, tests were conducted in such a way that no one knew the identity of any sample until the test was completed. The midrib of each coded leaf was excised and weighed. Individual midribs were frozen in liquid nitrogen and triturated in TBS 20 × of tissue weight. A second dilution of 10^{-1} was made from the first extract for each sample. They were left undisturbed in test tubes for 5 min at room temperature. For each sample collected, both dilutions were applied in replica on nitrocellulose membranes. When the test was completed, results were interpreted by the fourth person (H.T.H.).

RESULTS

Hybridomas and monoclonal antibodies. Screening for antibody activities and selection of MLO antigen-specific

TABLE 1. Plant pathogenic mycoplasmalike organism (MLO)-specific hybridomas produced by fusion of P3/NS1/1-Ag4-1 myelomas with immune splenocytes derived from mice which had been injected neonatally with host plant constituents prior to immunization[a]

Reaction to	"N" spleen[b]	"A" spleen[c]	Total
MLO extract	14	6	20
Host constituents	27	68	95
None of the above	153	321	474

[a] Normal plant antigens (about 15–20 μl of healthy plant extracts) were injected intraperitoneally first within 24 hr after birth and then 7 days later. Immunogens were injected into peritoneal cavities when mice were 8 wk old. Splenectomy was done 3 days later.

[b] "N" spleen was from a mouse immunized by injection with 200 μl of MLO-enriched preparation.

[c] "A" spleen was from a mouse immunized with an MLO-enriched preparation equivalent to the amount used to immunize "N" mouse but cross-absorbed with host plant-specific mouse antiserum prior to injection.

hybridomas continued for 2–3 wk until the availability of MLO antigens was nearly exhausted. Although growth of new hybrid cells continued in 96-well tissue culture plates, selection and screening of hybridomas was terminated. The two spleen cell suspensions prepared from two immunized mice each contained about the same number of cells.

Of 960 wells seeded with cells from each fusion, 395 wells contained hybridomas for the fusion A made from the mouse immunized with the MLO preparation that had been absorbed with host-plant specific antiserum; and 194 wells contained hybrid cells for fusion N using spleen cells of the mouse immunized with the MLO-enriched suspension without prior cross-absorption (Table 1). When all hybridomas were tested with healthy plant extracts, 68 were positive to host plant constituents from the fusion A and 27 from the fusion N. Six hybridomas and 14 hybridomas secreting MLO-specific antibodies were produced from fusion A and fusion N, respectively (Table 1).

Of twenty hybridoma cultures selected, four were cloned. Hybridoma cell lines 3C8B11, 4E5C2, and 4G5E10 secrete IgM antibodies, whereas hybridoma 10B3G11 secretes IgG2a antibodies. When hybridomas were injected into mice that were primed with pristane, all four cell lines stimulated production of ascitic fluid in mice 2–3 wk after injection. Dilution end points for MLO-specific monoclonal antibodies produced in mouse ascitic fluids were greater than 2×10^6 for 10B3G11 and about 5×10^5 for the others, when assayed on nitrocellulose membranes (data not shown). ELISA end points of ascitic fluids were difficult to determine because of low A_{405} readings. The maximum A_{405} values for ascitic fluid antibodies while still retaining low background values (0.05 or less) for normal plant antigens were about 0.2. A_{405} values of control wells increased when dilutions of ascitic fluids were 2×10^{-3} or lower (data not shown).

Assays of MLO. Dot-blot immunoassays were compared with indirect ELISA for titrations of the MLO-enriched preparation. Using 4E5C2 ascitic fluid at a 1:20,000 dilution or 10B3G11 at a 1:50,000 dilution, positive reactions were obtained for the enriched MLO-preparation at about 1:640 to 1:1,280 dilutions (Fig. 1). No false positive reactions were observed in control healthy samples tested at 1:5 dilutions. The apparent positive spots on the membrane applied with control samples in Figure 1 were due to green pigment in the preparation.

Assays of MLO by indirect ELISA were difficult because of the higher background in negative controls. Ascitic fluids at a 1:5,000 dilution gave the lowest background reaction to healthy plant controls. At this dilution, the end point for positive result with the MLO-enriched preparation was between 1:40 and 1:80 (Fig. 2). The maximum A_{405} readings, even for the most concentrated MLO preparation used (1:5 dilution), was about 0.2.

Although it was difficult to quantify the absolute amount of MLO in the immunogen preparation, it was possible to compare dilution end points for both MLO and host constituents in the preparation by dot-blot immunoassays using a 1:20,000 dilution of MLO-specific 10B3G11 monoclonal antibodies and a 1:500 dilution of polyclonal antiserum raised against healthy plant extracts, respectively. The quantities of serologically reactive materials in the immunogen preparation differed by eight- to 16-fold (Fig. 3). Without a means to quantitatively measure antigens in the preparation, this serological method is the best estimate for the ratio of MLO/plant constituents. In this way, it was estimated that approximately 10% of the immunogen preparation used in the study was MLO antigen and the remainder was host contaminants.

Preparations of MLO-infected samples. Extensive treatments of samples with enzymes, sonication, clarification, and concentration used in method I produced suspensions with the least green color among the three methods used. It also left the least amount of green pigment on membranes (Fig. 4, rows A and B). The color shown on black and white photographs in Figure 4 for healthy controls is actually green. MLO-infected and healthy control samples prepared by the method II of enzyme digestion and sonication treatment or the method III of grinding liquid nitrogen-frozen tissues retained green color in suspensions, and green color was still visible when these samples were applied at 1:120 and 1:240 dilutions, respectively, (rows I and J for method II, and rows E and F for method III of Fig. 4).

All three methods used in preparing antigens from the tomato big bud agent infected plant tissues were satisfactory for detection of MLO infection (Fig. 4). On nitrocellulose membranes, MLO-infected tissue extracts developed strong dark purple color (rows C, D, G, H, K, and L of Fig. 4). Tissue extracts prepared by method II (rows K and L of Fig. 4) contained more MLO antigens than those prepared by either method I or method III.

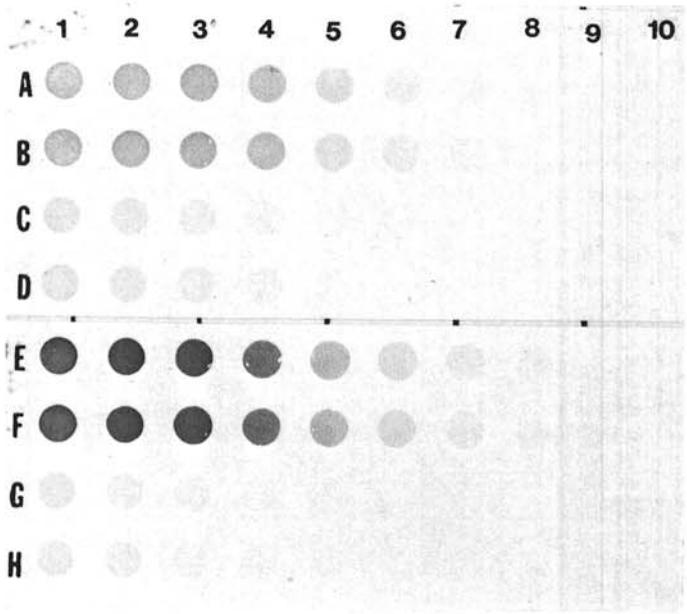

Fig. 1. Dot-blot (alkaline phosphatase) immunoassays of a mycoplasmalike organism (MLO)-enriched preparation on nitrocellulose membranes. Twofold dilutions of MLO-enriched preparation (rows A, B, E, and F), and normal plant antigens (rows C, D, G, and H) were made in replicates starting with 1:5 (w/v) dilutions on lane 1 from the left. The upper panel (rows A–D) was determined by using a 1:20,000 dilution of ascitic fluid of hybridoma antibody 4E5C2; the lower panel (rows E–G) was assayed by using a 1:50,000 dilution of ascitic fluid of hybridoma antibody 10B3G11.

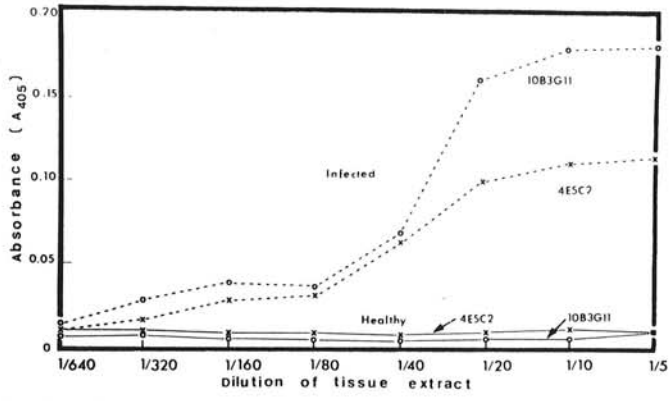

Fig. 2. Indirect enzyme (alkaline phosphatase)-linked immunosorbent assays of a mycoplasmalike organism (MLO)-enriched preparation on polyvinyl chloride plates. Samples of each dilution used in the assay were made from the same preparation from which dot-blot immunoassays were performed in Figure 1. Twofold dilutions of MLO-enriched preparation and normal plant antigens were made starting 1:5 (w/v) dilutions in 0.05 M carbonate buffer, pH 9.8, and 50 μl per well of each dilution was added in triplicates to polyvinyl chloride microtiter plates. The plates were incubated at 4 C overnight. Ascitic fluid at a 1:5,000 dilution was used to react with antigens, and antibodies were detected by alkaline phosphatase-labeled goat anti-mouse immunoglobulins.

Blind tests of MLO-infection. In the dot-blot immunoassay of the 20 samples tested, the 10 samples that gave positive reactions were all collected from infected plants, whereas the 10 negative samples were all from healthy controls.

DISCUSSION

Mouse hybridomas secreting monoclonal antibodies reactive to extracts of an MLO-infected plant were successfully generated using mice first injected neonatally with nontarget healthy plant extracts followed by one single injection of MLO-enriched crude antigen preparation in which only about 10% of immunogen preparations consisted of MLO. Although it was not determined in these studies, it was demonstrated that immunological tolerance to plant antigens could be induced in mice by injecting the newborns with plant extracts (10). Induction of tolerance in mice to plant proteins of *Nicotiana occidentalis* Wheeler 37B could also be accomplished by weekly injections of soluble plant proteins into the young (A. Schots, *personal communications*). An advantage of this method is that it does not require the different sources of MLO-enriched preparations that were needed in the production of hybridomas for the aster yellows agent (15). This is important since more than 300 plant diseases have been reported to be caused by MLOs, but vectors of only a few of these are known (18).

In the current study, a total of 20 hybridomas secreting antibody to an MLO were produced from two fusions. More hybridomas secreting MLO-specific antibodies may have been selected had the supply of antigens for selection not become limited and had the dot-blot immunoassay been utilized instead of ELISA.

The results of this work show that assays of MLO antigens by dot-blot immunoassays on nitrocellulose membranes are sensitive. The sensitivity is about 1.5 orders of magnitude greater than that of indirect ELISA. The level of detection of MLO infection by dot-blot immunoassays can be further increased by applying a larger volume of more dilute samples onto nitrocellulose membranes, an advantage that is not available with ELISA (Y. C. Wang and H. T. Hsu, *unpublished results*). This is a significant benefit of using dot-blot immunoassays for detection of MLO infection in which the small tissue sample can be triturated in a larger volume of buffer and applied into a single well. This method of preparing samples is easier than grinding large amounts of tissue in smaller volumes of buffer in order to keep concentrations above sensitivity threshold of detection for ELISA.

Mechanical disruption of liquid nitrogen-frozen tissues provides adequate means of extraction of MLO-antigens for dot-blot immunoassays, although green pigments from plant tissues may make test results misleading with black and white photographs. However, in actual tests, the green color of healthy controls provides a contrast to the dark purple spots of positive reactions. Further clarification and concentration following enzyme digestion and sonication of MLO suspension, however, caused some loss of antigens by differential centrifugation. It should be noted that methods I and II, both employing enzyme treatments, required sterile operations and an extra 24 hr of time for enzymes to digest the tissue, whereas method III took only a few min to prepare samples for tests.

Extensive purification in order to obtain an antigen preparation pure enough for antibody production may not prove to be suitable for MLOs (3). Our studies also show that for an added step during the course of antigen preparation, there were some losses of MLO antigens. It was suggested that during purification, some degradation may also occur to MLO structure and result in fewer active antigens remaining in sufficient quantity to be recognized by an immune system (3). If this is the case, production of monoclonal antibodies using immune splenocytes derived from mice first immunologically induced tolerant-to-host plant antigens at the neonatal stage before injection with a crude preparation of immunogens at the adult stage (10) may provide better means to produce antibodies to diversity of different epitopes, because immunogens used in current studies received very minimum treatments as compared to those studied by Clark et al (3).

The difficulty of generating hybridomas for MLOs, on one hand, and the feasibility of using monoclonal antibodies for studies of the agent, on the other hand, are well documented (2,5,15,16). With methods of suppressing immune response to nontarget antigens in a crude immunogen preparation (1,10) and the improved methods of immunogen preparation (3,11), the production of hybridoma antibodies for fastidious phloem-limited prokaryotes may be greatly enhanced.

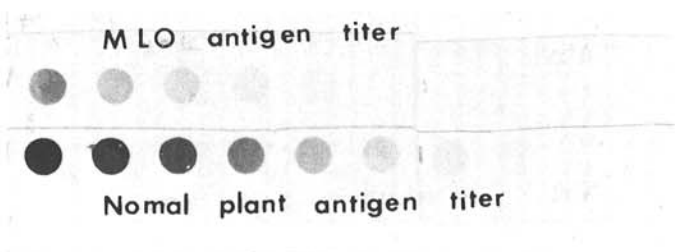

Fig. 3. Determination of the mycoplasmalike organism (MLO) titer and the host plant constituent titer of an MLO-enriched immunogen preparation by dot-blot (alkaline phosphatase) immunoassays on a nitrocellulose membrane. Fifty μl per well of twofold dilution of the MLO-enriched preparation was made starting with a 1:40 (w/v) dilution on the left. MLO titer (the top row) was determined by using mouse monoclonal antibodies 10B3G11 at a 1:20,000 dilution, whereas host plant constituent titer (the bottom row) was measured by using a 1:500 dilution of mouse polyclonal antiserum prepared for normal plant antigens.

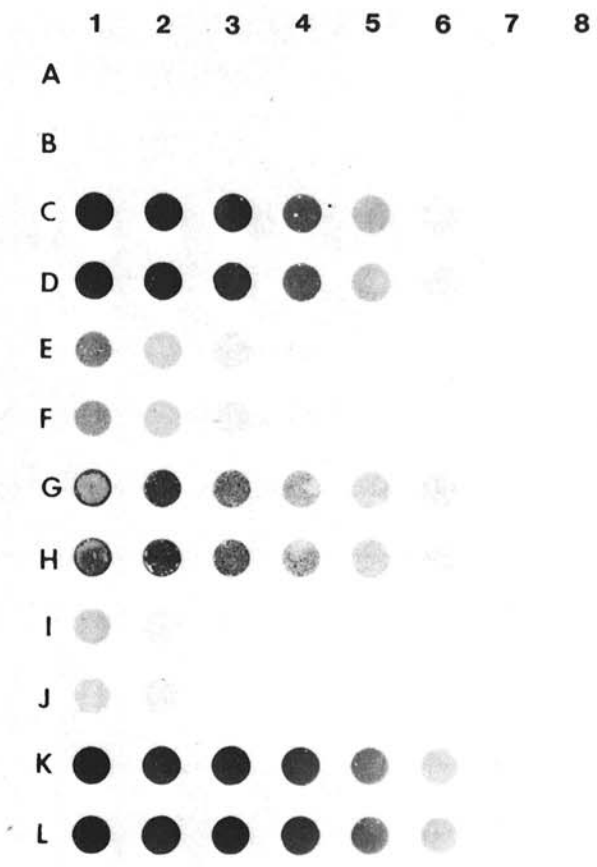

Fig. 4. The effect of sample treatments on the determination of mycoplasmalike organism (MLO) by dot-blot (alkaline phosphatase) immunoassays. Samples in rows A–D were partially purified by enzyme digestions, sonication, and differential centrifugations. Samples in rows I–L were prepared by enzyme digestions followed by sonication; samples in lanes E-H were ground in liquid nitrogen. Fifty μl per well of twofold dilutions of infected tissue extracts (rows C, D, G, H, K, and L) and normal plant antigens (rows A, B, E, F, I, and J) were applied in replica starting with 1:15 dilutions on lane 1 from the left.

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