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REPORTS

Influenza B Virus in Seals

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Influenza B virus is a human pathogen whose origin and possible reservoir in nature are not known. An influenza B virus was isolated from a naturally infected harbor seal (*Phoca vitulina*) and was found to be infectious to seal kidney cells in vitro. Sequence analyses and serology indicated that influenza virus B/Seal/Netherlands/1/99 is closely related to strains that circulated in humans 4 to 5 years earlier. Retrospective analyses of sera collected from 971 seals showed a prevalence of antibodies to influenza B virus in 2% of the animals after 1995 and in none before 1995. This animal reservoir, harboring influenza B viruses that have circulated in the past, may pose a direct threat to humans.

Influenza A, B, and C viruses cause influenza in humans (*1*). Influenza A viruses have been isolated from many species, and aquatic birds may form the natural reservoir from which pandemics and subsequent epidemics in humans have originated (*2*). In contrast, influenza C virus has been isolated from humans and pigs, and influenza B virus infection is supposed to be restricted to humans. Previous reports of influenza B virus infection of a dog and a pig did not meet the established criteria to prove infection (*3, 4*). The origins and possible natural reservoirs of influenza B and C viruses, as well as their potential to infect other species, are unknown (*1*).

Herpes-, morbili-, calici-, and poxviruses have been identified as causes of significant morbidity and mortality among pinniped species (*5–11*). In addition, influenza A viruses of avian origin, including four different antigenic subtypes (H3N3, H4N5, H4N6, and H7N7), have caused outbreaks of influenza among seals (*12–15*).

Seals stranded on the Dutch coast are admitted to the Seal Rehabilitation and Research Center (SRRC) in Pieterburen, the Netherlands, for rehabilitation. In the spring of 1999, 12 juvenile harbor seals with respiratory problems, but not infected with phocine herpesvirus (PHV) or phocine distemper virus (PDV), were tested for influenza virus infection. Cytopathic changes were noted within 3 days of inoculation of a throat swab sample from an 8-month-old animal (seal 99-012) in Madine Darby canine kidney (MDCK) cells (*16*). The supernatant of this cell culture agglutinated turkey erythrocytes in a hemagglutination (HAI) assay (*16*). Negative contrast electron microscopic analysis of the hemagglutinin (HA)–positive culture supernatant showed the presence of orthomyxovirus-like particles (*17*). To our surprise, the presence of influenza B virus was identified by reverse transcription polymerase chain reaction (RT-PCR) analysis of RNA isolated from the culture supernatant (*18*). A semi-quantitative RT-PCR performed with the original throat swab obtained from seal 99-012 confirmed the presence of influenza B virus at levels equivalent to approximately 400 50% tissue culture infectious dosis ($TCID_{50}$) of virus, present in the supernatant of MDCK cell culture infected with influenza B virus (Fig. 1). This influenza virus, B/Seal/Netherlands/1/99, could be propagated in primary seal kidney cell cultures (*7, 19*).

To exclude sample contamination in the laboratory, serum samples collected from seal 99-012 before and after virus isolation were tested for the presence of antibodies that inhibit hemagglutination (HAI) (*20*) of B/Seal/Netherlands/1/99. Samples collected 44 days before and at the day of virus isolation were negative (HAI titer <6), whereas the sample collected 83 days after virus isolation showed a HAI titer of 192, confirming that seal 99-012 had been infected with influenza B virus. Sera from seven other juvenile harbor seals, kept in the same basin as seal 99-012, were also tested for the presence of HAI. The serum of one seal, 99-041, collected 61 days after the day of virus isolation from seal 99-012, showed a HAI titer of 48. All the other sera from this and the other animals were negative (HAI titer <6). We were unable to detect influenza B virus by RT-PCR in the throat swabs collected from seal 99-041 at 34 days before and 61 days after virus isolation from seal 99-012 (*17*).

Quantitative analyses of immunoglobulin G (IgG) antibodies to nucleoprotein (NP) (*17*) and envelope glycoproteins HA and neuraminidase (HA/NA) of influenza B virus, as well as IgM antibodies to NP as an indicator for primary infection, were performed by enzyme-linked immunosorbent assay (ELISA) (*20, 21*) (Fig. 2). In seals 99-012 and 99-041, IgG antibodies to HA/NA and NP were detected, which increased over time and correlated with virus-neutralizing and HAI antibodies (*17*). Both harbor seals displayed decreasing levels of IgM antibody to

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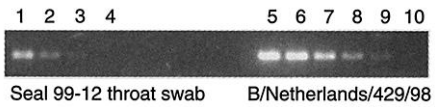

Fig. 1. RT-PCR analysis of influenza B virus in a throat swab from seal 99-012. RNA was isolated from 20, 2, 0.2, and 0.02 μl (lanes 1 through 4, respectively) of a throat swab soaked in 1 ml of transport media and from 800, 80, 8, 0.8, 0.08, and 0.008 $TCID_{50}$ (lanes 5 through 10, respectively) of human influenza virus B/Netherlands/429/98. RNA was then used for RT-PCR. Samples were analyzed by agarose gel electrophoresis and ethidium bromide staining (*18*).

NP from the day of admission onward; virus was isolated from the throat swab of seal 99-012 at day 44 after admission. The kinetics of IgM responses generally observed upon primary virus infection in mammals (*22*) suggest that both seals had been infected days (seal 99-012) or weeks (seal 99-041) before admission.

We next amplified part of the *nonstructural* (*NS*) gene segment and the first domain of the *HA* gene (*HA1*) from B/Seal/Netherlands/1/99, as well as from the original throat swab, by RT-PCR and then sequenced the amplified fragments. The sequences from the throat swab and from the virus isolate were identical. The *NS* fragment of 201 nucleotides displayed 99 to 100% nucleotide identity to many human influenza B viruses. Comparison of the *HA1* sequence from B/Seal/Netherlands/1/99 with human influenza B virus sequences revealed that the seal isolate closely resembled B/Harbin/7/94-like strains (Fig. 3). In fact, one *HA1* sequence in the database (B/Argentina/4105/95) proved to be identical to that of B/Seal/Netherlands/1/99 (*23, 24*). Because this isolate had never been used in our laboratory and no reagents were used that were not fully accounted for in terms of their origin, contamination of our samples by B/Argentina/4105/95 is highly unlikely. The high antibody titers to influenza B virus in seal 99-012 support this conclusion. The homology between B/Seal/Netherlands/1/99 and 1995-like strains is surprising, as HA from human influenza B undergoes significant antigenic and genetic change over time, which indeed occurred from 1995 until 1999 in humans (see the genetic analysis in Fig. 3 of B/Harbin-like strains in 1995 versus B/Yamanashi-like strains in 1999). Moreover, influenza A viruses are known to undergo rapid adaptation to their new host upon zoonotic transmission (*2*).

A HAI assay was performed with serum from seal 99-012 and B/Seal/Netherlands/1/99 as well as B/Netherlands/384/95 and B/Netherlands/429/98, which are prototypes of the 1994–1995 and 1998–1999 epidemics. Serum from seal 99-012 consistently showed an approximate twofold better recognition of homologous and 1995 viruses than of the 1998 virus in independently repeated HAI assays. Although twofold differences in HAI titers are generally not considered significant, these data do suggest that seal 99-012 was indeed infected with a 1995-like virus (*17*).

Because both seals seemed to have been infected in the wild, we tested the hypothesis that a human influenza B virus was introduced in the seal population in 1995 and had circulated since. We screened sera obtained from 580 seals before 1995 and from 391 seals during or after 1995 for HAI activity against B/Seal/Netherlands/1/99 and confirmed the findings by ELISA using HA/NA and NP as antigens. For seals stranded in 1995 or later, both the serum sample taken at the moment of admission to the SRRC and the last sample before release to the wild were included, to exclude seroconversion during rehabilitation. None of the seals admitted to the SRRC before 1995 were seropositive, whereas 8 (including 99-012 and 99-041) out of 391 seals admitted from 1995 onward had HAI antibody titers between 24 and 768 ($P < 0.001$) (Fig. 4A). The six additional seals were four harbor seal pups and two grey seal (*Halichoerus grypus*) pups. Harbor and grey seals are known to share the same habitat in the Dutch coastal waters. None of these animals had IgM antibodies to NP, and all of them displayed decreasing levels of IgG antibodies to HA/NA (*17*) and NP (Fig. 4B) over time. This decrease correlated with a decrease in virus-neutralizing and HAI antibody levels (*17*). This is in contrast to seals 99-012 and 99-041, which had decreasing IgM titers and increasing IgG titers. The estimated half-life of approximately 25 days for NP-specific antibodies during the first 2 months of life in these six pups is in accordance with the half-life of maternal antibody titers to PDV observed in seal pups (*17*), which suggests that the detected antibodies are of maternal origin. On the basis of available seal population data in the Netherlands (*17*), it may be estimated that between 0.5 and 2% of animals in the wild have experienced influenza B virus infection since 1995.

Our data do not allow conclusions about the pathogenesis of influenza B virus infections in

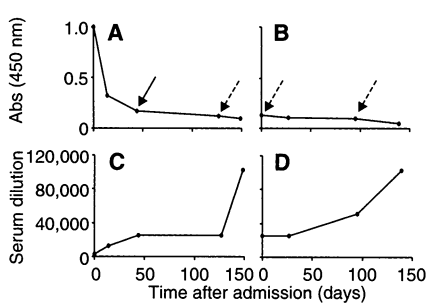

Fig. 2. Antibody response in seals infected with influenza B virus. Sera from seals 99-012 (**A** and **C**) and 99-041 (**B** and **D**) were tested for the presence of IgM antibodies to NP [(A) and (B)] and IgG antibodies to HA/NA proteins [(C) and (D)] (*21*). Arrows indicate the time points when virus detection in throat swab samples was attempted, with isolate B/Seal/Netherlands/1/99 represented as a solid arrow and negative results as dashed arrows. Abs, absorbance.

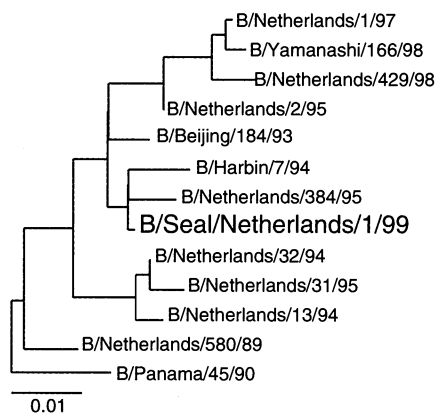

Fig. 3. Phylogenetic analysis of influenza B virus vaccine strains and Dutch epidemic strains from 1989 to 1999 and of B/Seal/Netherlands/1/99. A maximum likelihood tree was generated on *HA1* nucleotide sequences (*23, 29*) by means of the DNAML program of the Phylip 3.5 software package with 100 bootstraps and three jumbles, revealing that B/Seal/Netherlands/1/99 is a 1995-like influenza B virus. Sequence data are available from GenBank under accession numbers AF217214 through AF217223.

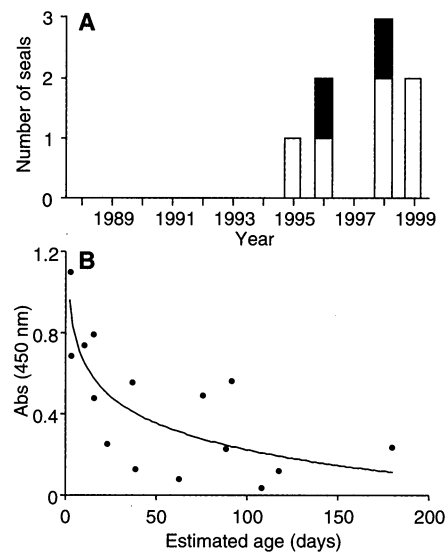

Fig. 4. Frequency of seals seropositive for influenza B virus admitted at the SRRC over time and the decline of antibody titers in seropositive seals. Serum samples obtained from seals in the past 10 years (580 seals before 1995 and 391 seals from 1995 and later) were screened for antibodies against influenza B virus by HAI and confirmed by ELISA with NP and HA/NA proteins (A) (*20, 21*). White bars represent harbor seals; black bars represent grey seals. Levels of IgG antibody to NP from longitudinal samples obtained from the six seropositive seals admitted to the SRRC from 1995 to 1998 (**A**) were determined by ELISA (*21*) and plotted against seal age (**B**). Abs, absorbance.

seals. Although the two harbor seals with proven influenza B virus infection displayed respiratory symptoms during their rehabilitation period, this occurred at a time when many of the admitted juvenile seals suffered from lungworm (*Otostrongylus circumlitus* and *Parafilaroides gymnurus*) infections (*17*). Association of lungworm infections in pigs with influenza A virus pathogenesis and transmission has been described (*25*), but the evidence was considered weak (*26*).

The combined serological and virological data obtained from seal 99-012 indicate that shedding of influenza B virus in seals was prolonged as compared to shedding in humans (*27, 28*) and that IgG antibody responses to NP and HA/NA were delayed. Possible explanations for this apparent suboptimal immune response upon infection may be associated with xenobiotic-related immunosuppression (*11*) or the therapeutic use of corticosteroids to combat the lungworm infections (*17*). Prolonged virus shedding in addition to the limited spreading of influenza B virus among seals (as shown in the SRRC and indicated by the limited seroprevalence of specific antibodies in the wild) may explain why little or no genetic and antigenic drift of influenza B virus is observed in seals.

Our data not only highlight the fact that influenza B virus infections can emerge in seal populations but also show that seals may constitute an animal reservoir from which humans may be exposed to influenza B viruses that have circulated in the past.

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18. RNA was isolated by means of a high pure RNA isolation kit (Boehringer-Mannheim). RNA was used for RT-PCR analysis to amplify a 240–base pair fragment of the influenza B virus *NS* gene segment using primers 5′-ATG GCC ATC GGA TCC TCA AC-3′ and 5′-TGT CAG CTA TTA TGG AGC TG-3′, and AMV reverse transcriptase, Amplitaq DNA polymerase, recombinant ribonuclease inhibitor (Promega) in the presence of 50 mM tris-HCl, 50 mM NaCl, 2 mM dithiothreitol, 7 mM $MgCl_2$, 1 mM dNTP, and 400 nM each of primer. Cycling parameters were 30 min at 42°C, 4 min at 95°C, 1 min at 45°C, and 3 min at 72°C once; and then 1 min at 95°C, 1 min at 45°C, and 3 min at 72°C, repeated 39 times. PCR fragments were sequenced with a DYEnamic ET terminator cycle sequencing premix kit (Amersham) on an ABI-373A apparatus (Perkin Elmer).
19. Seal kidney cells were plated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% L-glutamine, penicillin, and streptomycin at 1×10^5 cells per well in 24-well plates. Cells were inoculated with 1×10^5 $TCID_{50}$ of influenza virus B/Seal/Netherlands/1/99 in DMEM supplemented with 4% bovine serum albumin, 1% L-glutamine, penicillin, and streptomycin. Influenza B virus infection was detected by immunofluorescence with influenza B NP-specific antibodies, which were labeled with fluorescein isothiocyanate (IMAGEN Influenza A+B, DAKO Diagnostics) after 24 hours. Cytopathic changes and HA activity (titer = 32) were detected in the culture cell after 48 hours.
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21. Antibody titers were determined with a recombinant fusion protein between maltose-binding protein (MBP) and NP or with HA/NA proteins purified from virions (both proteins were derived from B/Harbin/7/94). IgG antibody levels to HA/NA and NP proteins were determined by an indirect ELISA with antigen-coated plates and peroxidase-labeled protein A for detection. IgM antibody levels to NP were determined by means of an antibody-capture ELISA with goat anti-dog IgM–coated plates and peroxidase-labeled MBP-NP antigen for detection. The goat anti-dog IgM antibody preparation specifically captures seal IgM, as was shown in routine serological tests for PDV and PHV.
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29. *HA1* sequences were amplified with M13-tagged primers P1 (5′-29M13-GCA TTT TCT AAT ATC CAC AA-3′) and P4 (5′-21M13-TTT GGG AAG CCA CCA ATC TG-3′) or primers P3 (5′-29M13-CCT ATA ATG CAC GAC AGA AC-3′) and P6 (5′-21M13-AAA CCA GCA ATA GCT CCG AA-3′), followed by sequencing with primers 29M13 (5′-CAG GAA ACA GCT ATG ACC-3′) and 21M13 (5′-TGT AAA ACG ACG GCC AGT-3′) using a DYEnamic ET terminator cycle sequencing premix kit (Amersham) and an ABI-373A sequencing apparatus (Perkin Elmer).

30. We are grateful to L. van der Kemp, G. de Mutsert, and M. van der Bildt for technical assistance; J. Habova for electron microscopy; Solvay Pharmaceuticals, Weesp, the Netherlands, for providing HA/NA proteins from B/Harbin/7/94; and the SRRC staff for taking care of and samples from seals. R. F. is a fellow of the Royal Netherlands Academy of Arts and Sciences.

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Noxa, a BH3-Only Member of the Bcl-2 Family and Candidate Mediator of p53-Induced Apoptosis

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A critical function of tumor suppressor p53 is the induction of apoptosis in cells exposed to noxious stresses. We report a previously unidentified pro-apoptotic gene, *Noxa*. Expression of Noxa induction in primary mouse cells exposed to x-ray irradiation was dependent on p53. *Noxa* encodes a Bcl-2 homology 3 (BH3)–only member of the Bcl-2 family of proteins; this member contains the BH3 region but not other BH domains. When ectopically expressed, Noxa underwent BH3 motif–dependent localization to mitochondria and interacted with anti-apoptotic Bcl-2 family members, resulting in the activation of caspase-9. We also demonstrate that blocking the endogenous Noxa induction results in the suppression of apoptosis. Noxa may thus represent a mediator of p53-dependent apoptosis.

The mechanism of p53-induced apoptosis has been extensively studied in the context of tumor suppression (*1*). p53-dependent apoptosis is regulated, at least in part, by transcriptional activation of its target genes (*1*), and this process is dependent on the Apaf-1/caspase-9 activation pathway (*2*). Among the identified target genes of p53, *Bax* encodes a pro-apoptotic Bcl-2 family of proteins that can activate this pathway (*3*). However, in *Bax*-deficient mice, DNA damage–induced apoptosis occurs normally in thymocytes, and apoptosis induced by treatment with anticancer drugs is only partly inhibited in mouse embryo fibroblasts (MEFs) expressing the adenovirus oncoprotein E1A (*4*). Furthermore, thymocytes from *p53*-defi-