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Congo Red-Mediated Regulation of Levels of *Shigella flexneri* 2a Membrane Proteins

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The ability of *Shigella* spp. to bind Congo red from agar medium is generally correlated with their virulence properties. We used a metabolically active culture of *Shigella flexneri* 2a to determine the effect of Congo red on its membrane protein profiles. Virulent *S. flexneri* grown in the presence of Congo red at 37°C showed increased levels of three proteins with M_rs of 43,000, 58,000, and 63,000 (43K, 58K, and 63K proteins) in the Sarkosyl-soluble membrane fractions. The observed phenomenon was temperature dependent. At 30 or 42°C the protein levels remained unaffected by the presence of Congo red. Similar regulation of the levels of the 43K, 58K, and 63K membrane proteins was also observed with *Shigella dysenteriae* 1 and enteroinvasive *Escherichia coli*, but not with enteropathogenic *E. coli*. The cellular uptake of Congo red seemed to be essential, but not sufficient, for regulation. All three proteins reacted with human convalescent-phase sera in immunoblots of *S. flexneri* 2a Sarkosyl-soluble membrane fractions. Using the 43K-specific antiserum as the primary antibody, by indirect immunofluorescence studies, we detected an increase in the level of the 43K protein in *S. flexneri* which had invaded epithelial cells. These observations strongly indicate that the 43K, 58K, and 63K proteins are virulence associated. We propose that the observed regulatory effect of Congo red on membrane proteins of *S. flexneri* is mediated through induction. Since the same regulatory effect was also observed during the invasion of epithelial cells by *S. flexneri*, it is suggested that Congo red mimics some host tissue factor in vitro.

Bacillary dysentery caused by shigellae is a major health problem among infants all over the world. *Shigella* virulence is a multistep process, the primary event being the invasion of colonic epithelial cells by the organisms (11). This is followed by intracellular multiplication, epithelial cell death, tissue destruction, leading to ulceration, and bloody diarrhea. The genetic loci determining *Shigella* virulence are located on the chromosome and on a 120- to 140-megadalton plasmid (19, 20). The plasmid-encoded virulence factors have been implicated in the ability of shigellae to invade epithelial cells. The expression of virulence factors and, hence, *Shigella* virulence are temperature dependent (13); bacteria grown at 37°C are virulent, whereas organisms grown at 30°C are avirulent.

The property that virulent *Shigella* strains bind Congo red (CR) from solid agar media has been exploited as a rapid test for distinguishing the virulent from the avirulent strains (15). The virulent strains form red colonies, whereas the avirulent forms appear as white colonies on plates containing CR. This difference between virulent and avirulent strains is also seen in other invasive organisms such as *Yersinia pestis* (23) and *Aeromonas salmonicida* (10). Moreover, in shigellae the loss of virulence, as determined by the Sereny test (22), correlates well with the loss of the ability of the bacteria to bind CR on solid agar (21). Recently, Sakai et al. (16) and others (3, 4, 17) have cloned the genes conferring the ability to bind CR. The gene products encoded have apparent molecular weights of 24,000 (3), 21,000, 27,000, and 30,000 (17) (24K, 21K, 27K, and 30K proteins). The 30K protein has subsequently been shown to be a positive regulatory factor at the transcriptional level for some of the virulence-associated proteins (18).

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The biochemical basis for CR binding and the virulence phenotype is, however, not clear. To understand the relationship between CR binding and the expression of virulence in *Shigella flexneri* 2a, we investigated CR uptake and its relation to the expression of virulence-associated factors. In the existing liquid binding assay method (5), bacteria are allowed to bind the dye for a brief period, during which there is no significant growth of the organisms. This is not comparable to the growth of the bacteria on the solid phase (CR agar). An analogous situation in liquid medium would be to grow the cells in the presence of CR. Therefore, we exposed the metabolically active cells to CR for a prolonged period by adding the dye, together with the inoculum, to the growth medium and allowed the bacteria to grow to the stationary phase. We present evidence to show that there is specific regulation of the levels of at least three membrane proteins of *Shigella* spp. when grown in the presence of CR. The same phenomenon is also observed with enteroinvasive *Escherichia coli* (EIEC). Furthermore, we provide evidence to show that the proteins regulated by CR in vitro appear to have relevance to the pathogenicity of *S. flexneri* 2a in vivo.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Shigella* and enteropathogenic *E. coli* (EPEC) strains used in this work were obtained from St. John's Medical College Hospital, Bangalore, India, as clinical isolates. The strains were isolated on selective medium and serotyped. The invasive strains were subjected to the Sereny test and appropriately labeled as virulent or avirulent. The avirulent clinical isolate of *S. flexneri* 2a was invasion negative (Inv^-) in tissue culture assay and it also failed to bind CR (Pcr^-) in solid medium. However, it harbored the megaplasmid whose mobility was indistinguishable from that obtained from the virulent strain.

EIEC (serogroup O143 and O152) strains originally received from the Disciplina de Microbiologia, Escola Paulista de Medicina, Sao Paulo, Brazil, were obtained from R. Macaden, St. John's Medical College Hospital. Bacteria were routinely grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.). For the growth of cells in the presence of CR (Sigma Chemical Co., St. Louis, Mo.), the dye was added to a final concentration of 0.01% (wt/vol) to the medium during inoculation.

Sereny test and preparation of corneal smears for Giemsa and immunofluorescence staining. An overnight culture of the bacteria, suspended at a density of 10^{10} viable cells in 20 μl of phosphate-buffered saline (PBS), was dripped into one of the eyes of guinea pigs. The other eye served as the control. Conjunctivitis of the cornea developed at between 10 and 24 h with the virulent strains. For microscopic studies corneal epithelial cells were scraped from both eyes at 10 h after infection, using ophthalmic implements, and suspended in PBS. Smears were prepared from the suspension and processed for Giemsa and immunofluorescence staining. In contrast to the normal eye, we generally observed very few epithelial cells and many pus cells in the infected eye. Even the few epithelial cells seen were usually lysed.

Preparation of membrane and cytosol fractions. Sarkosyl-soluble and -insoluble membrane fractions were prepared essentially by the method of Carlone et al. (2). Briefly, cells were washed with 0.01 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Sigma; pH 7.5) containing 0.01 M NaCl, suspended in 0.01 M HEPES (pH 7.5), and disrupted by sonication (Branson Sonic Power Co., Danbury, Conn.), employing four 1-min pulses with an interval of 1 min between the pulses and at a setting of 6 (0°C). Lysed cells were centrifuged at 16,000 × *g* for 2 min to pellet the cell debris. The supernatant was centrifuged at 16,000 × *g* for 30 min to get the total membrane (pellet) and the cytosol. The membrane pellet was washed with 0.01 M HEPES (pH 7.5), suspended in an appropriate volume of 0.01 M HEPES (pH 7.5)–0.1 mM phenylmethylsulfonyl fluoride containing 1% sodium lauroylsarcosine (Sarkosyl, Sigma), and kept at room temperature for 1 h with gentle stirring. The Sarkosyl-insoluble membrane fraction was then pelleted by centrifugation at 16,000 × *g* for 30 min, washed with 0.01 M HEPES (pH 7.5) containing 1% Sarkosyl, and then suspended in an appropriate volume of 0.01 M HEPES (pH 7.5)–0.1 mM phenylmethylsulfonyl fluoride. The cytosol and membrane fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

PAGE. The electrophoretic analysis of the cytosol and different membrane fractions was performed by the method of Laemmli (12). Both gradient (9 to 15%) and homogeneous gel (9%) systems were used in a discontinuous buffer system. Whenever quantitation was required, stained gels were scanned at 600 nm in a DU-8B spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). Known amounts of standard proteins served as the standards for molecular weight estimation and to calculate the amount of proteins from areas under the peaks. Protein concentrations were estimated by the method of Lowry.

Concentration-dependent uptake of CR. Cells were grown overnight in the presence of various concentrations of CR (10, 20, 50, and 100 μg/ml) at 37°C, as well as at 30 and 42°C. Cytosol and membrane fractions were prepared as described above. The amount of CR in the cytosol and in the spent medium was measured spectrophotometrically at 480 nm.

Preparation of 43K antiserum. The 43K protein was isolated from the Sarkosyl-soluble membrane fraction of virulent shigellae grown in the presence of CR at 37°C. The membrane fractions were subjected to preparative SDS-PAGE, and the single prominent band corresponding to the 43K region was excised after incubation of the gels in cold 0.25 M KCl for visualization. The protein was then electroeluted and analyzed by SDS-PAGE for homogeneity. Preparations that gave a single band in gel runs were pooled and used for raising antibodies in rabbits. Rabbits were injected subcutaneously at multiple sites and boosted after 4 weeks, and the blood was collected after an additional 2 weeks. The antiserum showed a high titer and was specific for the 43K protein, as revealed by Western blot (immunoblot) analysis of the Sarkosyl-soluble membrane fractions of shigellae at an antiserum dilution of 1:1,000. The antiserum similarly reacted with the 43K protein of *Shigella dysenteriae* 1, *Shigella sonnei*, and EIEC but not with any proteins of EPEC and other common laboratory strains of *E. coli*.

Immunoblotting of Sarkosyl-soluble membrane proteins. Membrane proteins separated by SDS-PAGE were electroblotted onto nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.). The blots were blocked with 0.1 M Tris hydrochloride (pH 7.5) containing 1 M NaCl, 0.01 M $MgCl_2$, 0.05% (vol/vol) Triton X-100, and 3% bovine serum albumin for 30 min. Human sera (1:100 dilution) or the specific antiserum raised in rabbits against the electroeluted *Shigella* membrane protein (43K; 1:1,000 dilution) was added to the blocking buffer and the mixture was incubated for 30 min at room temperature with the blot. The blots were subsequently given four 10-min washes with wash buffer (blocking buffer without bovine serum albumin). Washed blots were incubated with ^{125}I-labeled staphylococcal protein A in blocking buffer for 30 min. After three quick rinses and five 10-min washes the blots were exposed to X-ray film (AGFA-GEVAERT-CURIX RP1) overnight or longer. Normal sera for control experiments were received from the serum bank of the Common Cold Unit, Salisbury, United Kingdom, through R. Macaden. Since shigellosis is not endemic in the United Kingdom, these sera were used in the present studies.

Infection of Henle 407 cells. The established Henle 407 human intestinal epithelial cell line (Flow Laboratories, Inc., McLean, Va.) was maintained in Eagle minimum essential medium (Himedia Laboratories, India) with 10% fetal calf serum (Sera Labs, United Kingdom), 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Cells were grown to confluency in plastic bottles (Nunc, Roskilde, Denmark) in an atmosphere containing 5% CO_2, trypsinized, and seeded at a concentration of approximately 3×10^5 per cover slip. Cover slips were incubated for 18 h in the presence of 5% CO_2 at 37°C. At 3 h before exposure to shigellae, the above medium was replaced with antibiotic-free Eagle minimum essential medium. For infection of monolayers, bacteria were harvested from a 3-h log-phase culture grown in the absence of CR. They were washed with PBS, suspended in Eagle minimum essential medium, and subsequently added to monolayers. After 1 h of infection at 37°C, the cover slips were washed three times with Earle balanced salt solution and incubated further for up to 2 h in Eagle minimum essential medium containing 200 μg of gentamicin per ml (to kill the extracellular bacteria). For microscopic studies the monolayers were washed with PBS and fixed in either methanol (Giemsa staining) or chilled acetone (immunofluorescence staining).

Immunofluorescence studies. The infected Henle 407 monolayers on cover slips and the corneal smears made from guinea pigs as described earlier were air dried and fixed with

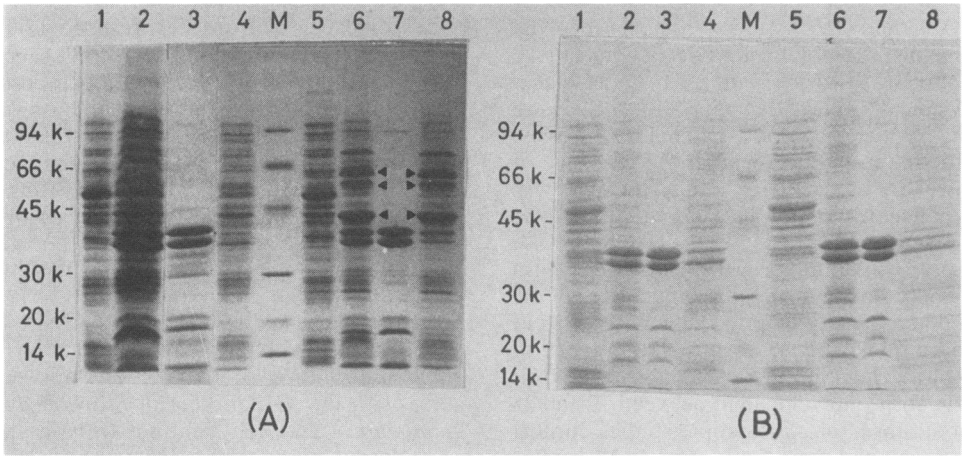

FIG. 1. SDS-PAGE (9 to 15% gradient gel) analysis of cytosol and membrane fractions of *S. flexneri* 2a. (A) Virulent *S. flexneri* grown in the absence (lanes 1 to 4) and in the presence (lanes 5 to 8) of CR at 37°C. Lanes: 1 and 5, cytosol; 2 and 6, total membranes; 3 and 7, Sarkosyl-insoluble membrane; 4 and 8, Sarkosyl-soluble membrane. (B) Avirulent *S. flexneri* grown in the absence (lanes 1 to 4) and in the presence (lanes 5 to 8) of CR at 37°C. Lanes: 1 and 5, cytosol; 2 and 6, total membrane; 3 and 7, Sarkosyl-insoluble membrane; 4 and 8, Sarkosyl-soluble membrane. M, Molecular weight markers; ◀, CR-regulated proteins. Protein (micrograms) loaded per lane: lanes 3 and 7 in panels A and B, 50; lanes 1, 4 to 6, and 8 in panel A and lanes 1, 2, 4 to 6, and 8 in panel B, 100; lane 2 in panel A, 150.

chilled acetone for 20 min. After three washes with PBS and blocking with 3% goat serum in PBS for 30 min, specimens were incubated with 43K-specific antiserum (1:2,000 dilution in PBS containing 1% goat serum) for 1 h in a humid atmosphere at room temperature. After three 5-min washes with PBS, goat anti-rabbit immunoglobulin G-fluorescein isothiocyanate conjugate (Sigma; 1:40 dilution) was added to the cover slips as the second antibody and the cover slips were incubated a further 45 min in the dark. They were then washed with PBS, mounted in buffered glycerol (pH 8) under a glass cover slip, and examined by epifluorescence microscopy (Diaplan epifluorescence microscope; E. Leitz Inc., Rockleigh, N.J.). For positive and negative controls, shigellae grown in the presence and in the absence of CR were similarly subjected to indirect immunofluorescence, using the 43K-specific antiserum.

RESULTS

Effect of CR on the membrane protein composition. A virulent isolate and an avirulent local isolate of *S. flexneri* 2a were grown overnight at 37°C in medium containing CR (0.01%, wt/vol). Upon visual examination the bacterial pellets obtained from the virulent strain looked red, whereas the pellets from the avirulent strain looked pale pink. There was a significant difference in the colors of the cytosols obtained from the two cell lysates. The cytosol of the virulent strain looked deep red, whereas that of the avirulent strain looked pale pink. SDS-PAGE of the cytosol and the Sarkosyl-soluble and Sarkosyl-insoluble membrane fractions of the above two strains of *S. flexneri* 2a grown in the presence and in the absence of CR showed a marked difference only in the Sarkosyl-soluble membrane protein profiles (Fig. 1). In the presence of CR, the Sarkosyl-soluble membrane fraction of virulent *S. flexneri* showed at least three proteins, the 43K, 58K, and 63K proteins, the levels of which were significantly higher compared with their levels in cells grown in the absence of CR (Fig. 1A, lane 8 versus lane 4). In addition, there were other minor differences between protein profiles of the bacteria grown in the presence and in the absence of the dye (see Discussion). The Sarkosyl-insoluble membrane fractions were apparently devoid of proteins with molecular weights of 43,000, 58,000, and 63,000 (Fig. 1A, lanes 3 and 7), and the cytosolic fraction of virulent *S. flexneri* grown in the presence and in the absence of CR did not show any significant difference (Fig. 1A, lane 5 versus lane 1). In the case of the avirulent strain, there were no apparent changes in the protein profiles of the cytosol and membrane fractions of cells grown in the presence or in the absence of the dye (Fig. 1B).

Regulation of the membrane protein pattern of *S. flexneri* 2a by CR is temperature dependent. Regulation of the specific membrane proteins of *S. flexneri* by CR suggested that the proteins may be associated with the virulence phenotype. If this is so, their regulation by CR could be temperature dependent. To test this possibility, the virulent isolate of *S. flexneri* 2a was grown in medium containing CR at 30 and 42°C (both are nonpermissive temperatures for invasion) and the Sarkosyl-soluble membrane protein profiles were compared with that obtained from cells grown at 37°C in the presence of the dye (Fig. 2). The SDS-PAGE protein profiles clearly showed that the 43K, 58K, and 63K proteins were present at increased levels in the membrane fractions of the bacteria grown at 37°C only (compare lanes 1 and 3 versus lane 2 in Fig. 2). The intracellular concentration of the dye in virulent *S. flexneri* grown at 37°C showed a direct relationship with its concentration in the medium (maximum concentration used, 100 μg/ml). The increase in the levels of the 43K, 58K, and 63K proteins was found to be linear with the concentration of CR in the medium up to 50 μg/ml. At 30°C the intracellular concentration of CR in the bacteria was insignificant, which correlated with the absence of any regulation of specific membrane proteins. However, at 42°C the dye was taken up by the bacteria and its intracellular concentration was comparable to that of organisms grown at 37°C in the presence of CR, but there was no regulation of the membrane proteins (Fig. 2).

Specificity of the regulation of membrane proteins by CR. The regulation of membrane proteins by CR, as seen above, could be unique phenomenon observed with *S. flexneri* 2a or it could be shared by other *Shigella* species and also by other invasive and noninvasive enteropathogens. To verify this, we chose virulent strains of *S. dysenteriae* 1, EIEC, and

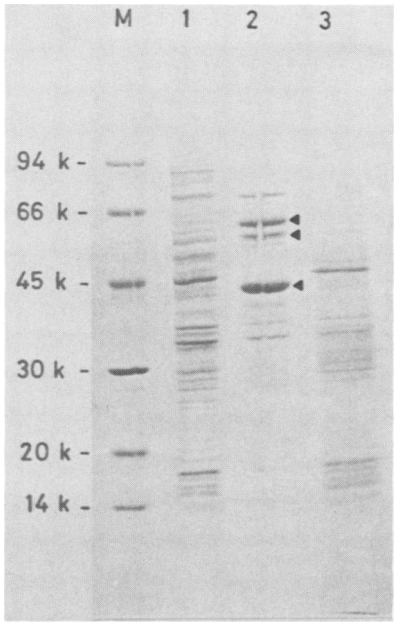

FIG. 2. SDS-PAGE (9 to 15% gradient gel) analysis of Sarkosyl-soluble membrane proteins (100 μg per lane) of virulent *S. flexneri* 2a grown in the presence of CR at 30°C (lane 1), 37°C (lane 2), and 42°C (lane 3). M, Molecular weight markers. Only at 37°C are regulated proteins (◀) seen.

EPEC and analyzed the Sarkosyl-soluble membrane protein profiles after growing the cells in the presence and in the absence of CR at 37°C (Fig. 3). Membrane fractions isolated from virulent *S. flexneri* 2a grown in the presence of CR at 37°C served as a positive control. Increased levels of the 43K, 58K, and 63K proteins were clearly identified in

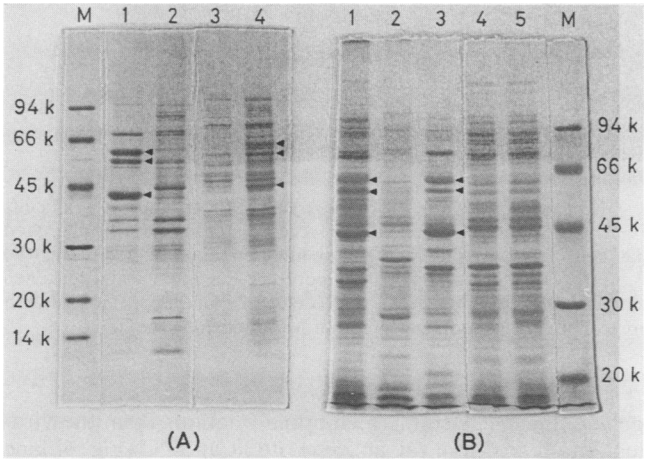

FIG. 3. Membrane protein profiles of invasive and noninvasive enteropathogens. (A) SDS-PAGE (9 to 15% gradient gel) analysis of Sarkosyl-soluble membrane proteins (100 μg per lane) of virulent *S. flexneri* 2a grown in the presence (lane 1) and in the absence (lane 2) of CR at 37°C and EIEC grown in the absence (lane 3) and in the presence (lane 4) of CR at 37°C. (B) SDS-PAGE (9% gel) analysis of Sarkosyl-soluble membrane proteins (100 μg per lane) of virulent *S. flexneri* 2a grown in the presence of CR (lane 1), *S. dysenteriae* 1 grown in the absence (lane 2) and in the presence (lane 3) of CR at 37°C, and EPEC grown in the absence (lane 4) and in the presence (lane 5) of CR at 37°C. This is a composite gel picture. Only invasive organisms exhibited a regulation of membrane proteins.

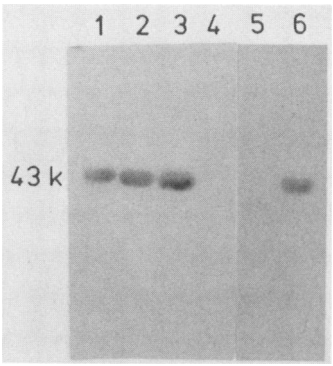

FIG. 4. Antigenic similarity of the 43K protein among various enteropathogens. Sarkosyl-soluble membrane fractions from *S. flexneri* 2a (lanes 1 and 6), *S. dysenteriae* 1 (lane 2), EIEC (lane 3), and EPEC (lane 4) and the Sarkosyl-insoluble membrane fraction from *S. flexneri* 2a (lane 5) grown in the presence of CR were treated with 43K-specific antiserum in Western blots. Protein loaded, 100 μg per lane.

SDS-PAGE profiles of Sarkosyl-soluble membrane preparations obtained from *S. dysenteriae* 1 (Fig. 3B, lane 3) and EIEC (Fig. 3A, lane 4) grown in the presence of CR. The similarity of at least the 43K protein among different *Shigella* species and EIEC was demonstrated in immunoblots of Sarkosyl-soluble membrane fractions of these organisms, using the 43K-specific antiserum (Fig. 4). In the case of EPEC, no such increase in the levels of the 43K, 58K, and 63K proteins was apparent under identical growth conditions (Fig. 3B, compare lane 5 versus lanes 1 and 3). In Western blots also the 43K-specific antiserum failed to detect any antigenically related protein (Fig. 4, lane 4). The cytosolic fractions of the above-mentioned invasive organisms appeared red, whereas that of EPEC looked pale pink.

Regulation of the 43K protein of *S. flexneri* 2a in infected epithelial cells as revealed by indirect immunofluorescence. To determine whether the CR-regulated membrane proteins of *S. flexneri* were also regulated during its invasion of epithelial cells, the Henle 407 intestinal cell line and Sereny test were used as in vitro and in vivo models for invasion, respectively (Fig. 5). *S. flexneri* grown in the absence of CR was used as the inoculum in both models. Giemsa staining detected the presence of the intracellular bacteria both in the infected Henle 407 monolayers and in the infected guinea pig corneal epithelial cells (Fig. 5A, a and c). When the duplicate cover slips from both the tissue culture invasion and the Sereny test were fluorescence stained to localize the presence of the 43K protein in the invaded bacteria, using the 43K-specific antiserum, fluorescent bacteria were clearly visible in large numbers in the infected monolayers (Fig. 5B, a) and also in significant numbers in the corneal epithelial cells (Fig. 5B, c). In our experience the pus cells always outnumbered the epithelial cells in the infected-eye scrapings, and hence the intensity of fluorescence was less compared with the Henle 407 cells. In control experiments we failed to detect any significant level of fluorescence associated with the bacteria grown in the absence of CR that were used for infecting the epithelial cells (Fig. 5C, b). However, *S. flexneri* grown in the presence of CR showed intense fluorescence associated with the bacteria (Fig. 5C, a). These results, therefore, clearly indicated that the level of the 43K membrane protein of *S. flexneri* was regulated similarly during the invasion of epithelial cells in vivo and by CR in vitro.

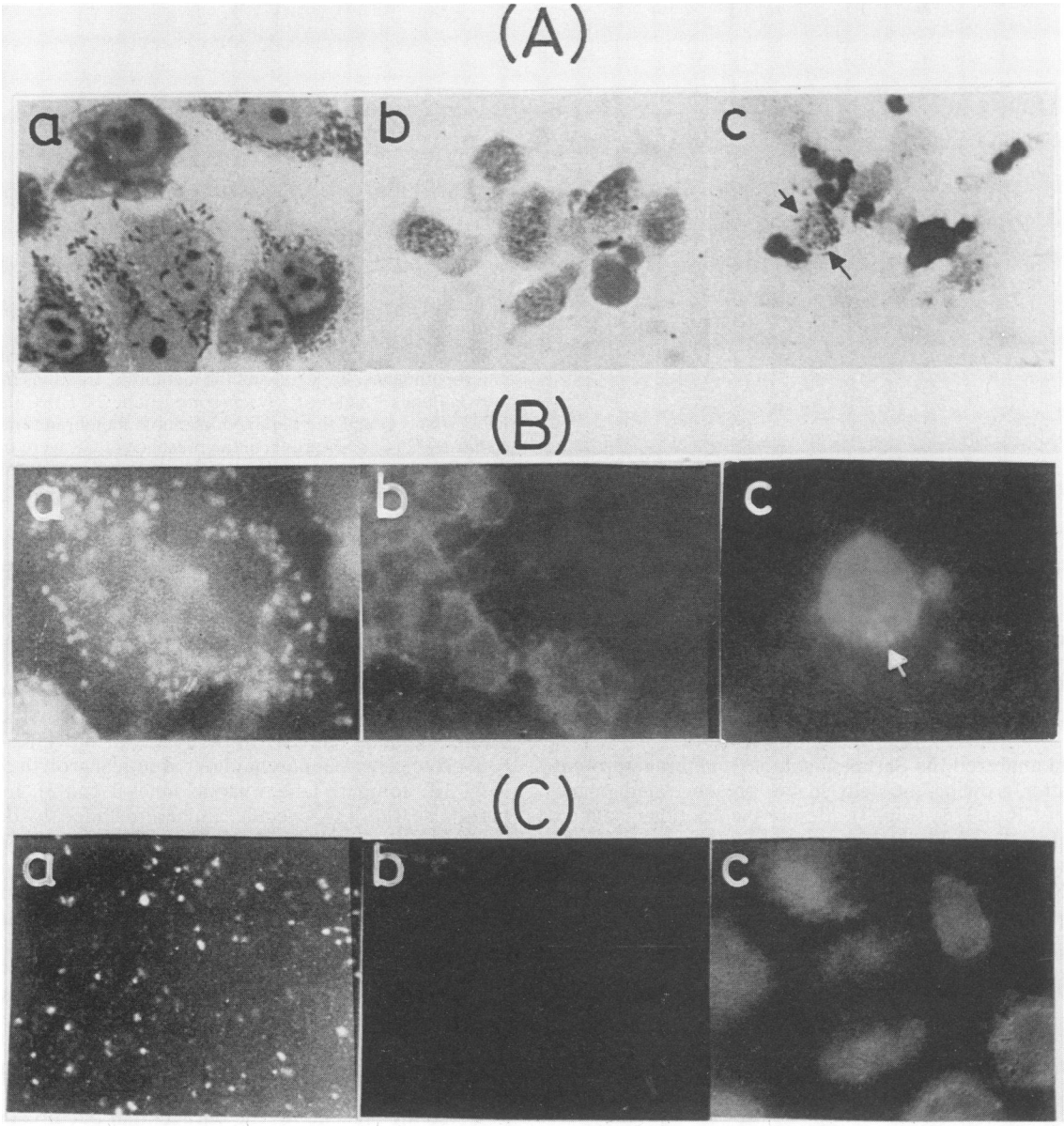

FIG. 5. Invasion of epithelial cells by *S. flexneri* 2a and detection of the 43K protein. (A) Henle 407 monolayers and corneal epithelial cells from guinea pigs were fixed in methanol and Giemsa stained. Details are given in Materials and Methods. a, Infected Henle 407 cells incubated for 120 min in the presence of gentamicin; b and c, corneal epithelial cells from an uninfected and infected eye, respectively. (B) Immunofluorescence labeling of respective duplicate samples of panel A. (C) Positive and negative controls for immunofluorescence experiments. a and b, *S. flexneri* grown in the presence and in the absence of CR, respectively; c, infected Henle 407 monolayers treated with normal rabbit serum as the primary antibody. Details of the immunofluorescence experiments are given in Materials and Methods.

Presence of circulating antibodies in the convalescent-phase sera for the CR-regulated proteins. Immunoblots of Sarkosyl-soluble membrane fractions of *S.flexneri* 2a grown in the presence of CR were probed with convalescent-phase serum from a 2-year-old child (*S. flexneri* 2a) and adult (*S. dysenteriae* 1 and *S. sonnei*) patients who had recovered from acute *Shigella* infection. All of the sera reacted prominently with the 63K and 43K antigens (Fig. 6, lanes 2, 5, and 6) and possibly with the 58K antigen with serum from *S. flexneri* 2a infection (Fig. 6, lane 2). In addition, the sera reacted variably with a few other protein antigens ranging in molecular weight from 20,000 to 90,000. Control sera did not show any detectable level of immunoreaction to any of the *Shigella* antigens. With the membrane fractions from the virulent strain grown in the absence of CR at 37°C (Fig. 6, lane 1) or in its presence at 30°C (Fig. 6, lane 3) and the avirulent strain grown in the presence of CR at 37°C (Fig. 6, lane 4), the convalescent-phase serum from *S. flexneri* 2a infection, as well as the other convalescent-phase sera, reacted, weakly, only in the 63K region. The low level of 43K antigen could, however, be detected in the membranes of virulent *S. flexneri* 2a grown in the absence of dye when reacted with the 43K-specific antiserum and with adult convalescent-phase sera from *S. dysenteriae*- and *S. sonnei*-infected patients (data not shown). However, neither the adult human sera nor the 43K-specific antiserum could detect the 43K

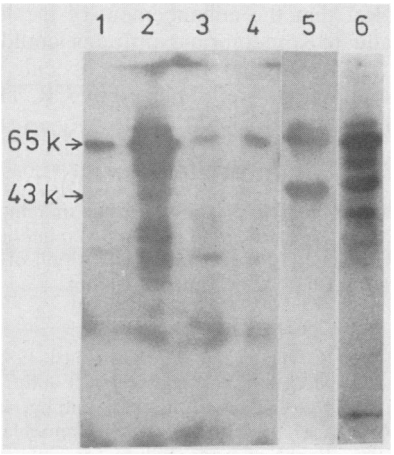

FIG. 6. Immunoblot analysis of Sarkosyl-soluble membrane antigens of *S. flexneri* 2a recognized by human convalescent-phase sera from acute *S. flexneri* 2a (lanes 1 to 4), *S. dysenteriae* 1 (lane 5), and *S. sonnei* (lane 6) infection. Lanes: 1, virulent *S. flexneri* grown at 37°C in the absence of CR; 2, 5, and 6, virulent *S. flexneri* grown at 37°C in the presence of CR; 3, the same strain grown in the presence of CR at 30°C; 4, avirulent *S. flexneri* grown in the presence of CR at 37°C. The positions of the 43K and 63K antigens have been indicated by arrows.

antigen in virulent *S. flexneri* 2a grown at 30°C in the presence of CR or the avirulent isolate grown at 37°C in the presence of the dye (data not shown).

DISCUSSION

When grown in the presence of CR at 37°C, virulent *S. flexneri* accumulated the dye intracellularly and the concentration of dye was directly proportional to that present in the medium. That the dye accumulated intracellularly was borne out by the fact that it remained in the soluble fraction of the cytosol when centrifuged at 100,000 × *g*. At the maximum concentration of CR present in the medium (100 μg/ml), virulent *S. flexneri* accumulated as much as 20 μg of dye per 4×10^9 cells. In comparison, the uptake of dye by the avirulent isolate was less significant (3 μg per 4×10^9 cells). Such a marked difference in the concentration of CR, however, was not apparent in the total membrane fractions of the virulent and avirulent strains of *S. flexneri* 2a. In the membranes it is possible that a considerable portion of the dye could have been bound nonspecifically (15) and leached out during the preparation of the cytosol, but when membrane preparations from both the virulent and the avirulent strains grown in the absence of CR were treated with exogenously added CR, both bound the dye avidly, the majority of which could not be removed by washing with 0.01 M HEPES (pH 7.5).

Sarkosyl-soluble membrane fractions isolated from virulent *S. flexneri* 2a grown in the presence of CR showed increased levels of the 43K, 58K, and 63K proteins, constituting about 25% of the total Sarkosyl-soluble membrane proteins. We had consistently observed a comparatively smaller number of protein bands in the Sarkosyl-soluble membrane isolates of *S. flexneri* grown in the presence of CR compared with those grown in the absence of the dye. This pattern was also variable. The enormous increase in the levels of the three specific proteins, however, could have affected the apparent levels of other membrane proteins. It is unlikely that these proteins could be transported from either the outer membrane or the cytoplasm to the inner membrane by the presence of CR in the medium because in the absence of CR we did not see any significant level of these proteins either in the outer membrane or in the cytosol (Fig. 1A). The possibility of the tryptic soy broth medium contributing to the observed effect of CR was ruled out because of the fact that a similar phenomenon was also observed when the medium was changed to Luria broth. From immunoblot experiments with 43K-specific antiserum, it seems unlikely that the 43K protein and, perhaps, the other two membrane proteins are degradation products because the autoradiograms did not reveal any higher-molecular-weight protein antigens.

The presence of the above three proteins in the Sarkosyl-soluble fractions of the total membrane of *S. flexneri* indicated that they were possibly inner membrane proteins. This was further supported by the fact that when *S. flexneri* grown in the presence of CR was treated with 43K-specific antiserum followed by ^{125}I-labeled protein A, the bacterium did not show any significant radioactivity associated with it. The same bacterium also did not show agglutination when treated with the 43K-specific antiserum. However, when the Sarkosyl-soluble and -insoluble membrane fractions immobilized on nitrocellulose were similarly treated with 43K-specific antiserum and ^{125}I-labeled protein A as above, only the Sarkosyl-soluble fraction showed significant counts associated with it. These observations were further confirmed by enzyme-linked immunosorbent assay (data not shown). Western blot analysis of Sarkosyl-insoluble membrane fractions of *S. flexneri* 2a grown in the presence of CR failed to reveal any protein in the 43K region when reacted with the 43K-specific antiserum (Fig. 4, lane 5). However, our experiments still do not rule out the possibility of the 43K protein antigen being inaccessible to the specific antiserum and Sarkosyl preferentially solubilizing it from the outer membrane of *S. flexneri*.

Although we had observed a good correlation between the uptake of CR and the regulation of the *Shigella* membrane proteins, an exception to these observations was seen when the bacteria were grown at 42°C. At this temperature, although the dye entered into the virulent bacterial cells and its intracellular concentration was comparable to that at 37°C, the membrane protein profiles of the bacteria remained unaltered. When an inoculum from the culture grown at 42°C in the presence of CR was grown at 37°C in the presence of the dye, the membrane protein profiles of the cells showed increased levels of the 43K, 58K, and 63K proteins, thereby indicating that the above effect was reversible. Taken together with the 30°C experimental results, it appears that the CR uptake by *S. flexneri* and the subsequent regulation of the specific membrane proteins were two independent thermoregulated phenomena, possibly controlled by separate genetic loci. The presence of intracellular CR was essential, but not sufficient, to regulate the membrane protein levels of *S. flexneri* 2a. Our observations are supported by the recent report of Maurelli and Sansonetti (14), where evidence presented indicates that the CR pigmentation and the other virulence properties of *Shigella* spp. (*vir* genes) were under different genetic controls. A chromosome-coded repressor was implicated in the thermoregulation of the *vir* genes.

The presence of the 43K, 58K, and 63K proteins in the membranes of *Shigella* spp. appeared to be associated with their virulence phenotype for the following reasons. (i) An avirulent isolate of *S. flexneri* 2a did not show any apparent change in its membrane protein profile when grown in the presence of CR, nor did it reveal a 43K protein when treated

with the 43K-specific antiserum or the convalescent-phase sera. The convalescent-phase sera could detect only a low level of the 63K antigen (Fig. 6). (ii) The expression of all three membrane proteins is thermoregulated. At 30 and 42°C the inclusion of CR in the growth medium did not alter the membrane protein profiles of *S. flexneri* 2a. When the above membrane fractions were treated with the 43K-specific antiserum in Western blots, even the low level of the 43K protein seen with cells grown in the absence of CR at 37°C was not detected (data not shown). (iii) In the immunoblot analysis of membrane preparations of *S. flexneri* grown in the presence of CR, we could detect antibodies to 43K and 63K protein antigens in the convalescent-phase sera of humans with acute shigellosis. However, in the case of the avirulent strain, the convalescent-phase sera detected a very low level of only the 63K protein. Although the Western blots revealed a few more protein antigens in the molecular weight range of 20,000 to 90,000, their levels in the membranes of *S. flexneri* 2a were not affected by CR. The fact that the levels of the three specific proteins were also regulated in EIEC but not in EPEC suggested that the regulation phenomenon of membrane proteins by CR could be characteristic of invasive organisms.

The megaplasmids of various *Shigella* strains and EIEC isolates carrying genetic traits for the invasion phenotype have been found to encode several outer membrane polypeptides ranging in molecular weight from 12,000 to 140,000 (7–9). At least six of these protein antigens, 39K, 43K, 57K, 62K, 78K, and 140K, have been shown to be strongly recognized by human and monkey convalescent-phase sera (6, 8). The 43K (*ipaC*) and 62K (*ipaB*) gene sequences have been published recently (1, 24). We have sequenced (Applied Biosystems protein sequencer, model 477A) the N-terminal 20 amino acid residues (MEIQNTKPTQILYTDI ETKE) and two internal peptides (YASALEEEEQLI SQASSK and SQNYQQIAA-IPLNVG) of the 43K protein. These sequences matched perfectly those derived from the published *ipaC* gene sequence, except for the residues underlined. It is probable that the 58K and 63K proteins reported by us in this paper could be related to the antigenic *Shigella* proteins reported in the literature (1, 6, 7, 18, 24). Furthermore, the relevance of the regulation by CR of at least the 43K protein in relation to the invasion of *Shigella* spp. was demonstrated by immunofluorescence staining, using the 43K-specific antiserum as the primary antibody. Both the invaded Henle 407 monolayers and the guinea pig corneal epithelial cells showed bacteria associated with specific fluorescence. During the initial period of infection of the Henle 407 monolayers by shigellae, bacteria, mostly extracellular, could be detected by Giemsa staining (data not shown). However, immunofluorescence staining of the monolayers, at the same time period, failed to detect any significant number of fluorescent bacteria. Evidence presented here, therefore, clearly indicates that the level of the 43K protein in shigellae is enhanced in response to the invasion of the host cells by the organisms. It is likely that the 58K and 63K proteins could also be regulated similarly during the invasion process of shigellae. The molecular basis of regulation on the levels of the membrane proteins by CR requires detailed experimentation. The observed increase in the levels could be the result of some stabilizing effect of CR on these proteins. Alternatively, CR might be regulating at the genetic level with or without the mediation of a protein factor(s). Our observations with invasion experiments, using immunofluorescence, together with the fact that CR regulation was seen only in an actively growing *Shigella* culture strongly suggest that the enhancement of the levels of the 43K, 58K, and 63K membrane proteins could be due to induction. It is likely that there are specific host tissue factors whose functions are mimicked by CR. The nature of these factors is yet to be determined.

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LITERATURE CITED

1. **Boudry, B., M. Kaczorek, and P. J. Sansonetti.** 1988. Nucleotide sequence of the invasion plasmid antigen B and C genes (*ipa* B and *ipa* C) of *Shigella flexneri*. Microb. Pathog. **4**:345–357.
2. **Carlone, G. M., M. L. Thomas, H. S. Rumschlag, and F. O. Sottnek.** 1986. Rapid microprocedure for isolating detergent-insoluble outer membrane proteins from *Haemophilus* species. J. Clin. Microbiol. **24**:330–332.
3. **Chambers, C. E., S. L. Stockman, and D. W. Niesel.** 1985. Thermoregulated expression of a cloned Congo red binding activity gene of *S. flexneri*. FEMS Microbiol. Lett. **28**:281–286.
4. **Daskaleros, P. A., and S. M. Payne.** 1985. Cloning the gene for Congo red binding in *Shigella flexneri*. Infect. Immun. **48**: 165–168.
5. **Daskaleros, P. A., and S. M. Payne.** 1987. Congo red binding phenotype is associated with hemin binding and increased infectivity of *Shigella flexneri* in the HeLa cell model. Infect. Immun. **55**:1393–1398.
6. **Dinari, G., T. H. Hale, S. W. Austin, and S. B. Formal.** 1987. Local and systemic antibody responses to *Shigella* infection in rhesus monkeys. J. Infect. Dis. **155**:1065–1069.
7. **Hale, T. L., and S. B. Formal.** 1986. Genetics of virulence in *Shigella*. Microb. Pathog. **1**:511–518.
8. **Hale, T. L., E. V. Oaks, and S. B. Formal.** 1985. Identification and antigenic characterization of virulence-associated, plasmid-coded proteins of *Shigella* spp. and enteroinvasive *Escherichia coli*. Infect. Immun. **50**:620–629.
9. **Hale, T. L., P. J. Sansonetti, P. A. Schad, S. Austin, and S. B. Formal.** 1983. Characterization of virulence plasmids and plasmid-associated outer membrane proteins in *Shigella flexneri*, *Shigella sonnei*, and *Escherichia coli*. Infect. Immun. **40**:340–350.
10. **Ishiguro, E. E., T. Ainsworth, T. J. Trust, and W. W. Kay.** 1985. Congo red agar, a differential medium for *Aeromonas salmonicida*, detects the presence of the cell surface protein array involved in virulence. J. Bacteriol. **164**:1233–1237.
11. **LaBrec, E. H., H. Schneider, T. J. Magnani, and S. B. Formal.** 1964. Epithelial cell penetration as an essential step in the pathogenesis of bacillary dysentery. J. Bacteriol. **88**:1503–1518.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
13. **Maurelli, A. T., B. Blackmon, and R. Curtis III.** 1984. Temperature-dependent expression of virulence genes in *Shigella* species. Infect. Immun. **43**:195–201.
14. **Maurelli, A. T., and P. J. Sansonetti.** 1988. Identification of a chromosomal gene controlling temperature-regulated expression of *Shigella* virulence. Proc. Natl. Acad. Sci. USA **85**: 2820–2824.
15. **Payne, S. M., and R. A. Finkelstein.** 1977. Detection and differentiation of iron-responsive avirulent mutants on Congo red agar. Infect. Immun. **18**:94–98.
16. **Sakai, T., C. Sasakawa, S. Makino, K. Kamata, and M. Yoshikawa.** 1986. Molecular cloning of a genetic determinant for Congo red binding ability which is essential for the virulence of *Shigella flexneri*. Infect. Immun. **51**:476–482.
17. **Sakai, T., C. Sasakawa, S. Makino, and M. Yoshikawa.** 1986. DNA sequence and product analysis of the *virF* locus responsible for Congo red binding and cell invasion in *Shigella flexneri* 2a. Infect. Immun. **54**:395–402.

18. **Sakai, T., C. Sasakawa, and M. Yoshikawa.** 1988. Expression of four virulence antigens of *Shigella flexneri* is positively regulated at the transcriptional level by the 30 kilodalton *virF* protein. Mol. Microbiol. **2**:589–597.
19. **Sansonetti, P. J., T. L. Hale, G. J. Dammin, C. Kapfer, H. H. Collins, Jr., and S. B. Formal.** 1983. Alterations in the pathogenicity of *Escherichia coli* K-12 after transfer of plasmid and chromosomal genes from *Shigella flexneri*. Infect. Immun. **39**:1392–1402.
20. **Sansonetti, P. J., D. J. Kopecko, and S. B. Formal.** 1982. Involvement of a plasmid in the invasive ability of *Shigella flexneri*. Infect. Immun. **35**:852–860.
21. **Sasakawa, C., K. Kamata, T. Sakai, S. Y. Murayama, S. Makino, and M. Yoshikawa.** 1986. Molecular alteration of the 140-megadalton plasmid associated with loss of virulence and Congo red binding activity in *Shigella flexneri*. Infect. Immun. **51**:470–475.
22. **Sereny, B.** 1955. Experimental *Shigella* conjunctivitis. Acta Microbiol. Acad. Sci. Hung. **2**:293–296.
23. **Surgalla, M. J., and E. D. Beesley.** 1969. Congo red agar plating medium for detecting pigmentation of *Pasteurella pestis*. Appl. Microbiol. **18**:834–837.
24. **Venkatesan, M. M., J. M. Bussy, and D. Kopecko.** 1988. Characterization of invasion plasmid antigen genes (*ipa* BCD) from *Shigella flexneri*. Proc. Natl. Acad. Sci. USA **85**:9317–9321.