Immunoblotting to Demonstrate Antigenic and Immunogenic Differences among Nine Standard Strains of Clostridium difficile

SHELLEY R. HEARD,† BARBARA RASBURN, RUTH C. MATTHEWS, AND SOAD TABAQCHALI*

Department of Medical Microbiology, St. Bartholomew's Hospital Medical College, West Smithfield, London EC1A 7BE, United Kingdom

Received 14 January 1986/Accepted 19 May 1986

The epidemiology of Clostridium difficile-associated disease is being elucidated with the development of typing schemes for the organism. We recently described a new typing scheme based on the incorporation of [35S]methionine into bacterial proteins followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Nine standard strains were identified. We report here some observations on the antigenic differences among these nine strains when studied by immunoblotting. Type-specific rabbit antiserum was raised against each of the nine standard strains. Immunoblotting of the strains with these antiserum demonstrated, in addition to the presence of shared, common proteins, a type-specific response with homologous antisera. When [35S]methionine-labeled C. difficile proteins were immunoblotted with homologous and heterologous antisera, both the immunoblots and the autoradiographs demonstrated the same strain-specific response. These strain-specific proteins, which have been so useful for epidemiological and typing purposes, were also immunogenic.

We previously reported a typing scheme for Clostridium difficile based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of [35S]methionine-labeled proteins followed by autoradiography. Nine distinct groups, A to E and W to Z, were described (9).

The application of this typing scheme in epidemiological studies suggested that the spectrum of disease produced by C. difficile may well be related to the strain isolated. Strains isolated from asymptomatic neonates were types A to D, and strains isolated from clinical outbreaks of antibiotic-associated colitis among adult patients were types X and E (9). More recently, Delmee et al. (1), using a serological typing scheme based on slide agglutination tests, confirmed this observation; they found that certain serotypes were isolated from outbreak cases and from adults and children with antibiotic-associated diarrhea, whereas different serotypes were carried by asymptomatic neonates.

Typing schemes may be of value not only in enabling differentiation between potentially pathogenic and less pathogenic strains but may also have important epidemiological applications. Wüst et al. (14) used a variety of methods to show that an outbreak of antibiotic-associated colitis in a hospital in Zurich, Switzerland, was caused by one C. difficile strain, and their findings have been corroborated (H. Hächler and J. Wüst, Letter, J. Clin. Microbiol. 20:604, 1984) with the use of the bacteriophage method for typing described by Sell et al. (8). Poxton et al. (6) used immunoblotting with antiserum raised against one C. difficile strain to demonstrate that a single strain was involved in a hospital outbreak in Sweden.

Rampling et al. (7) reported 20 patients with hematological malignancy and C. difficile-associated diarrhea, but typing of representative strains performed for them by Sell et al. (8) and Tabaqchali et al. (9), using their two different schemes, could not implicate an epidemic strain. These cases occurred intermittently over 3 years. We recently described a similar group of immunocompromised patients who were clearly cohorts of an extensive outbreak of the disease in our wards, involving 49 patients over 6 months, 35 of whom had the epidemic strain of the organism (type X). Nosocomial acquisition of the strain and cross infection with it were clearly demonstrated (2).

Our typing scheme is based on the demonstration of consistently present and easily identifiable radiolabeled protein bands which are strain specific (9). To determine whether these bands are both specific and immunogenic, we investigated the common and specific antigenic determinants of the nine standard groups of C. difficile (designated standard strains A, B, C, D, E, W, X, Y, and Z) by immunoblotting (10). Rabbit antiserum was raised against each strain and immunoblotted against homologous and heterologous antigen preparations from these nine strains and against Clostridium sordellii and Clostridium septicum. The aim was to provide corroborative evidence for the type-specific variations seen on [35S]methionine-labeled SDS-PAGE and to determine whether these antigens are immunogenic.

MATERIALS AND METHODS

C. difficile typing. The method for typing C. difficile by incorporation of [35S]methionine into soluble bacterial proteins was recently published (9, 9a). Briefly, C. difficile isolates were grown on horse blood agar (Oxoid Ltd., London, United Kingdom) for 48 h at 37°C under anaerobic conditions. Tubes containing 50 μl of prereduced methionine-free medium (Flow Laboratories, Inc., McLean, Va.) and 1 μl of [35S]methionine with approximately 10 μCi of radioactivity (Radiochemical Centre, Amer sham, United Kingdom) were inoculated with portions of two to three C. difficile colonies from the horse blood agar cultures. After incubation for 2 h at 37°C under anaerobic conditions, double-strength electrophoresis buffer (cracking buffer) was added in equal volumes to each tube. The tubes were then boiled for 2 min. After cooling, 25 μl of each sample was loaded onto a 12.5% polyacrylamide gel, and electrophoresis at 200 V for 3 to 4 h was performed. After being fixed and dried, the gels were autoradiographed.
Rabbit antiserum preparation. (i) Antigen preparation. Using the medium described by Nakamura et al. (5), each of the nine strains was inoculated from an anaerobic 48-h horse blood agar culture into 500 ml of prereduced broth containing 3% Proteose Peptone (Difco Laboratories, Detroit, Mich.), 0.5% yeast extract (Difco), 1% glucose, and 0.05% l-cysteine HCl (pH 7.2). After 7 h of incubation in an anaerobic chamber at 37°C, the broth cultures were centrifuged (Hi-Spin 21; MSE Scientific Instruments, United Kingdom) at 7,000 × g for 15 min. The deposit was then washed once in Dulbecco A (Oxoid) and suspended in 50 ml of 0.4% formol saline. These formalized antigens were stored at 4°C until use the next day.

(ii) Rabbit immunization. Nine New Zealand White rabbits (males; 3 to 3.5 kg in weight) were bled for preimmunization serum samples and then injected via ear veins at 3- to 4-day intervals with increasing doses (0.5, 0.75, 1.0, 1.5, 2.0, 2.5, and 3.0 ml) of one of the nine prepared formalized antigens. One week after the final injection, the rabbits were bled via cardiac puncture. Serum samples were separated and stored at −20°C until use.

Immunoblotting of rabbit antiserum. Preparations of the nine standard strains (A to E and W to Z) were immunoblotted against their homologous and heterologous antisera.

Each strain was grown for 48 h anaerobically on horse blood agar at 37°C. A standard loopful of the growth was inoculated into 400 μl of Eagle minimum essential medium (glutamine and methionine free; Flow Laboratories). Immunoabsorbent was prepared as previously described (4, 11). Briefly, each of the nine cultures was solubilized in cracking buffer, as described above, and boiled for 5 min. They were then frozen at −20°C before SDS-PAGE on a 10% gel at 40 mA. Transfer onto nitrocellulose membrane was performed in a transblotting chamber (Bio-Rad Laboratories, Richmond, Calif.) containing 20% methanol, 25 mM Tris, and 192 mM glycine buffer (pH 8.3) at 25°C for 90 min using a 90 V, 350 mA current. Free protein-binding sites were saturated by overnight incubation at 4°C in 3% bovine serum albumin in buffered saline (0.9% NaCl, 10 mM Tris [pH 7.4]). The nitrocellulose was incubated at 25°C for 2 h with rabbit antiserum raised against one of the nine strains, diluted 1:10 in 3% bovine serum albumin–0.05% Tween 20 (Bio-Rad) in buffered saline. After being washed five times with 0.05% Tween 20 in 0.9% NaCl over 30 min, it was incubated for 1 h at 25°C in alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Sigma Chemical Co., St. Louis, Mo.). The conjugate was diluted 1:1,000 immediately before use in 3% bovine serum albumin–buffered saline (0.9% NaCl, 10 mM Tris [pH 7.4]). After being washed, as described above, the nitrocellulose was incubated for 15 min at 25°C in a freshly prepared and filtered mixture of an equal volume of naphthol AS-MX phosphate (Sigma) (0.4 mg/ml in distilled water) and fast red TR salt (Sigma) (6 mg/ml in 0.2 M Tris [pH 8.2]). This technique was also applied to the [35S]methionine-labeled samples of each of the nine standard strains prepared as described above for typing the organism. These samples were electrophoresed on a 12.5% polyacrylamide gel run at 200 V for 3 to 4 h. Transfer to nitrocellulose membrane and immunoblotting were then performed, followed by autoradiography of the immunoblots.

RESULTS

The nine standard strains identified by [35S]methionine incorporation and SDS-PAGE were previously described (9, 9a). An autoradiograph of these standard strains is shown in Fig. 1. Representative immunoblots of three of the rabbit antisera against the nine standard strains of C. difficile are shown in Fig. 2 through 4.

There are three bands on the C. difficile immunoblots common to each of the nine strains (common bands 1 [a present only in strain Y, 2, and 3]). These bands are readily seen in representative immunoblots from strains W, X, and Y (Fig. 2 through 4) in which antiseria raised against each of these bands were used to demonstrate the type-specific response which characterizes each strain. These common bands were also present in the immunoblots of the remaining six standard strains (results not shown) but were not universally present on the [35S]methionine autoradiographs. A number of additional high- and low-molecular-weight bands were also shown by immunoblotting, but these varied considerably among strains over a molecular weight range of 30,000 to 130,000. These proteins were not recognized consistently for each strain by heterologous or homologous rabbit antiserum.

The representative immunoblots using antiseria raised against strains W, X, and Y show that the antiserum immunoblotted against its homologous strain had a distinct specificity for that strain and allowed the type-specific protein characteristic of that strain to be uniquely demonstrated. In addition, when a specific antiserum was im-

FIG. 1. Autoradiograph of the nine standard strains of C. difficile (A to E and W to Z) demonstrated by [35S]methionine incorporation into bacterial proteins and SDS-PAGE. The arrows indicate the major bands which distinguish each strain and are labeled A1, B1, B2, etc. MW, Molecular weight (106).

FIG. 2. Immunoblot of nine standard strains by using rabbit antiserum raised against strain W. The common bands (CB1 or CB1a, CB2, and CB3) are indicated. The type-specific bands of strain W (arrows) are clearly highlighted. MW, Molecular weight (106).
munoblotted against heterologous strains, the common bands were demonstrated, but strain specificity was lost. These observations held true for all nine strains. Thus, for example, when W antiserum was used (Fig. 2), the type-specific bands characteristic of that strain (W1 and W2) were shown, but none of the other strains were fully characterized with this antiserum. Similarly, the major antigenic bands associated with strain X (X1 and X2) were readily shown by using X antisera, but the type-specific bands of the other strains were lost to various degrees (Fig. 3). The same findings were observed for strain Y (Fig. 4) and for the remaining six standard strains (results not shown).

Bands common to all strains (common band 1 or 1a, 2, and 3) could be demonstrated by immunoblotting but were not seen in all strains by \( ^{35}S \) methionine incorporation (Fig. 1). The type-specific bands (A1, A2, B1, B2, etc.) could, however, be delineated by both techniques. The immunoblots of the nine standard strains labeled with \( ^{35}S \) methionine using homologous antiserum raised against each of these strains are shown in Fig. 5. The autoradiography of these immunoblots is shown in Fig. 6. The major bands (A1, B1, B2, etc.) used to describe the standard strains were clearly demonstrated on the autoradiograph of the immunoblot. The type-specific protein bands shown by immunoblotting were identical with the bands demonstrated by \( ^{35}S \) methionine typing.

Immunoblots of the specific antisera against two other strains of *Clostridium* sp., *C. sordellii* and *C. septicum*, showed cross-reactivity with *C. sordellii*, as might have been predicted (3), but showed none with *C. septicum*. The *C. sordellii* protein bands recognized by the strain-specific *C. difficile* antiserum were not, however, the same bands as those demonstrated when *C. difficile* was used as antigen in the blotting system. No *C. septicum* antigens were detected by using antisera raised against *C. difficile* (results not shown).

**DISCUSSION**

Since the original description of immunoblotting in 1979 (11), the technique has been used extensively to study a range of bacterial, viral, protozoal, and immunological diseases (10). The application of immunoblotting in this study demonstrated the presence of common and specific proteins among the nine standard strains of *C. difficile* described in our typing system (9, 9a). It also showed that the specific differentiating protein bands are immunogenic. Paxton et al. (6) used immunoblotting to show that 9 of 10 patients involved in an outbreak of *C. difficile*-associated colitis carried the same strain of the organism. By using an EDTA extract of the organism, differences between various strains were demonstrated, both by SDS-PAGE followed by stain-
ing with Coomassie blue and subsequently by immunoblotting the same antigens with rabbit antisera raised against a single strain of the organism. Although the same epidemiological conclusions could have been drawn from analysis of the Coomassie blue-stained gels alone, with the use of immunoblotting the differences between strains became more obvious. Indeed, Wexler et al. (13), reporting on the PAGE patterns produced by C. difficile, argued that for typing purposes complex protein patterns may be of less benefit than simple patterns.

The typing scheme developed by Tabaqchali et al. emphasizes this view, because the identification of the nine standard strains in this open-ended system is based on the presence of only one or two predominant specific protein bands (9, 9a). These bands are reproducible not only by [35S]methionine incorporation followed by SDS-PAGE but have now also been demonstrated by immunoblotting. The technique, as applied in this study, thus defines strain-specific proteins and characterizes them as immunogenic. This may provide some insight into the differences in pathogenicity among the strains observed by us previously (9, 9a), particularly if specific bands are found to be surface proteins, because it suggests the possibility that virulence factors such as adherence and motility are associated with these proteins.

The use of immunoblotting with reference to the standard [35S]methionine system of typing confers several advantages. First, it provides corroborative evidence for the strain specificity of the radiolabeled proteins shown by the [35S]methionine typing system. The demonstration of the strain-specific bands when homologous antisera were used and their absence when heterologous antisera were applied suggest that it is not possible to provide a comprehensive typing scheme based on the use of a single antiserum for immunoblotting. Although such an approach may be adequate for epidemiological purposes to show gross similarities or differences between clinical strains (6), it clearly cannot be used for the systematic typing of strains. Moreover, the present study has provided an explanation for the observation that extensive cross absorption is required before a serological panel can be developed, because the common antigens shared by the strains are clearly immunogenic and would be expected to cross-react with each other (1). Existing serological typing systems will therefore have difficulty in rapidly incorporating new strains, because only the specific strains allowed the differences characterized by serological cross-absorption studies will be recognized by the reference antiserum, whereas new strains can be expected to react with the common antigens of all identified strains. Thus, when a new strain appears, extensive cross-absorption studies using animal immunization procedures will be required. If, however, a combination of [35S]methionine typing and immunoblotting is used, new strains can be readily demonstrated by the radiolabel system and hence can be easily incorporated into that system, and immunogenic differences will be visualized and described by immunoblotting.

Finally, the results obtained from these rabbit immunization studies suggest that this technique is of value in considering the immune response elicited by patients with pseudomembranous colitis and C. difficile-associated colitis. Viscidi et al. (12), using an enzyme-linked immunosorbent assay to study the serum antibody response to the enterotoxin (toxin A) and the cytotoxin (toxin B) produced by C. difficile, demonstrated antibody to both toxins in children over 2 years of age and adults. Rising titers of immunoglobulin G in patients with C. difficile-associated colitis were also demonstrated. Very preliminary studies in this laboratory suggested that immunoblotting may be used to show an immune response to the organism which appears to be strain specific. If this is indeed the case, immunoblotting may serve as an important diagnostic indicator of recent infection with a particular strain and perhaps have prognostic implications in severe cases of pseudomembranous colitis.

ACKNOWLEDGMENTS

This work was supported by a grant from the Medical Research Council and by the Joint Research Board, St. Bartholomew's Hospital.

LITERATURE CITED