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Cry1Ac Protoxin from *Bacillus thuringiensis* sp. *kurstaki* HD73 Binds to Surface Proteins in the Mouse Small Intestine

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***Bacillus thuringiensis* (Bt), considered a safe insecticide, produces insecticidal proteins named Cry during sporulation, which possess exceptional immunological properties. In this work using an immunohistochemical test we demonstrated that Cry1Ac protoxin (pCry1Ac) binds to the mucosal surface of the mouse small intestine. Ligand blot assay allowed us to detect, under denaturing conditions, six pCry1Ac-binding polypeptides present in brush border membrane vesicles isolated from the small intestine. Moreover, this protein induced *in situ* temporal changes in the electrophysiological properties of the mouse jejunum. The data obtained indicate a possible interaction *in vivo* of Cry proteins with the animal bowel which could induce changes in the physiological status of the intestine.**

Bacillus thuringiensis produces inclusion bodies during sporulation, which are formed by proteins toxic to a group of important pests. These are called Cry proteins. The biochemical properties of Cry proteins are very peculiar: they have a high molecular weight, are resistant to proteolysis and are soluble at alkaline pH (1). Little is known about the physiological or immunological effects of Cry proteins on vertebrate organisms, despite the proven homology of Bt with the pathogenic *Bacillus cereus* species (2).

The few studies related with the immunological properties of Cry proteins have been limited to the protoxin. In previous reports, Prasad *et al.* suggested that these proteins have antitumoral activity against Yoshida ascites sarcoma in rats (3), and that they enhance the immune response to sheep red blood cells (4). Recently, we demonstrated that recombinant Cry1Ac protoxin (pCry1Ac) administered to mice by intraperitoneal or intragastric route induces systemic and mucosal antibody responses similar to those obtained with cholera toxin (5). Moreover, in adjuvanticity studies, pCry1Ac elicited serum antibodies to hepatitis B surface antigen and BSA when these antigens were co-administered via intragastric, and IgG antibodies in the intestinal fluid when administered by the intraperitoneal route (6).

Transgenic maize containing Cry proteins is being used in food elaboration (7). In this way, animals and man will be in contact with these toxins at the intestinal epithelium; however, we do not know if Cry1Ac produces any physiological effect on the mammalian bowels. In this report, we show that pCry1Ac from Bt HD73 binds *in situ* to the intestinal epithelium of mice and induces a transient hyperpolarization of the mucosal tissue.

MATERIALS AND METHODS

Organisms and culture conditions. Dr. Donald H. Dean from Ohio State University, Columbus, gently provided *Escherichia coli* JM103 [pOS9300] strain. The recombinant strain was grown in LB medium containing 50 μg of ampicillin per ml, and the induction of Cry1Ac protein production was performed using isopropyl-β-D-thiogalactopyranoside (IPTG) (8).

Purification of Cry1Ac protein. Recombinant Cry1Ac protoxin was purified from IPTG-induced *E. coli* JM103 [pOS9300] cultures (8). The cell pellet harvested by centrifugation was resuspended in TE buffer (50 mM Tris–HCl, pH 8, 50 mM EDTA) and sonicated (Fisher Sonic Dismembrator Model 300, U.S.A.) three times for 5 min on ice. Inclusion bodies were collected by centrifugation at 10,000*g* for 10 min. The pellets were washed twice with TE buffer and pCry1Ac was solubilized in CBP buffer (0.1 M Na_2CO_3 pH 9.6, 1% 2-mercaptoethanol, 1 mM PMSF). The particulate material was discarded by centrifugation at 10,000*g* for 10 min. Purified protoxin was examined by SDS–PAGE (9) and protein concentration was determined using the method of Bradford (10).

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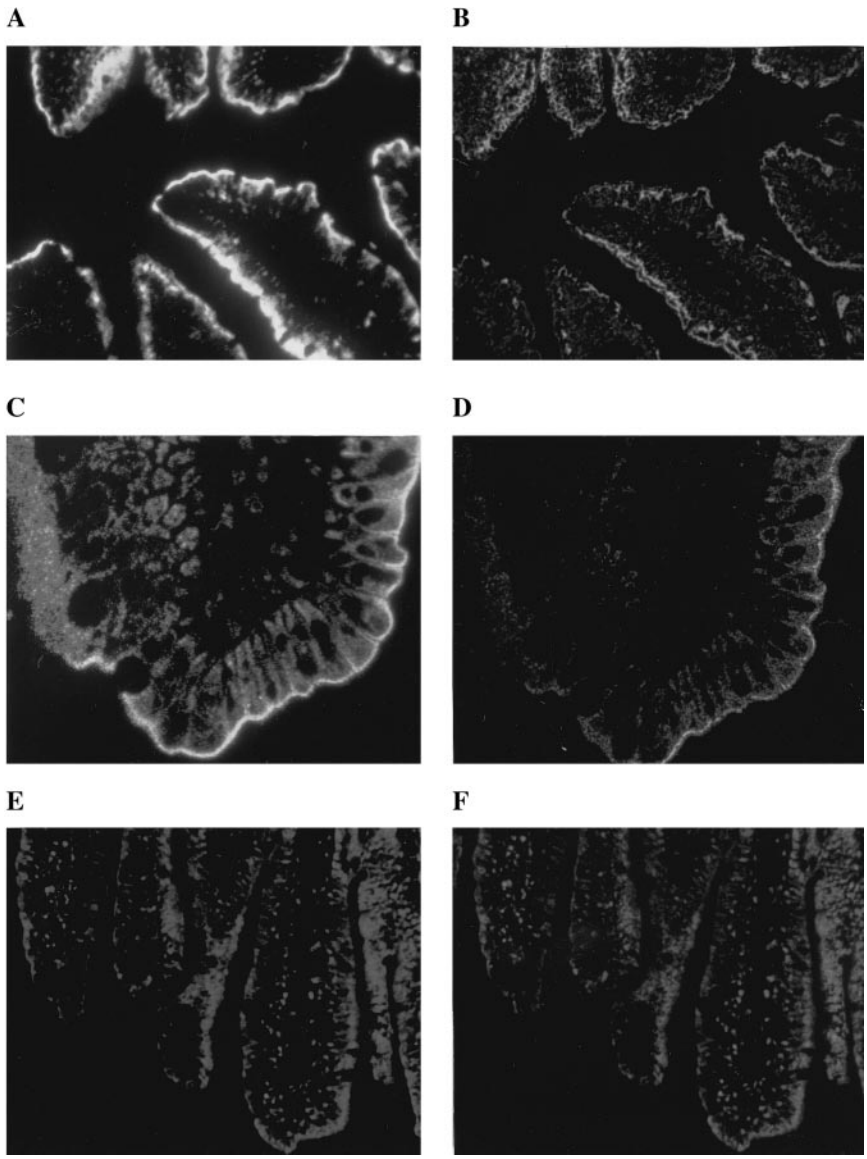

FIG. 1. Immunolocalization of pCry1Ac bound to mouse jejunum sections. The fixed small intestine from anesthetized mouse was extracted and cryosection into the thin layers was performed. The protoxin was added on the tissue section and the protein bound detected using an immuno-purified anti-Cry1 polyclonal antibody. A and C showed different intestine preparations treated with the protoxin. The same images converted to pseudocolor scale are shown in B and D. The image obtained from the section not treated with pCry1Ac is shown in E and F (pseudocolor scale).

Indirect immunofluorescence assay. The mouse intestines were fixed *in vivo* using cardiac perfusion with 4% formaldehyde in PBS (11). The jejunum of treated mice was then extracted, flushed out with ice-cold PBS and immersed into an osmotic solution (2.3 M sucrose in PBS). Frozen cut sections from fixed intestine were obtained using an IEC Minotone cryomicrotome (International Equipment Co., U.S.A.) and picked up on gelatin-coated slides. The preparations were stored at −20°C until use. For *in situ* binding assays intestine sections were thawed in PBS solution for 1 h at 37°C. Cry1Ac protoxin was added on the tissue at 0.1 mg/ml in 0.1 M Na_2CO_3, pH 9.6, during 2 h at 37°C. Unbound protein was removed by washing six times with PBS and the slides were incubated for 1 h at 37°C with an anti-Cry1Ac polyclonal antibody at 10 μg/ml in PBS (12). The anti-rabbit monoclonal antibody conjugated with rhodamine (Sigma Chemical Co., U.S.A.) was used at a 1:100 dilution. Immunofluorescence was recorded using a MRC-6000 confocal microscope (Bio-Rad, U.S.A.). Cry1Ac protoxin was omitted in the negative controls. The images were analyzed in its original form or were transformed to pseudocolor scale.

Electrophysiological experiments. The small intestine was removed from adult Balb/c male mice under sodium pentobarbital anesthesia. Segments of jejunum were placed on ice-cold Ringer's solution at 4°C and gassed with an O_2–CO_2 (95:5) mixture. Each segment was cut open along its mesenteric border, rinsed clean of luminal contents, and extended and kept in gassed Ringer's solution. Full-thickness segments were divided into two and mounted between the circular openings of two adjacent Ussing hemichambers (13). Each hemichamber was filled with 2.5 ml of gassed Ringer's solution and kept at 37°C while bubbling with the O_2–CO_2 mixture. The

protoxin (7.5 μg) was applied on the mucosal side of the preparation that had been mounted ten minutes before, and the transmural potential difference (PD) and short-circuit currents (Isc) were recorded each minute for sixteen minutes. Transmural resistance (R) values were obtained from PD/Isc ratios at each time point (13). Decay rates were calculated by regression analysis of the corresponding PD and Isc values.

Brush border membrane vesicles (BBMV) purification. Mouse intestinal BBMVs were prepared according the methodology reported by Biber *et al.* (1980) with some modifications (14). Briefly, small intestine from five anesthetized mice was extracted, rinsed with PBS and cut to small pieces. Intestinal fragments were immersed in 15 ml of ice-cold BBMV isolation buffer (300 mM D-mannitol, 5 mM EGTA, 12 mM Tris–HCl, pH 7.4) and homogenized at 4°C using a potter (B. Braund, Germany). The homogenate was mixed with 21 ml of ice-cold water and $MgCl_2$ was added to 12 mM. The mixture was incubated 15 min on ice and part of the precipitate was discarded by centrifugation at 4500*g*. The BBMVs suspended in the supernatant were then collected by centrifugation at 16,000*g* during 15 min at 4°C and stored at −70°C. The quality of BBMVs was tested by measuring the activity of alkaline phosphatase and β-glucoronidase enzymes (14). Protein concentration was determined by Bradford's method (10).

Ligand blot assay. Ligand blot assays were performed following the protocol reported by Hoffman *et al.* (15) for insect BBMVs (15). Different amounts (5, 10, 15, 20 μg) of mouse BBMV were dissolved in sample buffer (1% 2-mercaptoethanol, 1% SDS, 10% glycerol, 100 mM Tris–HCl pH 8) and applied on SDS–PAGE (9). The resolved proteins were immobilized onto Hybond C+ (Amersham, UK) using a submarine transfer (Bio-Rad, USA). Membranes were blocked with 5% dry milk in PBS for 1 h at 37°C, and incubated under similar conditions with 10 μg of pCry1Ac alone or with 10 mM of glucose, mannose, *N*-acetyl-galactosamine, or biotin. Cry1Ac protein binding to the membrane was detected using an anti-Cry1Ac polyclonal antibody (12). The immune complexes were revealed with an ECL Western blot kit (Amersham, UK).

RESULTS

Immunohistochemical detection of Cry1Ac binding after in situ exposure. Cry1Ac protoxin under alkaline conditions bound to the jejunum epithelium, being more intensive the binding to enterocytes. The processing of confocal images with pseudocolor scale showed that the protoxin binds mainly to the apical surface, including the brush border. The tissue sections to which pCry1Ac was not added did not emit intense red fluorescence (Fig. 1).

Study of Cry1Ac interaction with BBMV proteins from mouse small intestine. BBMVs isolated from the small intestine were separated and immobilized onto a nitrocellulose membrane. Six polypeptides with molecular weights of 102, 87, 62, 55, 51, and 39 kDa bound pCry1Ac in ligand blot assays (Fig. 2). The signal was saturated with more than 10 μg of BBMVs per lane. The assay performed with no protoxin did not show signal, which evidences the lack of antibody immunoreactivity with the membrane proteins.

A second experiment was performed to characterize the binding of pCry1Ac to intestinal surface proteins. The presence of biotin, glucose, mannose and *N*-acetyl-glucosamine in the reaction buffer of ligand blot assay did not inhibit the protoxin interaction with the BBMV proteins (Fig. 2).

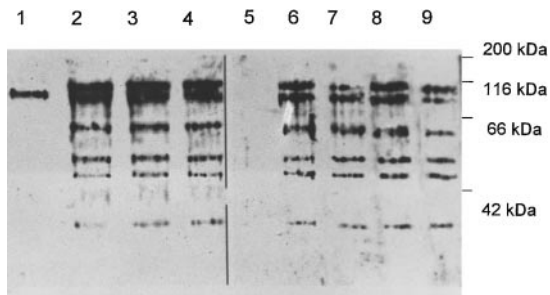

FIG. 2. Ligand blotting assay performed with BBMVs isolated from mouse small intestine. (Left) Amounts of BBMV proteins of 5 (1), 10 (2), 15 (3), and 20 μg (4) were loaded onto a SDS–PAGE and blotted to a nitrocellulose membrane. The membrane was incubated with 10 μg of pCry1Ac and the bound protoxin was detected using a polyclonal antibodies. (Right) Strips of nitrocellulose membrane, contained 15 μg of resolved proteins from mouse BBMVs, were incubated with pCry1Ac mixed with glucose (6), mannose (7), *N*-acetyl-galactosamine (8), or biotin (9). Line 5 was treatment without pCry1Ac and was used as a negative control.

Cry1Ac effect on electric properties of mouse small intestine. The PD and Isc values of full-thickness jejunum fragments mounted in the Ussing chamber decayed very slow with a rate of -0.015 mV min^{-1} and -0.016 μA cm^2 min^{-1}, respectively (Fig. 3). The R values of this tissue in Ringer's solution were constant during the experiments. Treatment of mice jejunum with pCry1Ac raised ΔIsc in 37% and ΔPD in 31% during the first 5 min, with the increased rates of 0.092 μA cm^2 min^{-1} and 0.096 mV min^{-1}, respectively. Seven minutes later, these electrical parameters decayed with a rate of -0.040 μA cm^2 min^{-1} and -0.020 mV min^{-1}. The resistance of treated jejunum did not change during the experiments (Fig. 3).

DISCUSSION

Cry1Ac protoxin induced a high immune response in mice by both oral and intraperitoneal route (5), and had an immunostimulant effect when coadministered with other antigen (6). In this study, we demonstrate that a Cry binding protein exists in mice, which could be related with an efficient capture and processing of Cry proteins by the antigen presenting cells in the mucosal tissue. Ligand blot and immunohistochemical assays allowed us to detect six Cry1Ac binding proteins probably placed in the apical membrane of the small intestine. The data obtained by us do not discard the possibility that Cry1Ac protein could bind to another molecule in the intestine mucosal surface. Other bacterial toxins with exceptional immunological properties like cholera toxin and *E. coli* labile enterotoxin from *E. coli* (LT), bind to gangliosides presents on the intestinal surface of vertebrates (16).

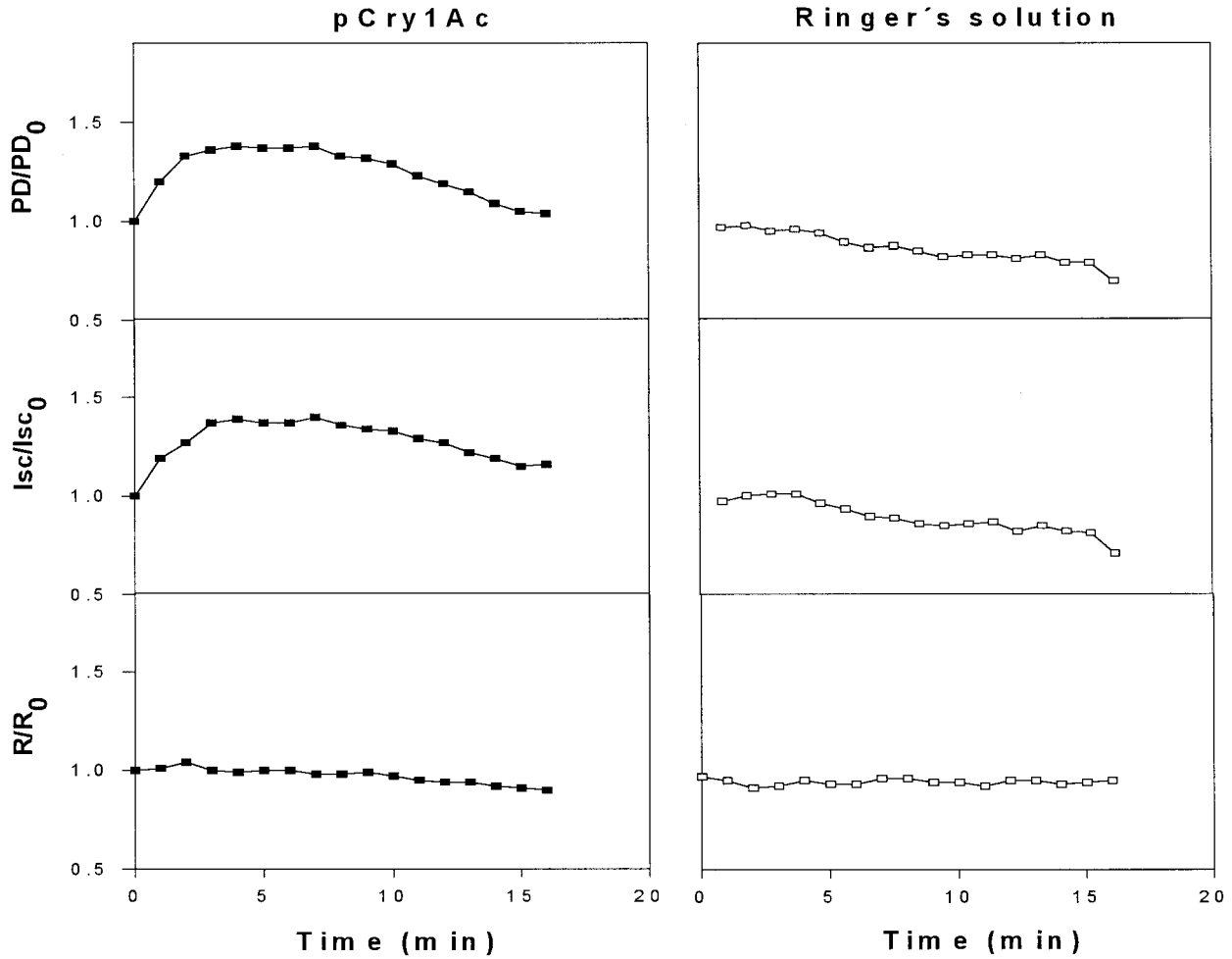

FIG. 3. Effects of pCry1Ac protoxin on the electrophysiological properties of mouse jejunum preparation. Both hemichambers contained Ringer's solution gassed with 95% O_2–5% CO_2, and 15 min later 7.5 μg of protoxin were added to each preparation on the mucosal side. The symbols represent the average normalized values of PD/PD_0, Isc/Isc_0, and R/R_0 ratios, i.e., (value for each time)/(initial values). Each point represents the mean of four experiments.

Putative Bt toxin receptors have been found in the apical membrane of the columnar cells present in the insect midgut, and have been identified in many cases as either 100- or 120-kDa N-aminopeptidases (17) or 200- to 220-kDa E-cadherine-like proteins (18). Similar proteins are present in vertebrate bowel playing important roles in nutrient absorption (19) and cellular adhesion (20). In contrast with the data reported for insect BBMVs, the interaction of Cry binding protein with the protoxin is not mediated neither by sugars nor biotin, thus electrostatic interactions involving other structures may occur (17, 18).

Cry1Ac not only binds to the intestinal surfaces, but also induces a temporal hyperpolarization of the intestine without apparent tissue damage. This phenomenon could be explained by supposing that pCry1Ac, or a locally produced toxin, form cationic channels like it happens in the insect midgut (21). In this way, a net ion flux could be generated across the enterocytes increasing the positive charges on the intestinal serose side. Many substances, from simple organic molecules to complex proteins, induce changes on the electric parameters of animal intestine (22). In contrast with LT and ciguatoxins, which produce hyperpolarization on the intestinal tissue, pCry1Ac does not induce electrolyte secretions (23, 24).

For many years, Bt-based formulations containing high concentrations of Cry proteins have been used as an example of totally safe insecticides. This idea is based on three important facts: (i) Bt-based insecticides have been used for many years with no reports of consistent hypersensitivity or toxicity (25); (ii) the toxicological tests performed to support registration of Bt-based insecticides show that several strains of this microorganism are innocuous when administered to animals and man by systemic and mucosal routes (26); and (iii) Bt strains isolated from food and human infected wounds are unable to produce illness in animals

(27, 28). However, there are no histopathological or immunotoxicological assays probing the absence of a microscopic effect on animal physiology.

The results obtained by us indicate that pCry1Ac could induce temporal changes in the intestinal membrane of mice without affecting the macroscopic physiological signals, perhaps due to the existence of a mechanism that eliminates or inhibits the toxin effect. We think that previous to commercialization of food elaborated with self-insecticide transgenic plants it is necessary to perform toxicological tests to demonstrate the safety of Cry1A proteins for the mucosal tissue and for the immunological system of animals. If pCry1Ac is a totally harmless protein that binds to the intestinal surface of animals it could be now used as a carrier of vaccinal antigens for oral immunization.

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