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Bacillus thuringiensis Cry1Ac Protoxin is a Potent Systemic and Mucosal Adjuvant

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Recently we demonstrated that recombinant Cry1Ac protoxin from *Bacillus thuringiensis* is a potent systemic and mucosal immunogen. In this study we compared the adjuvant effects of Cry1Ac and cholera toxin (CT) for the hepatitis B surface antigen (HBsAg) and bovine serum albumin (BSA). The antibody responses of intestinal secretions and serum were determined by ELISA in Balb/c mice immunized through the intragastric (IG) or intraperitoneal (IP) routes. When HBsAg was administered via IG, the anti-HBsAg intestinal response was not enhanced by either Cry1Ac or CT, whereas via IP Cry1Ac increased the anti-HBsAg intestinal immunoglobulin (Ig)G response and CT increased the intestinal IgA and IgM responses. Serum anti-BSA antibodies increased when BSA was co-administered with CT or Cry1Ac by both routes. Cholera toxin and Cry1Ac co-administered via IP increased the IgG anti-BSA response in fluid of the large intestine and CT also increased the IgA and IgM responses slightly. When co-administered via IP, CT and Cry1Ac did not affect the IgG anti-BSA response of the small intestine significantly. We conclude that Cry1Ac is a mucosal and systemic adjuvant as potent as CT which enhances mostly serum and intestinal IgG antibody responses, especially at the large intestine, and its effects depend on the route and antigen used. These features make Cry1Ac of potential use as carrier and/or adjuvant in mucosal and parenteral vaccines.

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INTRODUCTION

Oral antigen delivery systems that potentiate mucosal immune responses against adherent pathogens have received considerable attention because parenteral immunization usually elicits little or no mucosal immunity [1]. Vaccine strategies to improve mucosal immunity include the use of mucosal adjuvants, because most antigens are poor immunogens or induce tolerance when administered alone by the oral route or locally on mucosae [2].

Cholera toxin (CT) and the heat-labile enterotoxin (LT) from enteropathogenic *Escherichia coli* have strong adjuvant effects when they are administered to mice *per os* either mixed or conjugated with poor mucosal immunogens [3, 4]. Both increase antigen uptake and presentation by accessory cells [5, 6] as well as Th2-type immune responses [4] and decrease or eliminate suppressive responses [7]. However, they appear to be inappropriate for use in vaccines because of their toxicity and high production costs [2, 8]. Non-toxic CT and LT mutants that maintain their adjuvanticity [9, 10] would be more convenient but still expensive to produce.

Major features of the Cry proteins from *Bacillus thuringiensis* (*Bt*) are their high resistance to proteolysis, their stability in alkaline pH and the fact that they are innocuous to vertebrates [11]. Cry proteins are used as biopesticides and are massively and inexpensively produced through large-scale fermentation based on either *Bt* or transgenic micro-organism cultures [11, 12]. Recently we found that recombinant soluble Cry1Ac protoxin (Cry1Ac) administered to mice by the intraperitoneal (IP) or the intragastric (IG) route is a systemic and mucosal immunogen as potent as CT [13]. These results suggested to us that Cry1Ac may be of use as an antigen carrier.

The present study was undertaken to examine the systemic and mucosal adjuvanticity of recombinant Cry1Ac for the hepatitis B surface antigen (HBsAg) and for bovine serum albumin (BSA). Co-administered via IG, Cry1Ac enhanced the serum anti-HBsAg and anti-BSA antibody responses of the immunoglobulin (Ig)M, IgG and IgA isotypes, whereas via IP it enhanced mostly

intestinal IgG antibody responses. These results indicate that Cry1Ac is indeed a potent and inexpensive adjuvant of potential use in oral or parenteral vaccines.

MATERIALS AND METHODS

Organism and culture conditions. *Escherichia coli* JM103 (pOS4201) was kindly donated by Dr D. Dean, Ohio State University, Ohio, USA. The recombinant strain was grown in Luria Bertani (LB) medium containing 50 μg ampicillin per ml and the induction of Cry1Ac protein production was performed using isopropyl thiogalactoside (IPTG) [12].

Immunogens. Cholera toxin and BSA were purchased from Sigma Chemical Co. (St Louis, MO, USA). We purchased HBsAg from Heber Biotech (Havana, Cuba).

Recombinant Cry1Ac was purified from IPTG-induced *E. coli* JM103 (pOS4201) cultures [12]. Cell pellets were resuspended in TE (50 mM Tris-HCl, 50 mM EDTA, pH 8) buffer and sonicated (Fisher Sonic Dismembrator Model 300 Laboratory Equipment Company, Hayward, CA, USA) three times for 5 min in ice. Inclusion bodies were collected by centrifugation at 10 000 ***g*** for 10 min. The pellets were washed twice with TE buffer, solubilised in CBP (1 mM PMSF in CB) buffer (0.1 M Na_2CO_3, 1% 2-mercaptoethanol, 1 mM phenyl methyl sulphongl fluoride (PMSF), pH 9.6), and the particulated material was discarded by centrifugation. Purified proteins were examined by SDS-PAGE [14], and protein concentration was determined using Bradford's method [15].

Immunizations. In all experiments 8–10-week-old female Balb/c mice were used. Antigens were administered via IP in 0.1 ml phosphate-buffered saline (PBS), and via IG in 0.1 ml magnesium–aluminium hydroxide suspension (Maalox–Ciba Geigy, Mexico City, Mexico). Each experimental group contained five animals to which three antigen doses were applied on days 1, 7 and 14 either by the IP or IG route. Mice were sacrificed 7 days after the last immunization. The immunogens administered to determine the effect of Cry1Ac and CT on the immune response to HBsAg were: (1) 10 μg HbsAg; (2) 10 μg HBsAg plus 10 μg CT; (3) 10 μg HBsAg plus 10 μg Cry1Ac; and (4) 10 μg HBsAg plus 100 μg Cry1Ac. To determine the effect of Cry1Ac and CT on the immune response to BSA, mixtures containing 10 μg of BSA were administered. Control mice received 10 or 100 μg of Cry1Ac alone. Mice were killed on day 21 and serum and intestinal samples collected from them.

Sample collection. Fluids from the small and large intestine were collected as described by Moreno-Fierros *et al*. [16]. The contents of the small intestine were flushed out with 5 ml cold PBSC (1% casein in PBS) and those of the large intestine with 3 ml of the same solution. Each sample was centrifuged for 10 min at 8000 ***g*** and the supernatants were frozen immediately and stored at − 20°C. Serum samples were obtained from blood extracted by cardiac puncture from ether-anaesthetised mice.

ELISA. Antibody levels in sera and intestinal fluid were determined by an enzyme-linked immunosorbent assay (ELISA) [17]. Briefly, 96-well plates were coated with 100 μl of HBsAg (10 μg/ml), BSA (10 μg/ml), Cry1Ac (10 μg/ml) or CT (5 μg/ml) in carbonate buffer (0.1 M Na_2CO_3, pH 9.6). Plates were incubated for 2 h at 37°C and washed three times with 0.05% Tween 20 in PBS (PBST). Blocking was performed with PBSCT (1% casein in PBST) and further washing with PBST. Serial dilution of sera was done with PBSCT. Small and large intestinal fluid samples were diluted with ice-cold PBSCT and 100-μl volumes were added to microwells. The plates were incubated overnight at 4°C, washed with PBST and then anti-IgG, anti-IgM (Pierce, Rockford, IL, USA) or anti-IgA (Sigma) secondary antibodies (peroxidase-labelled goat anti-mouse) were added and incubated at room temperature for 2 h. The plates were washed and enzymatic reactions started by addition of substrate solution (0.5 mg/ml *o*-phenylendiamine, 0.01% H_2O_2 in 0.05 M citrate buffer, pH 5.2). After 15 min, the reactions were stopped with 2.5 N H_2SO_4 and absorbance at 492 nm (A_{492}) measured with an ELISA Multiskan reader (Labsystems Oy, Helsinki, Finland). Titres were defined as the reciprocal values of the highest end-point dilution of samples having an $A_{492} > 0.05$, and the specific antibody levels in the intestinal fluid were expressed as their corresponding A_{492} values.

Calculations and statistics. The significance of differences between antibody levels in the experimental groups used was determined with the Student's *t*-test. In the figures, bars represent the mean of antibody levels ± SD for each experimental group ($n = 5$).

RESULTS

Adjuvant effect of Cry1Ac on HBsAg immunization

To investigate Cry1Ac adjuvanticity, mice were immunized with HBsAg either alone or co-administered with Cry1Ac or CT. Intraperitoneal immunization with HBsAg alone induced high IgM and IgG antibody responses that were not increased by Cry1Ac co-administration. Cholera toxin increased IgG antibodies slightly ($P < 0.05$), but not IgM antibodies. In contrast, CT and Cry1Ac doubled the IgA anti-HBsAg titres ($P < 0.05$) (Fig. 1).

Intragastric administration of HBsAg alone failed to induce any antibody response. However, when co-administered with Cry1Ac or CT, a strong serum response was observed. The enhancement of anti-HBsAg IgG and IgA antibody titres after co-administration of 100 μg Cry1Ac, and those of IgM after co-administration of 10 μg Cry1Ac, were comparable to those caused by co-administration of 10 μg CT. Both doses of Cry1Ac enhanced the IgG anti-HBsAg antibodies ($P < 0.05$), but 10 μg stimulated the IgM response ($P < 0.05$) whereas 100 μg enhanced the IgA response ($P < 0.05$) (Fig. 1).

Intragastric immunization with HBsAg alone or co-administered with Cry1Ac or CT did not induce anti-HBsAg intestinal antibody responses (data not shown).

Intraperitoneal co-administration of HBsAg with CT increased IgA but not IgG anti-HBsAg responses in the small intestinal fluid. HBsAg co-administered with 10 or 100 μg Cry1Ac induced higher levels of specific IgG antibodies in the small intestine than those attained with antigen alone or co-administered with CT ($P < 0.05$). In contrast with CT co-administration, Cry1Ac co-administration did not affect the IgA anti-HBsAg intestinal response (Fig. 2).

Intraperitoneal co-administration of HBsAg with CT induced moderate levels of IgM- and IgA-specific antibodies in the fluid of the large intestine, whereas IgG anti-HBsAg antibodies of the same fluid increased only when the antigen was co-administered with 10 μg of Cry1Ac ($P < 0.05$) (Fig. 2).

Adjuvant effect of Cry1Ac on BSA immunization

We also used BSA as an antigen to analyse the adjuvant properties of Cry1Ac. Whereas BSA alone via IG did not induce detectable specific serum antibodies, BSA co-administered

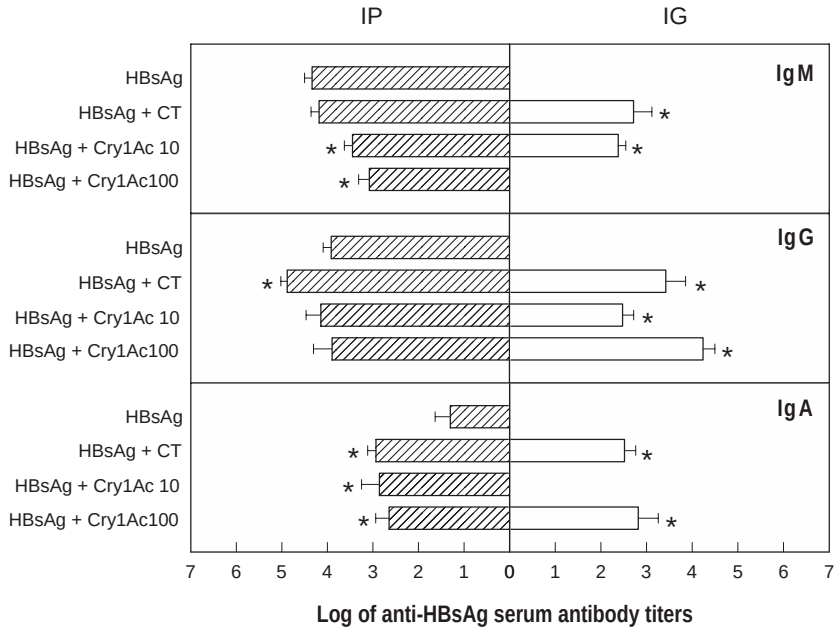

Fig. 1. Anti-HBsAg serum antibody titres. Mice were immunized via IP or IG with 10 μg HBsAg alone or co-administered with CT or Cry1Ac at doses of 10 μg (Cry1Ac 10) or 100 μg (Cry1Ac 100). The anti-HBsAg IgA, IgG and IgM serum antibody titres are expressed as the logarithm of their end-point dilution. Asterisks indicate significant differences ($P < 0.05$) with the group of mice immunized with antigen alone.

with Cry1Ac or CT increased the serum antibody responses. Anti-BSA IgG, IgM and IgA antibody levels induced after Cry1Ac co-administration via IP or IG were comparable or even higher than those attained by CT co-administration (Fig. 3).

Bovine serum albumin alone by both routes was unable to induce a mucosal immune response. Only IgG anti-BSA antibodies were detected in the fluid of the small intestine after IP co-administration of BSA with CT ($P < 0.05$) (Fig. 4). Cry1Ac co-administration via IP enhanced the IgG anti-BSA response in the large intestine in a similar way to CT co-administration ($P < 0.05$). In contrast to CT, 100 μg Cry1Ac enhanced anti-BSA IgA and IgM antibodies in the fluid of the large intestine ($P < 0.05$). Intragastric immunization with BSA alone or with Cry1Ac or CT did not induce detectable anti-BSA antibody responses in the small intestine. However, both CT and Cry1Ac enhanced IgG anti-BSA antibodies in the large intestine; CT co-administration also induced anti-BSA IgA antibodies (data not shown).

Antibody response to Cry1Ac

As expected, administration of Cry1Ac alone or its co-administration with HBsAg or BSA by both routes induced serum

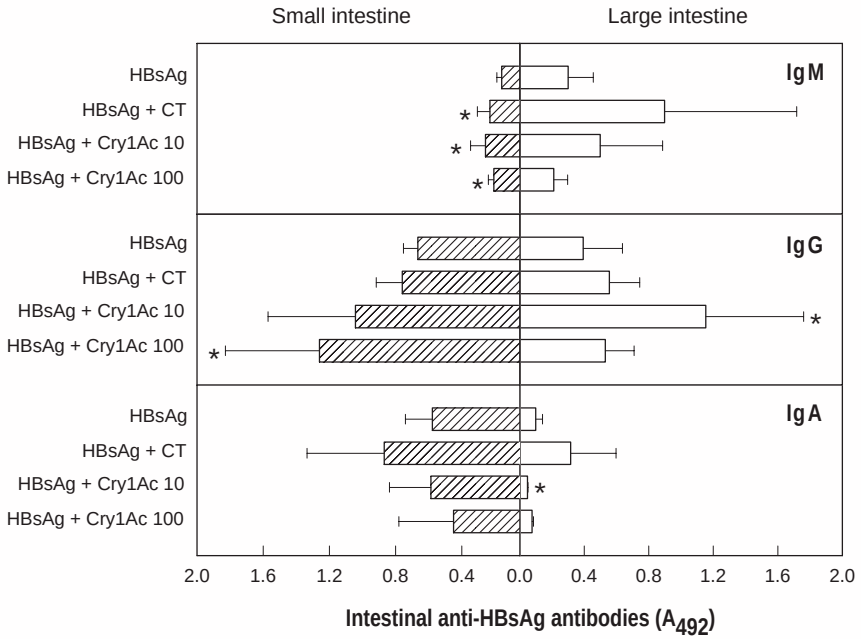

Fig. 2. Anti-HBsAg antibody levels in the fluid of the small and the large intestines. Mice were immunized via IP with 10 μg of HBsAg alone or co-administered with CT or Cry1Ac. Anti-HBsAg IgA, IgG and IgM coproantibody levels were determined by ELISA and their values expressed as A_{492} readings. Asterisks indicate significant differences ($P < 0.05$) with the group of mice immunized with antigen alone.

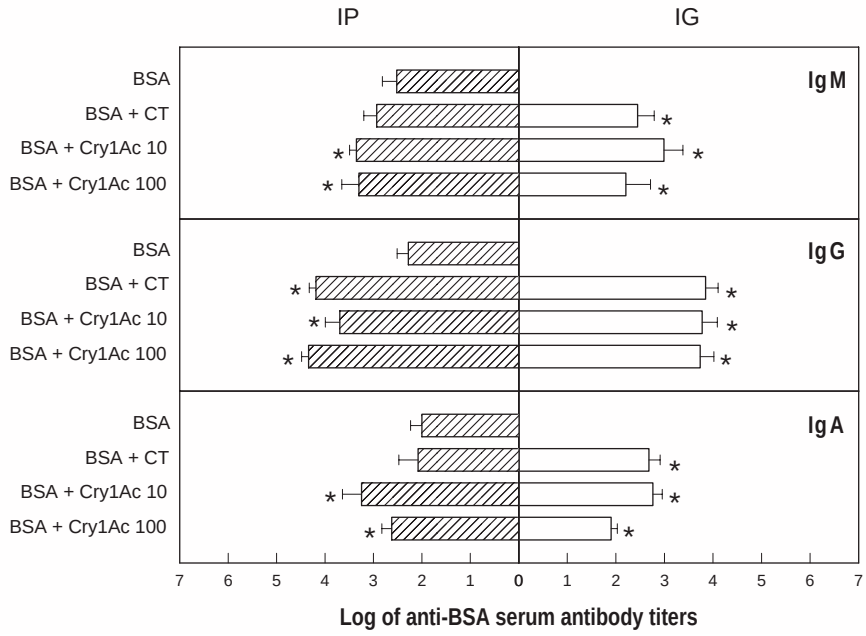

Fig. 3. Anti-BSA serum antibody titres. Mice were immunized via IP or IG with 10 μg of BSA alone or co-administered with CT or Cry1Ac at doses of 10 μg (Cry1Ac 10) or 100 μg (Cry1Ac 100). Anti-BSA IgA, IgG and IgM serum antibody titres are expressed as the logarithm of their end-point dilution value. Asterisks indicate significant differences ($P < 0.05$) with the group of mice immunized with antigen alone.

anti-Cry1Ac antibodies; the highest titres were attained with the highest protoxin dose (Table 1).

The immune response to Cry1Ac was affected by the antigen co-administered and the route used. Slightly enhanced serum IgG anti-Cry1Ac antibody levels were found when 100 μg of the protoxin were co-administered with 10 μg of HBsAg via IP. However, protoxin co-administration with BSA via IP significantly decreased IgA and IgG anti-Cry1Ac serum antibody responses ($P < 0.05$). Administration of BSA with 100 μg Cry1Ac via IG also decreased the anti-Cry1Ac serum IgA antibody response ($P < 0.05$). A remarkable decrease of the IgM anti-Cry1Ac antibody response was found when HBsAg was co-administered with 10 μg of the protoxin via IG ($P < 0.05$) (Table 1).

Co-administration of HBsAg with Cry1Ac via IP significantly enhanced the content of IgG, IgA, and IgM anti-Cry1Ac antibodies in the fluid of the small and the large intestines (Table 2). Anti-Cry1Ac IgG antibody levels were highest in the fluid of the large intestine with both doses ($P < 0.05$) and in the fluid of the small intestine with 100 μg Cry1Ac ($P < 0.05$). Anti-Cry1Ac IgA

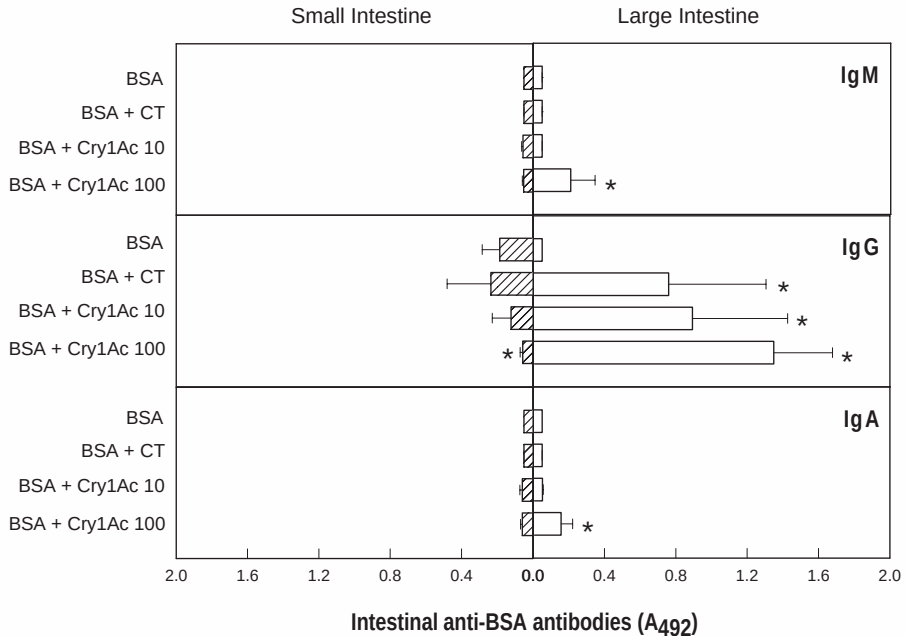

Fig. 4. Anti-BSA antibody levels in the fluid of the small and the large intestines. Mice were immunized via IP with BSA alone or co-administered with CT or Cry1Ac. Anti-HBsAg IgA, IgG and IgM coproantibody levels were determined by ELISA and their values expressed as A_{492} readings. Asterisks indicate significant differences ($P < 0.05$) with the group of mice immunized with antigen alone.

Table 1 Anti-Cry1Ac IgA, IgG and IgM antibodies in sera from mice to which Cry1Ac was co-administered intraperitoneally (IP) or intragastrically (IG) with BSA or HBsAg

Route	Cry1Ac dose	Co-administered antigen*	Anti-Cry1Ac antibody titres†		
			IgA	IgG	IgM
IP	10 μg	None	$(2.7 \pm 1.6)\ 10^3$	$(4.2 \pm 1.6)\ 10^4$	$(6.4 \pm 0.9)\ 10^3$
		BSA	$(6.0 \pm 6.2)\ 10^2$‡	$(2.2 \pm 0.8)\ 10^5$‡	$(5.6 \pm 4.2)\ 10^4$‡
		HBsAg	$(1.9 \pm 2.6)\ 10^3$	$(1.0 \pm 1.7)\ 10^6$	$(2.2 \pm 0.9)\ 10^3$‡
	100 μg	None	$(5.1 \pm 1.7)\ 10^3$	$(1.8 \pm 2.1)\ 10^6$	$(3.2 \pm 0)\ 10^3$
		BSA	$(1.8 \pm 0.4)\ 10^2$‡	$(7.6 \pm 2.8)\ 10^4$	$(1.9 \pm 0.9)\ 10^4$‡
		HBsAg	$(2.6 \pm 0.8)\ 10^3$‡	$(3.3 \pm 1.7)\ 10^6$	$(3.2 \pm 0)\ 10^3$
IG	10 μg	None	$(2.4 \pm 2.2)\ 10^3$	$(5.0 \pm 7.4)\ 10^3$	$(2.3 \pm 1.5)\ 10^3$
		BSA	$(2.0 \pm 1.4)\ 10^2$‡	$(1.8 \pm 2.0)\ 10^4$	$(1.2 \pm 1.3)\ 10^3$‡
		HBsAg	$(1.2 \pm 0.4)\ 10^2$‡	$(5.0 \pm 3.0)\ 10^2$	0.1 ± 0.1‡
	100 μg	None	$(5.0 \pm 6.2)10^2$	$(2.6 \pm 5.6)\ 10^4$	0.1 ± 0.1
		BSA	0.1 ± 0.1‡	$(3.0 \pm 2.0)\ 10^3$	$(0.5 \pm 0.3)\ 10^2$
		HBsAg	$(7.6 \pm 7.6)\ 10^2$	$(2.5 \pm 2.6)\ 10^4$	0.1 ± 0.1

*Three weekly 10 μg doses.

†Values are the mean ± SD of anti-Cry1Ac titres for antibody isotypes, determined by an end-point dilution ELISA in each group of mice ($n = 5$).

‡$P < 0.05$, compared with the group of mice which received corresponding dose of Cry1Ac alone.

antibody levels increased significantly in the fluid of the small and large intestines when HBsAg was co-administered with 10 μg Cry1Ac ($P < 0.05$), and in that of the large intestine only when HBsAg was co-administered with 100 μg of Cry1Ac ($P < 0.05$). A higher level of anti-Cry1Ac IgM antibodies was induced by HBsAg co-administration with 10 μg Cry1Ac in the fluid of the large intestine ($P < 0.05$) and with 100 μg Cry1Ac in fluids of both the large and the small intestines ($P < 0.05$).

Table 2 A_{492} readings for Anti-Cry1Ac IgA, IgG and IgM antibody isotypes detected by ELISA in the fluids of the small and large intestine from mice to which Cry1Ac was co-administered intraperitoneally (IP) or intragastrically (IG) with BSA or HBsAg

Route	Cry1Ac dose	Co-administered antigen*	Anti-Cry1Ac antibody levels†					
			IgA		IgG		IgM	
			Small intestine	Large intestine	Small intestine	Large intestine	Small intestine	Large intestine
IP	10 μg	None	0.06 ± 0.01	0.05 ± 0.004	0.06 ± 0.009	0.53 ± 0.11	0.06 ± 0.004	0.09 ± 0.04
		BSA	0.02 ± 0.001	0.02 ± 0.001	0.025 ± 0.04	0.02 ± 0.002‡	0.02 ± 0.001	0.02 ± 0.001
		HBsAg	0.93 ± 0.32‡	0.12 ± 0.03	0.45 ± 0.34‡	1.10 ± 0.13‡	0.04 ± 0.01	0.20 ± 0.09‡
	100 μg	None	0.40 ± 0.27	0.15 ± 0.05	0.45 ± 0.19	0.65 ± 0.54	0.03 ± 0.003	0.03 ± 0.001
		BSA	0.25 ± 0.03	0.04 ± 0.03	0.025 ± 0.045‡	0.04 ± 0.04‡	0.10 ± 0.06	0.02 ± 0.06
		HBsAg	0.15 ± 0.06	0.40 ± 0.34	1.50 ± 0.20	1.50 ± 0.10‡	0.60 ± 0.50	0.60 ± 0.27
IG	10 μg	None	0.06 ± 0.005	0.08 ± 0.02	0.07 ± 0.02	0.22 ± 0.37	0.07 ± 0.01	0.06 ± 0.02
		BSA	0.11 ± 0.02	0.05 ± 0.05	0.04 ± 0.02	0.05 ± 0.05	0.04 ± 0.004	0.05 ± 0.05
		HBsAg	0.24 ± 0.10‡	0.08 ± 0.06	0.10 ± 0.03	0.20 ± 0.12	0.04 ± 0.009	0.05 ± 0.01
	100 μg	None	0.20 ± 0.20	0.07 ± 0.02	0.10 ± 0.04	0.42 ± 0.35	0.04 ± 0.01	0.03 ± 0.005
		BSA	0.05 ± 0.03	0.05 ± 0.03	0.05 ± 0.05	0.04 ± 0.04‡	0.05 ± 0.05	0.04 ± 0.04
		HBsAg	0.13 ± 0.06	0.15 ± 0.16	0.10 ± 0.10	0.13 ± 0.18	0.03 ± 0.003	0.04 ± 0.01

*Three weekly 10 μg doses.

†Values are the mean ± SD of anti-Cry1Ac direct A_{492} readings for antibody isotypes, determined by ELISA in each group of mice ($n = 5$).

‡$P < 0.05$, compared with the group of mice which received corresponding dose of Cry1Ac alone.

Co-administration of HBsAg with Cry1Ac via IG did not affect the intestinal anti-Cry1Ac antibodies except for the increase of anti-Cry1Ac IgA antibody level in the fluid of the small intestine using the lowest Cry1Ac dose (Table 2). Co-administration with BSA had a negative effect on the anti-Cry1Ac mucosal immune response. The antibody levels detected in the fluids of the large and small intestines of mice immunized with Cry1Ac plus BSA were similar or lower than those of mice immunized with Cry1Ac alone (Table 2).

Antibody response to CT

Cholera toxin induced a strong anti-CT serum antibody response when co-administered with HBsAg or BSA by both routes. Anti-CT antibody titres were similar to anti-Cry1Ac titres. Anti-CT IgA antibodies predominated in the small intestine and anti-CT IgG antibodies in the large intestine (data not shown).

DISCUSSION

This work confirms our previous findings [13] that Cry1Ac is highly immunogenic and induces a mucosal (intestinal) immune response when administered via IG or IP. It also shows that Cry1Ac is a mucosal and systemic adjuvant as potent as CT.

Bt protoxins have been widely studied to determine their bioinsecticidal mechanisms [18] and the Cry genes encoding them have been cloned and transferred to bacteria and plants to generate auto-pesticidal organisms [19, 20]. However, there are few studies on the physiological or immunological effects of the Cry family proteins on vertebrate organisms, despite the known homology of *Bt* with the pathogenic *B. cereus* species [21]. Cry proteins induce high serum antibody titres in mice and rabbits [22] and appear to have anti-tumour activity against sarcoma cells possibly related to their ability to enhance general immunity [23]. The same authors showed that insecticide crystals from *Bt* spores enhance the immune response to sheep red blood cells [24].

We found that the adjuvant potency of Cry1Ac was similar to that of CT, but the first enhanced mainly IgG whereas the second enhanced IgA antibody responses. The adjuvant properties of both proteins depended on the immunization route, the antibody isotype and the site where the response was analysed, and the antigen co-administered. Anti-HBsAg and anti-BSA serum antibodies of the IgM, IgG and IgA isotypes increased significantly when both antigens were co-administered with Cry1Ac via IG, and the intestinal immune responses to HBsAg and BSA were enhanced when both antigens were co-administered with Cry1Ac via IP. In contrast with BSA, HBsAg alone or co-administered with both adjuvants via IG failed to induce a local immune response. Wilson *et al*. [25] observed a similar effect when ovalbumin was orally co-administered with CT. These findings suggest that oral Cry1Ac turns on extra-intestinal but not GALT antibody-secreting cells and that the adjuvant effects of Cry1Ac and CT are restricted to specific antigens.

Since adjuvant effects vary with the antigen [26] and the immunization route [27], the striking differences in the levels of serum IgM and IgA against HBsAg but not BSA administered with Cry1Ac via IG could be due to a higher antigenic lability of HBsAg in the gastrointestinal tract.

Parenteral HBsAg, the antigen on which effective commercial anti-hepatitis vaccines are based [28], induced specific IgG and IgA antibody secretion in the small and the large intestine that was enhanced by Cry1Ac co-administration. If this effect is also produced in humans, Cry1Ac would be a good adjuvant in anti-hepatitis B vaccination.

The mechanism by which Cry1Ac acts as an adjuvant to orally and parenterally administered antigens is unclear. There are no known functional or structural similarities between Cry1Ac and CT or LT. The latter two bacterial enterotoxins, which have the highest known mucosal adjuvant potency, act on the immune system by increasing antigen uptake by the intestine [5] and/or stimulating the activity of antigen-presenting cells [6] and eliminating the suppressive immune response induced by orally administered antigens [3, 7].

The toxicity and elevated production costs of CT or LT have limited their application in human vaccines [2, 8]. In contrast, δ-exotoxin-free *Bt* preparations containing Cry proteins have no significant toxicity for mammals [29] and large-scale production of recombinant Cry1Ac protein is easy and cheap [11, 12]. These facts and the findings described in this paper suggest that Cry1Ac may indeed be a convenient systemic and mucosal carrier and adjuvant for use in human and animal vaccines.

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REFERENCES

1 Michalek SM, Eldridge JH, Curtis R III, Rosenthal KL. Antigen delivery systems: new approaches to mucosal immunization. In: Ogra PL, Lamm ME, McGhee JR, Mestecky J, Strober W, Bienestock J, eds. Handbook of Mucosal Immunology. New York: Academic Press, 1994:373–6.

2 Elson CO, Dertzbaugh MT. Mucosal adjuvants. In: Ogra PL, Lamm ME, McGhee JR, Mestecky J, Strober W, Bienestock J, eds. Handbook of Mucosal Immunology. New York: Academic Press, 1994:391–401.

3 Clements JD, Hartzog NM, Lyon FL. Adjuvant activity of *Escherichia coli* heat labile enterotoxin and effect on the induction of oral tolerance in mice to unrelated protein antigens. Vaccine 1988; 6:269–77.

4 Lycke N, Holmgren J. Strong adjuvant properties of cholera toxin on gut mucosal immune responses to orally presented antigens. Immunol 1986;59:301–8.

5 Lycke N, Karlsson A, Sjôlander A, Magnusson KE. The adjuvant action of cholera toxin is associated with an increased intestinal permeability for luminal antigens. Scand J Immunol 1991;33:691–8.

6 Bromader A, Holmgren J, Lycke N. Cholera toxin stimulates IL-1 production and enhanced antigen presentation by macrophages *in vitro*. J Immunol 1991;146:2908–14.

7 Elson CO, Holland S, Woogen S. Preferential inhibition of the $CD4^+$ T cell subset by cholera toxin (CT) and its B Subunit (CT-B). FASEB J 1990;4:A1864.

8 Spangler BD. Structure and function of cholera toxin and related *Escherichia coli* heat labile enterotoxin. Microbiol Rev 1992; 46:622–47.
9 Yamamoto S, Kiyono H, Yamamoto M *et al.* A nontoxic mutant of cholera toxin elicits Th2-type response for enhanced mucosal immunity. Proc Natl Acad Sci USA 1997;94:5267–72.
10 Dickinson BL, Clements JD. Dissociation of *Escherichia coli* heat-labile enterotoxin adjuvanticitiy from ADP-ribosyltransferase activity. Infect Immun 1995;63:1617–23.
11 Höfte H, Whiteley H. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol Rev 1989;53:242–55.
12 Ge AZ, Pfister RM, Dean DH. Hyperexpression of a *Bacillus thuringiensis* delta-endotoxin-encoding gene in *Escherichia coli*: properties of the product. Gene 1990;93:49–54.
13 Vázquez-Padrón RI, Moreno-Fierros L, Neri-Bazan L, de la Riva GA, Lopez-Revilla R. Intragastric and intraperitoneal administration of Cry1Ac protoxin from Bacillus thuringiensis induces systemic and mucosal antibody responses in mice. Life Sci 1999;64:1897–1972.
14 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
15 Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
16 Moreno-Fierros L, Domínguez-Robles MA, Enríquez-Rincón F. *Entamoeba histolytica*: induction and isotype analysis of antibody producing cell responses in Peyer's patches and spleen after local and systemic immunization in male and female mice. Exp Parasitol 1995;80:541–9.
17 Hornbeck P. Indirect ELISA to detect specific antibodies. In: Coligan JE, Kruisbeek AM, Margulies DH, Saevach ET, Struber W, eds. Current Protocols in Immunology. New York: Wiley, 1996:2.1.3–6.
18 Knowles B. Mechanism of action of *Bacillus thuringiensis* insecticidal delta-endotoxin. Adv Insect Physiol 1994;24:275–3076.
19 Herrera G, Snyman S, Thompson J. Construction of a bioinsecticidal strain of *Pseudomonas fluorescens* active against the sugarcane borer, *Eldana saccharina*. Appl Environ Microbiol 1994;60:682–90.
20 Perlak FJ, Deaton RW, Armstrong TA *et al.* Insect resistant cotton plants. Biotechnol 1990;8:939.
21 Carlson CR, Caugant DA, Kolsto AB. Genotypic diversity among *Bacillus thuringiensis* and *Bacillus cereus* strains. Appl Environ Microbiol 1994;60:1719–25.
22 Vázquez RI, de Prieto D, la Riva G, Selman-Housein G. Development of an immunoradiometric assay for quantitative determination of CryIA (b) protein in transgenic sugarcane plants. J Immunol Meth 1996;196:33–9.
23 Prasad SSSV, Shethna YI. Antitumor immunity against YAS after treatment with proteinaceous crystals of *Bacillus thuringiensis*. Indian J Exp Biol 1976;14:285–90.
24 Prasad SSSV, Shethna YI. Enhancement of immune response by the proteinaceous crystal of *Bacillus thuringiensis*. Biochim Biophys Res Commun 1975;62:517–21.
25 Wilson AD, Clarke CJ, Stokes CR. Whole cholera toxin and B subunit act synergistically as an adjuvant for the mucosal immune response of mice to keyhole limpet haemocyanin. Scand J Immunol 1990;31:443–51.
26 Wilson AD, Stokes CR, Bourne FJ. Adjuvant effect of cholera toxin on the mucosal immune response to soluble proteins. Scand J Immunol 1989;29:739–45.
27 Hopkins S, Krahenbuhl J-P, Schödel F *et al.* A recombinant *Salmonella typhimurium* vaccine induces local immunity by four different routes of immunization. Infect Immun 1995;63:3279–86.
28 Milich DR, McLachlan A, Thornton GB, Hughes JL. Antibody production to the nucleocapsid and envelope of the hepatitis B virus primed by a single synthetic T cell site. Nature 1987;329: 547–9.
29 McClintock JT, Schaffer CR, Sjoblad RD. A comparative review of the mammalian toxicity of *Bacillus thuringiensis*-based pesticides. Pestic Sci 1995;45:95–105.