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SHORT COMMUNICATION

Virulence and Immunogenicity in Experimental Animals of *Bacillus anthracis* Strains Harbouring or Lacking 110 MDa and 60 MDa Plasmids

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A comparative study was made of the virulence and immunogenicity in mice or guinea pigs of *Bacillus anthracis* strains harbouring 110 MDa and/or 60 MDa plasmids. Strains cured of the 110 MDa or the 60 MDa plasmid were more than 100-fold less virulent to mice than were the parental strains harbouring these plasmids. Guinea-pigs immunized with plasmid-free derivatives of the non-encapsulated vaccine strain 34F2 showed no resistance to challenge with strain 17JB, which harbours both 110 MDa and 60 MDa plasmids, suggesting that the derivative strains had lost their immunizing ability against anthrax.

INTRODUCTION

Bacterial plasmids confer a wide variety of phenotypic characteristics on their host, including virulence properties (Elwell & Shipley, 1980; Shipley *et al.*, 1979). Two kinds of virulence-associated plasmids with molecular masses of 110 MDa and 60 MDa have been reported in *Bacillus anthracis*, the causative agent of anthrax (Green *et al.*, 1985; Mikesell *et al.*, 1983; Uchida *et al.*, 1985). Mikesell *et al.* (1983) and Vodkin & Leppla (1983) demonstrated that the production of protective antigen in *B. anthracis* was mediated by a temperature-sensitive 110 MDa plasmid, and they suggested that Pasteur's attenuation of this organism by repeated subculture at 43 °C may have occurred as a result of curing. Recently, Uchida *et al.* (1985) and Green *et al.* (1985) reported that *B. anthracis* strains harboured two kinds of plasmids with molecular masses of 110 MDa and 60 MDa and that the encapsulation of this organism may be associated with the presence of a 60 MDa plasmid. Little information is available, however, on the correlation between the presence of these plasmids and the phenotypes of *B. anthracis* strains in experimental animals. This report describes a comparison of the virulence and immunogenicity of *B. anthracis* strains carrying 110 MDa and/or 60 MDa plasmids with those of their derivative strains cured of one or both of these plasmids.

METHODS

Bacterial strains. These are listed in Table 1. *B. anthracis* strains were cured of the 110 MDa plasmid by serial passage in Penassay broth for 10 consecutive days at 42·5 °C as described by Mikesell *et al.* (1983). Strains cured of the 60 MDa plasmid were obtained by producing non-encapsulated variants as described previously (Uchida *et al.*, 1985).

Plasmid isolation. Plasmid DNAs were isolated as described previously (Uchida *et al.*, 1985). The samples were electrophoresed through a horizontal 0·8% agarose gel at 100 V for 5 h, using Tris/borate buffer (89 mM-Tris, 2·5 mM-EDTA, 89 mM-boric acid). The gel was stained for 15 min in ethidium bromide (0·5 μg ml^{-1}) and then washed for 15 min in distilled water.

Table 1. *Bacterial strains and plasmids*

Strain	Plasmid size (MDa)	Remarks	Origin or reference	LD_{50}†
17JB	110, 60	Pasteur vaccine no. 2	CVL*	$10^{3.5}$
#17JB	60	Cured derivative of 17JB isolated after passages at 42·5 °C	This study	$>10^{6.3}$
17JB-NCV	110	Non-encapsulated variant of 17JB	Uchida *et al.* (1985)	$10^{5.5}$
#17JB-NCV	None	Cured derivative of 17JB-NCV isolated after passages at 42·5 °C	This study	$>10^{7.5}$
34F2	110	Non-encapsulated vaccine strain	Ando *et al.* (1961); CVL	$10^{5.5}$
#34F2-1	None	Cured derivatives of 34F2 isolated after passages at 42·5 °C	This study	$>10^{7.5}$
#34F2-2	None			$>10^{7.5}$
Davis	60	Asporogenous strain	Ando *et al.* (1961)	$>10^{7.3}$
Carbozoo	110, 60	Pasteur vaccine no. 2	Ando *et al.* (1961)	Not tested

* CVL, Central Veterinary Laboratory, Weybridge, UK.

† A group of five mice was injected subcutaneously with 0·2 ml spore suspension. The number of dead animals was recorded and the LD_{50} was calculated.

Test for virulence. Comparative studies of the virulence of derivative strains lacking the 110 MDa or the 60 MDa plasmid were made by subcutaneous injection of spore suspensions into 5-week-old female ICR specific pathogen-free (SPF) mice. Suspensions containing between 10^3 and 10^7 spores were used to determine the dose that killed 50% of the mice (LD_{50}). The number of dead animals was recorded during 10 d after injection.

Test for immunogenicity. Guinea-pigs were immunized subcutaneously with 0·2 ml suspension containing $10^{7.0}$ spores of the immunizing strain. Three weeks after primary inoculation, all the immunized animals and the controls were challenged subcutaneously with 0·2 ml suspension containing $10^{7.0}$ spores of strain 17JB. Animals were observed for 10 d after this challenge.

RESULTS AND DISCUSSION

All the derivative strains cured of the 110 MDa plasmid (#17JB, #17JB-NCV, #34F2-1, #34F2-2) were about 100-fold less virulent to mice than the parental strains (Table 1). The LD_{50} of the laboratory strain Davis, which is asporogenous and is widely used as a propagative strain for the bacteriophage of *B. anthracis*, corresponded to a dose of more than $10^{7.3}$. The derivative strain 17JB-NCV, which harboured the 110 MDa plasmid but had lost the 60 MDa plasmid, was also about 100-fold less virulent than its parental strain, 17JB.

The immunizing ability of strain 34F2, which is widely used as a non-encapsulated vaccine strain, was compared with that of its derivatives lacking the 110 MDa plasmid (#34F2-1, #34F2-2). Strain 34F2 possessed a high immunizing ability: all the seven guinea-pigs immunized with this strain were fully resistant to the challenge with spores of strain 17JB. In contrast, all the seven guinea pigs immunized with either of the two derivative strains (#34F2-1, #34F2-2) died within 10 d of the challenge, as did the non-immunized control animals. Strain 17JB was recovered from samples of heart blood, spleen and inoculated sites of the dead animals.

Characters such as toxin production and encapsulation have been considered to be virulence factors of *B. anthracis* (Smith, 1960). This organism produces three distinct toxins, i.e. protective antigen, oedema factor and lethal factor (Beall *et al.*, 1961; Stanley & Smith, 1961). The protective antigen is the most important factor for immunogenicity: it is closely associated with virulence and also confers a protective immunity against anthrax by itself or in combination with another toxin component (Fish *et al.*, 1968; Stanley & Smith, 1963). On the other hand, encapsulation is related to the protection of the organism from phagocytosis and lytic antibody (Preisz, 1909). The results obtained in this study revealed a close correlation between the presence of 110 MDa and 60 MDa plasmids and the virulence phenotype for mice of *B.*

anthracis. Our finding that the loss of the 110 MDa plasmid from the non-encapsulated vaccine strain 34F2 resulted in the loss of immunogenicity in guinea-pigs strongly suggests that the 110 MDa plasmid plays an important role in the virulence and immunogenicity phenotypes of this organism.

The question arises, however, as to why the attenuated Pasteur vaccine strains (17JB, Carbozoo) examined in this study possessed the 110 MDa plasmid. Mikesell *et al.* (1983) did not find the 110 MDa plasmid in the Pasteur vaccine strains (ATCC 4229, ATCC 6602) that they examined, and speculated that the plasmid might have been cured during repeated subcultures at 43 °C. Although the reason for this discrepancy is unknown, assuming that protective antigen production is mediated by 110 MDa plasmid, our finding that the Pasteur vaccine strains and the non-encapsulated vaccine strain 34F2 still harboured this plasmid may account for the immunizing ability of these strains against anthrax. The data obtained in this study suggest that the 110 MDa plasmid could be used as a genetic marker for the immunogenicity of anthrax vaccine strains such as 34F2.

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