Dual Binding Sites for Peanut Lectin on Rhizobia

By A. A. BHAGWAT AND JOSEPH THOMAS*

Biology and Agriculture Division, Bhabha Atomic Research Centre,
Trombay, Bombay 400 085, India

(Received 13 March 1979; revised 23 August 1979)

Purified peanut lectin (peanut agglutinin, PNA) labelled with fluorescein isothiocyanate (FITC-PNA) or iodine-125 (^{125}I -PNA) bound to *Rhizobium* B. TG-3 and *Rhizobium* 5a, which nodulate peanut, but did not bind to *R. japonicum* or *R. meliloti*, which do not nodulate peanut, or to the non-nitrogen-fixing bacteria *Escherichia coli* and *Bacillus subtilis*. Washing the rhizobia in phosphate-buffered saline markedly decreased PNA binding. The decrease in binding could be correlated with removal of exopolysaccharides during washing. Dialysed culture filtrate could be labelled with ^{125}I -PNA and the radioactivity was recovered in an acetone-precipitable exopolysaccharide fraction, showing that the lectin complexed with exopolysaccharides. Residual binding which remained after washing could not be removed by further washing. Moreover, rhizobia could be labelled with ^{125}I -PNA after removal of exopolysaccharides and the radioactivity was recovered by extraction with hot phenol, indicating that the lectin complexed also with lipopolysaccharide on the bacterial cell wall. Rhizobial lipopolysaccharide not only inhibited haemagglutination by PNA but also dispersed preagglutinated human erythrocytes into free cell suspensions. The results demonstrate the presence of dual binding sites for PNA on rhizobia. Exopolysaccharides are the major sites and the remainder are lipopolysaccharides.

INTRODUCTION

Plant lectins, proteins characterized by their carbohydrate-binding property, are thought to be determinants in the host range specificity of microbial pathogens and of legume–*Rhizobium* symbioses (Bauer, 1977). A number of recent studies have indicated that lectins are probably involved in the recognition of compatible rhizobia by their respective legume hosts. Bohlool & Schmidt (1974) demonstrated that most of the rhizobia which nodulated soybean were also bound by soybean lectin, which had been labelled with fluorescein isothiocyanate (FITC), whereas the lectin did not bind to rhizobia incapable of nodulating soybean. A common antigen has been found (Dazzo & Hubbell, 1975a) on the *Rhizobium trifolii* cell surface and clover root hair, indicating immunological specificity in the legume–*Rhizobium* symbiosis. Trifoliin, an agglutinin purified from white clover by Dazzo *et al.* (1978), showed binding specificity towards its symbiont *R. trifolii*.

Evidence on the exact nature of the receptor sites for lectin binding on the bacterial cell surface has been conflicting. The binding site in the *Rhizobium*–clover symbiosis was shown to be sensitive to 2-deoxyglucose (Dazzo *et al.*, 1976), which specifically inhibited the selective adsorption of *R. trifolii* capsular polysaccharide (exopolysaccharide) to receptor sites on clover root hairs (Dazzo & Brill, 1977). The studies of Bhuvaneswari *et al.* (1977) indicated that in the soybean–*Rhizobium* system the biochemically specific lectin binding sites are transient components of the cell surface of *R. japonicum*. Subsequently, electron microscopic evidence favouring exopolysaccharides of *R. japonicum* as sites of soybean

lectin binding was obtained in the same laboratory (Calvert *et al.*, 1978). Wolpert & Albersheim (1976), who isolated lectins from four different legumes that bound specifically to the respective rhizobia, found that the lectin interaction was with the bacterial lipopolysaccharides rather than with the capsular polysaccharides. Maier & Brill (1978) found a surface antigen associated with the O antigen portion of the lipopolysaccharide in nodulating strains of *R. japonicum* but not in non-nodulating mutants.

Results suggesting lack of specificity in lectin binding have also been obtained (Dazzo & Hubbell, 1975*b*; Chen & Phillips, 1976). Recently, soybean lines lacking lectin have been reported to be effectively nodulated by *R. japonicum* strains (Pull *et al.*, 1978).

The investigations cited above dealt mainly with the legume-*Rhizobium* symbioses in soybean and clover. We have examined the role of lectin in the peanut-*Rhizobium* symbiosis. Our experiments, using FITC- and ^{125}I -labelled peanut agglutinin (PNA) show specificity in PNA binding to its rhizobial symbiont. The results indicate that there are dual binding sites on the *Rhizobium* cell surface, and afford a plausible interpretation for the apparently ambiguous results in the literature about the nature of receptor sites for lectin binding.

METHODS

Organisms. *Rhizobium* B.TG-3 was isolated from nodules of a large pod mutant of *Arachis hypogaea* developed at Trombay (Patil, 1973) called var. TG-3. *Rhizobium* 5a is a peanut-nodulating strain provided by Dr Peter Dart, International Crops Research Institute for the Semi-Arid Tropics, Hyderabad, India. *Escherichia coli* K12 JC9239 was isolated by Mahajan & Datta (1977) and *Bacillus subtilis* BS-40 by Kamat & Kumta (1974) of this Research Centre.

Cultures. Stock cultures of the *Rhizobium* strains were stored at 4 °C on slants of yeast mannitol agar and liquid cultures were grown in the defined medium described by Vincent (1962). The formation of nodules on *Arachis hypogaea* var. TG-3 was used to verify the infectivity of the *Rhizobium* strains. *Escherichia coli* and *Bacillus subtilis* were grown in nutrient broth. Bacterial cell populations were determined using a Petroff-Hauser bacterial counting chamber.

Purification of peanut agglutinin. PNA was purified from seeds of *Arachis hypogaea* var. TG-3. The initial phase of the purification procedure was as described by Lotan *et al.* (1975). The $(\text{NH}_4)_2\text{SO}_4$ precipitated PNA was loaded on a DEAE-cellulose column (Whatman DE-52; 25.0 × 0.85 cm), which had been equilibrated with phosphate buffer (0.05 M, pH 7.2) and was eluted with a linear gradient of NaCl (100 ml, 0 to 1 M) in the same buffer. Fractions eluting at 15.0 mM-NaCl and showing haemagglutination of neuraminidase-treated group B+ red blood cells (Lotan *et al.*, 1975) were pooled (2.5 ml) and lyophilized. The material was then dissolved in phosphate-buffered saline [PBS; 0.05 M-phosphate buffer in 0.9% (w/v) NaCl, pH 7.2] and partitioned on a Sephadex G-150 column (23 × 1.2 cm) by eluting with PBS. Fractions were monitored for absorbance at 280 nm and for haemagglutinating activity. Purified PNA was collected in a single symmetrical peak. The molecular weight of the PNA was determined by comparison with the elution volumes of standard proteins (bovine γ -globulin, bovine serum albumin, ovalbumin, lactoperoxidase, malate dehydrogenase, lactate dehydrogenase, chymotrypsinogen) on Sephadex G-150 (52 × 1.5 cm).

Preparation of FITC-labelled PNA. The method of Udenfriend (1962) was followed in preparing PNA labelled with fluorescein isothiocyanate (FITC-PNA). To 1 mg PNA (obtained by dissolving in PBS the lyophilized material after DEAE-cellulose column chromatography during the PNA purification procedure) in 10 ml 0.05 M-sodium bicarbonate buffer (pH 9.0) containing 0.1 M-D-galactose, 0.05 mg FITC was added and stirred for 2 h at room temperature. The FITC-PNA complex formed was dialysed against 1 l PBS (three changes) at 5 °C and the complex was concentrated by lyophilization.

Preparation of ^{125}I -labelled PNA. Radioactively labelled PNA (^{125}I -PNA) was prepared by the chloramine-T oxidation procedure for labelling proteins (Sela *et al.*, 1971). Carrier-free ^{125}I [1 mCi, sp.act. 14 mCi μg^{-1} (518 MBq μg^{-1}), The Radiochemical Centre, Amersham] was added as NaI to 200 μl PBS containing adequate PNA (5.0 mg ml^{-1} , obtained after the DEAE-cellulose column chromatography stage of the PNA purification procedure). Iodination was initiated by adding 5 μl (25 μg) chloramine-T in water. The reaction, which was allowed to proceed in an ice bath for 60 s, was stopped by adding 10 μl (100 μg) sodium metabisulphite. The iodinated PNA was separated from unreacted and damaged PNA and other proteins by filtration on Sephadex G-150 (23 × 1.2 cm). Fractions showing peak haemagglutinating activity were pooled and the free ^{125}I was removed either by dialysis against PBS or, preferably, by the use of Dowex anion exchange resin (chloride form, 1-X8, 50/100; a few mg of the resin was added to the pooled fraction and after 15 min at 5 °C the resin was removed by centrifugation for 5 min at 5000 g). Absence of free ^{125}I in the ^{125}I -PNA

sample was confirmed by electrophoresis on cellulose acetate strips in 0.05 M-phosphate buffer (pH 8.6). The ^{125}I -PNA was diluted with unlabelled PNA to give solutions (in PBS) containing $200\text{ }\mu\text{g PNA ml}^{-1}$ and having $3 \times 10^5\text{ c.p.m. ml}^{-1}$. The specific activity of the PNA product was $1.43\text{ }\mu\text{Ci mg}^{-1}$.

FITC-PNA binding. FITC-PNA ($25\text{ }\mu\text{g}$) was added to 2×10^7 cells suspended in 1.0 ml PBS and incubated at $25\text{ }^\circ\text{C}$ for 30 min. Cells were pelleted by centrifuging at 12000 g for 15 min and resuspended in PBS. FITC-labelling was examined using a fluorescence microscope (Carl Zeiss, Oberkochen, W. Germany) equipped with phase contrast objectives. The phase objectives helped in confirming fluorescent material as bacteria.

^{125}I -PNA binding to unwashed rhizobia. Rhizobial cultures (64 h-old) were harvested by centrifugation for 10 min at 12000 g and suspended in PBS at $2 \times 10^9\text{ cells ml}^{-1}$. Saturation curves for ^{125}I -PNA binding to rhizobial populations indicated that $10\text{ }\mu\text{g }^{125}\text{I}$ -PNA ml^{-1} was adequate. In the binding experiments reported here, $20\text{ }\mu\text{g }^{125}\text{I}$ -PNA was added to 1 ml of the suspension and incubated for 30 min at $37\text{ }^\circ\text{C}$. The suspension was then centrifuged at $4\text{ }^\circ\text{C}$ (12000 g ; 10 min) and the supernatant was discarded. The radioactivity of the pellet was determined to obtain the initial value for the bound ^{125}I -PNA. The pellet was then washed four times in PBS to determine the release of labelled material into the supernatant. The pooled supernatants and the pellet were counted for radioactivity on a Radioimmune Assay Counter (Electronics Division, Bhabha Atomic Research Centre, Bombay, India) equipped with a sodium iodide-thallium crystal scintillation system.

^{125}I -PNA binding to washed rhizobia. Washed cells were prepared by repeating resuspension and centrifugation (12000 g ; 10 min, $4\text{ }^\circ\text{C}$) in PBS four times. Washed suspensions ($2 \times 10^9\text{ cells ml}^{-1}$) were incubated at $37\text{ }^\circ\text{C}$ with ^{125}I -PNA ($20\text{ }\mu\text{g ml}^{-1}$) for periods ranging from 1 to 60 min followed by centrifugation (12000 g) at $4\text{ }^\circ\text{C}$. The radioactivity of the pellet was determined as described above.

Precipitation of exopolysaccharide- ^{125}I -PNA complex. Suspensions of *Rhizobium* B.TG-3 were centrifuged (12000 g ; 10 min, $4\text{ }^\circ\text{C}$) and the pellets were washed four times by resuspension in PBS followed by centrifugation. The pooled supernatants were vacuum dialysed and then dialysed twice against 1 l PBS. The material after dialysis was designated exopolysaccharide (EXP). The sugar content of EXP was determined by the anthrone test (Ashwell, 1957). To $500\text{ }\mu\text{g}$ (glucose equivalents) EXP in 1 ml PBS, $20\text{ }\mu\text{g }^{125}\text{I}$ -PNA was added and the material was incubated at $37\text{ }^\circ\text{C}$ for 30 min. The polysaccharide-PNA complex was then precipitated in acetone according to the procedure of Sutherland & Wilkinson (1971). Solubility of non-complexed ^{125}I -PNA in acetone was ascertained by the following procedure: $20\text{ }\mu\text{g }^{125}\text{I}$ -PNA ml^{-1} in water was diluted with acetone to give a final concentration of 70% (v/v) acetone. This material was processed as for EXP precipitation. The material was then centrifuged at 12000 g to ascertain the formation of precipitate. In addition, the material was filtered through a Millipore filter ($0.2\text{ }\mu\text{m}$ pore size) and the filter and filtrate were separately examined for radioactivity.

Isolation of lipopolysaccharide- ^{125}I -PNA complex. *Rhizobium* B.TG-3 cultures were washed four times in PBS, resuspended in PBS at $10^{10}\text{ cells ml}^{-1}$ and incubated with $20\text{ }\mu\text{g }^{125}\text{I}$ -PNA ml^{-1} at $37\text{ }^\circ\text{C}$ for 30 min. The bacteria were collected by centrifugation and the lipopolysaccharide (LPS)- ^{125}I -PNA complex was extracted using hot phenol according to the procedure of Maier & Brill (1978). The quantity of LPS- ^{125}I -PNA complex extracted was estimated in the radioimmune assay counter. In some experiments, after phenol extraction the aqueous phase (phenol-free) was lyophilized. The dried material was dissolved in water and placed on to a column of Dowex anion exchange resin (1-X8, 50/100, acetate form). The column was eluted with water to yield LPS material designated LPS fraction A. The column was then eluted with 1 M-NaCl to yield the acidic LPS designated LPS fraction B. The sugar content of the LPS fractions was determined by the anthrone test. The competitive PNA binding property of these fractions was then assessed by their ability to inhibit, as well as reverse, haemagglutination.

Inhibition of haemagglutination by rhizobial lipopolysaccharide. Fresh human blood (type B) from one of us (A.A.B.), collected in the presence of heparin (100 units ml^{-1}), was washed three times in 0.9% (w/v) NaCl and resuspended to give 2% (v/v) blood cell concentration. The suspension was treated with 20 units neuraminidase ml^{-1} for 60 min at $37\text{ }^\circ\text{C}$ and then washed four times with PBS. PNA ($20\text{ }\mu\text{g}$ in 0.2 ml PBS) was incubated for 45 min in the presence of either 100 mM-galactose, lactose, galactosamine, or $30\text{ }\mu\text{g}$ of the A or B fraction of rhizobial LPS. The resulting mixture was stirred with 0.1 ml of the neuraminidase-treated erythrocytes and incubated for 60 min at $37\text{ }^\circ\text{C}$. The suspension was then examined for agglutination. In some experiments, the neuraminidase-treated erythrocytes were preagglutinated by incubation for 45 min with $20\text{ }\mu\text{g PNA ml}^{-1}$. The preagglutinated material (0.2 ml) was then incubated further for 60 min in the presence of $30\text{ }\mu\text{g}$ of the LPS fraction or 100 mM-sugar to test for reversion to free cell suspension.

Fig. 1. Pattern of elution from Sephadex G-150 of ^{125}I -labelling and haemagglutinating activity of partially purified PNA: $\circ$, A_{280} ; $\bullet$, radioactivity; $\triangle$, haemagglutinating activity.

RESULTS AND DISCUSSION

The peanut lectin was eluted as a symmetrical peak from the Sephadex G-150 column (52×1.5 cm). Its molecular weight, estimated from the comparative elution sequence of the PNA with standard proteins (see Methods), was nearly 112000, i.e. close to the value of 110000 previously determined (Lotan *et al.*, 1975) for another peanut variety from data on sedimentation, diffusion and gel filtration.

When the ^{125}I -labelled proteins, obtained by iodination of the active portion separated on the DEAE-cellulose column, were resolved on Sephadex G-150, the haemagglutinating activity was located in a narrow symmetrical peak (Fig. 1). The purified PNA was about 60-fold more active than crude extracts.

Rhizobium strains B.TG-3 and 5a, which nodulate peanut roots, bound both FITC- and ^{125}I -labelled PNA. *Rhizobium japonicum* and *R. meliloti*, which do not nodulate peanut, and the non-nitrogen-fixing bacteria *E. coli* and *B. subtilis*, did not bind the labelled lectin. These results, demonstrating the specificity of PNA binding to the rhizobia capable of nodulating peanut, agree with similar legume-*Rhizobium* specificity found previously for soybean (Bohloul & Schmidt, 1974; Bhuvaneswari *et al.*, 1977) and clover (Dazzo *et al.*, 1978). Only Bhuvaneswari *et al.* (1977) had previously demonstrated specificity using radioactively labelled lectin.

Washing in PBS removed polysaccharides from the cell surface. After four such washings, no more sugar was released. Moreover, when the washed bacterial suspensions were treated for 10 min in boiling water and then centrifuged, the resulting supernatant did not contain sugar, indicating that rhizobial EXP could be completely dissolved from the cell surface by washing in PBS.

A major portion of the bound radioactive lectin on the rhizobia could also be removed by washing the suspensions in PBS (Table 1) indicating the EXP nature of the binding sites. The EXP content of *Rhizobium* B.TG-3 increased with the age of the culture (Fig. 2). After incubation of the EXP with ^{125}I -PNA, most of the added radioactivity could be precipitated with 70% (v/v) acetone showing that the EXP complexed with ^{125}I -PNA. When ^{125}I -PNA was subjected to the same process in the absence of EXP, no radioactive complex could be detected as a precipitate, either in the form of a sediment on centrifugation or on filtration. The amount of ^{125}I -PNA complexed with EXP and the sugar content of the EXP preparation

Table 1. Effect of washing on ^{125}I -PNA binding to rhizobia

Details of experiments are given in Methods. Values in parentheses are percentages with respect to the number of molecules initially bound.

^{125}I -PNA	$10^{-3} \times \text{No. of } ^{125}\text{I}\text{-PNA molecules bound per } \textit{Rhizobium} \text{ cell}$	
	<i>Rhizobium</i> B.TG-3	<i>Rhizobium</i> 5a
Initially bound	34.7 (100)	44.2 (100)
Retained after washing	7.5 (22)	14.7 (33)
Removed by washing	27.7 (80)	28.9 (66)

Fig. 2

Fig. 2. Binding of ^{125}I -labelled PNA to exopolysaccharide (EXP, $\blacksquare$) and lipopolysaccharide (LPS, $\square$) of *Rhizobium* B.TG-3. The EXP content ($\bullet$) and cell numbers ($\circ$) are also shown. All polysaccharide concentrations are expressed as glucose equivalents.

Fig. 3

Fig. 3. Time-course of binding of ^{125}I -labelled PNA to washed suspensions of *Rhizobium* B.TG-3.

followed the same pattern with the age of the culture. These results clearly demonstrate binding of PNA to the exopolysaccharides of the rhizobia.

Although washing in PBS removed most of the bound lectin, a significant amount of the ^{125}I -PNA was still bound to the rhizobia (Table 1). Since washing removed EXP, the remaining activity was probably due to binding of ^{125}I -PNA to LPS. Transfer of EXP-bound ^{125}I -PNA to LPS may occur while LPS is newly exposed during washing. However, at 4°C (the temperature maintained during washing), the risk of re-equilibration of ^{125}I -PNA is minimized. For instance, agglutination of neuraminidase-treated red blood cells took nearly 20 min at 4°C as against 3 min required at 37°C .

The capacity of LPS to bind PNA is clearly demonstrated by the fact that *Rhizobium* B.TG-3 cultures showed significant binding of ^{125}I -PNA even after removal of EXP by four washings in PBS (Fig. 3). The time course showed that added lectin was rapidly bound to the washed bacteria. The bound activity could not be removed by further washing in PBS but was extractable with hot phenol (Fig. 2). The binding of phenol-extractable lectin decreased markedly in old cultures.

LPS extracted with hot phenol, after removal of EXP by washing in PBS, could be separated into two fractions, A and B, using a Dowex column. Fraction A inhibited haemagglutination of human erythrocytes, an effect which was also caused by galactose,

lactose and galactosamine. Moreover, fraction A and the sugars caused unequivocal reversion of preagglutinated erythrocytes into free cell suspensions. Fraction B also inhibited haemagglutination, but was somewhat less effective.

Our results indicate that there are dual binding sites for PNA on the rhizobial cells: the EXP and the LPS. The discrepancies in the earlier literature, assigning either EXP (capsular polysaccharide) (Dazzo & Brill, 1977), LPS (Wolpert & Albersheim, 1976; Maier & Brill, 1978) or a transient component on the rhizobial cell surface (Bhuvaneswari *et al.*, 1977) as the receptor site for lectin binding, may have been due to differences in the experimental conditions employed. Age of culture and conditions for processing the bacterial cultures for assay would greatly influence the results (Fig. 2). Although Bhuvaneswari *et al.* (1977) concluded that soybean lectin binding sites are transient components on the rhizobial cell surface, they found that the soybean lectin binding curves were biphasic and hinted at the possibility that two types of receptor structures might exist on the rhizobial cell surface. Several features of our results are consistent with the presence of dual receptor sites on the rhizobia which nodulate peanut. The quantitative results on the binding of ^{125}I -PNA demonstrate that EXP are the major lectin binding sites on these rhizobia; LPS are probably secondary sites and the observation that lectin binds readily to LPS after removal of EXP may be of ecological significance. EXP are known to be secreted and the quantity of EXP available as receptors on the bacterial cell surface may become negligible depending on the bacterial growth phase and the micro-environment. Under such circumstances LPS may play a major role in the legume-*Rhizobium* recognition process. It may also be that EXP helps in casual binding of the rhizobia to the root wall lectin and that the more specific binding is achieved by LPS.

We are grateful to Dr R. S. Mani of Isotope Group, BARC, for providing the facilities, to Miss Meera Subramanian for the help in preparing ^{125}I -labelled peanut lectin and to Dr R. S. Kamat, Haffkine Institute, Bombay, for facilities and help with fluorescence microscopy.

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