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Evaluation of Diverse Antisera, Conjugates, and Support Media for Detecting *Bradyrhizobium japonicum* by Indirect Enzyme-Linked Immunosorbent Assay

ABATENI AYANABA,†* KAREN D. WEILAND, AND ROBERT M. ZABLOTOWICZ‡
Crop Science Laboratory, Syracuse Research Laboratory, Allied-Signal Inc., Solvay, New York 13209

Received 2 April 1986/Accepted 3 August 1986

We evaluated three antisera and four enzyme conjugates for the detection of *Bradyrhizobium japonicum* by an indirect enzyme-linked immunosorbent assay in microtiter plates. Nitrocellulose membrane sheets were then evaluated as an alternative support medium by using some combinations. Partially purified immunoglobulin G (IgG) or unpurified antisera to strain USDA 110 raised in rabbits, goats, or sheep was reacted in microtiter plates with alkaline phosphatase conjugated to protein A, goat anti-rabbit (GAR), sheep anti-rabbit (SAR), or rabbit anti-goat (RAG) IgG. Cultures or nodules containing homologous rhizobia were detected with equal sensitivity when protein A, GAR, or SAR was reacted with 5 μg of protein IgG per ml or a 1:800 titer of antisera from rabbits, but not goats or sheep. RAG reacted with IgG or antisera from goats or sheep. The detection limit was 2×10^5 rhizobia per well. Rhizobia were spotted on nitrocellulose sheets as an alternative support medium, followed by soaking in 5 μg of protein per ml as IgG and 1:4,000 dilutions of protein A or GAR conjugate. Rhizobia in serogroup 110 were detected with the dye combination Nitro Blue Tetrazolium–5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP), and rhizobia in serogroup 122 were detected with fast red-naphthol phosphate (FR-NP). At the conclusion of the 5-h assay, purple (NBT-BCIP) or red (FR-NP) spots were visible in positive reactions. The sensitivity of detection was about 1,000 rhizobial cells or 3 μg of nodules tissue.

The enzyme-linked immunosorbent assay (ELISA) is gaining prominence as a technique for detecting rhizobia in ecological studies. Both the direct and the indirect ELISA have been used to detect fast- and slow-growing rhizobia (1–3, 10, 11, 19, 20). Although both forms of ELISA are equally effective (2), the indirect ELISA appears to be more amenable for ecological work than the direct ELISA, because one enzyme conjugate is used to detect several strains.

Much effort has been devoted to simplifying the indirect ELISA to make it a more useful ecological tool. Recently, Kishinevsky and Maoz (12) described the use of protein A, produced by *Staphylococcus aureus*, for detecting peanut rhizobia. There have been no reports of the use of protein A to detect other rhizobia, such as *Bradyrhizobium japonicum*. A report by Fuhrmann and Wollum (8) indicated that crude antisera, like isolated immunoglobulin G (IgG), can be used in ELISA, while maintaining sensitivity levels adequate for ecological work. We are not aware of reports where non-rabbit antisera have been used for the detection of rhizobia by ELISA. Here, we report on experiments in which we compared protein A with second antibody conjugates as well as crude antisera with partially purified IgG from rabbits, goats, or sheep for the detection of *B. japonicum*.

Further improvements of the indirect ELISA should include shorter incubation periods and ways of eliminating false-positive data without compromising specificity and sensitivity. Nitrocellulose membranes have been used for the quantitative transfer of proteins prior to immunological detection (9, 21). The basic mechanism of adsorption of proteins to nitrocellulose is similar to that in microtiter plates in which polystyrene surfaces or similar, solid phases are coated with proteins or lipoproteins (23). Thus, Blake et al. (5) used the immunoblot-alkaline phosphatase system with nitrocellulose to detect the rabbit or mouse immune complex and Mandrell and Zollinger (16) detected meningococcal outer membrane proteins with nitrocellulose. It seemed that nitrocellulose should be examined as an alternative support for ELISA. We report on the application of a procedure which uses colored dyes for visualizing proteins on nitrocellulose for the detection of rhizobia in culture and from nodules.

MATERIALS AND METHODS

Antigens. Rhizobia used in this study were from the Allied-Nitragin collection. Cultures were grown in the yeast extract mannitol medium described by Vincent (22), washed, and boiled. Working stocks of about 10^9 cells per ml were prepared and kept at 4°C. Nodules were collected from plants grown either in the glasshouse in a sand-vermiculite mixture (1:1) or in field soil for 4 to 5 weeks and stored at −20°C until required.

Preparation of antisera and isolation of IgG. *B. japonicum* USDA 110 was grown in a defined medium (4) to mid-logarithmic phase and used to inject rabbits, goats, or sheep, by the procedure of Vincent (22). The animals were sacrificed, and antisera were collected only when titers were over 1,600. Partial purification of IgG was achieved by using octanoic (caprylic) acid and sodium acetate (15). Rabbit anti-USDA 122 IgG was similarly obtained. All IgG samples were adjusted to 1 mg of protein per ml (optical density at

* Corresponding author.
† Present address: Del Monte Corporation, Agricultural Research Center, Box 36, San Leandro, CA 94577.
‡ Present address: Allelix Inc., Mississauga, Ontario, Canada L4U1P1.

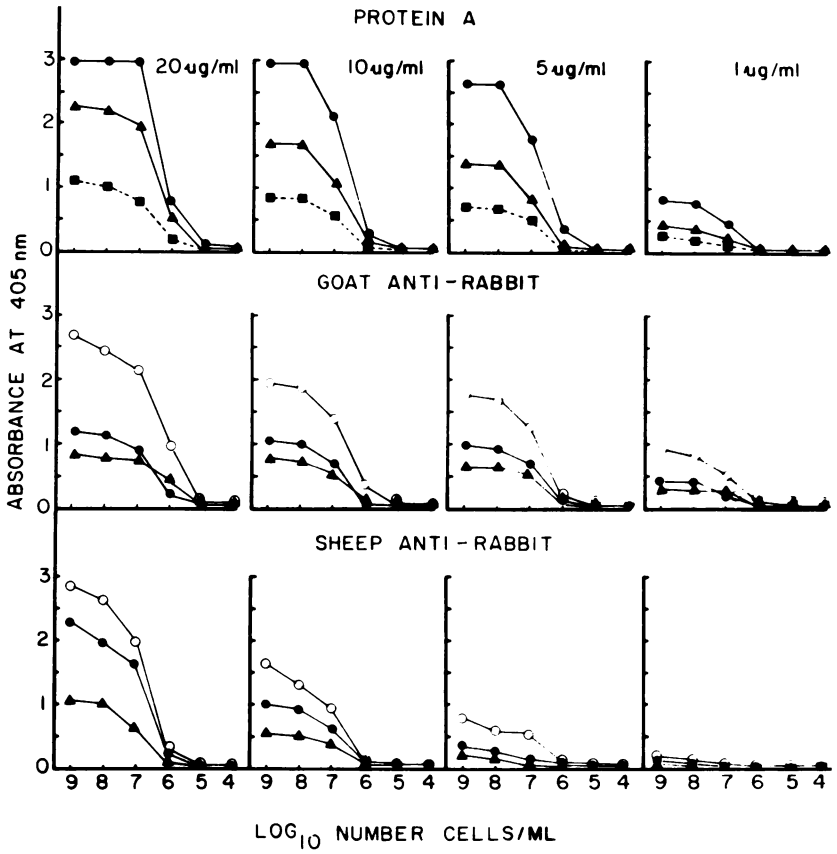

FIG. 1. Absorbances obtained when four concentrations (20, 10, 5, and 1 µg of protein per ml) of rabbit anti-USDA 110 IgG were reacted with alkaline phosphatase conjugates of protein A, GAR or SAR IgG. Conjugate dilutions were 1:1,000 (○), 1:2,000 (●), 1:4,000 (▲), and 1:8,000 (■). A suspension of USDA 110 containing 10^9 cells per ml was serially diluted to obtain the desired densities.

280 nm, 1.4) and frozen in 0.5- to 1.0-ml fractions until required.

Enzyme conjugates. Protein A (Sigma Chemical Co., St. Louis, Mo.) was conjugated to alkaline phosphatase by the two-step glutaraldehyde procedure (7, 12). Alkaline phosphatase conjugated to goat anti-rabbit (GAR), sheep anti-rabbit (SAR), and rabbit anti-goat (RAG) IgGs were purchased from Sigma. All conjugates were stored at 4°C.

Evaluation of antisera and enzyme conjugates. Checkerboard titrations by indirect ELISA (6, 24), with modifications (12), were performed to determine the optimum concentrations of IgG and conjugates for detecting the fewest rhizobia. USDA 110 cultures at about 10^9 cells per ml were serially diluted 10-fold to provide from 10^4 to 10^8 cells per ml in phosphate-buffered saline (PBS) (15). The suspensions were added to microtiter plates (Nunc, Vineland, N.J.) and left at room temperature overnight. PBS with 0.05% Tween 20 (PBS-Tween) was used to dilute the IgG samples to yield 1, 5, 10, or 20 µg of protein per ml; and the conjugates were diluted to 1:1,000, 1:2,000, and 1:4,000 for GAR, SAR, and RAG or 1:2,000, 1:4,000, and 1:8,000 for protein A. Incubations were at 28°C for IgG and conjugates. Phosphatase substrate (*p*-nitrophenyl phosphate; Sigma) was used at the concentration of 0.6 mg/ml. After 1 h at room temperature, the yellow color was read at 405 nm in a microplate reader (model EL 310; Bio-Tek Instruments, Burlington, Vt.), with a maximum A_{405} of 2.999.

Detection of rhizobia in culture and nodules. Anti-USDA 110 IgG and conjugates that gave positive reactions were used to detect rhizobia in nodules containing strains L-11Nal, I-110ARS, or AN-16 or seven strains in culture. Nodules were crushed en masse in PBS at the rate of 0.4 ml per nodule; the yield was 140 mg of nodule tissue per ml for AN-16 and 17 mg of tissue per ml for the others. For comparison, unpurified antisera from the three animals were also used for the detection of the rhizobia.

Immunoblot (paper) assay. Checkerboard titrations were performed by an immunoblot assay (procedure for GAR-AP, product no. 170-6509; Bio-Rad Laboratories, Inc., Richmond, Calif.). The procedure was essentially like that for the indirect ELISA. The antigens evaluated were (i) cultures of strain USDA 110; (ii) cell extracts of strain I–110ARS, and (iii) a culture of strain AN-16. Antigens were added in duplicate, at 1 µl per spot, to 10 spots on strips (0.8 by 10 cm) of pure nitrocellulose membrane sheets (catalog no. 162-0114; BioRad) that had been soaked in Tris-buffered saline (TBS) for 5 min. The strips were air-dried, and the assay was completed in a small incubation tray with 25 troughs (Bio-Rad). The tray was shaken for 1 h in a blocking solution consisting of 3% gelatin in TBS. After two 5-min washes in TBS containing 0.05% Tween 20 (TTBS), dilutions of IgG were added and the tray was shaken for 1.5 h. Rabbit anti-USDA 110 IgG was diluted to 1, 5, 10, and 20 µg/ml of protein, and 5 ml at the appropriate dilution was added to each trough. This was followed by washing with TTBS, and then conjugates were added and the tray was shaken for 1 h.

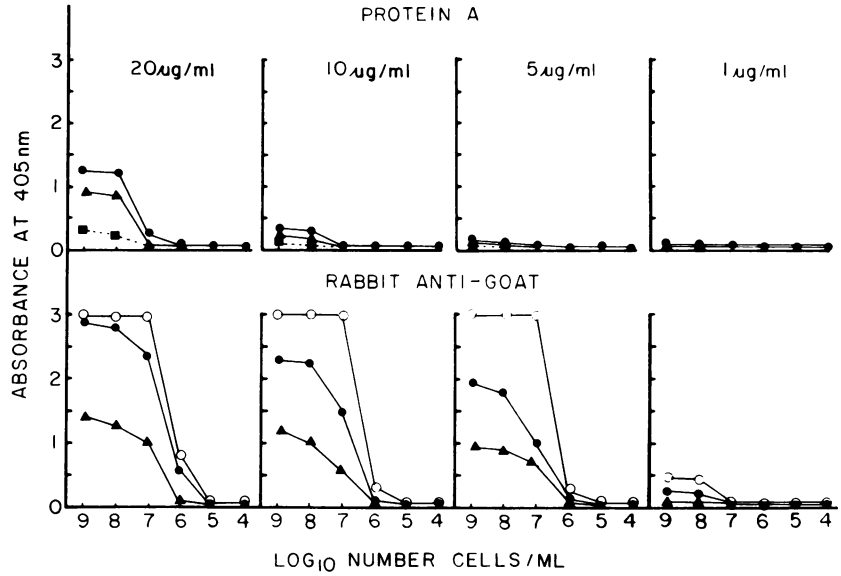

FIG. 2. Absorbances obtained when four concentrations (20, 10, 5, and 1 μg of protein per ml) of goat anti-USDA 110 IgG were reacted with alkaline phosphatase conjugates of protein A or RAG IgG. Key to symbols and cell densities are given in the legend to Fig. 1.

GAR-alkaline phosphatase conjugate (Bio-Rad) and protein A-alkaline phosphatase conjugate prepared as described above were compared at dilutions of 1:2,000, 1:4,000, and 1:8,000. Unreacted conjugates were washed off by washing twice with TTBS and once with TBS. For color development, a mixture consisting of 0.3 mg of Nitro Blue Tetrazolium (NBT) per ml and 0.15 mg of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) per ml was added to the troughs. Positive reactions were visible within 30 min as purple spots. The strips of nitrocellulose were air-dried and photographed.

Following the checkerboard titration, rabbit anti-USDA 110 and anti-USDA 122 IgG, at 5 μg of protein per ml, were used to probe for single antigens with protein A or GAR conjugates at 1:4,000 dilutions. Antigens (1 μl per spot) were spotted in quadruplicate on grids (1 by 1 cm) on nitrocellulose sheets (8.0 by 7.5 cm). There were 56 spots per sheet. The sheets were placed in square petri dishes (100 by 15 cm; Becton Dickinson Labware, Oxnard, Calif.) maintained on a rotary shaker. The assay was performed as described above, except that petri dishes received 25 ml of the appropriate reagents. To probe for two antigens, test antigens were immobilized (1 μl per spot) in identical locations on each of four squares. Cell cultures of USDA 110, USDA 122, and AN-16 and squashed nodules of L-110Nal, 122SR, and AN-16 were spotted either alone or in equal mixtures. Two of the sheets were probed for 110 and 122 serotypes the same day; the other two sheets were stored at room temperature (ca. 23°C) for 7 days and similarly probed. For double

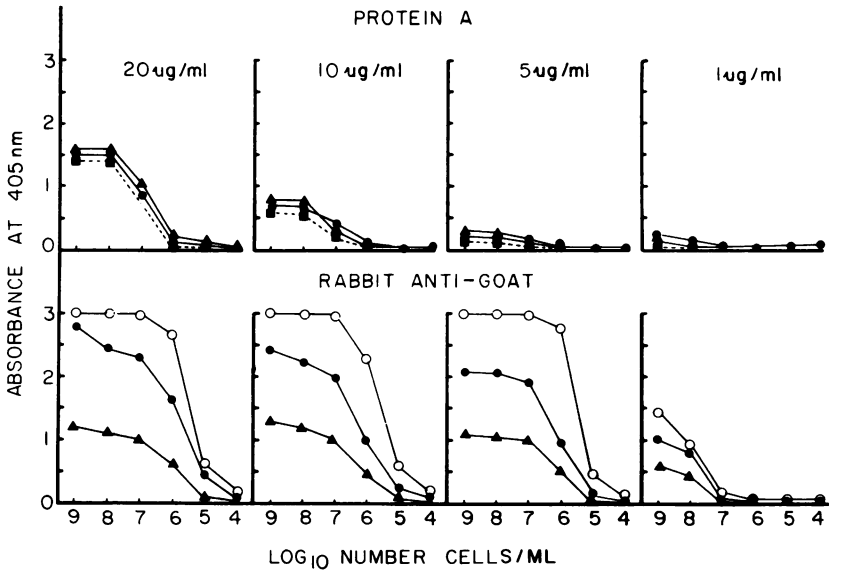

FIG. 3. Absorbances obtained when four concentrations (20, 10, 5, and 1 μg of protein per ml) of sheep anti-USDA 110 IgG were reacted with alkaline phosphatase conjugates of protein A or RAG IgG. Key to symbols and cell densities are given in the legend to Fig. 1.

TABLE 1. Evaluation of four enzyme conjugates and IgG isolated from antisera raised against *B. japonicum* USDA 110 in rabbits, goats, or sheep

Antigens	Serotype	PBS control	A_{405} for the following sources:						
			Rabbit IgG			Goat IgG		Sheep IgG	
			Protein A	GAR	SAR	Protein A	RAG	Protein A	RAG
Cultures									
PBS control		0.000	0.011	0.004	0.002	0.001	0.000	0.000	0.020
USDA 110	110	0.000	2.999	2.999	2.999	0.014	2.870	0.002	2.999
I-110	110	0.000	2.500	2.558	2.055	0.016	2.266	0.012	2.999
L-110Nal	110	0.000	2.999	2.684	2.021	0.040	2.999	0.004	2.999
AN-6	110	0.000	2.828	2.188	1.948	0.010	2.272	0.003	2.999
AN-16	c-3	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000
USDA 83	c-3	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000
USDA 122	122	0.000	0.003	0.093	0.063	0.000	0.093	0.002	0.353
USDA 123	123	0.000	0.003	0.032	0.020	0.000	0.024	0.010	0.101
Nodules									
AN-16	c-3	0.000	0.005	0.099	0.082	0.068	0.079	0.004	0.053
I-110ARS	110	0.000	1.724	0.275	0.234	0.099	0.128	0.008	0.354
L-110Nal	110	0.000	0.014	0.122	0.089	0.036	0.087	0.003	0.219

probes, both sheets were first probed for 110 serotypes as described above. One of the sheets was then used to probe for 122 serotypes by first shaking in TBS for 5 min. For convenience, the sheet was left in water overnight and the wash with TBS was performed the next day. The sheet was then incubated in deactivation buffer (pH 2.9) for 10 min to irreversibly denature the alkaline phosphatase bound to 110 IgG. The sheet was shaken in blocking solution for 10 min. The other steps were as described above for single probes, except that USDA 122 IgG was used and the color was developed by shaking for 10 min in a dye mixture of 3 mg of fast red (FR) per ml and 0.5 mg of naphthol phosphate (NP) per ml.

RESULTS

Antisera yields. Over 500 ml of antisera per animal was collected from goats and sheep. Rabbits gave about 50 ml per animal.

Evaluation of antisera and enzyme conjugates. Protein A-alkaline phosphatase conjugate used in conjunction with 5 or 10 μg of rabbit anti-USDA 110 IgG per ml was suitable for detecting rhizobia in culture at 10^6 cells per ml (Fig. 1). This represents about 2×10^5 cells per well. Protein A was more useful at much higher dilutions than GAR or SAR. Protein A could not be used to detect USDA 110 when IgG was isolated from antisera raised in goats or sheep (Fig. 2 and 3). Sheep and goat anti-USDA 110 IgG reacted well with RAG, poorly with protein A (Fig. 2 and 3), and not at all with GAR or SAR (data not shown).

Combinations of 10 μg of IgG per ml and the conjugates were used to detect several rhizobial strains in culture and from nodules (Table 1). USDA 122 gave unexpectedly high absorbances when tested against sheep IgG and RAG; similar results were obtained in two other experiments (data not shown). Nodules of strain I-110ARS were readily detected, but the absorbances were low for the less-effective strain L-110Nal (Table 1). The amount of nodule tissue detected in this system was 3 to 6 mg per well. Lower levels were not tested.

Crude antisera and the appropriate conjugates enabled detection of homologous antigens of USDA 110 but not the heterologous antigens (Table 2). For identical treatments, background absorbance was higher with crude antisera than with isolated IgG. Nodules of heterologous rhizobia gave unusually high absorbance values (cf. Tables 1 and 2).

TABLE 2. Evaluation of four enzyme conjugates and crude antisera raised against *B. japonicum* USDA 110 in rabbits, goats, or sheep

Antigens	Serotype	PBS control	A_{405} for the following sources:						
			Rabbit IgG			Goat IgG		Sheep IgG	
			Protein A	GAR	SAR	Protein A	RAG	Protein A	RAG
Cultures									
PBS control		0.000	0.143	0.753	0.398	0.024	0.247	0.053	0.215
USDA 110	110	0.000	2.999	1.667	1.977	2.999	1.670	1.530	0.813
I-110	110	0.000	2.999	1.699	2.067	2.908	2.007	1.896	0.916
L-110Nal	110	0.000	2.999	1.615	1.858	2.999	1.830	1.419	0.829
AN-6	110	0.000	2.999	2.006	2.172	2.999	1.962	1.951	0.953
AN-16	c-3	0.000	0.165	0.489	0.688	0.033	0.204	0.045	0.301
USDA 83	c-3	0.000	0.141	0.554	0.594	0.047	0.154	0.064	0.712
USDA 122	122	0.002	1.314	1.629	2.033	0.210	0.495	0.784	0.962
USDA 123	123	0.000	0.915	0.943	1.246	0.376	0.543	0.081	0.701
Nodules									
AN-16	c-3	0.008	0.500	1.354	1.372	0.226	1.185	0.121	1.114
I-110ARS	110	0.000	0.947	1.369	1.679	0.217	1.265	0.146	1.193
L-110Nal	110	0.000	0.657	0.905	1.100	0.186	0.988	0.131	0.943

Immunoblot assay. Homologous antigens from cultures and nodules were readily detected in checkerboard titrations on nitrocellulose strips, with as little as 1 μg of protein of rabbit anti-USDA 110 IgG per ml and a 1:4,000 dilution of either GAR or protein A conjugates (Fig. 4). At dilutions of 5×10^5 to 1×10^6 cells per ml, positive results were obtained, indicating that as few as 500 to 1,000 rhizobia within a spot could be detected. GAR-treated samples had a high background when the sample was in excess. As expected, heterologous rhizobia gave negative reactions.

USDA 110, USDA 122, or their homologs were detected with 5 μg of IgG and either GAR or protein A conjugate per ml (Fig. 5). Nonspecific reactions with heterologous cultures or nodules were observed with GAR. IRc 540, a *Rhizobium* species of unknown serotype from West Africa (A. Ayanaba, unpublished data) cross-reacted with 110 IgG.

We probed for two antigens by using the NBT-BCIP (purple) dyes for 110 serotypes and the FR-NP (red) dyes for 122 serotypes (Fig. 6a and b). One week following the immobilization of antigens on nitrocellulose, the double probe was also successful (Fig. 6c and d). The lowest detection limit for nodule tissue was 3.6 μg for USDA 110 (Fig. 6a and c, spot D5) and 3.1 μg for 122SR (Fig. 6b and d, spot E6).

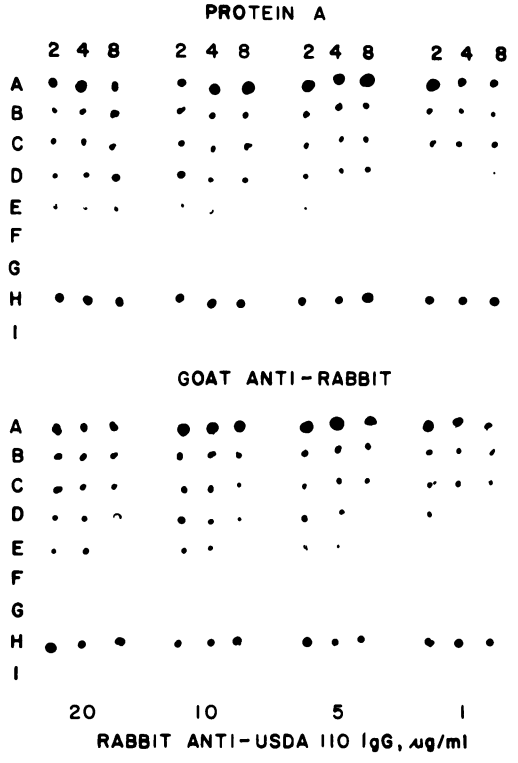

FIG. 4. Detection of *B. japonicum* on nitrocellulose strips, using 1 μl of antigen suspensions, four concentrations (20, 10, 5, and 1 μg of protein per ml) of rabbit anti-USDA 110 IgG, and three dilutions (2, 4, and 8 representing 1:2,000, 1:4,000 and 1:8,000 dilutions, respectively) of alkaline phosphatase conjugates of protein A or GAR IgG. Antigens in spots of rows A through G are dilutions of a USDA 110 culture containing 1×10^8, 1×10^7, 5×10^6, 1×10^6, 5×10^5, 1×10^5, and 5×10^4 cells per ml, respectively; row H contains a crude cell extract of USDA 110 as a positive control; and row I is a culture of 10^9 cells of AN-16 per ml as a negative control.

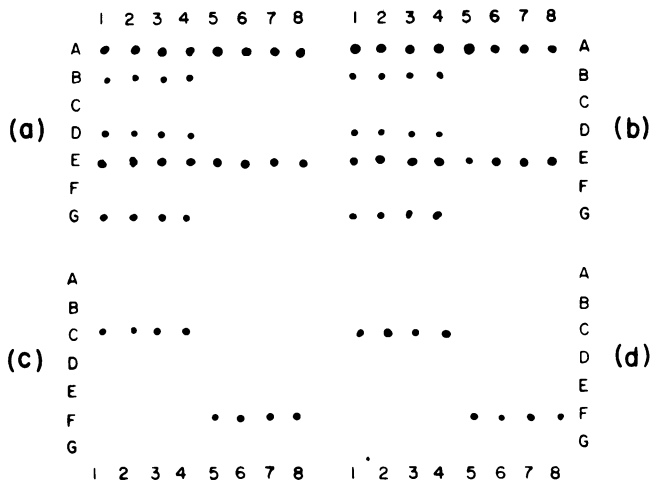

FIG. 5. Single probe for vegetative cells or bacteroids on nitrocellulose with the dyes NBT-BCIP. Antigens were applied in quadruplicate and reacted with 5 μg of IgG per ml and 1:4,000 dilution of conjugates. (a) Anti-USDA 110 IgG reacted with protein A; (b) anti-USDA 110 IgG reacted with GAR; (c) anti-USDA 122 IgG reacted with protein A; (d) anti-USDA 122 IgG reacted with GAR. Antigens tested were cultures of *B. japonicum* USDA 110 (spotted in grids A1 to A4; see text), L-110Nal (grids A5 to A8), AN-6 (grids B1 to B4), AN-16 (grids B5 to B8), USDA 122 (grids C1 to C4), and IRj 2101 spc (grids C5 to C8); *Rhizobium* spp. IRc 540 (grids D1 to D4) and NC 92 (grids D5 to D8); and squashed nodules of *B. japonicum* AN-3 (grids E1 to E4), I-110ARS (grids E5 to E8), AN-25 (grids F1 to F4), 122SR (grids F5 to F8), L-110Nal (grids G1 to G4), and AN-16 (grids G5 to G8); USDA 110, L-110Nal, AN-3, AN-6, and I-110ARS belong to serogroup 110, whereas USDA 122 and 122SR belong to serogroup 122.

DISCUSSION

Yields of antisera from goats or sheep were substantially higher than those from rabbits. Because similar titers of the antisera gave similar ELISA absorbance values and the maintenance cost of the larger animals was not significantly greater than that for the rabbits, the larger yields of antisera are an advantage.

When used with rabbit antisera, protein A conjugate gave positive reactions at much higher dilutions than did the second antibody conjugates. In preliminary work, we observed positive results at a dilution of 1:16,000 (data not shown). These results on the usefulness of protein A agree with those of Kishinevsky and Maoz (12) on peanut rhizobia. Protein A, produced by *S. aureus*, reacts with the Fc portion of IgG from all mammalian species (14). It is chiefly this lack of species specificity that has made protein A attractive as a conjugate in ELISA studies (7, 12). However, our results show that protein A did not react well with antisera of IgG isolated from goats or sheep. This is in agreement with the report by Kronvall et al. (14) that protein A produces an inhibition reaction with IgG from certain members of the mammalian order *Artiodactyla*, such as goats and sheep.

Fuhrmann and Wollum (8) indicated in a recent report that unpurified antisera could be used to detect *B. japonicum* without the loss of sensitivity. This is true of pure cultures. With bacteroids, high backgrounds and nonspecific reactions were observed (Table 2) and could compromise the sensitivity of the assay.

Although alkaline phosphatase has been used in ELISAs for some time, it is only recently that it has been used to

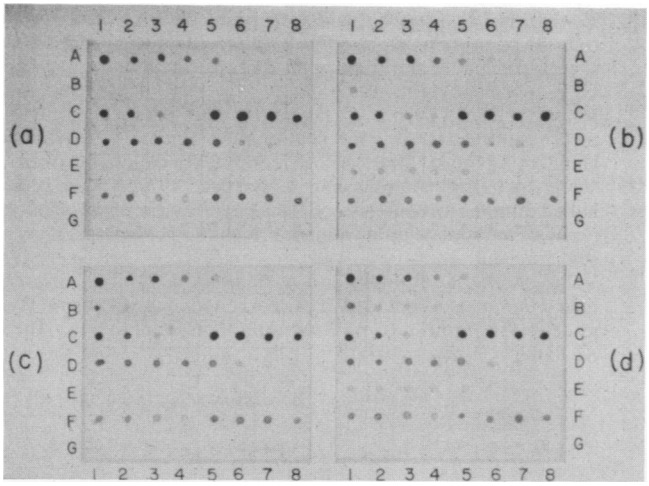

FIG. 6. Double probing for 110 and 122 serotypes on nitrocellulose with 5 μg of rabbit anti-USDA 110 IgG per ml and 1:4,000 dilution of protein A conjugate. Test antigens (1 μl) were immobilized in identical locations on each of four squares, as follows: rows A1 to A8, USDA 110 cells at 1×10^8, 1×10^7, 5×10^6, 1×10^6, 5×10^5, 1×10^5, 5×10^4, and 1×10^4, respectively; rows B1 to B8, USDA 122 at the same densities as USDA 110, respectively; rows C1 to C8, equal mixtures of USDA 110/USDA 122 at $10^8/10^8$, $10^7/10^8$, $10^6/10^8$, $10^5/10^8$, $10^8/10^5$, $10^8/10^6$, $10^8/10^7$, $10^8/10^8$, respectively; rows D1 to D8, L-110Nal nodule tissue at 17.8, 8.9, 5.9, 4.5, 3.6, 1.8, 0.9, and 0.4 mg/ml, respectively; rows E1 to E8, 122SR nodule tissue at 31.0, 15.5, 10.3, 7.8, 6.2, 3.1, 1.6, and 0.8 mg/ml, respectively; rows F1 to F8, equal mixtures of L-110Nal/122SR nodule tissue (mg/mg) at 17.8/31.0, 8.9/31.0, 3.6/31.0, 1.8/31.0, 17.8/3.1, 17.8/6.2, 17.8/15.5, and 17.8/31.0, respectively; rows G1 to G4, cultures of AN-16 at 10^9 cells/ml; and G5 to G8, nodule tissue of AN-16 at 142 mg/ml. On the same day that antigens were immobilized, a probe was performed for 110 serotypes with the dyes NBT-BCIP (a), and a second probe was performed for 122 serotypes with the dyes FR-NP (b). Seven days after immobilization, probes for 110 (c) and 122 (d) serotypes were similarly performed.

detect antibody-antigen complexes on nitrocellulose (18). Modifications of the basic procedure involving *p*-nitrophenyl phosphate as the substrate for alkaline phosphatase has led to the use of other substrates such as the dyes NBT, BCIP, FR, and 4-chloro-1-napthol (5, 13, 17, 18). Our results indicate that nitrocellulose is a good support for immobilizing rhizobia in an indirect ELISA. The assay was routinely completed within 5 h, representing savings in time over the microtiter plate assay. Moreover, the results are immediately visible to the naked eye. Because antigens were spotted on the same sheet, they received uniform treatment, thereby reducing variability. There was no loss in specificity or sensitivity compared with the plate assay. Indeed, there was a 200-fold gain in sensitivity with cells. With nodule tissue, the gain was 1,000-fold. Other advantages of nitrocellulose over microtiter plates have been reported (17).

The purple spots produced from NBT-BCIP and the red spots from FR-NP dye substrates make the paper assay extremely useful for typing nodules for double or multiple occupancy, as shown in our studies with USDA 110 and USDA 122. When a probe for a particular antigen is negative, continued deactivation followed by probes with various antisera could lead to eventual detection of the nodule occupant(s).

We detected antigens which had been immobilized for 7 days (longer periods were not tested) prior to probing. The potential for immobilizing antigen on nitrocellulose in the field for later completion of the assay, or to perform the entire assay in the field, makes this an attractive ecological tool that awaits exploitation.

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