ENHANCEMENT OF IMMUNE RESPONSE BY THE PROTEINACEOUS CRYSTAL OF *BACILLUS THURINGIENSIS* VAR *THURINGIENSIS*

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SUMMARY: The proteinaceous crystal of *Bacillus thuringiensis* var *thuringiensis* was found to enhance humoral immune response in rats and guinea pigs immunised with sheep red blood cells. The enhancement was due to the increased levels of both 19S and 7S antibodies in the sera of the treated animals. A novel synthesis of 7S haemolytic antibodies was observed in case of crystal treated animals.

INTRODUCTION

Nonspecific immunoenhancement is brought about by a number of agents like the heat killed cells of BCG (1,2), *Corynebacterium parvum* (3), lipopolysaccharide endotoxins of *Enterobacteriaceae* (4), *Bordetella pertussis* vaccine (5), zymosan from yeast cell walls (6) and the complexes of synthetic nucleotides like poly A and U, poly C conjugated with methylated bovine serum albumin (7). However, little is known regarding the immunoenhancement effect of pure protein components of bacterial origin. Purified L-asparaginase from *Escherichia coli*, a well known antitumour agent has been found to suppress immunity (8). We report here the enhancement of immune response by the proteinaceous crystals of *Bacillus thuringiensis*.

During sporulation *Bacillus thuringiensis* produces a crystalline material which is liberated into the medium when the sporangium is lysed (9). This unique crystal is composed of an alkali soluble protein having a molecular weight of 200,000 which is highly toxic for Lepidopterous insect larvae (10) with least toxicity for mammals (11,12). Recently we have reported antitumour activity against Yoshida ascites sarcoma with high therapeutic index for this crystal preparation (12).

Further the purification, crystallization and partial characterization of the biologically active protein subunit has been achieved (13). The crystals also induce long-lasting immunity among the protected animals against further challenge with tumor cells. Antitumour immunity in these animals was demonstrated by tumour rejection and detection of enhanced humoral antibodies in the sera against cell-free extract from Yoshida ascites sarcoma cells (14). This observation initiated us to study the effect of this crystal preparation on humoral immune response in rats. The preliminary results of these studies have been presented recently (15).

MATERIALS AND METHODS

Bacillus thuringiensis var thuringiensis serotype I was kindly supplied by Dr. H. de Barjac of the Institut Pasteur, Paris. The pure cystal preparation was obtained as described earlier (13). The proteinaceous crystal suspensions were prepared freshly by mixing the freeze-dried material in sterile saline such that the required concentrations were contained in 0.2 ml of suspension.

Inbred Wistar A/Iisc rats of either sex, weighing 100-120 gms were used for immunisation. The guinea pigs used for obtaining complement were randomly bred and maintained in our laboratory. Sheep blood collected in Alsever's solution, was centrifuged at 1,000 g and washed thrice with sterile physiological saline to separate red blood cells. Fresh guinea pig serum, preadsorbed with sheep erythrocytes at 4 C was used as the source of complement.

Rats were immunised with 3×10^{10} sheep red blood cells (SRBC) intraperitoneally (i.p) on day zero. Different doses of the crystal preparations were administered 24 hr after antigenic stimulus as a single or divided doses. Untreated immunised and treated unimmunised rats served as the controls. Animals were sacrificed 7 days after antigenic stimulus, blood collected, serum separated, inactivated at 56 C for 30 min and stored frozen until further use. The dose of antigen used and the time of bleeding were earlier found to elicit maximum antibody response (16). Antibodies in the sera were quantitatively estimated by haemagglutination and haemolysin titrations (17).

RESULTS AND DISCUSSION

The haemagglutination and haemolysin titers of the animals treated with the crystals showed a significant enhancement over the immunised untreated controls (Table 1). A dose of 1 mg/Kg which was earlier observed to be the minimum effective dose against Yoshida ascites

Table 1. EFFECT OF PROTEINACEOUS CRYSTAL OF *B. THURINGIENSIS* ON HAEMOLYSIN AND HAEMAGGLUTINATION TITERS OF ANTISERA FROM RATS IMMUNISED WITH SRBC

Dose of crystal*	Haemagglutination titer**	Haemolysin titer units/ml**
0.1	2560	4835
1	2560	4794
2.5	5120	9641
5	2560	4217
10	2560	3892
1 x 5+	5120	8774
10 x 5+	5120	7336
Untreated immunised	320	1341
Treated unimmunised	0	100

* mg of crystal/Kg body weight administered i.p. 24 hrs after antigenic stimulus.
** Average of six animals.
\+ One dose/day for 5 days.

Haemagglutination and haemolysin titrations were carried out according to Campbell *et al.* (13). The reciprocal of highest dilution showing haemagglutination was taken as haemagglutination titer. The haemolysin titrations were performed with a slight modification wherein the final reaction volume was reduced to 1 ml. The data were analysed by Vankregh's equation and the haemolysin titer units/ml obtained.

sarcoma (12) also showed enhancement of immune response. A divided dose of 1 mg/Kg/day for five days, showed more pronounced enhancement than a single dose of 10 mg/Kg. The possibility that the crystals as such or the breakdown products might bring about the immunoenhancement was ruled out, as the animals administered with crystals alone without prior immunisation with SRBC did not show haemagglutination or haemolysis.

Maximum enhancement in haemagglutination and haemolysin titers

Table 2. EFFECT OF TIME OF ADMINISTRATION OF CRYSTAL OF *B. THURINGIENSIS* ON HAEMAGGLUTINATION AND HAEMOLYSIN TITERS OF ANTISERA FROM RATS IMMUNISED WITH SRBC.

Time of crystal administration after antigenic stimulus*	Haemagglutination titers**	Haemolysin titer units/ml**
- 24 hrs	320	1344
0 hr	5,120	7039
+ 24 hrs	10,240	12760
+ 48 hrs	5,120	8774
+ 72 hrs	640	1930
+ 96 hrs	640	1495
Untreated immunised	320	1553

* 2.5 mg of crystal/Kg body weight was administered i.p. to all the animals.
** Average of six animals.

were obtained when the crystals were administered 24 hr after antigenic stimulus (Table 2). The effect of crystal on the immune response of other experimental animals immunised with SRBC is presented in Table 3. The crystal was found to be equally effective in case of guinea pigs whereas mice and rabbits showed a poor response.

To verify whether the crystal mediated enhancement of immunity is due to increased levels of 19S and 7S antibodies, the SRBC immunized rat serum was analysed by Sephadex G-200 gel filtration. The elution profiles along with haemagglutination and haemolysin titers of the individual fractions are depicted in Fig. 1 (a) and (b). It is evident that SRBC immunised and crystal treated sera showed enhanced levels of 19S (IgM), and 7S (IgG) antibodies which are also associated with increased haemolysin and haemagglutination titers.

It is known that the 19S antibodies are much more effective in

Table 3. EFFECT OF PROTEINACEOUS CRYSTAL OF B. THURINGIENSIS ON HAEMOLYSIN AND HAEMAGGLUTINATION TITERS OF ANTISERA FROM OTHER EXPERIMENTAL ANIMALS IMMUNISED WITH SRBC.

Animal	Dose of crystal*	Haemagglutination titer**	Haemolysin titer units/ml**
Guinea pig	2.5	2560	4560
	1 x 5+	1280	2086
	Nil	320	1240
Mice	2.5	1280	2420
	1 x 5+	640	1892
	Nil	320	1110
Rabbit	2.5	160	778
	1 x 5+	320	1042
	Nil	160	642

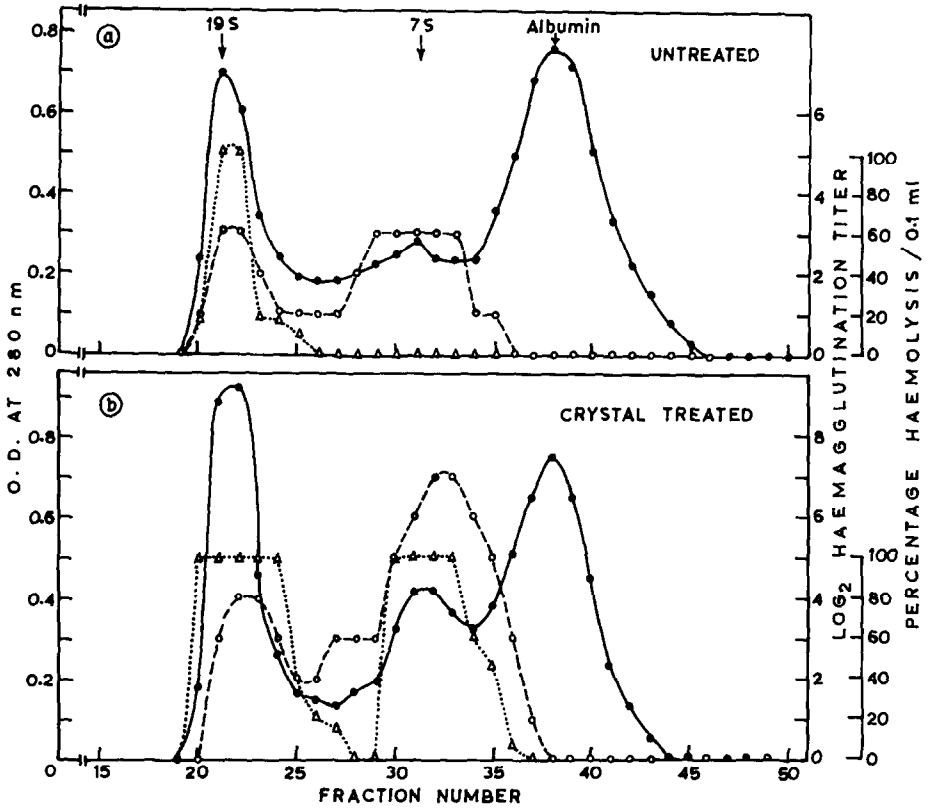
The weights of guinea pigs, Swiss mice and rabbits used were 400, 20 and 1200 gms respectively.

* mg of crystal/Kg body weight administered 24 hrs after antigenic stimulus. The doses were selected on the basis of results seen in Table 1.

** Average of six animals.

+. One dose/day for 5 days.

inducing haemolysis than 7S antibodies (18). An interesting observation here is that the 7S antibodies obtained from the crystal-treated immunised sera showed good haemolytic activity in contrast to untreated immunised sera, indicating that the crystals induce a novel synthesis of 7S haemolytic antibodies in the treated animals. This observation was confirmed by treating the IgG fraction from crystal treated sera with 0.1M 2-Mercaptoethanol (2-ME) at 37 C for 30 min to inactivate the contaminating IgM fraction (19). Mercaptoethanol was removed by gel filtration on Sephadex G-25 and the fractions assayed for haemolytic and haemagglutination titers. The same procedure was repeated with the IgM fractions. The results was that the IgG fraction from



ELUTION PROFILES OF CRYSTAL TREATED AND UNTREATED SERA ON SEPHADEX G-200 COLUMN

0.5 ml of serum from untreated (a) and treated (b) animals was put on Sephadex G-200 column (2 x 50 cm) and eluted with 0.15 M phosphate-saline buffer at pH 7.4. Three ml fractions were collected. Absorbance at 280 nm, haemolysis and haemagglutination titers of the individual fractions were determined as described earlier. ● O.D. at 280 nm, Δ Haemolysis, O Haemagglutination titer.

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the crystal treated sera retained the haemolytic activity whereas the IgM fraction had lost the haemolytic activity after treatment with 2-ME. The possibility that the contaminating IgM might be responsible for the haemolytic activity exhibited by IgG fractions of treated sera was thus ruled out.

These results suggest that the enhancement of immune response by the crystal of B. thuringiensis may be one of the way by which tumour regression and long lasting immunity against Yoshida ascites sarcoma

is brought about. The major drawback with the well known adjuvants like BCG, endotoxins of *Enterobacteriaceae*, *Bordetella pertussis* vaccine is their toxic side effects (20). The proteinaceous crystal of *B. thuringiensis* might prove itself as an ideal adjuvant in view of its known least toxicity for mammalian system.

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