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Serological Identification of Pilus Antigen and Other Protein Antigens of *Bacteroides nodosus* Using Electro-Blot Radioimmunoassay after Electrophoretic Fractionation of the Proteins on Sodium Dodecyl Sulfate Polyacrylamide Gels

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Abstract

Proteins of various strains of *B. nodosus* were fractionated by polyacrylamide gel electrophoresis in buffer containing sodium dodecyl sulfate. Transfer of these proteins to activated paper was carried out electrophoretically (Electro-Blot). Subsequent sequential reaction of these proteins with sera from sheep which had been naturally infected with a particular strain of *B. nodosus* showed that there were antibodies to many (10–15) components. Antibodies to pilus proteins could be recognized but the most predominant antibody in natural infections was to antigens in the region of molecular weight approximately 75 000. Assessment of the paper-bound antigens by successive reactions with antisera from sheep infected with other strains of *B. nodosus* gave a semiquantitative picture of cross-reactions.

Introduction

Interest in the nature of the protective immunogens of *Bacteroides nodosus* has been generated by the demonstration that homologous *B. nodosus* vaccines induced protective and curative effects in sheep vaccinated against footrot (Egerton and Burrell 1970; Egerton and Roberts 1971). A substantial amount of evidence has now accumulated indicating that the major homologous protective immunogen is associated with the bacterial pilus (Stewart 1978*a*; Every 1981; Skerman *et al.* 1981; Stewart *et al.* 1981, 1982; Thorley and Egerton 1981). *B. nodosus* can be classified into serotypes on the basis of specific K antigens (Egerton 1973) which actually represent the pilus (Stewart 1973; Walker *et al.* 1973; Every 1979). A multiple number of serotypes are known to exist (Thorley 1976; Schmitz and Gradin 1980; Claxton 1981).

There are other antigens of *B. nodosus*, shared by at least some serotypes, which have not been studied as intensely as the pili. These include O antigens (Egerton 1973), protease antigens (Egerton and Merritt 1973) and lipopolysaccharide antigens (Stewart 1978*b*).

The variety of serological tests to identify *B. nodosus* antigens which have been used include agglutination, indirect haemagglutination, fluorescent antibody, double immunodiffusion and immunoelectrophoresis.

In the work reported here the Electro-Blot technique has been used in the study of infection of sheep by *B. nosodus* to demonstrate (*a*) that a limited number of the proteins of *B. nodosus* are antigenic in infected sheep, and that those of molecular weight approximately 75 000 are the predominant ones; (*b*) the purity of a preparation

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of pilus antigen used for vaccination studies in sheep; (*c*) that cross-reacting antigens (of the same molecular weight) can be detected by this method with various pilus serotypes; sometimes it is the pilus antigen but at other times it is other antigens. The other serological methods listed above cannot be used to study these aspects of *B. nodosus* infection as well as can the Electro-Blot method because other methods do not distinguish individual antigens.

The Electro-Blot technique recognizes only those antigens which, on transfer from a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel, bind to the solid media (in the present work to APT paper) and regain (or retain) their antigenicity when SDS is removed. However, with increasing use of the method by many workers it has been found that a large number of antigens do, in fact, regain their antigenicity when the denaturing agents SDS and mercaptoethanol are removed. For instance, it has been shown (Anderson *et al.* 1982) that 24 out of 25 proteins in whole plasma were specifically revealed after transfer of whole plasma patterns to nitrocellulose and use of appropriate antiserum; in addition, six previously unidentified proteins were identified for the first time using this technique.

Materials and Methods

Bacteria

Bacteroides nodosus strains 198, 305, 312 and 332 which contain serologically distinct pilus agglutinogens (K antigens) were from the CSIRO Animal Health culture collection. Strains 198, 305, 312 and 332 were grown on trypticase–arginine–serine (TAS) agar (Skerman 1975) plates containing 1·5% (w/v) Bacto agar (Difco) and 0·3% (w/v) elastin particles. Bacteria were washed off the agar surface with sterile saline. The strain 198 cells that were used to prepare pili were grown on TAS agar medium, in an atmosphere of 10% carbon dioxide and 90% hydrogen by volume.

Preparation of Purified Pili

The purified pili from *B. nodosus* strain 198 used in the present work was originally prepared for a pilus dose vaccination experiment. Cells were grown on TAS agar for 3 days and harvested with sterile physiological saline. The cell suspension was blended in a Waring blendor for 1·5 min at maximum speed, the pH adjusted to 4·5, and the suspension left overnight at 4°C. It was then centrifuged at 12000 *g* for 30 min, which removed all the pili from the supernatant as shown by fluorescence microscopy. The deposit containing the pili was then resuspended in phosphate-buffered saline (PBS, pH 7·3) and centrifuged at 14000 *g* for 30 min. The resulting supernatant was free of cells and contained a large amount of pili, as shown by fluorescence microscopy, whereas the deposit contained cells and only a small amount of pili. Precipitation by the addition of 0·1 M $MgCl_2.6H_2O$ was carried out twice and each time the precipitate was dissolved in PBS with the insoluble material being removed by centrifugation. The final pili preparation was exhaustively dialysed against distilled water and its purity was assessed by electron and fluorescence microscopy, and SDS–PAGE.

Preparation of B. nodosus *Suspensions and Pili for SDS–PAGE*

A 2 ml suspension of *B. nodosus* was concentrated by precipitation with 200 µl 1 M sodium acetate at pH 4·5 and centrifugation at 12000 *g* for 15 min. Tris–glycine buffer (200 µl containing 0·1% (w/v) SDS, 0·025 M Tris, 0·192 M glycine, pH 8·3, i.e. Laemmli electrode buffer) was added to this precipitate (or to 200 µg of dried pili) together with 25 µl β-mercaptoethanol, and 20 µl of dilute bromophenol blue in 50% (v/v) glycerol. After heating in a boiling water bath for 3–5 min the solution was analysed by SDS–PAGE.

Fractionation of B. nodosus *Extracts and Purified Pili Preparations by SDS–PAGE*

Fractionation was carried out in 12·5% (w/v) polyacrylamide gels (1·5 mm thick) using SDS in the buffer according to the method of Laemmli (1970) in a Bio-Rad apparatus model 220. Samples

of 5–10 μl were loaded in each 4-mm slot. Low molecular weight standards (Pharmacia, Sweden) were run on the outside wells of the gel for staining with Coomassie Blue. ^{14}C-labelled proteins from Amersham (England) were used as standards when radioautographs were to be obtained after transfer to activated paper.

Preparation of Aminophenylthioether (APT) paper

Preparation of APT paper was done using Whatman No. 54 paper as described by Reiser and Wardale (1981). The derivatized paper was diazotized according to the instructions of Alwine *et al.* (1977).

Transfer of Separated Proteins from SDS–PAGE Gels to Activated Paper and Subsequent Probing for the Presence of B. nodosus *Antigens with Sera from Naturally Infected Sheep*

The electrophoretic transfer of proteins separated by SDS–PAGE was carried out basically according to the instructions and apparatus design of Bittner *et al.* (1980) (see O'Donnell *et al.* 1982). The gels after electrophoresis were given three 20-min washes in transfer buffer (0·025 M phosphate) at pH 6·5 containing 0·1% (w/v) SDS. After transfer overnight at 27 V and approximately 1 A (in the cold room) the paper was treated with ethanolamine–gelatin–Tris buffer, and then gelatin–Tris–Nonidet P-40 buffer (NP40 buffer) according to the method of Renart *et al.* (1979). The paper (*c.* 12 by 14 cm) was probed for *B. nodosus* antigens by incubation with antisera (25 μl) in 10 ml NP40 buffer for 1 h at room temperature with shaking in a polyethylene bag sealed with a Calor Bag Sealer (France). The paper was washed with tap water and then shaken at 37°C for two periods of 30 min with 30 ml NP40 buffer. The paper was then treated for 30 min with 3 μl rabbit antisheep immunoabsorbed immunoglobulin G in 10 ml of NP40 buffer at room temperature and washed as before with water and NP40 buffer at 37°C. Following this the paper was treated for 30 min at room temperature with 37 kBq [^{125}I]protein A, labelled by the Bolton-Hunter method (Radiochemical Centre, Amersham, U.K.) in 10 ml NP40 buffer. After further washing with water it was shaken for 90–120 min at 37°C with 30 ml Sarkosyl buffer (Renart *et al.* 1979), washed with tap water, blotted and radioautographed for 24–60 h at −70°C using Fuji Rx Medical X-ray film and Ilford Fast Tungstate Intensifying Screens. The antibody and protein A were removed with urea–mercaptoethanol (Renart *et al.* 1979; Symington *et al.* 1981) from the paper prior to reprobing with another antiserum. We confirmed that all antibody and [^{125}I]protein A were removed by this treatment.

Results and Discussion

It was shown for the first time (Fig. 1*A*,*b*) that there are from 10 to 15 antigens recognized by the sera of sheep naturally infected with *B. nodosus*, their molecular weights ranging from approximately 10000 to approximately 90000. The pilus antigens were not readily distinguished in whole bacterial extracts (e.g. in Fig. 1*A*,*b*) run on SDS–PAGE. That antibodies to them do exist in naturally infected sheep was readily seen when a purified pilus preparation was run on SDS–PAGE and subsequently probed with serum from a sheep naturally infected with strain 198 (e.g. see Fig. 1*A*,*a*). Of the antigens revealed by the Electro-Blot technique only the location of the pilus antigen is known. It is possible that any one of these antigens may have some host-protective effect, but to date the only one studied in any detail is the pilus antigen (of molecular weight approx. 18000—see Fig. 1*A*, band 2). The fact that antigens other than the pilus antigen may have some host-protective effect has been suggested by Thorley and Egerton (1981) and Skerman (1981) and recently demonstrated by Stewart *et al.* (1983). In order to ascertain the usefulness of a particular antigen for vaccine purposes it is necessary that it be used in a pure form. The demonstration (cf. Fig. 1*A*,*a*) of reaction with serum 198 in the band 1 position of a pilus 198 preparation used for vaccine trials showed that the Electro-Blot technique described in this paper is a discerning means of assessing purity of antigen.

In Fig. 1*B* it can be seen that the sheep infected with *B. nodosus* strain 305 has produced a serum which reacts strongly with the pilus antigen from strain 198 (Fig. 1*B*,*a* and *b*) and strain 305 (Fig. 1*B*,*c*) but does not react as intensely with most of the other separated antigens as do sera from sheep infected with strains 198, 312 and 332. This shows that a sheep can respond preferentially to a particular antigen. The Electro-Blot technique is unique in being able to provide this type of information.

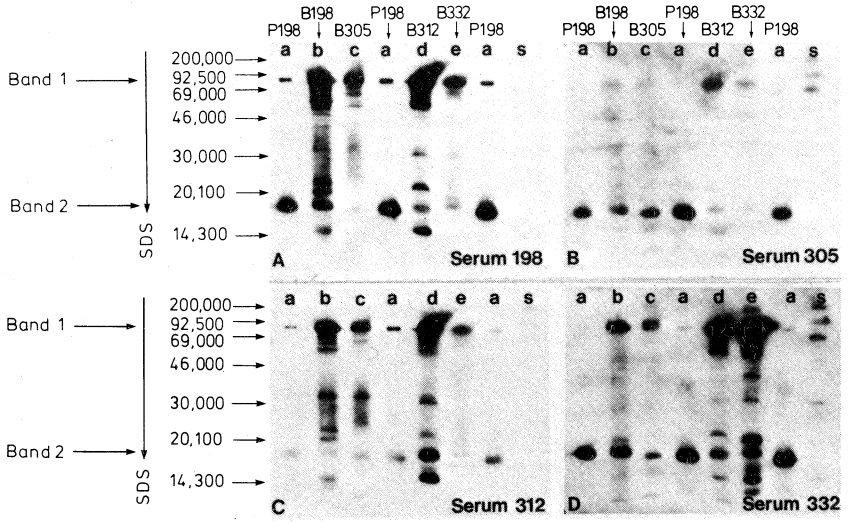

Fig. 1. *A–D*, autoradiographs of pili and four strains of *B. nodosus* cells after SDS–PAGE, transfer to APT paper (Electro-Blot), and successive probings with antisera from sheep naturally infected with these strains. The bands which bound antisera were detected with [^{125}I]protein A. P198, pili from *B. nodosus* strain 198; B198, B305, B312, B332, cell extracts of *B. nodosus* strains 198, 305, 312 and 332; *S*, ^{14}C-labelled standard proteins. The strain specificity of the serum used for probing is indicated in the lower right-hand corner of each part of the figure; for example, 'serum 198' was from a sheep naturally infected with *B. nodosus* strain 198.

When four serotypes of bacteria (strains 198, 305, 312 and 332) (as well as a pilus preparation of strain 198) were studied by the Electro-Blot technique using serum 198 from a sheep naturally infected with strain 198, the pattern in Fig. 1*A* was obtained. This radioautograph demonstrates that there was some cross-reaction between antiserum to strain 198 and pili 312, but very little with pili 305 and pili 332. Different cross-reactions were seen when the Electro-Blot was successively reacted with antisera to strains 305, 312 and 332 (Figs 1*B*,1*C*,1*D*) and this is not surprising in view of the results of Claxton (1981) who serogrouped various strains of *B. nodosus* using agglutination reactions. The band 1 proteins also cross-reacted to differing extents, e.g. antiserum to strain 198 reacted strongly with its homologous band 1 proteins (Fig. 1*A*,*b*) and with strain 312 band 1 protein (Fig. 1*A*,*d*) but less strongly with band 1 protein from strain 305 (Fig. 1*A*,*c*) and strain 332 (Fig. 1*A*,*c*). Cross-reactions were also detected among antigens other than band 1 and band 2 proteins (Figs 1*A*–1*D*).

The value of these cross-reactions among the various *B. nodosus* antigens in eliciting cross-protection in vaccinated sheep is currently under study. The pilus is responsible for inducing high levels of protection against footrot caused by homologous *B. nodosus* serotypes (Stewart 1978*a*; Every 1981; Skerman *et al.* 1981; Stewart *et al.* 1981, 1982;

Thorley and Egerton 1981). However, Egerton *et al.* (1978, 1979) have shown that a substantial curative effect can be obtained with heterologous whole-cell vaccines despite very low levels of anti-pilus agglutinating antibody to the infecting strains of *B. nodosus*. Furthermore, Thorley and Egerton (1981) and Stewart *et al.* (1983) obtained cross-protective immunity against a heterologous pilus serotype in sheep immunized with a whole-cell vaccine. These results indicate that antigen(s) other than those detected in conventional pilus agglutination tests are responsible for inducing cross-protective immunity in vaccinated sheep, or that cell-mediated immune reactions with antigenic specificities broader than humoral immune responses are involved.

The predominant antigens revealed by the Electro-Blot technique with serum from sheep naturally infected with *B. nodosus* were in the region of molecular weight 75000 (Fig. 1, band 1). Such antigens have not been recognized before. That the band 1 proteins described here are not related to pilus proteins was shown when rabbit antisera raised using acrylamide gel slices of band 1 and band 2 proteins only cross-reacted minimally in electro-blots of the original pilus preparations. We are at present investigating whether these band 1 proteins of *B. nodosus* are located in the outer membrane and their value in host protection (see Stewart and Egerton 1979; Every and Skerman 1980).

In infections of humans with *Neisseria gonorrhoeae*, antibodies have been recognized against outer membrane proteins (see Buchanan and Hildebrandt 1981) as well as pilus proteins (see Tramont *et al.* 1981).

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