Endogenous Lectins from Cultured Soybean Cells:
Isolation of a Protein Immunologically Cross-reactive with Seed Soybean Agglutinin and Analysis of Its Role in Binding of *Rhizobium japonicum*

Siu-Cheong Ho, Shahnaz Malek-Hedayat, John L. Wang, and Melvin Schindler

Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824

Abstract. Incubation of *Rhizobium japonicum* with the cultured soybean cell line SB-1, originally derived from the roots of *Glycine max*, resulted in specific adhesion of the bacteria to the plant cells. This binding interaction appears to be mediated via carbohydrate recognition, since galactose can inhibit the heterotypic adhesion but glucose cannot. Affinity chromatography, on a Sepharose column derivatized with N-caproyl-galactosamine, of the supernatant fraction of a SB-1 cell suspension after enzymatic removal of cell wall yielded a single polypeptide ($M_r \sim 30{,}000$) on immunoblotting analysis with rabbit antibodies

directed against seed soybean agglutinin. Fluorescently labeled rabbit anti–seed soybean agglutinin also yielded specific immunofluorescent staining on the cell wall and plasma membrane of the SB-1 cells. These results suggest that one likely candidate that may mediate the recognition between the *Rhizobium* and the soybean cells is the endogenously produced SB-1 lectin. This notion is supported by the observation that rabbit anti–seed soybean agglutinin blocked the *Rhizobium*–soybean cell adhesion, whereas control antibodies did not.

ANY eukaryotic and microbial cells can recognize and interact specifically with other cells, either cells of the same type (homotypic) or cells of a different type (heterotypic). Our understanding of the molecular components mediating specific cell recognition and adhesion has advanced mainly as a result of several paradigms: (*a*) yeast sexual agglutination (28); (*b*) sea urchin fertilization (38); (*c*) slime mold aggregation (30); (*d*) cell–cell adhesion in embryonic tissues of the chick (33); and (*e*) recognition, by lymphoid cells, of target cells bearing foreign antigens (39). Among plant systems, one area that has attracted much attention is the binding of the bacterium *Rhizobium* to the root cells of leguminous plants, leading to the nitrogen-fixing symbiosis (2). Clearly, a key event in the establishment of the legume–*Rhizobium* symbiosis is the initial binding between the bacterium and the host cell.

Studies directed at analysis of this event have been guided, for the most part, by the "lectin recognition" hypothesis, first proposed by Krüpe (20) and later revived by Hamblin and Kent (15) and by Bohlool and Schmidt (6). According to this hypothesis, legume lectins control host specificity by interacting with polysaccharide components on the bacterial symbiont. There have been many inconsistencies and experimental deficiencies in various studies that purport either to support or to refute the lectin recognition hypothesis (2, 29). Many of these difficulties may be associated with the facts that the studies have been carried out, in general, with in vivo root systems in which the initial binding event may be hard to assay and manipulate and that many of the assays are

long term "end point" assays, i.e., successful nodule formation. While these studies are, for the most part, informative and directly relevant to biological nitrogen fixation, it seems that a defined tissue culture system is much more amenable to study the initial binding event.

We have identified and isolated a lectin produced endogenously by the soybean cell line, SB-1, originally derived from roots of *Glycine max* (12). Immunofluorescence and biochemical evidence indicated that the lectin may be localized on the cell wall and therefore is accessible to the external environment. These results prompted us to determine whether *Rhizobium* binds specifically to the cultured SB-1 cells and whether the endogenous lectin plays a role in this adhesion. The results of our studies on these issues are reported in the present communication.

Materials and Methods

Cell Culture and Protoplast Isolation

The SB-1 cell line, derived from soybean roots (*Glycine max* [L.] Merr. cv. Mandarin) (12), was kindly provided by Dr. G. Lark (Department of Biology, University of Utah, Salt Lake City, UT). Cultures were grown in 125-ml Erlenmeyer flasks containing 30 ml of solution at 27°C on a gyratory shaker in the dark. Liquid cultures were subdivided every 3–4 d by transferring 10 ml of culture to 30 ml of fresh 1B5C medium (basic medium plus 1 ppm of 2,4-dichlorophenoxyacetic acid and 2 g/liter of casein hydrolyzate, pH 5.5). Suspensions of SB-1 cells were centrifuged for 4 min at 460 *g*; 1 ml of packed cells usually yielded $\sim 10^7$ cells by direct counting.

Protoplasts were prepared by a modified procedure of Constabel (9). Actively growing SB-1 cells (24–48 h after transfer) were digested with an

equal volume (20 ml) of enzyme solution containing 400 mg cellulysin (Calbiochem-Behring Corp., La Jolla, CA), 200 mg pectinase (Sigma Chemical Co., St. Louis, MO), and 2 g D-sorbitol (Sigma Chemical Co.), pH 5.5. After 2 h, the protoplast suspension was filtered through a 48-μm nylon filter and pelleted by centrifugation for 4 min at 460 g. The pelleted protoplasts were washed by gentle resuspension and centrifugation using 5 ml of protoplast medium (9), which was modified by substituting 30 g D-sorbitol for glucose. After three washes, the protoplasts were resuspended in 5 ml of the same protoplast medium. Fluorescence microscopy after Calcofluor staining and scanning electron microscopy of the protoplasts showed neither cell wall material nor cellulose microfibrils, respectively. The details of these analyses have been reported previously (25).

Seed Soybean Agglutinin and Antibody Reagents

Seed soybean agglutinin (SBA)[1] was isolated and purified by affinity chromatography on Sepharose column derivatized with N-caproyl-galactosamine (Gal-Sepharose) (1). Soybean meal was defatted with petroleum ether (1:10 wt/vol). The defatted meal was stirred with phosphate-buffered saline (PBS) overnight at 4°C, centrifuged at 9,000 g for 15 min, and the supernatant was applied to the Gal-Sepharose column. Bound SBA was eluted from the column with 0.2 M D-galactose (Gal) in PBS at 4°C.

Antibodies directed against seed SBA were raised in rabbits (New Zealand White, female). The primary injection consisted of 1 mg of protein in Freund's complete adjuvant (Difco Laboratories Inc., Detroit, MI). Booster injection of 1 mg protein in Freund's incomplete adjuvant were administered at weekly intervals. Antiserum was collected 1 wk after boosting. Monospecific antibodies directed against seed SBA were isolated by affinity chromatography. Purified seed SBA (14 mg) was coupled to cyanogen bromide-activated Sepharose 4B beads (3 ml; Pharmacia Fine Chemicals, Piscataway, NJ) and further cross-linked with 0.1% (vol/vol) glutaraldehyde (19). Antiserum raised against seed SBA was fractionated over this column; the bound fraction eluted with 0.1 M glycine-HCl, pH 3.0 was designated as rabbit anti-seed SBA.

The labeling of antibodies with ^{125}I was carried out with chloramine T following the procedure described by Ho et al. (17). Free ^{125}I was removed by passing the labeled material over a column (3.0 × 0.5 cm) of Dowex AG1×8 (Bio-Rad Laboratories, Richmond, CA). The specific activities of the products were: rabbit anti-seed SBA (1.5 × 10⁶ cpm/μg) and normal rabbit immunoglobulin (1.4 × 10⁶ cpm/μg).

We have also generated a rabbit antiserum that binds to the cell wall of SB-1 cells; this antiserum serves as a control for certain experiments carried out with rabbit anti-seed SBA. SB-1 cells (15 g wet wt) were homogenized in 30 ml water at 4°C, using a Waring blender at maximum speed for 5 min. After centrifugation at 3,000 g for 5 min, the pellet was extracted three times with 30 ml of 1% SDS in 20 mM Tris-HCl, pH 7.4. The ruptured cells were homogenized in a Potter-Elvehjem tissue grinder and then extracted successively with methanol (three times, each with 30 ml) and water (three times, each with 30 ml). The final pellet was suspended in water as a 10% (vol/vol) suspension. Microscopic observation revealed "cell wall fragments" and no cytoplasmic organelles.

Equal volumes of a 2% (vol/vol) suspension containing cell wall fragments and complete Freund's adjuvant were sonicated and used for immunization of female New Zealand white rabbits. Booster immunizations were given at biweekly intervals using the same antigen fraction emulsified in incomplete Freund's adjuvant. 2 mo after the initial immunization, serum derived from the rabbit was fractionated on protein A-Sepharose; the immunoglobulin fraction obtained bound to intact SB-1 cells as well as to the fraction containing cell wall fragments as revealed by indirect immunofluorescence (see below). This immunoglobulin fraction will be referred to as rabbit anti-cell wall fragments.

PAGE and Immunoblotting

PAGE in the presence of SDS was done as described (21) using 10% and 4% (wt/vol) acrylamide concentrations in the running and stacking gels, respectively. After electrophoresis, the proteins were revealed by staining with Coomassie Brilliant Blue or by immunoblotting after transfer to nitrocellulose paper. For immunoblotting, the proteins were transferred to nitrocellulose paper (Schleicher & Schuell, Inc., Keene, NH) by elec-

trophoresis (200 mA, 3 h, 25°C) (36). After transfer, the blots were washed overnight in Tris-buffered saline (20 mM Tris, 500 mM NaCl, pH 7.5) plus 0.05% Tween 20 which contained 5% (wt/vol) BSA. The nitrocellulose membrane was incubated with rabbit anti-seed SBA (40 μg/ml) for 6–8 h and then washed three times with 10-min incubations in Tris-buffered saline. The membrane was then incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Bio-Rad Laboratories; 1:2,000 dilution, 90 min, 25°C) and washed three times with Tris-buffered saline. The immunoreactive material was revealed via horseradish peroxidase activity according to the Bio-Rad procedure with 4-chloro-1-napthol and hydrogen peroxide as substrates.

Isolation of Antibodies by Specific Adsorption to a Polypeptide on Nitrocellulose

This procedure was used to isolate monospecific antibodies specifically directed against the 30-kD polypeptide of seed SBA. Purified seed SBA (0.5 mg) was subjected to SDS PAGE and transferred to nitrocellulose membrane. The nitrocellulose membrane was incubated with rabbit anti-seed SBA (40 μg/ml) for 6–8 h at 25°C. After washing, a strip of the membrane was cut out and used to determine the position of the 30-kD polypeptide band by immunoblotting as described above. The position corresponding to the 30-kD region was excised from the unstained membrane and the bound antibodies were eluted as described by Smith and Fisher (34). This preparation was designated monospecific antibodies directed against the 30-kD polypeptide of seed SBA.

Assays for SB-1 Cell Components Reactive with Rabbit Anti-Seed SBA

The presence of material reactive with rabbit anti-seed SBA on the surface of SB-1 cells or protoplasts was assayed as follows: cells (1 × 10⁶/ml) were washed twice in 0.55 M sorbitol, 10 mM Tris-HCl, 10 mM CaCl₂ (Tris buffer), pH 5.5. The cells were then incubated with 1 ml of Tris buffer alone, or Tris buffer containing 0.1 M D-glucose (Glc), Gal, or N-acetyl-D-galactosamine (GalNAc) for 10 min at room temperature. The cells were washed twice by centrifugation (460 g for 4 min) and resuspended in Tris buffer, and the extraction was repeated. After the final wash, the cells were resuspended in Tris buffer with 0.3% (vol/vol) normal goat serum, pH 7.2, and washed once. Monospecific rabbit anti-seed SBA or control rabbit immunoglobulin was added to a final concentration of 30 μg/ml. The samples were incubated for 1 h at 4°C and washed three times in Tris buffer with serum (pH 7.2). Fluorescein-conjugated goat anti-rabbit immunoglobulin (10 μl of 1:100 dilution; Gibco, Grand Island, NY) was added to the cells and incubated for 30 min at 4°C. The cells were washed three times in Tris buffer with serum and the fluorescence staining observed under a Leitz fluorescence microscope, equipped with a Leitz KP 490 dichroic mirror. Micrographs were taken with Kodak Tri-X film, which was pushed to ASA 3200.

The binding of rabbit anti-seed SBA to SB-1 cells and protoplasts was also assayed with ^{125}I-labeled antibody. Cells (1 × 10⁶/ml) were washed three times with 1 ml of Tris buffer, pH 7.2, containing 0.3% (vol/vol) normal rabbit serum, by centrifugation (460 g for 4 min) and resuspension. These samples were incubated on an orbital shaker (100 rpm) with ^{125}I-labeled rabbit anti-seed SBA or normal rabbit immunoglobulin for 2 h at 4°C. After three washes by centrifugation and resuspension of the cells in Tris buffer with rabbit serum, the radioactivity in the cells was determined. A similar binding experiment with ^{125}I-labeled antibodies was performed after SB-1 cells or protoplasts were extracted with 0.1 M Glc, Gal, or GalNAc as described above.

Isolation of Lectin Activity from SB-1 Cells

The cell wall was degraded by a modified procedure of Constabel (9). Actively growing SB-1 cells (4-d-old) were washed with fresh 1B5C medium by centrifugation (460 g, 4 min) and resuspension. The pelleted cells were then readjusted to the same volume with fresh 1B5C medium and digested with an equal volume (100 ml) of enzyme solution containing 0.8 g pectinase (Sigma Chemical Co., St. Louis, MO), 1.6 g cellulysin (Calbiochem-Behring Corp.), and 10.0 g D-sorbitol (Sigma Chemical Co.), pH 5.5. After 2 h incubation at 37°C, the digestion mixture was centrifuged (10,000 g, 30 min, 4°C). The supernatant was adjusted to pH 7.4 and purified by affinity chromatography on Gal-Sepharose columns. Material bound to the column was eluted with 0.2 M Gal, concentrated by Amicon ultrafiltration, and dialyzed against 0.1% SDS.

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1. *Abbreviations used in this paper:* Gal, D-galactose; GalNAc, N-acetyl-D-galactosamine; Gal-Sepharose, Sepharose derivatized with N-caproyl-galactosamine; Glc, D-glucose; SBA, soybean agglutinin.

Rhizobium Culture and SB-1 Cell Binding

Rhizobium strains used: R. japonicum 110d was obtained from Dr. Barry Chelm, R. fredii PRC 205 str (a fast growing strain originally derived from R. japonicum) was obtained from Dr. Kenneth Nadler, and R. meliloti 102F28, R. leguminosarum 128C56, and R. trifolii 0403. were obtained from Dr. Frank Dazzo. Various Rhizobium strains were maintained on yeast extract-mannitol-sodium gluconate medium at 30°C as described previously (5). Inocula were grown to mid-exponential phase. The bacterial strains were centrifuged at 8,000 rpm for 15 min and washed once with 20 ml of sterile 1B5C medium. The concentration was adjusted to 0.8 unit (1 unit = absorbance of 1.0 at 620 nm, 0.03 unit = 1 × 10⁸ cells).

SB-1 cells (2-d-old cultures) in 1-ml suspensions (5 × 10⁶ cells) were placed in 25-mm culture dishes, and 0.1-ml aliquots of the washed Rhizobium cultures (2.6 × 10⁸ cells) were added. To examine the inhibition of Rhizobium binding by saccharides, a final concentration of 0.1 M of different saccharides in 1B5C medium was added to the cell suspension. Similarly, inhibition studies were also carried out using various concentrations of rabbit anti–seed SBA, normal rabbit immunoglobulin, or rabbit anti–cell wall fragments.

The co-cultures of bacterial and soybean cells were incubated at 26°C for 2 h or 24 h in the dark without shaking. At the end of the incubation, the cell suspensions were transferred to polystyrene tubes. The SB-1 cells were washed three times by centrifugation (460 g for 4 min) with 2 ml of 1B5C medium to remove unbound bacteria. The binding of Rhizobium to SB-1 cells was observed with a Leitz microscope with phase-contrast optics.

## Histological Studies of SB-1 Callus Infected with Rhizobium

SB-1 callus cultures were grown in 1B5C medium with 0.8% agar. 1 wk after the transfer of the callus to new plates, it was inoculated with 50 μl of various Rhizobium strains (1.3 × 10⁸ cells) that were grown at the mid-exponential phase. After incubation in the dark for 1 wk at 26°C, the callus was transferred to another agar plate containing LNB5 medium with 0.8% agar. The LNB5 medium is the 1B5C medium with the omission of 2,4-dichloro-phenoxyacetic acid and casein hydrolysate. The callus was further cultured for 2 wk; then individual callus was taken out and fixed in FAA fixatives (37% formaldehyde/glacial acetic acid/70% ethanol in ratio of 5:5:90) for 2 d at room temperature. The samples were dehydrated in a series of steps of ethanol and xylene, and then embedded in paraffin. 8-μm thick sections were obtained and stained with Gram stain (22) or hematoxylin-eosin stain (24).

Results

Characterization of Antibodies against Seed SBA

Because the conclusions derived from our studies depended on the specificity of the rabbit anti–seed SBA antibodies used, extensive efforts were devoted to the characterization of the purity of the antigen used for immunization and of the specificity of the resulting antiserum. Seed SBA was isolated by affinity chromatography on Gal-Sepharose columns (1). On SDS PAGE under reducing conditions, this purified preparation yielded a single band (M_r ~30,000) (Fig. 1 A, lane 2), corresponding to the molecular weight reported for the polypeptide subunit of seed SBA (23).

Rabbit antiserum was raised against this highly purified preparation of seed SBA. When extracts of soybean seeds were analyzed by SDS PAGE and immunoblotting with rabbit anti–seed SBA, one major polypeptide band (M_r ~30,000) was observed (Fig. 1 B, lane 1). There was also a minor band (M_r ~60,000), which accounted for no more than 1% of the total material (Fig. 1 B, lane 1). These two bands were the only polypeptides detected by the antibody, among a host of many other polypeptides observed by Coomassie Blue staining (Fig. 1 A, lane 1). Similar results were obtained when purified seed SBA, the antigen used for immunization, was subjected to parallel immunoblotting
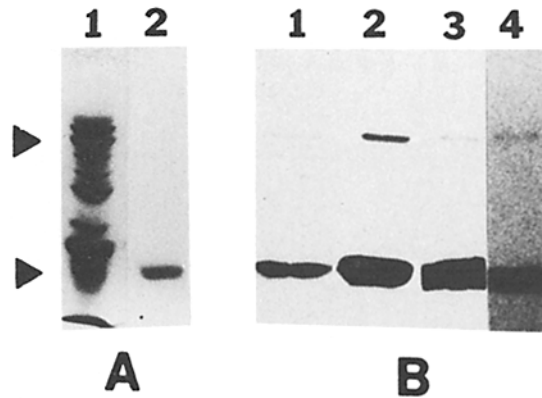

Figure 1. SDS PAGE analysis of extracts of soybean seeds, purified seed SBA, and SB-1 lectin. (A) Polypeptides of the SDS gels were revealed by Coomassie Blue staining. Lane 1, extract of soybean seeds (~100 μg total protein); lane 2, purified seed SBA (10 μg). (B) Polypeptides of the SDS gel were transferred to nitrocellulose paper and immunoblotted with rabbit anti–seed SBA and horseradish peroxidase–conjugated goat anti–rabbit immunoglobulin. Lane 1, extract of soybean seeds; lane 2, purified seed SBA; lane 4, SB-1 lectin derived from the cell wall of SB-1 cells (~20 g) purified by affinity chromatography on Gal-Sepharose column. In lane 3, purified seed SBA (10 μg) was subjected to SDS PAGE and immunoblotted with monospecific antibodies directed against the M_r 30,000 polypeptide observed in lane 2. The arrows on the left indicate the positions of migration of polypeptides of M_r 30,000 and M_r 60,000, relative to known molecular weight markers.

analysis (Fig. 1 B, lane 2). No polypeptide was observed when preimmune serum was used for such an analysis.

The position of migration of the predominant band (at 30 kD) in the immunoblots (Fig. 1 B, lanes 1 and 2) corresponded to the subunit molecular weight of seed SBA (M_r ~30,000). To test whether antibodies that bound to the 30-kD band also recognized the 60-kD polypeptide, the following experiment was performed. First, seed SBA was subjected to electrophoresis and transferred to nitrocellulose paper. The paper was incubated with rabbit anti–seed SBA. After washing to remove unbound antibodies, the region of the nitrocellulose paper corresponding to the 30-kD polypeptide was excised and those antibodies that bound the 30-kD polypeptide were re-eluted from the nitrocellulose strip. Finally, these monospecific antibodies directed against the 30-kD material were used to immunoblot another sample of seed SBA after SDS PAGE. The results showed that antibodies bound and eluted from the 30-kD region of the original gel recognized both the 30- and the 60-kD polypeptides (Fig. 1 B, lane 3).

We have also carried out comparative peptide mapping analysis on the 30- and 60-kD polypeptide bands of seed SBA. Limited digestion with V-8 protease of the two polypeptide bands yielded identical peptide maps. Together with the immunoblotting results, these data strongly suggest that the 60-kD polypeptide is a dimeric form of the SBA subunit. More importantly, it does not appear that the 60-kD polypeptide was an irrelevant protein contaminating the SBA preparation. We concluded from these series of experiments that the seed SBA preparation used as immunogen was pure and that the rabbit anti–seed SBA antibody was highly specific for the lectin. This conclusion forms the basis for subsequent studies reported in this paper.

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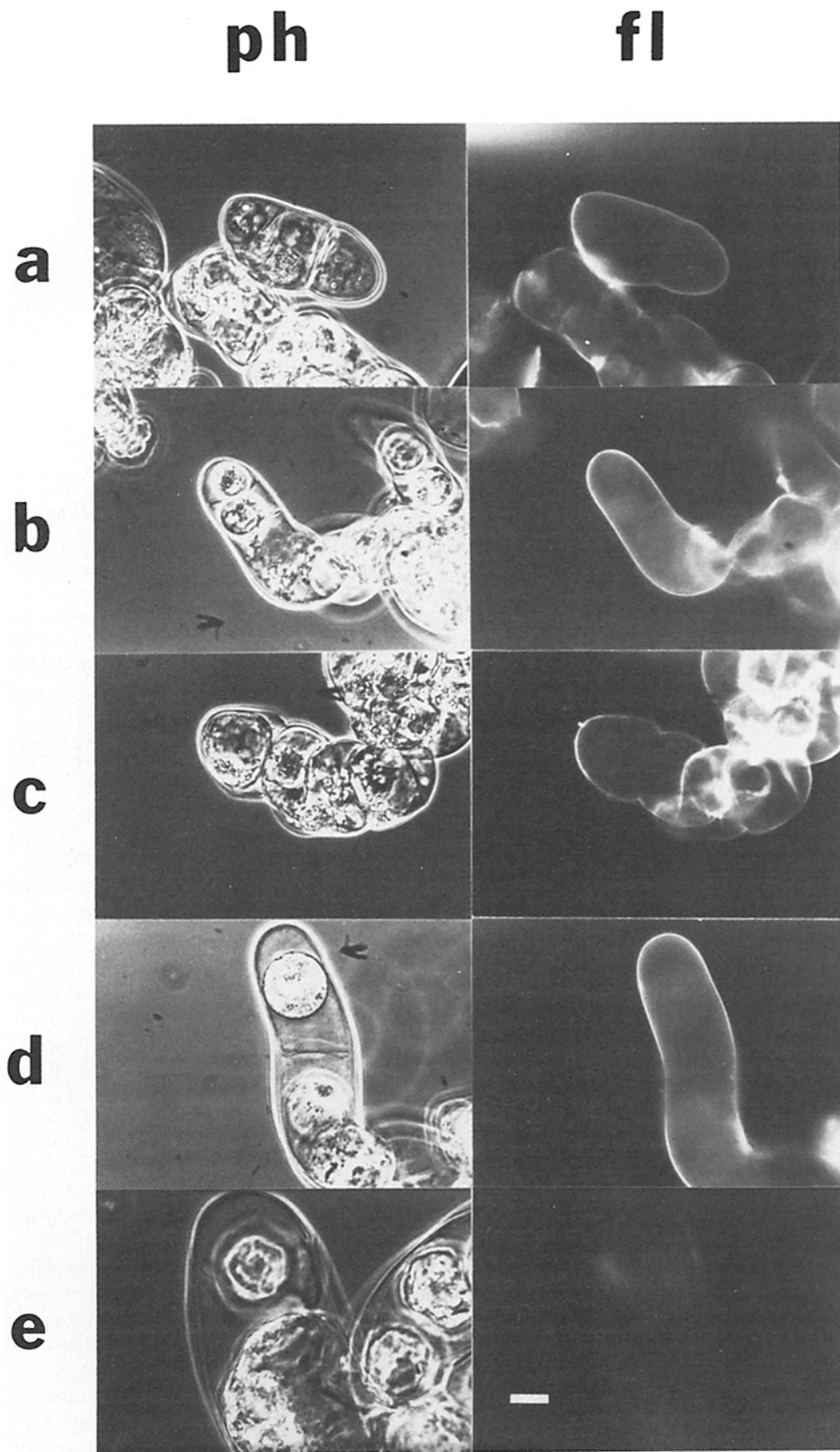
Figure 2. Fluorescence staining patterns of SB-1 cells treated for 1 h at 4°C with rabbit anti–seed SBA (30 µg/ml) or normal rabbit immunoglobulin (30 µg/ml), followed by fluorescein-conjugated goat anti– rabbit immunoglobulin (1:100 dilution; 30 min at 4°C). (*a*) SB-1 cells treated with rabbit anti–seed SBA; (*b*) SB-1 cells washed with Gal (0.1 M), then treated with rabbit anti–seed SBA; (*c*) SB-1 cells washed with GalNAc (0.1 M), then treated with rabbit anti–seed SBA; (*d*) SB-1 cells washed with Glc (0.1 M), then treated with rabbit anti–seed SBA; (*e*) SB-1 cells treated with normal rabbit immunoglobulin. *ph*, phase contrast microscopy; *fl*, fluorescence microscopy. Bar, 5 µm.
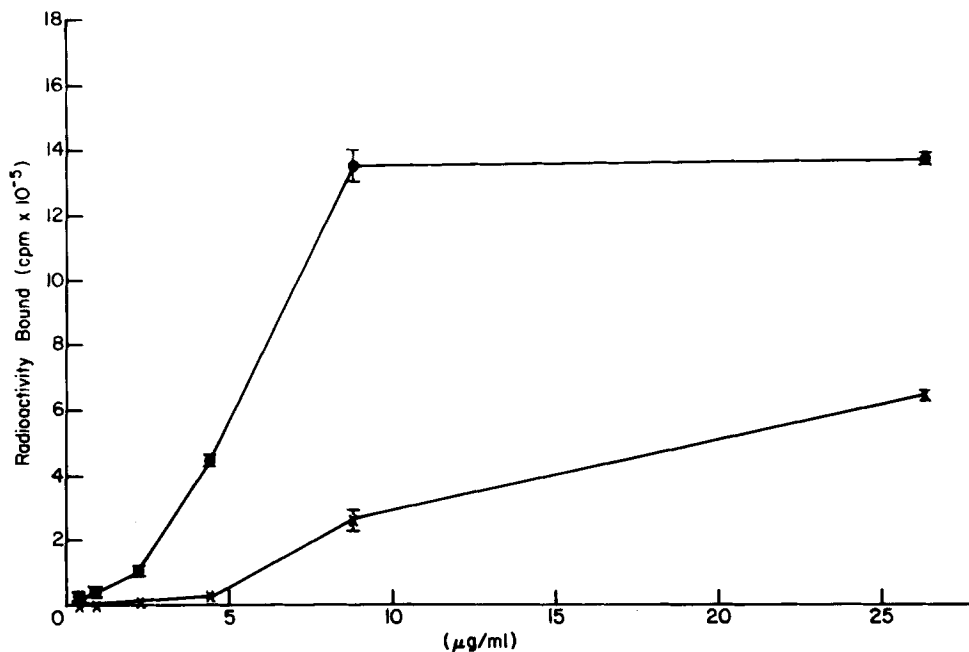
Figure 3. Dose-response curve for the binding of ^{125}I-labeled rabbit anti–seed SBA to SB-1 cells. The binding studies were carried out with 10^6 cells at 4°C for 2 h, as detailed in Materials and Methods. ●, ^{125}I-labeled rabbit anti–seed SBA (1.5×10^6 cpm/μg); x, ^{125}I-labeled normal rabbit immunoglobulin (1.4×10^6 cpm/μg). Data points are the averages of triplicate determinations.

Binding of Antibodies Directed against Seed SBA to SB-1 Cells

Incubation of SB-1 cells with rabbit anti–seed SBA resulted in the binding of the antibodies, as indicated by staining with fluorescein-derivatized goat antibodies directed against rabbit immunoglobulin (Fig. 2 *a*). The fluorescence was localized around the outer periphery of individual cells, suggesting that the antibodies were bound to the outer surface. Parallel incubations of SB-1 cells with preimmune rabbit immunoglobulin, followed by fluorescein-labeled goat anti-rabbit immunoglobulin, failed to yield the same bright staining (Fig. 2 *e*).

A similar conclusion can be derived from studies in the binding of ^{125}I-labeled rabbit anti–seed SBA. In the experiments shown in Fig. 3, monospecific affinity-purified rabbit antibodies directed against seed SBA were labeled with ^{125}I and used in the binding studies. The binding of rabbit anti–seed SBA to SB-1 cells was concentration dependent. The binding curve saturated at a concentration of ~10 μg/ml, suggesting that there was a finite number of antigenic sites exposed at the outer surface (Fig. 3). These results suggest that a molecule, immunologically cross-reactive with seed SBA, was present on the cell wall of SB-1 cells.

When the binding studies were carried out on SB-1 cells preincubated with saccharides such as Gal and GalNAc (0.1 M), neither the indirect immunofluorescence (Fig. 2, *b* and *c*) nor the radiolabeled binding results (Table I) were affected, qualitatively or quantitatively. Similarly, preincubation of SB-1 cells with the saccharide Glc also failed to change the results (Fig. 2 *d* and Table I). These data indicate that the molecule on the cell surface that binds anti–seed SBA was not anchored via its carbohydrate-binding properties.

Isolation of SB-1 Lectin after Cell Wall Digestion

SB-1 cells were digested with cellulase and pectinase to remove cell wall material. The digestion mixture was subjected

to affinity chromatography on a Gal-Sepharose column. The bound material was eluted with 0.2 M Gal and, upon SDS PAGE and immunoblotting, yielded a predominant band (M_r ~30,000) and a minor band (M_r ~60,000) (Fig. 1 *B*, lane *4*). This pattern was identical to that obtained on immunoblots of seed SBA (Fig. 1 *B*, lane *2*). Essentially the same results were obtained when the digestion mixture was subjected to affinity chromatography on columns derivatized with rabbit anti–seed SBA. The immunoreactive material yielded a predominant polypeptide band (M_r ~30,000) and a minor component (M_r ~60,000).

We shall designate this affinity-purified material as SB-1 lectin. Preliminary studies indicate that SB-1 lectin is similar if not identical to seed SBA when compared by gel filtration under nondenaturing conditions and by peptide mapping analysis. These results suggest that SB-1 cells produce an endogenous lectin that binds to galactose-containing glycoconjugates and that the SB-1 lectin may be on the cell wall and could be released upon degradation of the wall. This conclusion is consistent with our observations on the immunofluorescence staining of the cell wall with rabbit anti–seed SBA.

Table I. Binding of ^{125}I-labeled Rabbit Anti–Seed SBA to SB-1 Cells and Protoplasts Prewashed with Various Saccharides*

Treatment	Cells	Protoplast
Control	100 ± 11	100 ± 6
Glc (0.1 M)	87 ± 3	102 ± 8
Gal (0.1 M)	89 ± 6	107 ± 7
GalNAc (0.1 M)	80 ± 5	100 ± 9

* The binding experiments were carried out with 10^6 cells or protoplasts at 4°C for 2 h, as described in Materials and Methods. The data represent percent specific binding (binding observed for ^{125}I-labeled rabbit anti–seed SBA minus the binding observed for ^{125}I-labeled normal rabbit immunoglobulin of the same specific activity and concentration). Averages of triplicate determinations ± standard error are shown for each ligand.
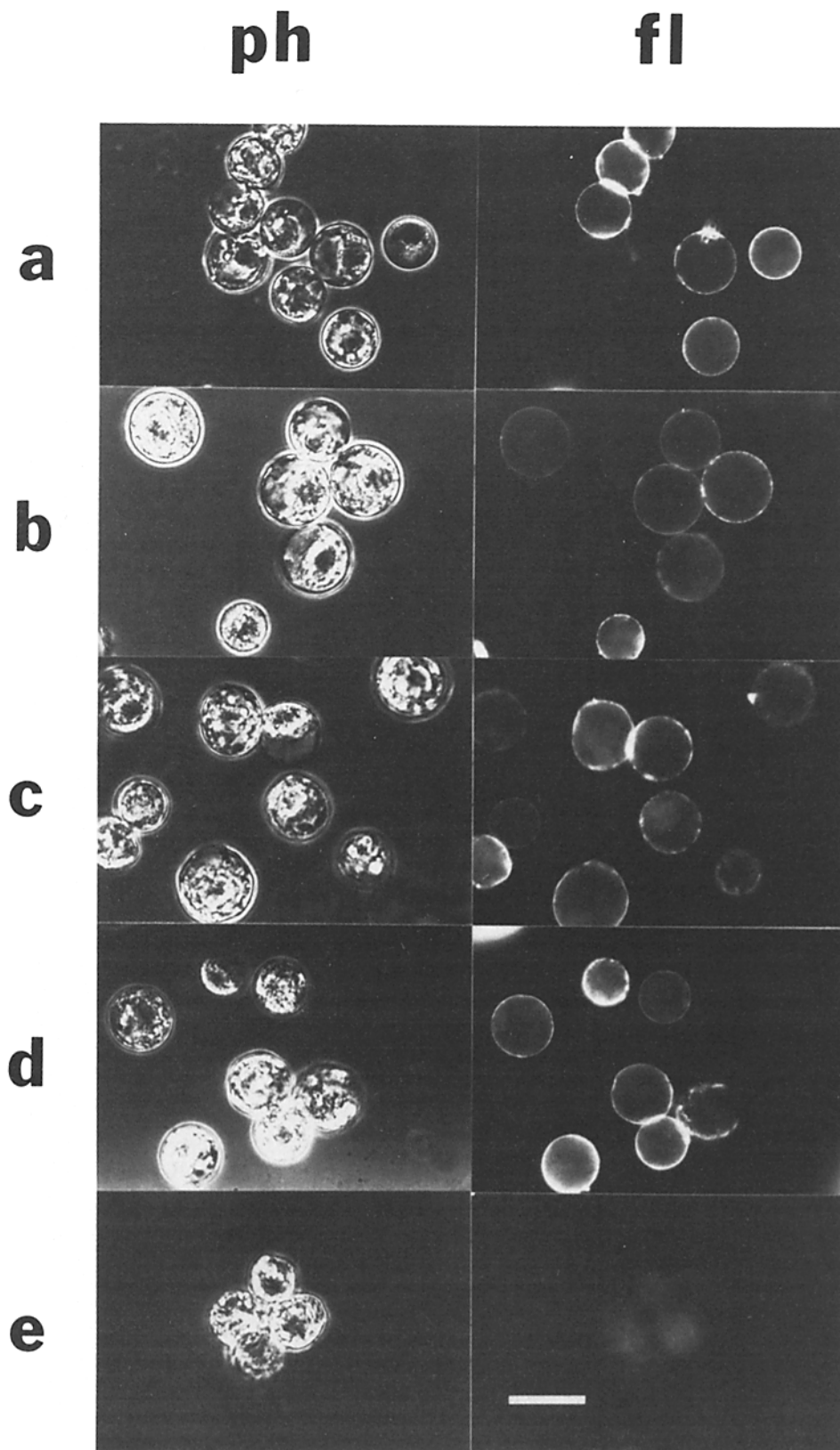
Figure 4. Fluorescence staining patterns of protoplasts derived from SB-1 cells treated for 1 h at 4°C with rabbit anti–seed SBA (30 μg/ml) or normal rabbit immunoglobulin (30 μg/ml), followed by fluorescein-conjugated goat anti–rabbit immunoglobulin (1:100 dilution; 30 min at 4°C). (*a*) Protoplasts treated with rabbit anti–seed SBA; (*b*) protoplasts washed with Gal (0.1 M), then treated with rabbit anti–seed SBA; (*c*) protoplasts washed with GalNAc (0.1 M), then treated with rabbit anti–seed SBA; (*d*) protoplasts washed with Glc (0.1 M), then treated with rabbit anti–seed SBA; (*e*) protoplasts treated with normal rabbit immunoglobulin. *ph*, phase-contrast microscopy; *fl*, fluorescence microscopy. Bar, 5 μm.
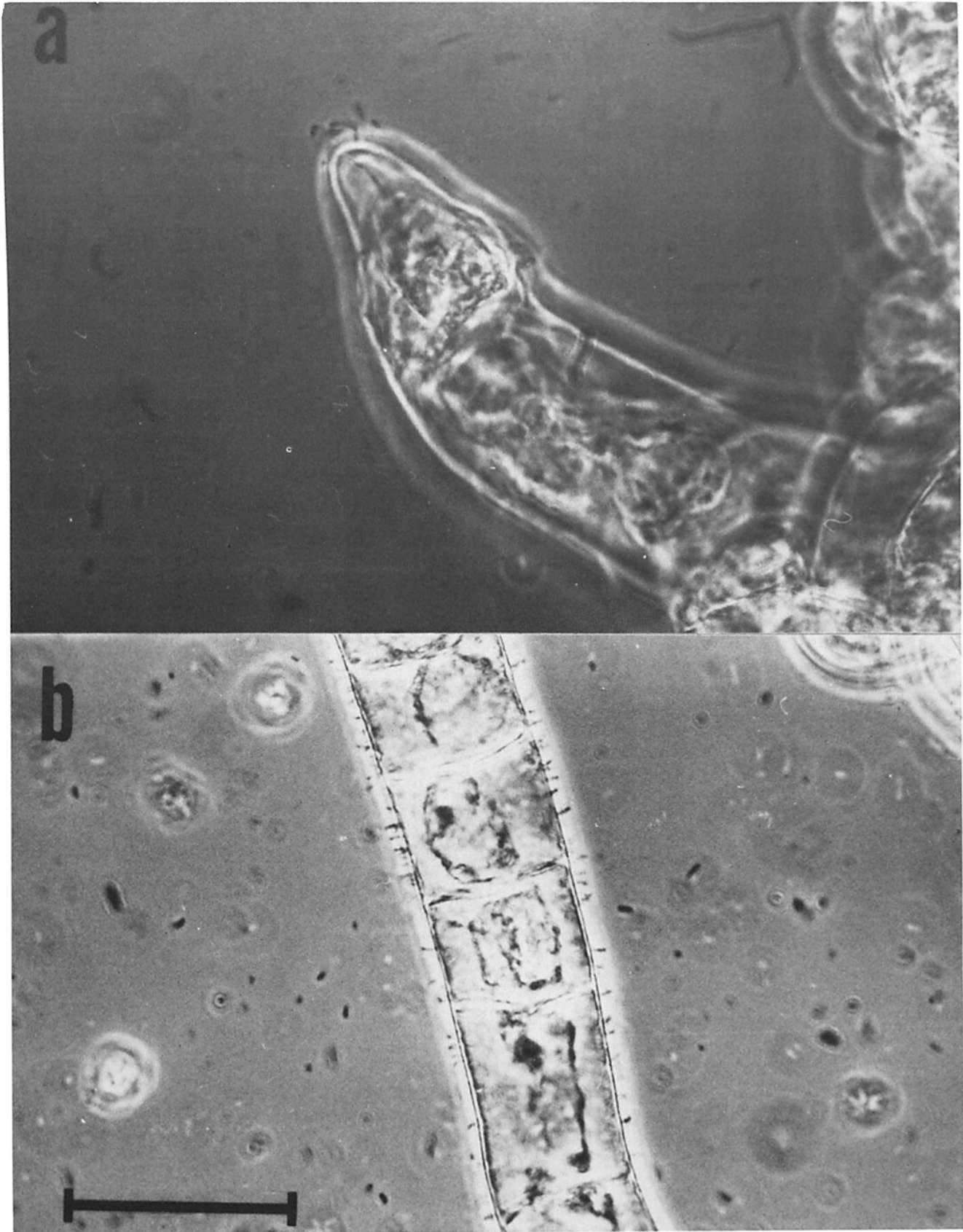
Figure 5. Representative photomicrographs showing the adhesion of *Rhizobium japonicum* (R110d) to SB-1 cells after (*a*) 2 h and (*b*) 24 h of co-culture at 26°C in the dark. Bar, 10 μm.

## Binding of Antibodies Directed against Seed SBA to Protoplasts

Rabbit antibodies directed against seed SBA also bind to protoplasts derived from SB-1 cells. Indirect immunofluorescence revealed ring-like staining, outlining the periphery of the cell and characteristic of surface staining patterns obtained with other spherical objects (Fig. 4 a). This suggests that the antigenic determinant recognized by rabbit anti–seed SBA is diffusely distributed on the plasma membrane. Preincubation with saccharides failed to alter the staining pattern (Fig. 4, b–d). Preimmune rabbit immunoglobulin yielded little or weak staining (Fig. 4 e). Similar results were obtained using the radiolabeled antibody probes. In these respects, the molecule immunologically cross-reactive with seed SBA on the plasma membrane appears to be very similar to that found on the outside of the cell wall (see below, however, for possible differences in Rhizobium-binding properties).

Binding of Rhizobium to SB-1 Cells

When SB-1 cells were mixed with Rhizobium japonicum (R110d) at 26°C for several hours, washed, and sampled under a microscope, the bacteria adhered to certain soybean cells (Fig. 5). Initially, there was little bacterial binding and the binding was limited to the tips of some plant cells. A representative photomicrograph, taken after 2 h of co-culture, is shown in Fig. 5 a. Between 12 and 24 h, however, there appeared to be a sorting out process. When the co-culture of SB-1 cells and Rhizobium was sampled after 24 h, a striking "polar" mode of binding was observed; the Rhizobium adhered to the plant cells in an "end-to-end" fashion (Fig. 5 b).

To test whether this interaction between Rhizobium and SB-1 cells leads to penetration and infection by the bacteria into the soybean cell, we carried out histological staining on SB-1 cells in callus culture that had been incubated with Rhizobium for 3 wk. Using the Gram stain to reveal the presence of bacteria, we observed staining within the cell wall of certain cells (indicated by the dark arrow in Fig. 6 a), suggesting bacterial infection of these cells. In addition, there was also staining in the interstitial spaces between cells (indicated by the open arrow in Fig. 6 a). These observations, particularly the presence of bacteria in areas between adjacent cells, are reminiscent of similar observations on
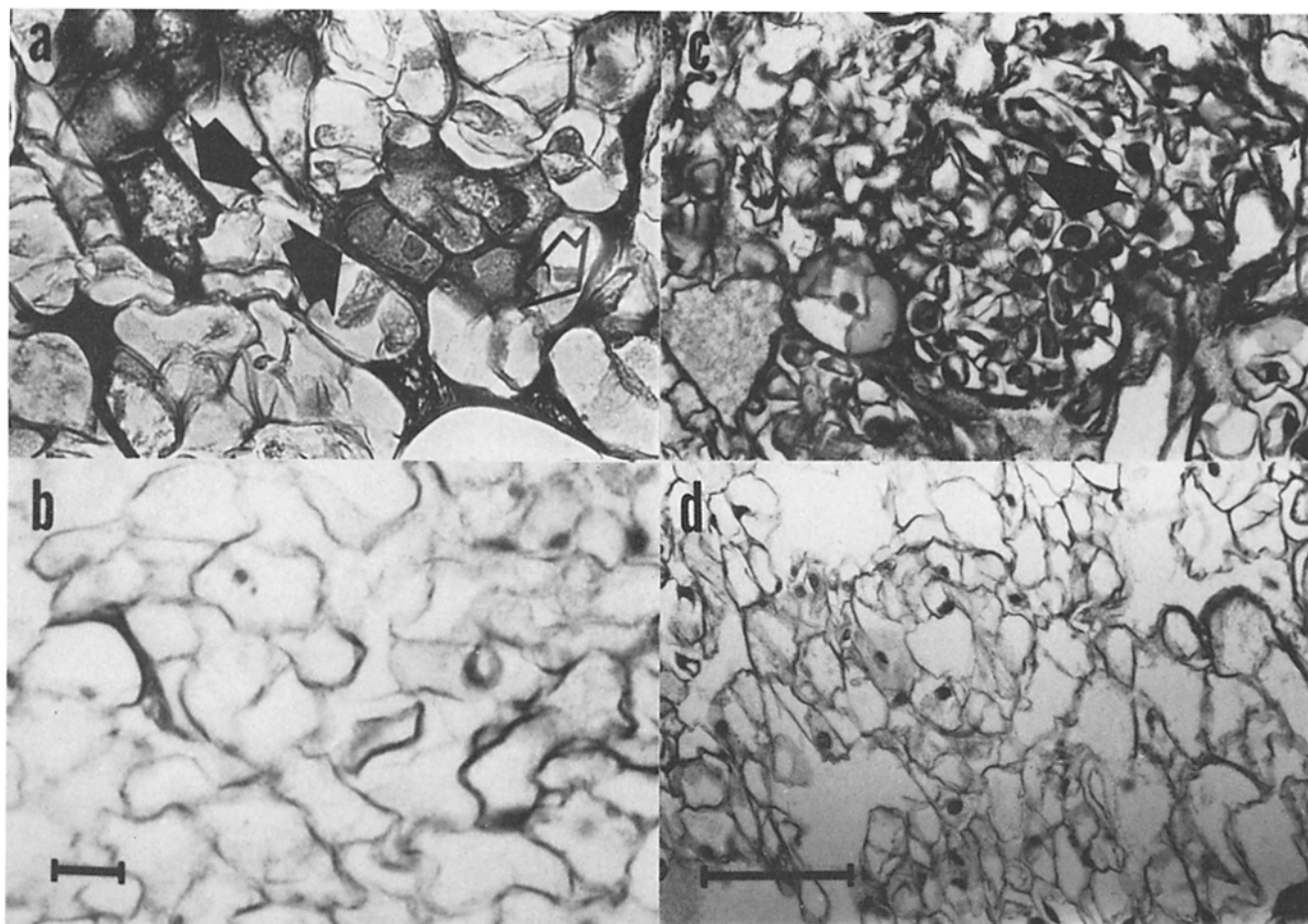
Figure 6. Histological staining of sections derived from callus cultures of SB-1 cells with and without Rhizobium japonicum (R110d). The SB-1 callus was cultured with the bacteria for 3 wk, fixed, sectioned, and stained as described in Materials and Methods. (a) SB-1 callus culture plus Rhizobium stained with Gram stain. The black arrows point to infected cells, containing bacteria within the cell wall. The open arrow shows bacteria in the interstitial space between cells, mimicking a pseudo-infection thread. (b) Control SB-1 callus culture without Rhizobium stained with Gram stain. Bar, 2.5 μm. (c) SB-1 callus culture plus Rhizobium stained with hematoxylin-eosin. The black arrow highlights a focal region of proliferative cells. (d) SB-1 callus culture without Rhizobium stained with hematoxylin-eosin. Bar, 10 μm.

pseudo-infection threads in the establishment of *Rhizobium*-soybean symbiosis (18). Control sections, derived from cultures without *Rhizobium*, failed to show bacterial staining (Fig. 6 *b*).

We have also stained sections of the *Rhizobium*-SB-1 callus co-culture with hematoxylin-eosin. These sections showed the positions of the nucleus and cytoplasm of the plant cell instead of the bacteria. In cultures containing *Rhizobium*,

there were focal regions containing many cells stained with the reagent, revealing prominent nuclei (Fig. 6 *c*). In contrast, control sections contained many areas that were not stained, most probably because these areas of the cell were filled with vacuoles (Fig. 6 *d*). These observations are similar to those reported previously on the in vivo infection of soybean roots by *Rhizobium* (2), in which the infection process stimulated cell division.
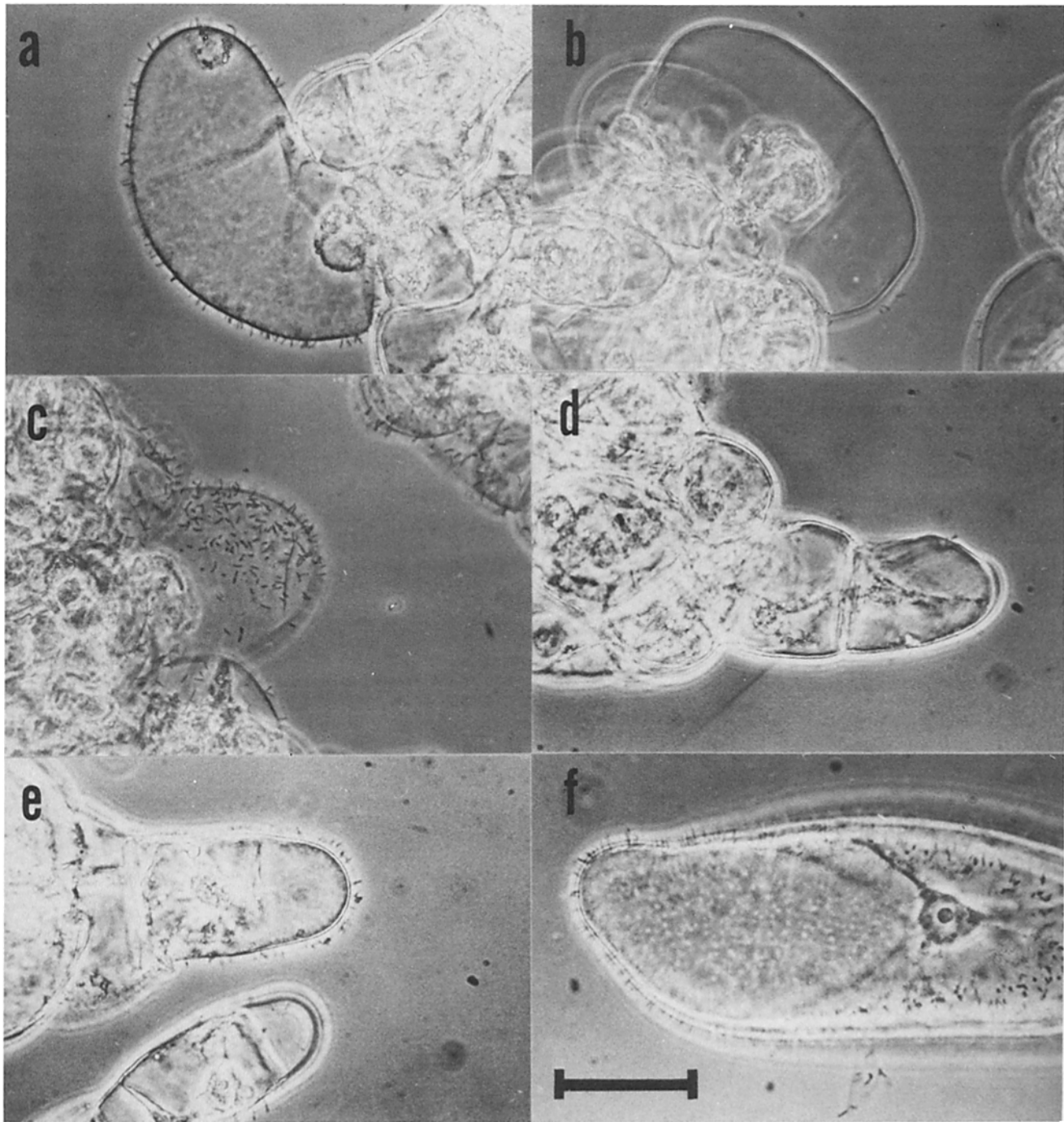

Figure 7. Representative photographs showing the adhesion of *Rhizobium japonicum* (R110d) to SB-1 cells after 24 h of co-culture at 26°C in the dark. (*a*) Co-culture; (*b*) co-culture in the presence of Gal (0.1 M); (*c*) co-culture in the presence of Glc (0.1 M); (*d*) co-culture in the presence of rabbit anti–seed SBA (10 µg/ml); (*e*) co-culture in the presence of normal rabbit immunoglobulin (1 mg/ml); and (*f*) co-culture in the presence of rabbit anti–cell wall fragments (1 mg/ml). Bar, 10 µm.

Evidence for Specificity and Role of Lectin in Rhizobium Binding to SB-1 Cells

Several aspects of the specificity of the binding interaction between Rhizobium and SB-1 cells were checked. First, the inclusion of certain saccharides such as Gal during the co-culture inhibited the binding of the Rhizobium to SB-1 cells when the observed polar adherence was assayed at 24 h (Fig. 7, a and b). This inhibition was observed at a Gal concentration as low as 3 mM. Similar results were observed with the disaccharide lactose (Table II). In contrast, other saccharide epimers of Gal, such as Glc (0.2 M), failed to yield the same inhibitory effect (Fig. 7 c). Melibiose, the α anomer of lactose, did not show inhibition. GalNAc was also not inhibitory at the concentration tested (Table II). These results raise the possibility that the adhesion of Rhizobium to SB-1 cells may be mediated via a highly specific carbohydrate recognition system.

Second, since we have identified on the cell wall and plasma membrane of the SB-1 cells a lectin that is specific for galactose residues, it was of interest to test whether antibodies reactive against the cell wall lectin could block Rhizobium adhesion. We found that inclusion of rabbit anti-seed SBA (10 μg/ml) during the co-culture inhibited the polar binding of the bacteria to the SB-1 cells (Fig. 7 d). Normal rabbit immunoglobulin did not yield the same effect (Fig. 7 e).

We also wished to test whether any ligand bound to the cell wall of SB-1 cells would block Rhizobium adhesion. To accomplish this, we took advantage of the availability of rabbit anti-cell wall fragments. This immunoglobulin fraction showed immunofluorescence staining of both intact SB-1 cells and the fraction containing cell wall fragments, but it did not yield any positive reaction with seed SBA or SB-1 lectin on immunoblots. More importantly, the binding of this immunoglobulin on the outer surface of SB-1 cells did not inhibit Rhizobium binding (Fig. 7 f).

These results provide strong evidence for the specificity and the role of the SB-1 lectin in mediating the initial recognition and adhesion between the Rhizobium and SB-1 cells. However, it should be noted that not all of the soybean cells bound Rhizobium. For example, Fig. 7 a shows one cell with many bacteria bound, but several adjacent cells devoid of any Rhizobium. In addition, we also found that Rhizobium did not bind to protoplasts derived from SB-1 cells after cell wall

removal. Therefore, even though the plasma membrane of SB-1 protoplasts contained a lectin reactive with rabbit anti-seed SBA, no binding of Rhizobium was observed.

Correlation between Rhizobium Binding and Establishment of In Vivo Symbiosis

The polar binding of bacteria to the SB-1 cells was also specific in terms of the bacterial cells used in the co-culture (Table III). Rhizobium japonicum bound specifically but Escherichia coli did not. Moreover, the binding was restricted to Rhizobium japonicum and Rhizobium fredii, two strains of bacteria that normally infect soybean roots to form a nitrogen-fixing symbiosis. In contrast, Rhizobium meliloti, Rhizobium trifolii, and Rhizobium leguminosarum did not bind to the SB-1 cells.

Discussion

The data documented in the present study indicate: (a) Incubation of Rhizobium with a cultured cell line derived from roots of Glycine max (SB-1) results in specific adhesion of the bacteria to the plant cell. (b) This binding interaction appears to be mediated via carbohydrate recognition, since Gal can inhibit the heterotypic adhesion whereas Glc failed to inhibit. (c) One likely candidate that may mediate such an interaction is a lectin identified on the cell wall and plasma membrane of the SB-1 cells. This notion is supported by the observation that rabbit anti-seed SBA blocked the Rhizobium-soybean cell adhesion, whereas control rabbit immunoglobulin did not.

These results are consistent with the lectin recognition hypothesis that suggests carbohydrate recognition as a basis for determining legume host-bacterial symbiont interactions (6, 15, 20). This hypothesis has been supported by experiments carried out in the soybean system (2, 3, 13, 14, 35) and in the clover system (10). There are, however, a number of experiments from various laboratories arguing against the acceptance of the hypothesis that lectins play a specific and indispensable role in legume-Rhizobium symbiosis; in the case of soybeans, this viewpoint has been put forth succinctly by Pueppke (29). In light of these circumstances, it is important to discuss our data on the SB-1-Rhizobium interaction with respect to the following key points.

First, we have obtained definitive evidence for the presence of a lectin in the SB-1 cells. This endogenously produced lectin has been purified to apparent homogeneity on the basis of its carbohydrate-binding activity. Immunofluorescence and binding studies carried out with ^{125}I-labeled antibodies indicate that the lectin is found on the cell wall. Treatment of SB-1 cells with the haptens for seed SBA, Gal,

Table II. Saccharide Inhibition of Rhizobium japonicum Binding to SB-1 Cells

Saccharide*	Inhibition of polar binding to SB-1 cells
Control	−
Galactose‡	+
N-acetyl-galactosamine	−
Lactose‡	+
Galacturonic acid‡	+
Gluconic acid	−
Mannose	−
Glucose	−
Melibiose	−
Glucuronic acid	−
Xylose	−

* Saccharide concentrations varied from 3 mM to 0.2 M.
‡ At a saccharide concentration ≥3 mM, polar binding of Rhizobium to SB-1 cells was inhibited.

Table III. Correlation between Bacterial Binding and Symbiotic Infection

Bacterium	Normal host	Polar binding to SB-1 cells
E. coli	?	−
R. japonicum R110d	Soybean	+
R. fredii PRC 205 str	Soybean	+
R. meliloti 102F28	Alfalfa	−
R. trifolii 0403	Clover	−
R. leguminosarum 128C56	Pea	−

and GalNAc failed to remove the soybean lectin from the cell wall. This implies that the lectin may be anchored on the cell wall with its carbohydrate-binding sites unoccupied and therefore can mediate recognition and binding of external ligands (e.g., *Rhizobium*). Therefore, the requirement for the presence of lectin molecule at the proximal point of interaction has been fulfilled.

Second, it should be noted that the mere presence of the lectin is not sufficient for *Rhizobium* binding. Two observations make this point particularly clear. The lectin of SB-1 cells is found on the cell wall of all cells examined by immunofluorescence. Yet, only certain cells out of a given population have *Rhizobium* adsorbed on them after coculture of the plant cells and bacteria. This may be related to the growth phase of the SB-1 cells in culture or other phenomena associated with transient susceptibility of root cells to be nodulated by *Rhizobium* in vivo (4). In addition, protoplasts also have SB-1 lectin exposed outside the plasma membrane but these protoplasts do not bind *Rhizobium* at all under conditions used to assay the adhesion of the bacteria to SB-1 cells. These results suggest that lectin–carbohydrate interactions may be a necessary but not sufficient condition for adhesion of the cells. A requirement for dual recognition (involving another set of complementary molecules) has been persuasively demonstrated in the interaction between lymphoid cells and target cells bearing foreign antigens (39).

Third, it is important to realize that lectin–carbohydrate binding need not be the only, or even the main, determinant of specificity in soybean root cell–*Rhizobium* interactions. The notion of dual recognition, invoking other sets of complementary molecules, is consistent with the less absolute lectin recognition hypothesis. In any case, the demonstration of saccharide and antibody specificity in blocking *Rhizobium* adhesion to SB-1 cells strongly suggest that at least one required component is a carbohydrate-binding protein. In this connection, it should be noted that GalNAc, a known hapten for seed SBA (23), did not inhibit *Rhizobium* adhesion to SB-1 cells. This may reflect a difference between SB-1 lectin and seed SBA. Alternatively, it may reflect the fact that the lectin anchored on the cell wall does not bind GalNAc.

Because our studies have been carried out in a defined cell culture system, one issue is whether this *Rhizobium*-SB-1 cell binding is relevant to the in vivo symbiosis. Several phenomenological observations suggest that our system mimics at least the early phase of the process of nodule formation in soybean roots. First, the binding of *Rhizobium* is polar as had been observed in a number of systems of *Rhizobium* binding to root cells (7, 11, 37). Second, there is preliminary evidence, based on histological staining, for the presence of bacteria in the interstitial spaces mimicking a pseudo-infection thread (18). The staining with hematoxylin and eosin also suggest an increase in the size of the nucleus and possibly cell division (2). These observations at the light microscope level must now be extended to the ultrastructural level to confirm that the *Rhizobium* initially bound to SB-1 cells actually penetrate and infect the target cells. Finally, correlative studies between *Rhizobium* binding to SB-1 cells and establishment of in vivo symbiosis indicate the specificities of the *Rhizobium* strains and their hosts.

There have been several previous reports on the binding of *Rhizobium* to cultured cells derived from callus of soybean roots (8, 16, 18, 26, 27, 31, 32). In some of these systems, the

interaction of *Rhizobium* with the soybean cells ultimately led to infection of the plant cell and the generation of a nitrogen-fixing symbiosis, as characterized by ultrastructural studies and enzymatic assays. It remains to be demonstrated that our present *Rhizobium* adhesion to SB-1 cells will lead to a symbiosis and activation of nitrogenase.

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