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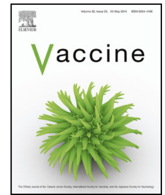

Immunisation with bacterial expressed VP2 and VP5 of bluetongue virus (BTV) protect α/β interferon-receptor knock-out (IFNAR$^{-/-}$) mice from homologous lethal challenge

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ABSTRACT

BTV-4 structural proteins VP2 (as two domains: VP2D1 and VP2D2), VP5 (lacking the first 100 amino acids: VP5$_{\Delta 1-100}$) and full-length VP7, expressed in bacteria as soluble glutathione S-transferase (GST) fusion-proteins, were used to immunise Balb/c and α/β interferon receptor knock-out (IFNAR$^{-/-}$) mice. Neutralising antibody (NAbs) titres (expressed as $\log_{10}$ of the reciprocal of the last dilution of mouse serum which reduced plaque number by ≥50%) induced by the VP2 domains ranged from 1.806 to 2.408 in Balb/c and IFNAR$^{-/-}$ mice.

The immunised IFNAR$^{-/-}$ mice challenged with a homologous live BTV-4 survived and failed to develop signs of infection (ocular discharge and apathy). Although subsequent attempts to isolate virus were unsuccessful (possibly reflecting presence of neutralising antibodies), a transient/low level viraemia was detected by real time RT-PCR. In contrast, mice immunised with the two VP2 domains with or without VP5$_{\Delta 1-100}$ and VP7, then challenged with the heterologous serotype, BTV-8, all died by day 7 post-infection.

We conclude that immunisation with bacterially-expressed VP2 domains can induce strong serotype-specific NAb responses. Bacterial expression could represent a cost effective and risk-free alternative to the use of live or inactivated vaccines, particularly if viruses prove to be difficult to propagate in cell culture (like BTV-25). A vaccine based on bacterially expressed VP2 and VP5 of BTV is also DIVA-compatible.

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1. Introduction

Bluetongue virus is the type species of genus *Orbivirus*, family *Reoviridae* [1,2]. Bluetongue viruses (BTV) are transmitted by adult *Culicoides* midges, causing 'bluetongue' (BT), a non-contagious but economically important disease of ruminants (sheep, cattle and some species of deer) [3,4]. Currently 26 BTV serotypes have been identified, 10 of which (BTV-1, 2, 4, 6, 8, 9, 11, 14, 16 and 25) have been detected in Europe since 1998 [5–7]. It is estimated that over one million sheep have died during repeated BT incursions into the Mediterranean basin between 1998 and 2005 [5]. An outbreak caused by BTV-8 that started in the Netherlands during 2006, subsequently spread across most of Europe, causing high levels of mortality in sheep (15–32%, reaching ~50% is some areas), as well as significant clinical signs but low mortality (<1%) in cattle [8–13]. However, inactivated-virus vaccines were used successfully, leading to the rapid eradication of BTV-8 from the region. These inactivated vaccines, which were made available for serotypes 1, 2, 4 and 8 of BTV are thought to work primarily through generation of a protective serotype-specific neutralising-antibody response targeting the VP2 antigen [2,14–21].

The BTV particle is made of seven structural proteins (VP1–VP7) [2,22,23]. VP2 represents a primary target for neutralising antibodies [1,2,16,17] and determines virus serotype [24]. VP2 shows 22.4–73% aa sequence variation between BTV serotypes [24]. VP5 of BTV, the second most variable BTV protein (aa identity of 41–79% between BTV serotypes [25,26]) enhances neutralising antibody response to VP2 [1,2,14,27].

Selected structural-proteins of BTV-4, including two domains of VP2 (aa 63–471 and 555–956), VP5 (from which a coiled-coil

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sequence [amino acids 1–100] was deleted to improve solubility) and full-length VP7, were expressed in bacteria as soluble fusion-proteins with glutathione S-transferase (GST). We report the use of these proteins to immunise mice, generating neutralising-antibodies to the homologous BTV serotype.

2. Materials and methods

2.1. Cell lines, viruses and mice

KC cells (*Culicoides variipennis*) were grown at 28 °C in Schneider's Drosophila medium, supplemented with 10% foetal bovine serum (FBS). BHK-21 cells (European Collection of Animal cell Cultures: ECACC – 84100501), or BSR cells (a clone of BHK-21 a gift from Dr. Noel Tordo, Institut Pasteur) were grown at 37 °C in Glasgow's Minimum-Essential-Medium supplemented with 10% FBS.

BTV-4(SPA2003/01) was from blood of sheep showing severe clinical disease (Spain 2003). The virus was isolated in embryonated eggs then adapted to BHK-21 cells (E1/BHK4). BTV-4(SPA2003/01) was used for RNA extraction/cDNA synthesis for the purpose of generating protein expression constructs.

BTV-4-Italy03 and BTV-8-France-28 were isolated in embryonated eggs, from sheep-blood (Italy), or cow-blood (France), then adapted to BHK-21 cells (BTV-4-E1/BHK4 or BTV-8-E1/BHK2). These isolates were used for homologous and heterologous challenge of IFNAR$^{-/-}$ mice.

Six weeks-old female Balb/cByJ mice were obtained from Charles River laboratories. Groups of six animals were immunised with proteins to assess NAb production.

Six weeks-old female IFNAR$^{-/-}$ mice (genetic background: A129SvEvBrd) were obtained from B&K Universal Ltd. Groups of six animals were used for immunisation with soluble expressed-proteins followed by homologous or heterologous challenge with live BTV.

Immunisation protocols were approved by ethics committees at the Pirbright Institute (license number 70/6133) and ANSES (license number 12/04/11-5).

2.2. Construction of expression plasmids

Previous analysis has indicated that BTV-VP2 is potentially made of two related domains [18]. We used BTV-4(SPA2003/01) VP2 domains which encompassed amino acid sequences 63–471 (44.5 kDa) and 555–956 (46 kDa) (nucleotide positions: 187–1326 and 1663–2868, Genbank accession: KJ700442).

VP5 lacked aa 1–100 (used sequence encompassed nucleotide positions 289–1581, Genbank accession: AJ783908) while the full-length aa sequence of VP7 was used (nucleotide positions: 1–1050, Genbank accession: KJ700443). All cDNAs were cloned into pGEX-4T-2 (expressing GST). The resulting plasmids are pGEX-BTV4VP2D1, pGEX-BTV4VP2D2, pGEX-BTV4VP5 and pGEXBTV4VP7. Their sequences were confirmed by comparison to parental virus sequences. Theoretical sizes of the GST-fused proteins are 70.5 kDa (VP2 domain 1), 72 kDa (VP2 domain 2), 73 kD (VP5 lacking aa 1–100) and 64.5 kDa for the VP7. The full-length ORFs of VP2, VP5 and VP7 were also cloned in the mammalian-expression plasmid pCIneo (pCIneo-BTV-4VP2, pCIneo-BTV-4VP5, or pCIneo-BTV-4VP7).

2.3. Protein expression and purification

pGEX-BTV4VP2D1, pGEX-BTV4VP2D2, pGEX-BTV4VP5 and pGEXBTV4VP7 were used to transform C41 bacteria, known to improve solubility of expressed proteins [28]. Overnight bacterial cultures were grown in 2XYT medium at 37 °C. On the day of expression bacterial cultures were grown until OD_{600} reached 0.6, then fusion-protein expression was induced by addition of 0.5 mM IPTG and incubation of the cultures at 28 °C for 4 h with shaking at 200 rpm. Bacteria were pelleted (3250 g) and lysed by sonication. Soluble proteins were purified from bacterial lysates by glutathione-affinity chromatography as previously described [29], then analysed by sodium-dodecyl-sulphate (SDS) polyacrylamide gel electrophoresis (PAGE). GST-fused proteins from inclusion bodies (insoluble fraction) were dissolved in a CAPS buffer (CAPS 50 mM, DTT 1 mM and Sarkosyl 0.3%), hence denaturing the proteins [30]. The dissolved and denatured protein was dialyzed overnight against 20 mM Tris–HCl pH 8.5. Insoluble proteins dissolved in CAPS buffer/dialysed are referred to as 'CAPS-denatured proteins' throughout the text. Purified proteins were quantified by two different methods: (i) a Bradford assay at 595 nm and (ii) UV spectrophotometry at 280 nm (extinction coefficient determined from aa sequences of each fusion protein). Concentration measurements were consistent using both methods. Relative amounts of proteins to be injected were based on copy number considerations in a BTV particle, as determined by X-ray crystallography (780 copies for VP7, 360 copies for VP5 and 180 copies for VP2 [1]).

2.4. Immunisation of mice with soluble bacterial expressed proteins

2.4.1. Immunisation of Balb/c

Seven groups of six Balb/c mice were injected subcutaneously at days 0, 14 and 28 with 100 μl of soluble protein/Montanide ISA50V emulsion (Table 1).

Three groups of six Balb/c mice were injected subcutaneously at days 0, 14 and 28 with 100 μl of CAPS-denatured protein/Montanide ISA50 V emulsion (Table 1).

A group of six Balb/c mice were injected subcutaneously at days 0, 14 and 28 each with 100 μl of Zulvac-4® Bovis. Sera were used for normalisation of ELISA results.

A group of six control Balb/c mice which were not immunised with any of the antigens was also included.

2.4.2. Immunisation and challenge of IFNAR$^{-/-}$ mice

Six groups of six IFNAR$^{-/-}$ mice were injected subcutaneously at days 0, 14 and 28 with: a mixture of VP2 domain 1 (VP2D1) and VP2 domain 2 (VP2D2) in Montanide, then challenged with (i) BTV-4 or (ii) BTV-8; or a mixture of VP2D1 + VP2D2 + $VP5_{\Delta 1-100}$/Montanide, then challenged with (iii) BTV-4 or (iv) BTV-8; or a mixture VP2D1 + VP2D2 + $VP5_{\Delta 1-100}$ + VP7/Montanide, then challenged with (v) BTV-4 or (vi) BTV-8 (Table 1). Blood samples were collected at day 0 and day 28.

The mice received an intravenous lethal [31] challenge on day 40, with 10^3 pfu of BTV-4-italy03 (homologous-challenge), or 10 pfu of BTV-8-28 (heterologous-challenge). Blood was collected on the day of challenge (day 40), then at days 2, 3, 4, 5, 7, 10 and 12 p.i. Sera were tested for anti-VP2, anti-VP5 and anti-VP7 antibodies by ELISA and immunofluorescence and for NAbs by PRNT.

Two groups of six IFNAR$^{-/-}$ mice were injected subcutaneously with $VP5_{\Delta 1-100}$ on days 0, 14 and 28. These groups were not challenged with BTV-4 or BTV-8. Two additional groups of six IFNAR$^{-/-}$ mice were immunised with VP7 on days 0, 14 and 28, then challenged at day 40 with either BTV-4 or BTV-8.

Two groups of non-immunised mice were used as positive controls, to confirm lethality of BTV-4 or BTV-8 challenge-strains. Blood from these two groups was taken at days 0 and 40 (just before challenge) in order to assess reactivity of sera with BTV-4 ELISA and for PRNT.

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