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Intranasal immunisation against tetanus with an attenuated *Bordetella bronchiseptica* vector expressing FrgC: improved immunogenicity using a Bvg-regulated promoter to express FrgC

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Abstract

Mice were immunised intranasally with live *Bordetella bronchiseptica aroA* strains possessing plasmids encoding fragment C (FrgC) of tetanus toxin. FrgC was expressed either from a constitutive *tac* promoter (strain GVB120) or the Bvg-dependent *fhaB* promoter (strain GVB1543). Serum anti-FrgC antibody titres were detected in all mice immunised with GVB1543 and GVB120 but the average titres were higher and the responses to FrgC were more consistent in GVB1543 immunised animals. This was reflected in the protective immunity conferred by the different strains: 100% of GVB1543 immunised mice were protected against tetanus toxin challenge whereas only 60% of animals immunised with GVB120 survived tetanus challenge. Viability of the *B. bronchiseptica* vector strain was shown to be critical to its efficacy as a vector for FrgC.

Keywords: Attenuated *Bordetella bronchiseptica*; Mucosal tetanus vaccines

1. Introduction

Mucosal vaccines have a number of potential benefits over their parenterally administered counterparts. These include superior stimulation of mucosal immune responses and avoidance of the potential to spread bloodborne pathogens (such as HIV and Hepatitis B) through the reuse of contaminated needles [1,2]. Delivery of heterologous antigens to the gastrointestinal mucosa by attenuated bacterial pathogens has been studied extensively, particularly using attenuated *Salmonella* sp. [3–7]. Using attenuated bacterial pathogens to deliver heterologous antigens to the respiratory mucosa is by comparison less well developed [8,9].

The *Bordetellae* are a group of highly related Gram-negative bacteria some of whom cause respiratory infections in mammals and birds. The most widely studied of these respiratory pathogens are *B. bronchiseptica* and *B. pertussis*. *B. bronchiseptica* has a wide host range and causes respiratory infections in domestic and companion animals and very occasionally humans [10,11]. *B. pertussis*, in contrast, exclusively infects humans and causes whooping cough in infants. *B. pertussis* and *B. bronchiseptica* produce an array of virulence factors, which for many the expression is controlled by the two-component regulator BvgAS [12,13]. Some of the Bvg-regulated factors, such as filamentous haemaglutinin (FHA), pertactin and fimbriae are important antigens for stimulating protective immunity against Bordetella in both humans and animals [12].

Parenteral immunisation of animals with killed whole cell and sub-unit vaccines to *B. bronchiseptica* can generate high titre serum antibodies and this has been shown to contribute to protection [10]. However some vaccines despite inducing high serum anti-*B. bronchiseptica* titres offer only low levels of protection after challenge [10,14]. Equally good immunity to *B. bronchiseptica* can be achieved in the absence of high serum anti-*B. bronchiseptica* titres [15]. It would appear that both local secretory antibodies and cell mediated immunity are important for full immunity to *B. pertussis* and *B. bronchiseptica* [15–21].

Unlike many other bacterial pathogens, *B. pertussis* and *B. bronchiseptica* are able to efficiently colonise healthy

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ciliated respiratory mucosa and as a consequence they are highly infectious. Therefore, it may be possible that live vaccines derived from this species may function as superior live vectors for delivering heterologous antigens to the respiratory tract of a range of mammalian species including humans.

Previously we have constructed a *B. pertussis aroA* mutant and demonstrated that this strain is highly attenuated in mice. We have also shown that when this strain is administered I/N it acts as an effective live mucosal vaccine which protects against wild type *B. pertussis* infection [22]. More recently we demonstrated that a *B. bronchiseptica aroA* mutant is also attenuated in the mouse respiratory tract and I/N immunisation with this strain enhanced clearance of wild type organisms from the respiratory tract of mice following challenge [23]. The *B. bronchiseptica aroA* mutant has also been used as a mucosal vaccine to deliver a heterologous antigen to the respiratory tract. A C-terminal non-toxic portion of tetanus toxin, Fragment C (FrgC), was expressed in the *B. bronchiseptica aroA* strain on a plasmid from a constitutive promoter (*tac*). Some of the mice immunise I/N with this strain (GVB120) developed high serum antibodies against tetanus toxin and approximately 40% of mice immunised I/N with this strain were protected from tetanus challenge [23]. Previously we have shown that environmentally regulated promoters are superior to the *tac* promoter for expressing heterologous antigens in Salmonella vector strains [7]. In this study we assess the efficacy of a *B. bronchiseptica aroA* strain expressing FrgC from an environmentally regulated promoter (*fhaB*) as an intranasal tetanus vaccine.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

B. bronchiseptica strains were grown on Bordet-Gengou (BG) agar base supplemented with 12% (v/v) sheep blood. *Escherichia coli* strains were routinely cultured on Luria-Bertani (LB) agar. For liquid culture *B. bronchiseptica* and *E. coli* strains were grown in LB broth. Media contained the following antibiotics as required; streptomycin (100 μg/ml), kanamycin (10 μg/ml) and gentamicin (50 μg/ml). All bacteria were grown aerobically at 37 °C. Wild type *B. bronchiseptica* BBC17 is a spontaneous streptomycin-resistant mutant of CN7531, which was isolated from a pig with atrophic rhinitis [24]. The *B. bronchiseptica aroA* mutant (BBC18) was derived from BBC17 and its construction has been described previously [23]. BBC18 and its derivatives were grown on BG agar supplemented with aromix. One hundred times aromix stock solution consisted of tryptophan (4 mg/ml), phenylalanine (4 mg/ml), dihydroxybenzoic acid (1 mg/ml) and *para*-aminobenzoic acid (1 mg/ml). pTET85 expresses FrgC regulated by the *tac* promoter and has been described previously [7]. pBBR1MCS-5 is a broad-host range plasmid vector [25].

2.2. DNA manipulations

Standard methods were used for preparation of plasmid and chromosomal DNA, restriction analysis and ligation of DNA [26]. All enzymes were purchased from Invitrogen (Paisley, Scotland). All PCR reagents were purchased from either ABgene (Epsom, UK) or Stratagene (The Netherlands). For amplification of *B. bronchiseptica* DNA, PCR master mixes were supplemented with DMSO at a final concentration of 6% (v/v).

2.3. Bacterial transformation

Bacteria were grown to log phase in LB broth, harvested and washed twice and then resuspended in sterile dH_2O to a density of 2×10^{10} CFU/ml. DNA (200 ng) was mixed with cells and introduced into bacteria by electroporation in a Bio-Rad (UK) gene pulsar with the following settings, 1.25 kV, 25 μF and 600 Ω for *E. coli*, 2.5 kV, 25 μF and 1000 Ω for *B. bronchiseptica*.

2.4. Construction of vectors for the expression of FrgC in B. bronchiseptica

The construction of a *B. bronchiseptica aroA* mutant and the expression of FrgC in this background from the *tac* promoter has been described in detail previously [23].

For expressing heterologous antigens in *B. bronchiseptica* strains under the regulation of a *bvg* dependent promoter the following plasmid was constructed. The FHA promoter from a *B. bronchiseptica* wild type strain (BBC17) was PCR amplified using primers FHA(P)1 (5′ GGGGTACCGAGGGCGCCCGCGGCGAGCCAGGG) and FHA(P)2 (5′ ACGCGTCGACATTCCGACCAGCGAAGTGAAGT). The PCR product was digested with restriction enzymes *Kpn*I and *Sal*I and ligated with pBBR1MCS-5 which had been similarly digested. DNA sequencing was carried out on the resulting vector (pFHAp) to confirm that the FHA promoter sequence was correct. The FrgC gene was amplified from pTET85 using primers FrgC1 (5′ CCCAAGCTTGGGATGAAAAACCTTGATTGTTG) and FrgC2 (5′ CGCGGAT CCGCGTTAGTCGTTGGTCCAACCT). The PCR product was digested with restriction enzymes *Hin*dIII and *Bam*HI and ligated with pFHAp which had been similarly digested. DNA sequencing was carried out on the resulting plasmid (pFHApFrgC) to confirm that the DNA sequence of the FrgC gene and flanking regions were correct. This plasmid was transformed into *B. bronchiseptica aroA* BBC18 to produce strain GVB1543. Expression of FrgC in this strain was confirmed by western blot using an anti-FrgC polyclonal rabbit serum as described below (Fig. 1). The control strain (GVB 1547) was produced by transforming BBC18 with pBBR1MCS-5 alone.

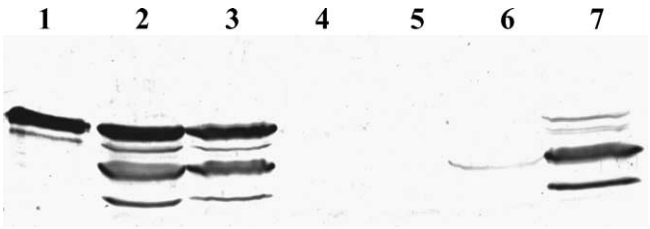

Fig. 1. Expression of FrgC in a *B. bronchiseptica aroA* mutant. Western blot of whole cell lysates from *B. bronchiseptica* strains (~2.6×10^8 CFU) probed with a specific anti-FrgC polyclonal rabbit sera. Cells were grown overnight at 37 °C in LB broth with or without 50 mM Mg_2SO_4: (1) recombinant FrgC (0.5 μg); (2) GVB120 + Mg_2SO_4; (3) GVB120; (4) GVB1547 + Mg_2SO_4; (5) GVB1547; (6) GVB1543 + Mg_2SO_4; (7) GVB1543.

2.5. Detection of B. bronchiseptica antigens and FrgC

Bacteria were grown in LB-broth ± 50 mM Mg_2SO_4. Bacterial cells were harvested into PBS, pelleted and resuspended in reducing SDS–PAGE sample buffer (Sigma, UK). Proteins were separated by SDS–PAGE [26] and transferred to PVDF membranes (Millipore, UK) by electroblotting. Proteins were visualised by incubating the membranes with a rabbit polyclonal antisera specific for FrgC, pertactin or FHA followed by anti-rabbit immunoglobulin horseradish peroxidase conjugate (Dako, UK) and 4-chloro-1-napthol substrate (Sigma).

2.6. Intranasal immunisation of mice

Bacterial suspensions were prepared by swabbing cells from 24 h cultures of *B. bronchiseptica* grown on BG blood agar plates with the appropriate supplements and antibiotics into PBS. For preparation of formalin inactivated *B. bronchiseptica*, cells were harvested as described above and were then treated with 10% formaldehyde at 37 °C for 16 h, cells were then washed in several changes of PBS. Cell suspensions were quantified by measuring their absorbance at 600 nm. CFU values for individual samples of live vaccine were determined after serial dilution in PBS by plating samples on BG agar with supplements. Groups of adult female BALB/c mice were mildly anaesthetised with halothane and immunised by placing 15 μl of bacterial suspension per nostril onto the external nares of the mouse where the inoculum was then inhaled. When required nasal cavities were lavaged with 1 ml of sterile PBS containing 0.1% (w/v) bovine serum albumin (BSA).

2.7. Measurement of the murine anti-B. bronchiseptica and anti-FrgC antibody response

Serum samples were taken from mice by venesection. Sera were assayed by ELISA for total anti-*B. bronchiseptica* and total anti-FrgC immunoglobulins using an anti-mouse immunoglobulin HRP antibody conjugate. Flat bottomed 96 well microtitre plates (Corning Costar, UK 3590) were coated with 50 μl per well of 1×10^8 CFU/ml formalised *B. bronchiseptica* or 2 μg/ml FrgC in PBS at 4 °C overnight. The wells were aspirated and washed three times with PBS containing 0.05% (v/v) Tween 20 (PBS-T) and blocked with 200 μl of PBS-T, 1% (w/v) BSA for 1 h at 37 °C. The wells were washed three times with PBS-T and incubated with 50 μl of test serum diluted in PBS-T, 0.1% (w/v) BSA for 2 h at 37 °C. Following washing, 50 μl of diluted rabbit anti-mouse HRP conjugate (Sigma) were added and incubated for 2 h at 37 °C. The wells were washed as described previously. After washing, plates were incubated for 30 min at room temperature with 50 μl of substrate per well (0.04% (w/v) *o*-phenylenediamine (Sigma) dissolved in phosphate–citrate buffer, pH 5.0; 24 mM citrate, 64 mM disodium hydrogen phosphate, containing 0.01% (v/v) hydrogen peroxide). The reaction was terminated by the addition of 50 μl of 1 M sulphuric acid. Plates were then read in a Multiskan Ascent microplate reader at 492 nm. The titre was calculated as the reciprocal of the highest dilution of serum that gave an A_{492} of 0.1. Statistical differences in the antibody titres of the different vaccine groups was analyzed by ANOVA.

3. Results

3.1. In vitro expression of FrgC in B. bronchiseptica aroA from the fhaB promoter

Previously we have shown that in *Salmonella* sp. expressing a heterologous antigen from certain in vivo regulated promoters rather than from constitutive promoters greatly enhances the immunogenicity of the foreign antigen [7]. In this study we attempt to improve the immune responses to FrgC by expressing this antigen from a regulated promoter. To achieve this the FrgC gene was cloned in front of the *B. bronchiseptica fhaB* promoter in plasmid pBBR1MCS-5. This plasmid, pFHApFrgC, was transformed into the *B. bronchiseptica aroA* strain BBC18 to produce strain GVB1543. (Fig. 1). When grown in LB, GVB1543 produced FrgC in amounts less than GVB120 (BBC18 which constitutively expresses FrgC from the *tac* promoter). In contrast, when 50 mM Mg_2SO_4 was added to the culture medium the amount of FrgC produced by GVB1543 but not GVB120 was reduced significantly. High concentrations of Mg_2SO_4 negatively regulates the activity of BvgAS, thus FrgC is expressed in a Bvg-dependent fashion in GVB1543. The FrgC produced by GVB1543 has the same band pattern as GVB120, but the lower molecular weight species predominates when FrgC is expressed from the *fhaB* promoter (Fig. 1).

3.2. Intranasal immunisation of mice with B. bronchiseptica aroA expressing FrgC from the FHA promoter

To investigate if expressing FrgC from *PfhaB* in *B.bronchiseptica aroA* improves its immunogenicity, mice

were immunised I/N with GVB1543, GVB120 or the control strain GVB1547 (BBC18 pBBR1MCS-5). Mice in all groups were immunised with the same regime: primary immunisation of three doses on days 1, 5 and 9 and a single booster dose on day 40. Mice received between ~1.1 × 10^7 and 4.5 × 10^7 CFU per dose of the relevant immunising strain. Additionally, to investigate the importance of vector viability, mice were immunised I/N, using the same regime, with formalin-killed GVB1543 at a dose equivalent to approximately 5 × 10^7 CFU per mouse per dose.

3.3. Antibody responses to FrgC

Serum samples were assayed for antibodies to FrgC and *B. bronchiseptica* by ELISA after primary immunisation and boosting. High anti-FrgC serum antibody titres were detected in mice which had been immunised I/N with GVB1543 after primary immunisation and boosting (Fig. 2). In fact anti-FrgC serum titres of greater than 10,000 were achieved for all mice in this group following I/N boosting with GVB1543. High anti-FrgC titres were also observed in animals which had been immunised with GVB120 as had been described previously [23] (Fig. 2). However, the mean log anti-FrgC titres of the GVB120 immunised mice were lower than that of the GVB1543 group following both the primary and booster immunisations although the difference was not significant. There was also a greater variation in the anti-FrgC titres in the GVB120 compared to the GVB1543 immunised group (Fig. 2). Immunisation with killed GVB1543 induces serum anti-FrgC antibody titres significantly above those of the control strain GVB1547 but

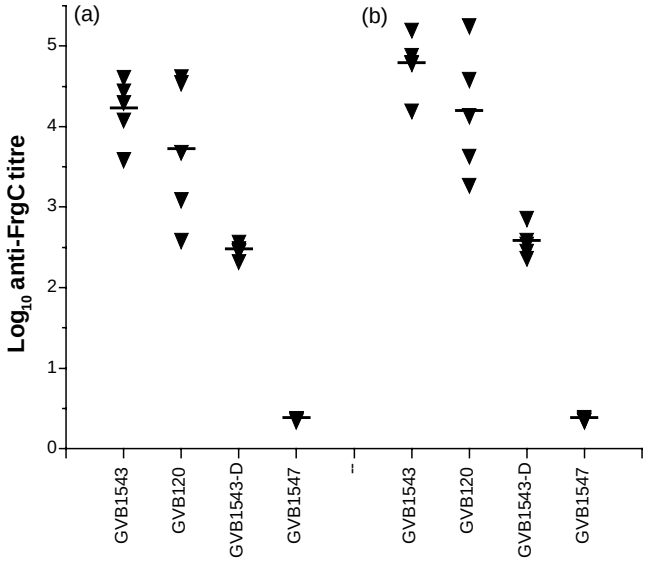

Fig. 2. Serum anti-FrgC antibody responses. The total serum anti-FrgC titres of intranasally immunised mice were measured (a) after primary immunisation and (b) after boosting (one dose) by ELISA. Each triangle represents the $\log_{10}$ titre of serum from an individual mouse. The horizontal bars represent the mean of the $\log_{10}$ titres for the group. GVB1543-D; formalin-killed GVB1543.

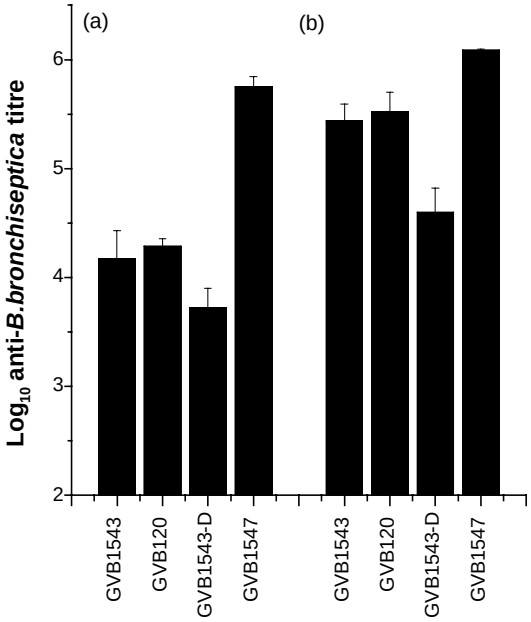

Fig. 3. Serum anti-*B. bronchiseptica* antibody responses. The total serum anti-*B. bronchiseptica* titres of intranasally immunised mice were measured (a) after primary immunisation and (b) after boosting (one dose) by ELISA. Each bar represents the mean $\log_{10}$ serum anti-*B. bronchiseptica* titre and the error bars the S.D.

the titres were considerably lower than those of mice immunised with the live vector strains (GVB1543 and GVB120) at both the pre and post-boost time points ($P < 0.01$). Nasal IgA anti-FrgC antibodies in all groups were low (titres < 10) (data not shown).

3.4. Antibody responses to B. bronchiseptica

High anti-*B. bronchiseptica* serum antibody titres were induced in mice immunised with all of the live *B. bronchiseptica aroA* strains (Fig. 3). In this case, the control strain with the plasmid vector alone induced significantly higher mean anti-*B.bronchiseptica* antibody titres following both the primary and booster immunisation ($P < 0.01$). Viability of the *B. bronchiseptica* strain was also important for induction of anti-*B. bronchiseptica* antibodies as although high anti-*B. bronchiseptica* titres were present in the sera of mice immunised I/N with the formalin-treated GVB1543 they were significantly lower ($P < 0.01$) than those of mice immunised with any of the live *B. bronchiseptica aroA* strains.

3.5. Tetanus challenge

To test whether mice were protected from tetanus they were challenged with 10 LD_{50} units of tetanus toxin and monitored for 5 days. All the mice immunised with the control strain GVB1547 and all the mice immunised I/N with killed GVB1543 developed tetanus. In contrast 100% of mice which had been immunised I/N with the live GVB1543 survived and had no signs of tetanus. I/N immunisation of

mice with GVB120 offered some protection from tetanus, in agreement with a previous study [23]. In this study 60% of mice which had been immunised I/N with GVB120 were protected from tetanus challenge.

4. Discussion

We have previously shown that inactivation of *aroA* highly attenuates both *B. bronchiseptica* and *B. pertussis* in mice [22,23]. Mice immunised I/N with either *B. pertussis* or *B. bronchiseptica aroA* strains clear these bacteria from their respiratory tract very rapidly. Mice immunised with either of these strains also efficiently clear the equivalent wild type bacteria from their lungs following I/N challenge [22,23]. The *B. bronchiseptica aroA* strain has also been used as a heterologous antigen carrier to deliver FrgC. Mice immunised I/N with a *B. bronchiseptica aroA* strain expressing FrgC from a constitutive *E. coli* promoter (*tac*) developed significant systemic antibody responses to FrgC and some protection from tetanus was observed following challenge [23]. Forty to sixty percent of mice which had been immunised I/N with this strain survived a tetanus challenge ([23], and this study). In this study we have used the Bvg-dependent promoter of *fhaB* to express FrgC in a *B. bronchiseptica aroA* mutant strain. FrgC expression is regulated as expected in vitro. In mice immunised I/N with *B. bronchiseptica aroA* expressing FrgC from the *fhaB* promoter (GVB1543) significant improvements in immune responses to FrgC were observed compared to mice that had been immunised with *B. bronchiseptica aroA* expressing FrgC constitutively from the *tac* promoter (GVB120).

The mean anti-FrgC serum titre of mice immunised with GVB1543 was higher than that of mice immunised with GVB120 (although not significantly so) and there was less variation in the individual titres the GVB1543 immunised group. This difference was functionally important as all the mice immunised I/N with GVB1543 were protected against tetanus whereas only 60% in the GVB120 immunised group were protected. Thus, expression of FrgC from a Bvg-dependent promoter in a *B. bronchiseptica aroA* carrier confers greater immunogenicity than expressing the same antigen from a constitutive promoter despite less expression of FrgC from the Bvg-dependent promoter in vitro.

Although the *B. bronchiseptica aroA* strain is rapidly cleared from the respiratory tract of mice, the strain must persist long enough to stimulate protective anti-FrgC serum antibodies. However despite the rapid clearance of the *B. bronchiseptica aroA* mutant from the respiratory tract, viability of the *B. bronchiseptica* carrier is crucial to its efficacy as a carrier. Despite, multiple I/N immunisation with formalin inactivated GVB1543, serum anti-FrgC antibody titres in these animals were much lower than those of mice immunised with viable GVB1543 and as a consequence all mice in the former group developed tetanus following challenge (Fig. 2).

Recently an attenuated *B. bronchiseptica* strain has been used as an antigen carrier to deliver truncated *Pasteurella multocida* toxin (PMT) to the respiratory tract of pigs [27]. The nature of the attenuation in this strain was not reported [27]. The rational for these experiments was to develop a single component mucosal vaccine which could be used against atrophic rhinitis in pigs. The part of PMT used was non-toxic and represented a protective epitope. Expression of this antigen was achieved using a promoter from a Bordetella heat-shock protein gene in a multi copy plasmid [27]. Despite multiple I/N immunisations mice failed to produce any serum or mucosal anti-PMT responses [27].

Mucosal immunisation against tetanus has also been studied in *B. pertussis* [28]. In these studies FrgC was fused to a fragment of FHA (Fha44). The plasmid encoding the FrgC/FHA fusion was found to be rapidly lost from *B. pertussis* carrier strains in vivo. When the fusion gene was integrated into the *B. pertussis* chromosome the level of expression of the FrgC/FHA protein was low and also the molecule was unstable [28]. Mice immunised intranasally with two doses of either PTX+ or PTX− strains of *B. pertussis* expressing FrgC/FHA, anti-FrgC antibodies could not be detected [28]. Mice were primed for an anti-FrgC response as they mounted a serum anti-FrgC antibody response when they were boosted I/N with purified FrgC [28]. In this study no animals were challenged in a tetanus protection study [28]. However, equivalent *B. pertussis* carriers have proved more effective for the delivery of other antigens [29–32].

Currently, at least for FrgC, multiple I/N immunisations with the *B. bronchiseptica aroA* strain are required to induce significant immune responses to the carried antigen. It is possible that alternative attenuating mutations may improve the immunogenicity of a *B. bronchiseptica* vector. However it should be noted that we have demonstrated that the *B. bronchiseptica aroA* strain is an efficacous I/N vaccine against *B. bronchiseptica* and as a live carrier for at least one heterologous antigen (FrgC). Further studies are therefore warranted to determine if this carrier strain has value for mucosal delivery of other heterologous antigens.

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