Original antigenic sin with human bocaviruses 1–4

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Human bocavirus (HBoV) 1 is a widespread parvovirus causing acute respiratory disease in young children. In contrast, HBoV2 occurs in the gastrointestinal tract and is potentially associated with gastroenteritis, whilst HBoV3 and -4 infections are less frequent and have not yet been linked with human disease. Due to HBoV1 DNA persistence in the nasopharynx, serology has been advocated as a better alternative for diagnosing acute infections. In constitutionally healthy children, we previously noted that pre-existing HBoV2 immunity in a subsequent HBoV1 infection typically resulted in low or non-existent HBoV1-specific antibody responses. A phenomenon describing such immunological events among related viruses has been known since the 1950s as ‘original antigenic sin’ (OAS). The aim of this study was to characterize this putative OAS phenomenon in a more controlled setting. Follow-up sera of 10 rabbit pairs, inoculated twice with HBoV1–4 virus-like particles (VLPs) or control antigens, in various combinations, were analysed with HBoV1–4 IgG enzyme immunoassays with and without depletion of heterotypic HBoV antibodies. There were no significant IgG boosts after the second inoculation in either the heterologously or the homologously HBoV-inoculated rabbits, but a clear increase in cross-reactivity was seen with time. We could, however, distinguish a distinct OAS pattern from plain cross-reactivity: half of the heterologously inoculated rabbits showed IgG patterns representative of the OAS hypothesis, in line with our prior results with naturally infected children. HBoVs are the first parvoviruses to show the possible existence of OAS. Our findings provide new information on HBoV1–4 immunity and emphasize the complexity of human bocavirus diagnosis.

Received 13 April 2015
Accepted 27 July 2015

INTRODUCTION

Human bocavirus 1 (HBoV1) is a recently identified human-pathogenic parvovirus, which was discovered in paediatric respiratory samples in 2005 (Allander et al., 2005). HBoV1 shows high infection rates globally, and has been associated with acute respiratory disease in infants and young children (Kesebir et al., 2006; Allander et al., 2007; Fry et al., 2007; Jartti et al., 2012; Paloniemi et al., 2014; Martin et al., 2015). Three additional types of human bocavirus, HBoV2, -3 and -4, were discovered in 2009–2010 (Arthur et al., 2009; Kapoor et al., 2009, 2010). Unlike HBoV1, HBoV2–4 usually occur in the gastrointestinal tract but rarely in the respiratory tract (Chieochansin et al., 2009; Kapoor et al., 2010; Paloniemi et al., 2014).

HBoVs cause asymptomatic to severe infections. HBoV1 infections have even been reported to cause life-threatening lower respiratory tract infections in children (Körner et al., 2011; Ursić et al., 2011, 2014; Edner et al., 2012; Mitui et al., 2012; Jula et al., 2013). In addition, Brebion et al. (2014) observed HBoV2 to be the only pathogen detected in a fatal disseminated infection of a 13-month-old child with subacute lymphocytic myocarditis in France. Trustworthy diagnosis of acute HBoV infections would be of great help in assessing the impact of HBoV in clinical cases.

PCR is the most common method used to detect acute infections of HBoV1 (Bastien et al., 2006; Kesebir et al., 2006; Allander et al., 2007; Fry et al., 2007; Schildgen et al., 2008). However, HBoV1 DNA has been shown to remain detectable by PCR in respiratory samples for several months after primary infection (Briere et al., 2008; Blessing et al., 2009; Lehtoranta et al., 2012; Martin et al., 2015). Thus, RT-PCR=Reverse Transcription PCR (polymerase chain reaction) (Proença-Modena et al., 2011; Christensen et al., 2013) and IgM and IgG enzyme immunoassays (EIAs) (Kantola et al., 2008; Lindner & Modrow, 2008; Söderlund-Venermo et al., 2009; Hedman et al., 2010;
Kantola et al., 2011; Guo et al., 2012) are more viable tools for the diagnosis of primary HBoV infection.

We have shown previously by unblocked and blocked EIAs that HBoV1–4 induce cross-reactive antibodies (Kantola et al., 2011; Guo et al., 2012). The structural protein 2 (VP2) amino acid sequences of HBoV2–4 are 22–23% divergent compared with those of HBoV1, and around 11% divergent compared with each other (Kantola et al., 2011; Shen et al., 2013). The VP2 proteins of HBoV1–4 share many antigenic epitopes (Zhou et al., 2014). We have, however, shown previously that it is possible to distinguish the specific HBoV1–4 IgG responses by blocking the antibodies against the shared epitopes (Kantola et al., 2011).

In our previous study of wheezing children, we detected less HBoV2–4-specific IgG in HBoV1 IgG-positive children than in negative children (Kantola et al., 2011). In another recent serological study of constitutionally healthy children followed for many years, several PCR-confirmed heterotypic secondary infections were observed with inefficient or no specific IgG responses against the second virus type. Instead, a vigorous recall response appeared against the first virus type (Kantola et al., 2015). Priming with one HBoV type may thus inhibit the subsequent generation of antibodies towards the unique epitopes of another HBoV type – a phenomenon known as ‘original antigenic sin’ (OAS) (Francis, 1960).

OAS was initially described in 1953 for antibody responses following sequential influenza infections (Davenport et al., 1953; Francis, 1953). These authors observed that human patients produced high-titre antibodies against a previous, rather than the current, virus type. OAS has since been reported for several viruses including influenza (Jensen et al., 1956; Fazekas de St Groth & Webster, 1966; Webster et al., 1976; Kim et al., 2009), dengue (Halstead et al., 1983; Mongkolsumapaya et al., 2003; Midgley et al., 2011), enterovirus (Tschiya et al., 2000) and human immunodeficiency virus (HIV) (Oxford et al., 1993; Kundu et al., 1998). OAS has also been observed in various animal models including rats, mice, ferrets and rabbits (Fazekas de St Groth & Webster, 1966; Webster, 1966; Virelizier et al., 1974; Angelova & Shvartsman, 1982; Kim et al., 2009).

The negative effect of OAS may manifest clinically as a more severe disease in the secondary infection (Guzmán et al., 2000; Alvarez et al., 2006). The presence of OAS may also diminish the efficacy of some vaccines (Fazekas de St Groth & Webster, 1966; Webster et al., 1976; Muller, 2004). Whilst the vaccine should be protective, it may instead inhibit the immune response of a natural primary infection by a related virus.

To determine the potential impact of OAS on HBoV infections, we wished to characterize the OAS phenomenon in a simple and controlled setting. Ten rabbit pairs were each inoculated twice with HBoV1–4 VP2 virus-like particles (VLPs) in various combinations, the second HBoV antigen 60 days after the first HBoV antigen. HBoV1–4 IgG EIAs, with and without homologous and heterotypic blocking, were employed to analyse follow-up serum samples from each rabbit, obtained at 5- to 20-day intervals from pre-inoculation up to 120 days post-inoculation (p.i.).

**RESULTS**

**Negligible background from High5 cell lysate**

To control for potential background absorbance caused by carryover of High5 insect-cell proteins, we inoculated one rabbit pair with proteins derived from uninfected High5 cells. None of the High5/High5 rabbit sera from days 0 to 120 p.i. were reactive in any of the HBoV1–4 IgG EIAs. Furthermore, none of the anti-VLP sera of day 120 p.i. reacted in a High5 cell-lysate EIA (data not shown). Thus, the High5 insect-cell lysate itself did not cause noticeable background in the HBoV IgG EIAs.

**Minimal interference by human parvovirus B19 (B19V) in HBoV1 ELISA**

Rabbits pre-inoculated with B19V VLPs before HBoV inoculation, and vice versa, were included as negative OAS controls, as B19V and HBoVs share <25% VP2 amino acid identity. Sera from days 0 to 120 p.i. of rabbit pairs HBoV1/B19V and B19V/HBoV1 were analysed by HBoV1 unblocked and heterologously blocked EIAs (Fig. 1).

The absorbance values of HBoV1/B19V (Fig. 1a) rabbit sera in HBoV1 IgG EIA decreased slightly after B19V blocking, but the end-point titres were at the same level (Fig. S1a). Moreover, there were no indications of OAS in the rabbits primed with B19V (Fig. 1b). These observations suggested that: (i) B19V VLP presented minimal interference in the HBoV1 EIA; (ii) B19V and HBoV VLPs may not share any major epitopes; and (iii) no OAS was visible with this non-related virus.

**No significant boost after sequential inoculation by the same antigen**

We inoculated rabbits with the homotypic combinations HBoV1/HBoV1 or HBoV2/HBoV2 to observe any possible increase in IgG titre. Quite unexpectedly, in HBoV1-primed rabbits, secondary HBoV1 inoculations were indistinguishable from B19V inoculations, yielding no obvious increase in HBoV1 IgG titre (Fig. 2). The same was true also for HBoV2/HBoV2 (data not shown).

**Five of 10 rabbits with various HBoV antigen combinations show possible OAS**

Rabbits inoculated with VLP antigens of different HBoV-type combinations (Table 1) were examined to elucidate the existence of OAS in HBoV1–4 immunology, as suggested by human patient data (Kantola et al., 2011).

We performed secondary HBoV antigen EIAs, both with and without heterologous blocking, on sera from day 120...
(120 days after the first and 60 days after the second inoculation) for antibody responses and cross-reactivities (Fig. 3). We considered each absorbance of the heterologously blocked EIA as a specific response to the secondary HBoV exposure, and that of the corresponding unblocked EIA as a shared response to both HBoV antigens, i.e. secondary and primary HBoVs. Those rabbits in which the difference in absorbance values between unblocked and heterologously blocked EIAs was pronounced, we considered possible candidates for OAS.

Unexpectedly, the rabbits inoculated with HBoV1/HBoV2 showed no sign of OAS (Fig. 3a), whilst in our previous study, some children with the same combination did present OAS (Kantola et al., 2015). This was shown by identical IgG curves in the blocked and unblocked EIAs (Fig. 3a). In contrast, in the HBoV2/HBoV1-inoculated rabbit b, the

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**Fig. 1.** IgG seroreactivity by HBoV1-VLP EIA of rabbits inoculated in the combinations HBoV1/B19V (a) and B19V/HBoV1 (b), at a 1 : 1000 dilution, either unblocked or blocked with soluble heterologous B19V VLPs. EP, End-point.

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**Fig. 2.** Unblocked HBoV1 IgG EIAs for sera of rabbits inoculated with HBoV1/B19V and HBoV1/HBoV1: absorbance at a 1 : 1000 dilution (a) and end-point titres in a series of threefold dilutions from 1 : 333 to 1 : 729 000 from days 0 to 120 (b). The rabbits in each pair were named by the combination of primary and secondary VLP antigen inoculations, e.g. rabbits HBoV1/B19V-a and HBoV1/B19V-b in the combination HBoV1/B19V in rabbit pair 2. EP, End-point.
end-point titre of day 120 was around 27-fold higher in the unblocked EIA than in the heterologously blocked EIA (Fig. 3b), in the HBoV4/HBoV1-inoculated rabbit a, it was 25-fold higher (Fig. 3d), and in the HBoV3/HBoV2-inoculated rabbit a, it was 50-fold higher (Fig. 3e). The significant difference in these three individual rabbits strongly suggested the existence of OAS in HBoVs.

In contrast to rabbit HBoV3/HBoV1-a, the HBoV3/HBoV1-inoculated rabbit b (Fig. 3c), in a blocked EIA (at the standard dilution 1:1000), showed half the absorbance of the unblocked EIA. Similar results were seen also for the HBoV4/HBoV1-inoculated rabbit b (Fig. 3d).

Taken together, among the 10 individual rabbits with five antigen combinations, three indicated clear OAS, and two presented partial OAS. All our results were reproducible, including the following results.

**Follow-up data of the five rabbits exhibiting OAS**

We analysed the absorbance values and end-point titres of HBoV IgG in sera at different time points pre- and post-inoculation of the five individual rabbits that showed OAS: HBoV2/HBoV1-b, HBoV4/HBoV1-a, HBoV3/HBoV2-b, HBoV3/HBoV1-b and HBoV4/HBoV1-b. The sera were studied by IgG EIA with and without heterologous HBoV VLP blocking, both at the standard dilution of 1:1000 (Fig. 4) and by titration with serial threefold dilutions (from 1:333 to 1:729,000) from pre-inoculation to day 120 (Fig. S2). We distinguished OAS from mere cross-reactivity by comparing the absorbance values and end-point titres of primary and secondary HBoV immune responses in heterologously blocked HBoV IgG EIAs.

To show the immune responses towards shared B-cell epitopes among HBoVs, we measured the IgG antibodies by unblocked EIA, and also those towards a third HBoV type not involved in inoculation, such as HBoV3 for the rabbit inoculated with HBoV2/HBoV1. As shown in Fig. 4, the cross-reactivity between two HBoV types increased with time, i.e. the acute-phase antibodies were more specific than the past-infection antibodies, as we have noted previously in children (Kantola et al., 2011).

The high and stable IgG responses after primary HBoV inoculations and the negligible boosters due to secondary HBoV inoculations in rabbits HBoV2/HBoV1-b (Fig. 4a), HBoV4/HBoV1-a (Fig. 4b) and HBoV3/HBoV2-b (Fig. 4c) showed that at least those three antigen combinations induced IgG results in line with OAS. In addition, comparing the primary and secondary responses by heterologously blocked EIA at 60 days after inoculation (i.e. day 60 for the primary HBoV type and day 120 for the secondary HBoV type), a ninefold higher titre of primary HBoV IgG in the HBoV3/HBoV1-inoculated rabbit b (Fig. S2d) and a 27-fold higher titre in the HBoV4/HBoV1-inoculated rabbit b (Fig. S2e), pointed to a partial OAS, i.e. the OAS phenomenon in these five rabbits occurred to variable degrees.

In the HBoV2/HBoV1-inoculated rabbit b (Fig. 4a), the absorbances of HBoV1-3 IgG in the unblocked EIAs suggested that, during the first 2 months, the immunological response was towards epitopes shared by HBoV2 and -3 rather than those towards HBoV1. After HBoV1 inoculation on day 60, the HBoV1 IgG level started to increase, also boosting HBoV3 IgG but not HBoV2 IgG. It is possible that the HBoV2 EIA had reached the saturation point.

**DISCUSSION**

Serology is highly useful in many virus infections in both the assessment of immunity and diagnosis of acute infection. Sequential immune responses against related viruses, however, can be more complicated than first thought, mostly due to cross-reactivity and OAS. In OAS of secondary infections, shared epitopes may activate the memory B-cells that have been primed by the initial antigen encounter, whilst the naive B-cells, destined for activation against the novel epitopes, are blocked. Such a non-specific immune response following a secondary virus encounter could potentially result in enhanced clinical severity. Paradoxically, the attenuated secondary antibody response could also reflect cross-protection between the two viruses, and instead lead to decreased clinical severity.

Despite the OAS phenomenon being known for over 60 years, its mechanism is still not fully understood. Undoubtedly, cross-reactivity due to antigenic similarity plays an essential role. HBoV1–4 are less divergent (compared with each other) (11–23 %) (Midgley et al., 2011). In our study, by blocking the heterotypic antibodies present in sera, we eliminated the cross-reactive antibodies towards shared epitopes. In this way, it was possible to examine type-specific immune responses.

Our recent study on humans, including HBoV1–3 IgM and IgG EIAs and real-time quantitative PCR performed on 109
Fig. 3. Unblocked and heterologously blocked IgG EIAs for the secondary HBoV antigens. Sera were collected on day 120 from rabbits inoculated with different HBoV combinations: HBoV1/HBoV2 (a), HBoV2/HBoV1 (b), HBoV3/HBoV1 (c), HBoV4/HBoV1 (d) and HBoV3/HBoV2 (e). Before being applied to plates, the sera were diluted in threefold dilutions from 1 : 333 to 1 : 729 000 in PBS/Tween 20 (PBST) alone if unblocked or in PBST with soluble unbiotinylated primary HBoV VLPs (30 μg ml⁻¹) for blocking. Each immobilized antigen and the blocking antigen are indicated on the figure. EP, End-point.
children each followed with, on average, 18 serum samples from birth up to 13 years of age, revealed many successive infections by two or even three bocaviruses. Half of these primed children showed diminished antibody responses against the secondary virus (Kantola et al., 2015). In several children showing OAS, the secondary infection was verified by simultaneous viraemia. These children experienced the HBoV infections in diverse combinations: HBoV1/HBoV2 or -3, HBoV2 or -3/HBoV1 and HBoV3/ HBoV2. However, other similarly infected children showed only moderate OAS, and some showed no signs of OAS at all. That not all individuals with sequential heterotypic infections presented with OAS may be due to several reasons, such as different age, gender, genetic disposition, past infectious events, living environment, infecting viral load and time intervals between the HBoV infections.

To minimize these confounding factors, male New Zealand rabbits with a uniform background were examined for the occurrence of OAS in the context of HBoV B-cell immunity. We wished to obtain evidence for or against OAS, with the rabbits sharing the same living environment, food, genetic heredity, age, gender and a minimal infection history. We also simplified the mode of virus encounter. The mode of inoculation used here – subcutaneous injection of non-replicating antigen in Freund’s adjuvant – is likely to elicit an immune response different from that elicited by natural HBoV infections, which in turn could occur by two different routes, respiratory or enteric.

Despite this simplified setting with uniformity of hosts, antigens and inoculation protocol, individual rabbits nevertheless diverged in the magnitude of OAS and extent of immune activation, even within the rabbit pairs with identical HBoV combinations, equal to the more complex naturally infected children. Among all five rabbits with signs of OAS, HBoV4/HBoV1 was the only combination in which both rabbits in the pair presented with OAS to some degree. Within the other three combinations, HBoV2/HBoV1, HBoV3/HBoV1 and HBoV3/HBoV2, only one of the two rabbits showed OAS. The reason for this variation is unknown.

Surprisingly, as opposed to our human study with natural HBoV infections, the homotypic inoculation of rabbits with HBoV1/HBoV1 failed to exert a detectable boost to the IgG titre, closely resembling the outcome of the cross-species inoculation HBoV1/B9V. The reasons for this discrepancy may be differing interval lengths versus host species, live virus infection versus antigen administration, or that the amount of antigen and effectiveness of adjuvant used had elicited the maximum B-cell activation and expansion possible in these animals. However, these rabbit data corroborate the prior notion that sequential doses of homotypic influenza A virus antigen do not increase the antibody responses in humans (Webster et al., 1976). These rabbit data further confirmed our previous results in humans of acute-phase specificity by demonstrating increasing cross-reactivity with time.

The water-in-oil adjuvants used in the current study have been shown not to denature proteins (Berzofsky et al., 1976). The antibodies induced in our rabbits by HBoV VP2 proteins were further able to recognize the same VLP antigens immobilized on the EIA wells, as did the human antibodies elicited by natural infection. They could furthermore be blocked by conformational VLPs in the liquid phase. With the homologous VLPs, this blocking was close to 100% (Fig. 4), substantiating the ability of the rabbit antibodies also to recognize conformational epitopes.

Induction of OAS has, in a number of animal models, been shown to be independent of time intervals and inoculation order (Fazekas de St Groth & Webster, 1966; Kim et al., 2009). In annual influenza vaccinations, it has been shown that a high pre-vaccination antibody level may negatively interfere with the revaccination response, whilst a low level leads to a better response (Gulati et al., 2005). However, there are different opinions on the effect of the inoculation dose on OAS. Kim et al. (2009) demonstrated that induction of OAS was independent of the dose of live viruses (0.01 or 0.1 LD50) in BALB/c mice. In contrast, Fazekas de St Groth & Webster (1966) showed that, irrespective of the primary vaccine dose in rabbits, both cross-reactive and specific antibodies to the secondary antigen are produced when a large dose [500 haemagglutination (HA) units] is used as the second inoculum, whilst a low dose of secondary antigen (31.6 HA units) induced more cross-reactive antibodies.

Researchers are now becoming more focused on how to reduce the negative impact of OAS, also shedding light on the possible mechanism of this phenomenon. Anderson et al. (2001) demonstrated, by HIV vaccination experiments, that when the immune system is primed with a wide range of epitope variants, it expands to a broad T-helper-cell response. Kim et al. (2012) further reported that influenza vaccines with three types of dendritic cell-activating adjuvants during the second viral exposure completely protected mice from a lethal challenge. It is possible that the administration of adjuvants may shift antigen presentation from memory B-cells to dendritic cells, which could enhance the cellular immune response, avoiding OAS (Kim et al., 2012). Besides, repeated
inoculation with the second virus strain induced robust responses, thus overcoming OAS. This may occur upon secondary inoculation through the selective activation of the second-antigen-specific B-cells. Wyrzucki et al. (2015) offered another strategy overcoming OAS for a pan-influenza virus vaccine by using epitopes inducing the otherwise rare HA stem-specific antibodies to elicit a broadly cross-neutralizing immune response. Currently, there are no data to support the suggestion that the above conjectures would be involved in the OAS phenomenon in HBoV inoculations or infections.

In conclusion, with five heterotypic HBoV VLP antigen combinations, five of 10 inoculated rabbits showed OAS to various degrees, in line with our results with naturally infected humans. To the best of our knowledge, this is the first report with detailed controlled analysis of the existence of OAS among parvoviruses in general, and among bocaviruses in particular. Our findings provide new insights into HBoV1–4 immunology and serology, and emphasize the complexity of HBoV diagnosis.

**METHODS**

**Animals and inoculation.** HBoV1–4 as well as B19V VP2 VLPs were produced by a baculovirus expression system in High5 insect cells and purified by CsCl gradient ultracentrifugation as described previously (Kantola et al., 2011); the High5 cell lysate used as a control underwent the same procedure. The VLPs in PBS were sent to GenScript Inc. for the rabbit inoculation process.

Twenty healthy male New Zealand rabbits (about 3 months old, 1.8–2.5 kg each) were used and divided into 10 pairs (Table 1), each pair receiving the same antigens. Using one HBoV VLP antigen at a time, 300 μg per rabbit was injected with complete Freund’s adjuvant in the first inoculation on day 0. Sera were collected on days 0, 5, 10, 15, 30, 45 and 60. The second inoculation, also with 300 μg of one VLP antigen but with incomplete Freund’s adjuvant, was done after collecting the sera on day 60. Sera were then collected on days 65, 70, 75, 90 and 110 from the final bleeding at day 120 p.i.. All sera (n=13 from each rabbit) were lyophilized before shipment and dissolved in water prior to serological testing (Table 2).

**Unblocked, heterologously and homologously blocked EIAs.** The HBoV IgG unblocked and blocked EIAs were conducted as described previously (Kantola et al., 2011), except that the secondary antibody was polyclonal swine anti-rabbit immunoglobulins conjugated to HRP, diluted 1:2000 (Dako).

The serum samples were examined by HBoV EIA both unblocked and blocked with either heterologous or homologous VLPs at 30 μg ml⁻¹. Sera were tested by unblocked EIA, for both the inoculating HBoV antigens and, for shared immunodominant epitopes, in another third-party HBoV. To detect specific antibodies for each involved HBoV type, the design of heterologous blocking depended on the specific combinations of the two inoculations, e.g. for the combination HBoV1/HBoV2, the samples were diluted in PBS/Tween 20 (PBST) with soluble HBoV2 antigen for HBoV1 IgG detection, or vice versa, pre-incubated for 1.5 h at 4 °C and then applied to the plate.

To assess the competition efficiency of sera, homologous blocking was also done by pre-incubating the serum samples with the same soluble unbiotinylated antigen as the immobilized antigen, before the specific HBoV IgG EIA. These residual absorbance values were typically very low (median 0.044; 90th percentile, 0.049). The net absorbance values were calculated by subtracting the residual absorbance from the raw absorbance readout at 492 nm. EIA IgG end-point titres were defined as reciprocal serum dilutions corresponding to two times the mean absorbance values obtained with pre-immune sera.

**ACKNOWLEDGEMENTS**

X. L. received a PhD fellowship from the China Scholarship Council (CSC) and K. K. from the Helsinki Biomedical Graduate School. This study was supported by grants from the Finnish Medical Foundation (FLS), the Sigrid Juselius Foundation, the Research Funds of the University of Helsinki, the Helsinki University Hospital Research and Education Fund, and the Academy of Finland (grant no. 1257964).

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