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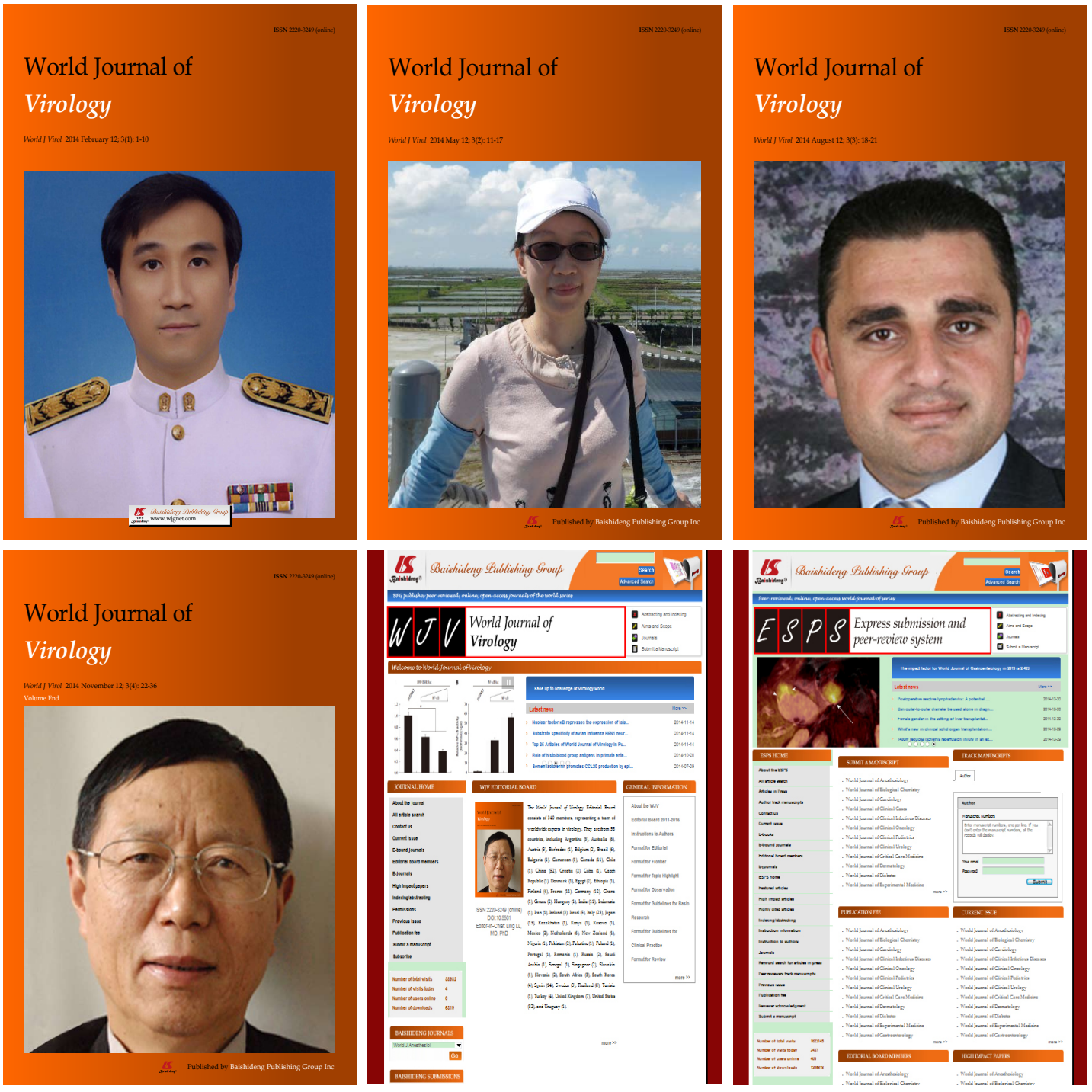

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MINIREVIEWS

Immunological-based assays for specific detection of shrimp viruses

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Abstract

Among shrimp viral pathogens, white spot syndrome virus (WSSV) and yellow head virus (YHV) are the most lethal agents, causing serious problems for both the whiteleg shrimp, *Penaeus (Litopenaeus) vannamei*, and the black tiger shrimp, *Penaeus (Penaeus) monodon*. Another important virus that infects *P. vannamei* is infectious myonecrosis virus (IMNV), which induces the white discoloration of affected muscle. In the cases of taura syndrome virus and *Penaeus stylirostris* densovirus (*Pst*DNV; formerly known as infectious hypodermal and hematopoietic necrosis virus), their impacts were greatly diminished after the introduction of tolerant stocks of *P. vannamei*. Less important viruses are *Penaeus monodon* densovirus (*Pm*DNV; formerly called hepatopancreatic parvovirus), and *Penaeus monodon* nucleopolyhedrovirus (*Pemo*NPV; previously called monodon baculovirus). For freshwater prawn, *Macrobrachium rosenbergii* nodavirus and extra small virus are considered important viral pathogens. Monoclonal antibodies (MAbs) specific to the shrimp viruses described above have been generated and used as an alternative tool in various immunoassays such as enzyme-linked immunosorbent assay, dot blotting, Western blotting and immunohistochemistry. Some of these MAbs were further developed into immunochromatographic strip tests for the detection of WSSV, YHV, IMNV and *Pemo*NPV and into a dual strip test for the simultaneous detection of WSSV/YHV. The strip test has the advantages of speed, as the result can be obtained within 15 min, and simplicity, as laboratory equipment and specialized skills are not required. Therefore, strip tests can be used by shrimp farmers for the pond-side monitoring of viral infection.

Key words: Shrimp viruses; Immunological assay; Western blot; Immunohistochemistry; Immunochromatographic strip test

Core tip: Monoclonal antibodies (MAbs) specific to various shrimp viruses were generated. The MAbs can be used to detect viral infection in shrimp by immunological assays such as Western blotting, dot blotting, and immunohistochemistry. Some of the MAbs were used to developed immunochromatographic strip tests for specific detection of white spot syndrome virus, yellow head virus, infectious myonecrosis virus, and *Penaeus monodon* nucleopolyhedrovirus formerly known as monodon baculovirus. The strip test has the advantages of speed, as the result can be obtained within 15 min, and simplicity, as laboratory equipment and specialized skills are not required.

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INTRODUCTION

According to the Food and Agriculture Organization of the United Nations FAO report, aquaculture is one of the fastest-growing animal food-producing sectors, and in the next decade, total production from both capture and aquaculture will exceed that of beef, pork or poultry[1]. In 2009, crustaceans contributed approximately 11.2 million tons to global fishery and aquaculture production. During the period of 2000-2008, crustacean production increased at an average annual rate of approximately 15%, faster than in the previous decade. The rapid increase largely reflected the remarkable increase in the whiteleg shrimp, *Penaeus vannamei*, in China, Thailand and Indonesia[2].

It has been estimated that 40% of tropical shrimp production is lost annually due to infectious diseases[3]. Approximately 60% of the disease-associated loss in shrimp production can be due to viral diseases and 20% to bacterial diseases. The remaining 20% of the loss was attributed to other pathogens, including parasites and fungi[4]. In the case of shrimp viruses, white spot syndrome virus (WSSV) is the most serious pathogen because it is lethal to all cultivated penaeid shrimp species, and mortality can be high and rapid. Another severe pathogen is yellow head virus (YHV), which can cause rapid mortality in *P. vannamei* and black tiger shrimp (*Penaeus monodon*). Other important viruses for *P. vannamei* but not for *P. monodon* are infectious myonecrosis virus (IMNV) and Taura syndrome virus (TSV). Less important viruses are *Penaeus monodon* densovirus [*Pm*DNV; formerly called hepatopancreatic parvovirus (HPV)] and *Penaeus monodon* nucleopolyhedrovirus [*Pemo*NPV; previously called monodon baculovirus (MBV)] because the proper washing of eggs and/or nauplii in the hatchery can eliminate the viruses. *Penaeus stylirostris* densovirus (*Pst*DNV), formerly known as infectious hypodermal and hematopoietic necrosis virus (IHHNV), can cause high mortality in American blue shrimp (*P. stylirostris*) and stunt growth in *P. vannamei*. At present, the commercial stocks of *P. vannamei* used in Asia are highly tolerant to TSV[4].

For the detection of shrimp viruses, the World Animal Health Organization (the OIE-Office International des Epizooties) recommends polymerase chain reaction (PCR)-based methods[5]. In this brief review, we present alternative assays for the detection of various shrimp viruses based on immunologically developed methods. In all cases, the immunological-based assays are virus specific with optimum sensitivity. Further development into immunochromatographic strip tests that can be used by shrimp farmers to monitor certain shrimp virus infections is discussed.

WSSV

WSSV is a causative agent of white spot disease, which is one of the most devastating diseases in cultured penaeid shrimp, including black tiger shrimp (*P. monodon*) and whiteleg shrimp (*P. vannamei*). WSSV infections results in the gross sign of white inclusions embedded in the shrimp cuticle at the late stages of infection[6]. This gross sign of infection was first recognized during an outbreak in *Penaeus japonicus* in 1993[7]. WSSV is a large, enveloped, rod-shaped, double-stranded DNA virus with a genome size of approximately 300 kbp. The WSSV genome encodes at least 181 open reading frames (ORFs), and most of the predicted gene products show no similarity to known proteins[8]. Due to its unique characteristics, the International Committee on Taxonomy of Viruses (ICTV) classified WSSV as the only member of the genus *Whispovirus* within a new family called *Nimaviridae*[9].

At present, at least 40 WSSV structural proteins have been identified, ranging in size from 68 to 6077 amino acid residues[8]. When WSSV virions were subjected to gradient SDS-PAGE analysis, major protein bands were identified and named VP664, VP28, VP26, VP24, VP19 and VP15. VP28 and VP19 were identified as enveloped proteins[10,11], whereas VP26 and VP24 were identified as tegument proteins[8]. Based on solubilization in salt-containing Triton X-100, the tegument protein may loosely associate with both the envelope and nucleocapsid[12]. The nucleocapsid protein VP15 demonstrates DNA-binding activity and may be involved in the packaging of the WSSV genome into the nucleocapsid[11,13]. VP664 is another major nucleocapsid protein with a calculated molecular mass of 664 kDa, and it is encoded by an intronless ORF of 18234 nucleotides. It is the largest known viral structural protein[14].

Because VP28 is the most abundant WSSV envelope protein[8], it is a good candidate protein for WSSV detection by immunological-based assays. In 2001, four monoclonal antibodies (MAbs) raised against purified WSSV were generated, and they were reacted with the VP28 protein by Western blot analysis. Two of the MAbs were selected for further development into various serological methods, *i.e.,* an immunohistochemical assay and Western blotting for WSSV detection. The two MAbs did not cross-react with hemolymph from shrimp infected with other viruses, including IHHNV, YHV and TSV. However, in that report, no comparative study with PCR was performed[15].

In 2002, three reports described the production of antibodies specific to WSSV VP28 protein. Liu *et al*[16] produced MAbs using a recombinant 6x-histidine-tagged VP28 as an antigen. The MAbs were used to develop an antigen-capture enzyme-linked immunosorbent assay (ELISA) (Ac-ELISA), and the results revealed that the sensitivity of the Ac-ELISA (400 pg of purified WSSV protein) was comparable to that of PCR (300 pg of DNA extracted from purified WSSV). You *et al*[17] generated polyclonal antibodies raised against a truncated histidine-tagged VP28 protein, but the antibody cross-reacted with a shrimp protein at 80 kDa. Anil *et al*[18] reported the production of MAbs against purified WSSV, and the obtained MAbs recognized both the 28 and 18 kDa WSSV proteins. The limit of detection of the immunodot test was 500 pg of the viral protein, which is similar to that of a 1-step PCR assay. However, the immunodot test de-

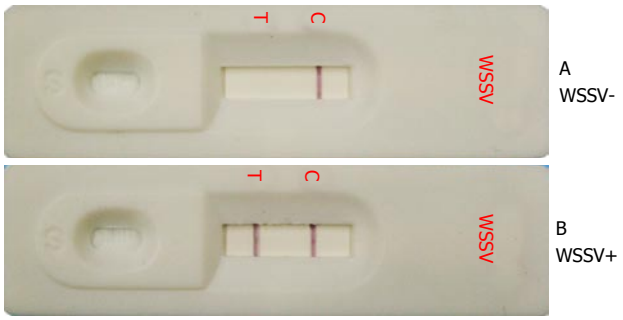

Figure 1 White spot syndrome virus immunochromatographic test strip. Gill homogenates from (A) uninfected *P. monodon* showing a negative result with only one reddish-purple band at the C line and (B) WSSV-infected *P. monodon* showing a positive result with two reddish purple bands at the T line and C line. T: Test line; C: Control line. WSSV: White spot syndrome virus.

tected WSSV in only 21 of the 22 PCR-positive samples.

Another study based on the production of MAbs using a truncated VP28 envelope protein lacking the N-terminal transmembrane region as an antigen has also been reported[19]. A MAb named W29 was used to develop immunohistochemical and dot blot assays for WSSV detection, and this MAb could be used to detect WSSV in experimentally infected *P. monodon* at 12 h. The W29 MAb was further used to develop an immunochromatographic strip test that can be used conveniently by shrimp farmers; the results of this test can be obtained within 15 min without the requirement of sophisticated tools. The strip test employed the W29 MAb conjugated with colloidal gold at a glass fiber located downstream of the sample pad and a rabbit anti-recombinant VP28 antibody combined with the W28 MAb at the test line (Figure 1). However, the sensitivity of the strip test was much lower than that of a 1-step PCR assay[20]. Another immunochromatographic assay called Shrimple® demonstrated lower sensitivity (34.7% of the inoculated shrimp) when compared with real-time PCR (100% of the specimens)[21].

The second generation of immunochromatographic strip tests, called the dual strip test, was developed for the simultaneous detection of WSSV and YHV[22]. MAbs designated W1 and W30 were raised against a truncated VP28 envelope protein and were used for the development of the dual strip test. The W30 MAb was conjugated to colloidal gold, and the W1 MAb was used at the test line (Figure 2). The detection of WSSV by the dual strip test was approximately 500-fold less sensitive when compared with a 1-step PCR assay. However, the dual strip test could remain functional under storage at room temperature for at least 2 years. At present, this dual strip test has been commercialized by Marine Leader Co. Ltd, Thailand.

In addition to VP28, other WSSV structural proteins, such as VP19 and VP26, have also been used as targets for WSSV detection. In the case of the VP19 envelope protein, polyclonal and MAbs were raised against a maltose-binding protein (MBP)-VP19 fusion protein[23,24]. The detection limit of MAbs specific to VP19, designated W25, was 1.2 fmole/μL of purified recombinant MBP-

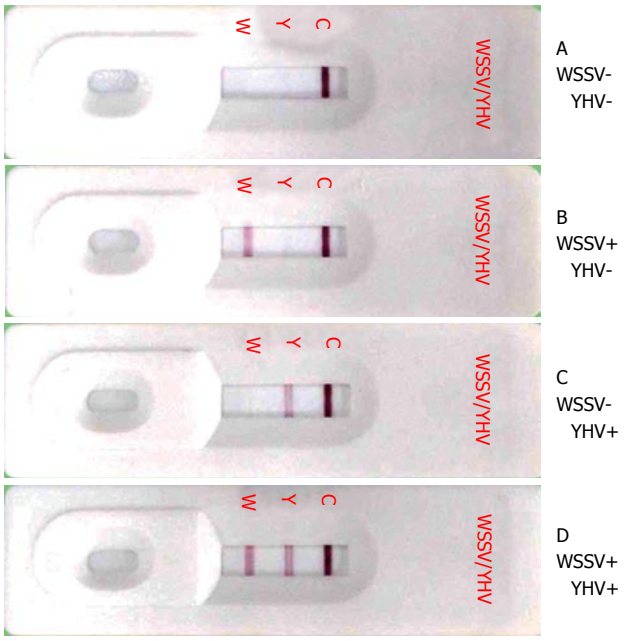

Figure 2 White spot syndrome virus and yellow head virus Dual test strip results. Pleopod homogenates samples from (A) uninfected *P. vannamei*, (B) WSSV infected *P. vannamei*, (C) YHV infected *P. vannamei* or (D) a combination of B and C were applied to the test strip. W: The test line for WSSV, Y: The test line for YHV and C: The control line. WSSV: White spot syndrome virus; YHV: Yellow head virus.

VP19 protein, as determined by dot blotting. It has been shown that the combination of MAbs specific to VP19 and VP28 (W29) results in twofold higher sensitivity than if either MAb is used alone. However, the sensitivity of the combined MAbs was still much lower than that of a 1-step PCR assay[24]. In the case of the VP26 structural protein, the polyclonal antibody raised against a truncated 6x-His-tagged VP26 protein demonstrated specific immunoreactivity to viral antigen, as determined by immunohistochemistry and Western blotting[25]. A MAb specific to the VP26 protein is under investigation in our laboratory.

As mentioned above, all of the target proteins for WSSV detection belong to viral structural proteins. However, one report has stated that the ICP11 protein, a non-structural protein, is likely to be a better indicator of WSSV infection. ICP11 is the most highly expressed protein in WSSV-infected gill tissue at 48 h post-infection[26]. ICP11 acts as a DNA mimic by binding to the histone H3 protein, thus disrupting nucleosome assembly[27]. MAbs specific to ICP11 were recently generated using C-terminally intein-tagged ICP11 (ICP11-intein) and N-terminally glutathione-S-transferase (GST)-tagged ICP11 (GST-ICP11) as antigens. The detection limit of the MAbs was approximately 0.7 fmole/μL of GST-ICP11 as determined by dot blotting. A combination of MAbs specific to ICP11, VP28 (W29) and VP19 (W25) increased the detection limit for WSSV to a sensitivity 250-fold lower than that of a 1-step PCR assay[28]. Thus, the development of a higher-sensitivity immunochromatographic strip test for WSSV detection could be achieved in the near future using these MAbs.

TSV

TSV is a major viral pathogen in cultured whiteleg shrimp (*P. vannamei*). It was first recognized in Ecuador in 1992[29] and later spread to many countries, including those in the Americas, Taiwan, China, Thailand and Indonesia[30-33]. TSV infections can cause gross pathology in *P. vannamei* that can occur in two phases, the acute and recovery phases. The acute phase can be characterized by a reddish necrotic area on the tail fan, whereas the recovery phase often includes black cuticular lesions in the regions where the acute phase necrosis occurred[6]. TSV is a small, nonenveloped icosahedron, positive-sense, single-stranded RNA virus with a diameter of 32 nm[34]. The viral genome is 10205 nucleotides and contains two large ORFs. ORF1 contains the sequence motifs for helicase, a protease and an RNA-dependent RNA polymerase (RdRp). ORF2 contains the sequences of structural proteins, including three major capsid proteins, VP1 (55 kDa), VP2 (40 kDa) and VP3 (24 kDa), and one minor capsid protein (58 kDa) (Mari 2002). TSV has been classified by the ICTV in the novel genus *Aparavirus* in a new family *Dicistroviridae* (in the Order *Picornavirales*)[35].

VP1 displays greater variation in its amino acid sequence (3.5%) than VP2 and VP3 (both 0.8%)[36]. Therefore, the VP1 region can be used to establish the genetic relationship among TSV isolates. At present, at least four genotypic variants have been identified according to the sequence of the VP1 (equivalent to capsid protein 2; CP2) structural protein. They are the Mexico, Southeast Asia, Belize/Nicaragua and Venezuela/Aruba lineages[37]. Therefore, strain variation may result in an inaccurate diagnosis of TSV infection. Chicken and mouse polyclonal antisera and MAbs against purified TSV antigens have been produced[38]. A MAb specific to VP1 (called 1A1) has been obtained and used to develop Western blot, dot blot and immunohistochemical assays for TSV detection. However, the 1A1 MAb does not recognize TSV isolates from Mexico, Nicaragua and Belize[39-41] and reacts weakly with TSV from Venezuela[42]. Moreover, the 1A1 MAb, raised against purified TSV, displays cross-reactivity to the hemolymph of shrimp infected with *Pst*DNV, YHV or WSSV[38].

In 2006, polyclonal antibodies against VP1 and VP3 were generated using recombinant VP1 and VP3 proteins as the antigens. The obtained MAbs demonstrated specificity to TSV by Western blot and immunohistochemistry[43]. Later, MAbs specific to the VP3 capsid protein were developed and shown to detect TSV infection by dot blot and Western blot without cross-reactions with other shrimp viruses[44]. To increase the sensitivity of TSV detection, MAbs raised against recombinant VP2 proteins were generated[45]. The combination of VP2 and VP3 MAbs can detect TSV infections in field samples of *P. vannamei* with a better detection limit than a single MAb[45]. Recently, MAbs specific to VP1 of TSV were produced and demonstrated dot blot sensitivity at 2 fmole/μL of GST-VP1[46]. In the near future, an immunochromatographic strip test using MAbs specific to VP1 and VP2 should be used to enhance the pond-side identification of TSV infection.

YHV

YHV is the causative agent of yellow head disease in penaeid shrimp. The virus was named after its gross signs of disease, including a yellowish cephalothorax and a very pale overall coloration of moribund, infected shrimp[6]. YHV first emerged in farmed black tiger shrimp (*P. monodon*) in Thailand in 1990[47] and caused a loss of shrimp production equivalent to 30-40 million USD[48]. The entire crop is typically lost within a few days after the appearance of the gross signs of this disease[49]. YHV is a bacilliform, enveloped, (+) single-stranded RNA virus classified in the new virus genus *Okavirus*, the new family *Roniviridae* and the order *Nidovirales*[50-52]. There are at least six distinct genetic lineages (genotypes) of YHV, but only YHV type-1 has caused major disease-related losses[53]. Gill-associated virus (GAV or YHV-type 2) has been linked to disease outbreaks with a less severe condition described as mid-crop mortality syndrome[54,55]. Other genotypes were detected exclusively as low level infections in apparently healthy shrimp[53].

Purified YHV virions contain three major structural proteins with molecular masses of 116 kDa (gp116), 64 kDa (gp64) and 20 kDa (p20). The gp116 and gp64 enveloped glycoproteins are encoded by ORF3, whereas the p20 nucleoprotein is encoded by ORF2[56,57]. For YHV detection, a dot blot assay using antiserum against purified YHV was first developed in the year 2000[58]. A MAb named V3-2B that is specific to gp116 was generated and demonstrated specificity to YHV-infected shrimp by dot blot, Western blot and immunohistochemistry[59]. Later, four groups of MAbs specific to gp116, gp64 and p20 were produced against purified YHV virions and were shown to detect YHV infection by dot blot and immunohistochemistry[60]. A single-chain variable fragment antibody directed against gp116 was generated, and a dot blot assay demonstrated its specificity to YHV without cross-reactivity with WSSV and TSV proteins[61].

A convenient immunochromatographic strip test was also developed using a MAb specific to p20 and polyclonal antibodies raised against recombinant p20 protein. The sensitivity of this strip test was approximately 500-fold lower than that of the 1st step reverse transcription (RT)-PCR assay. This kit can also be used to detect GAV infection because the MAb cross-reacts well with GAV[62]. Further improvement of this dual strip test for WSSV and YHV was performed. The sensitivity of the improved dual strip test was 1000-fold lower than an RT-PCR test for YHV and 500-fold lower than a one-step PCR test for WSSV. Although the dual strip test kit has a lower sensitivity than that of PCR, it has advantages in speed and simplicity, as it does not require equipment[22].

IMNV

The first disease outbreak caused by IMNV was reported

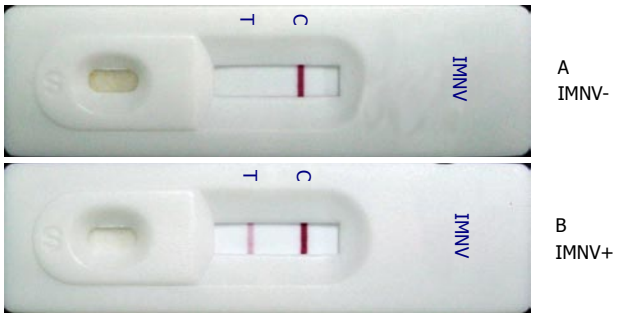

Figure 3 Infectious myonecrosis virus test strip results. Shrimp muscle homogenate samples from (A) an uninfected *P. vannamei* showing a negative result with only one reddish-purple band at the C line and (B) an IMNV infected *P. vannamei* showing a positive result with two reddish purple bands. IMNV: Infectious myonecrosis virus.

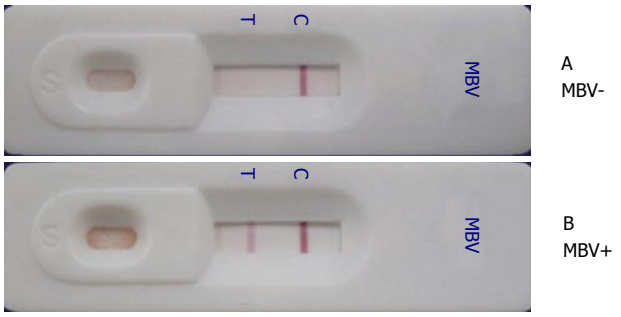

Figure 4 *Penaeus monodon* nucleopolyhedrovirus immunochromatographic strip test results. Homogenates of (A) uninfected *P. monodon* postlarvae showing a negative result with only one band at the C line and (B) *Pemo*NPV-infected *P. monodon* postlarvae displaying a positive result. *Pemo*NPV: *Penaeus monodon* nucleopolyhedrovirus; MBV: Monodon baculovirus.

in farmed Pacific white shrimp (*P. vannamei*) from Brazil in the year 2004. The gross signs of disease include focal to extensive necrotic areas in skeletal muscle tissues, primarily in the distal abdominal segments and the tail fan, and the appearance of white discoloration of affected muscle[63,64]. Mortality due to IMNV infection in cultivated *P. vannamei* can reach 70%[65]. IMNV is a nonenveloped, icosahedral virus with a diameter of 40 nm. The genome consists of a double-stranded RNA molecule of 7560 nucleotides containing two ORFs, ORF1 and ORF2. The first half of ORF1 encodes an RNA-binding motif, and the second half encodes a capsid protein with a molecular mass of 160 kDa. ORF2 encodes a putative RdRp[66].

For immunodiagnostic assays, MAbs against a recombinant capsid protein comprising amino acids 300-527 were generated. In an immunodot-blot assay and Western blotting, the MAbs effectively bound tissue extracts from shrimp naturally infected with IMNV. Viral inclusions can also be revealed by immunohistochemistry using these MAbs[67]. Another study on MAb production has also been reported. The gene encoding the capsid protein was amplified into three parts, namely CP-N (nucleotides 2248-3045), CP-I (nucleotides 3046-3954) and CP-C (nucleotides 3955-4953). Two MAbs (IMN7 and IMN12) specific to CP-N and one MAb (IMC1) specific to CP-C were then obtained. The detection sensitivities, as determined by dot blot, were 6 fmole/μL of purified recombinant CP-N protein and 8 fmole/μL of purified recombinant CP-C protein. A combination of all three MAbs resulted in a twofold increase in the detection limit compared to the use of any single MAb. However, the sensitivity is 10-fold lower than that of the a-step RT-PCR assay[68].

Subsequently, the MAbs IMN7 and IMC6, specific to CP-N and CP-C, respectively, were utilized for immunochromatographic strip test production (Figure 3). The sensitivity of the test was comparable to that of dot blot but was approximately 300-fold less sensitive than a one-step RT-PCR[69].

*Pemo*NPV

*Pemo*NPV was previously called MBV. It has been shown that *Pemo*NPV does not typically cause mortality in farmed *P. monodon*[70], but it has been linked to stunting, causing the mean length of *Pemo*NPV-infected shrimp to be significantly shorter than that of uninfected shrimp from the same pond[71].

In contrast to insect baculoviruses, little data exist on the genes and genomes of crustacean baculoviruses. However, the *Pemo*NPV genome size has been proposed to fall within the typical baculovirus range of 80 to 160 kb[72]. At present, the only characterized *Pemo*NPV gene is a polyhedrin-encoding gene, and its deduced amino acid sequence revealed no homology to other known proteins[73].

For use in an immunodiagnostic assay, MAbs were raised against a partially purified polyhedrin protein and demonstrated specific *Pemo*NPV detection by Western blot and immunohistochemistry[74]. Subsequently, MAbs raised against a recombinant polyhedrin protein were also produced. Dot blot, Western blot and immunohistochemical assays showed specific *Pemo*NPV detection without cross-reactivity with WSSV, TSV, YHV or *Pm*DNV. Dot blotting using a combination of four MAbs obtained from both studies was approximately 100-fold less sensitive than a 1-step PCR assay[75]. An immunochromatographic strip test using four MAbs raised against partially purified polyhedrin protein was developed and demonstrated a sensitivity 200-fold lower than that of a 1-step PCR assay[76] (Figure 4).

*Pm*DNV

*Pm*DNV, also called HPV, is a pathogen responsible for stunted growth in black tiger shrimp (*P. monodon*). Most *Pm*DNV-infected shrimp grow very slowly and stop growing at approximately 6 cm in length[6,71]. *Pm*DNV is a non-enveloped icosahedral virus that is 22-23 nm in diameter and contains linear ssDNA. It belongs to the family *Parvoviridae* in the densovirus group[77,78]. Two Asian types of *Pm*DNV have been characterized at the molecular level. One type has been identified in *P. chinensis* from South Korea[77], *P. monodon* from Madagascar, Mozambique and Tanzania and *P. merguiensis* from New Caledonia[79] and Australia[80]. The other type has been identified

in infected *P. monodon* from Thailand and India[78,81]. The complete genome of *Pm*DNV isolated from infected *P. monodon* in Thailand consists of 6321 nucleotides, representing three ORFs and two non-coding termini. ORF3 encodes a capsid protein of approximately 92 kDa, which may later be cleaved after the first or second arginine residue to produce a 57 kDa or 54 kDa structural protein, respectively[82].

MAbs raised against purified *Pm*DNV isolated from Thailand have been produced, four of which react with a 54 kDa protein by Western blotting and to intranuclear inclusion bodies in tubule epithelial cells in *Pm*DNV-infected tissue by immunohistochemistry[83]. The recombinant capsid proteins of *Pm*DNV have also been used to generate MAbs, and five MAbs have been obtained. The most sensitive MAb displayed a detection limit of 50 fmole/μL of a recombinant protein, as determined by dot blotting. However, the combination of three MAbs revealed a sensitivity 25000-fold lower than a one-step PCR assay[84].

*Pst*DNV

*Pst*DNV, formerly called IHHNV, is a viral pathogen that can cause mortality in juveniles and sub-adults of the blue shrimp *P. stylirostris*[85] and cuticular deformities and growth retardation (collectively called runt-deformity syndrome) in *P. vannamei*[86,87]. *Pst*DNV is a non-enveloped icosahedral virus that is 22-23 nm in diameter and contains 4.1 kb of linear ssDNA[85-89]. *Pst*DNV is classified in the family *Parvoviridae*, subfamily *Densovirinae* in the genus *Brevidensovirus*[90]. The *Pst*DNV genome consists of three large ORFs in which the left ORF encodes the non-structural protein, and the right ORF represents the capsid protein; the function of the middle ORF is still unknown[91].

An early report on MAb production described six IgM MAbs generated against purified *Pst*DNV. These MAbs displayed specificity to purified *Pst*DNV preparations in both ELISA and immunoblot assays. However, the ELISA using these MAbs reacted nonspecifically to shrimp samples that were negative for *Pst*DNV by histology and DNA hybridization[92]. In 2009, MAbs raised against a recombinant capsid protein were produced and demonstrated *Pst*DNV specificity in Western blot, dot blot and immunohistochemical assay without cross-reactivity with uninfected shrimp. The sensitivity was 300 pg/μL of recombinant capsid protein, as determined by immunodot-blotting; however, the sensitivity of the dot blot was 1000-fold lower than that of a one-step PCR assay[93].

*Mr*NV NODAVIRUS AND EXTRA SMALL VIRUS

*Mr*NV is an important viral agent that causes white tail disease (WTD) in the giant freshwater prawn *Macrobrachium rosenbergii*. WTD causes significant mortality in hatchery- and nursery-reared postlarvae[94]. The disease was first reported in Guadeloupe Island (French West Indies) in 1997[95] and was later reported in China[96], India[97], Thailand[98], Taiwan[99] and Australia[100]. *Mr*NV is a non-enveloped, icosahedral virus with a diameter of 26-27 nm and a genome comprised of two pieces of positive-sense ssRNA. RNA 1 is 3202 bp in length and encodes the RdRp, whereas RNA 2 is 1175 bp in length and encodes a viral capsid protein of 43 kDa[96,101]. Extra small virus (XSV) is usually associated with *Mr*NV. XSV is also a non-enveloped, icosahedral virus; it is 15 nm in diameter and contains a 796 bp ssRNA genome[101,102]. XSV has been hypothesized to be a satellite virus that depends on the RdRp of *Mr*NV for replication[96].

For use in immunodiagnostic assays, polyclonal antibodies were raised against a purified viral suspension. A sandwich ELISA was developed and successfully used to identify tissue extracts infected with *Mr*NV as well as purified viral extracts[103]. The purified virus was also used to generate MAbs that demonstrated specificity to the *Mr*NV 42 kDa capsid protein by Western blotting. A triple antibody ELISA (TAS-ELISA) has also been developed and shown to be more sensitive than an indirect ELISA[104]. Recently, a MAb raised against a recombinant *Mr*NV capsid protein was generated and demonstrated a sensitivity of 10 fmole/μL of recombinant protein by dot blotting. However, the sensitivity of this MAb in a dot blot assay using homogenate from naturally *Mr*NV-infected shrimp was 200-fold lower than that of a one-step RT-PCR assay. In the case of XSV, MAbs were raised against a recombinant XSV capsid protein. Four MAbs were obtained and demonstrated a detection limit of 10-20 fmole/μL of purified recombinant protein by dot blotting. Using an XSV-specific MAb and an *Mr*NV-specific MAb, immunohistochemistry on connective tissue sections from prawn with WTD revealed that XSV infection co-localized at varying densities with *Mr*NV infection[105].

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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CLINICAL TRIALS STUDY

Semen lactoferrin promotes CCL20 production by epithelial cells: Involvement in HIV transmission

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Abstract

AIM: To study the effect of seminal plasma on Chemokine (C-C motif) ligand 20 (CCL20) production by epithelial cells and its relationship with lactoferrin.

METHODS: HEC-1A cells, a cell line derived from a monostratified endocervical epithelium, were incubated with samples of seminal plasma (diluted 1:10 in culture medium) recovered from human immunodeficiency virus (HIV) seronegative (HIV-) or HIV seropositive (HIV+) subjects. Recombinant human interleukin 1 beta (IL-1β) was used as positive control, and culture medium only as negative control. The measurement of CCL20 production in the supernatants of HEC-1A cells and of lactoferrin in seminal plasma was determined by enzyme-linked immunosorbent assay techniques. A fractionation of seminal plasma proteins was performed by ion exchange chromatography on a pool of seminal plasma specimens from HIV- subjects. Each fraction was tested for its ability to stimulate the production of CCL20 by HEC-1A cells and for its lactoferrin concentration. The HIV viral load in seminal plasma samples from HIV+ patients was measured using the HIV-Monitor kit (Roche Diagnostic Systems, Branchburg, NJ, United States).

RESULTS: The positive control IL-1β was responsible for an increase of 11.36 ± 3.36 times in the production of CCL20. Stimulation of HEC-1A cells was performed in 34 seminal plasma samples (22 from HIV+ subjects and 12 from HIV- subjects). The mean production of CCL20 by HEC-1A in presence of seminal plasma from HIV- and HIV+ subjects was respectively 5.38 ± 0.91 and 7.57 ± 3.26 times higher than that obtained with the untreated cells ($P < 0.05$ between the two groups). Using the same 34 specimens of seminal plasma, no correlation was observed between the concentration of total proteins in seminal plasma and their ability to stimulate the secretion of CCL20 by HEC-1 cells. In contrast, the ability to produce CCL20 by HEC-1A cells correlated to the concentration of lactoferrin in the seminal plasma samples (r coefficient = 0.56; CI: 0.26-0.76; $P < 0.001$). After fractionation by ion exchange chromatography, the seminal plasma fractions exhibiting the highest concentrations of lactoferrin were responsible for the greatest stimulation of CCL20 production by HEC-1A cells (r coefficient = 0.89; CI: 0.78-0.95; $P <$

0.0001).

CONCLUSION: Lactoferrin present in seminal plasma correlated with an increased production of CCL20 by HEC-1A cells and therefore could facilitate HIV entry through the genital mucosa.

Key words: Human immunodeficiency virus/acquired immunodeficiency syndrome; Sexual transmission; Seminal plasma; CCL20; Lactoferrin; Endocervical epithelial cells

Lourenço AG, Komesu MC, Machado AA, Quintana SM, Bourlet T, Pozzetto B, Delézay O. Semen lactoferrin promotes CCL20 production by epithelial cells: Involvement in HIV transmission. *World J Virol* 2014; 3(2): 11-17 Available from: URL: http://www.wjgnet.com/2220-3249/full/v3/i2/11.htm DOI: http://dx.doi.org/10.5501/wjv.v3.i2.11

INTRODUCTION

Sexual transmission of human immunodeficiency virus type 1 (HIV-1) accounts for 60% to 90% of new infections, especially in developing countries. During male-to-female transmission, the virus is typically deposited in the vagina as cell-free or cell associated virions carried by semen[1]. In the absence of breaches in the genital mucosa, the epithelium crossing by HIV could occur through the recruitment of immune cells with migratory properties, such as macrophages, lymphocytes or Langerhans cells (LCs), the latter cells being considered as one of the first target for this virus[2]. HIV entry may be observed at different levels of the female genital tract including the vagina, the ectocervix and the endocervix. The epithelial architecture is variable in these regions. The epithelium of vagina and ectocervix is composed by multi-layered, pluristratified epithelial cells that do not form a polarized epithelium. In contrast, the epithelium of the endocervix is a single layer of polarized, columnar epithelial cells with tight junctions, dividing the epithelium into apical and basolateral domains[3]. These simple mono-layered epithelia provide a lower degree of protection.

The Chemokine (C-C motif) ligand 20 (CCL20) is liberated by epithelial cells from different tissues including skin[4,5], oral mucosa[6] and vaginal epithelium[7]. CCL20 is an important immune effector molecule that is chemotactic for immature dendritic cells (DCs) and lymphocytes[8,9]. DCs also likely contribute to the array of cells potentially involved in HIV entry into the vaginal and ectocervical mucosae. DCs efficiently capture, disseminate, and transmit viruses to mononuclear target cells; however, HIV does not productively infect the DCs themselves[3]. CCL20 secretion by human vaginal epithelial cells has been shown to be enhanced in the presence of semen resulting in chemoattraction of LCs that are permissive to HIV infection[10], but the compound(s) involved in this stimulation is (are) not yet characterized.

This study was performed for analyzing the ability of seminal plasma from HIV seronegative (HIV-) and HIV seropositive (HIV+) subjects to promote the production of CCL20 by monolayers of endocervical epithelium cells (HEC-1A cell line). This secretion correlated to the amount of lactoferrin present in the seminal plasma specimen.

MATERIALS AND METHODS

Seminal plasmas samples

Semen samples were collected from 22 HIV+ and 12 HIV- subjects. The patients gave their fully-informed written consent. The study was reviewed and approved by the Ethics Committee of the School of Medicine of Ribeirão Preto, University of São Paulo, Brazil (CHSMRP-USP No. 4926/2009). The inclusion criteria were as follows: being over 18 years old, having never undergone radiotherapy or chemotherapy treatment, and not having used antimicrobials or anti-inflammatory drugs during the last 6 mo. HIV- men were tested for the absence of common sexually transmitted diseases including syphilis, *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infection, herpes simplex virus infection and hepatitis B virus infection.

The participants were instructed to auto-perform an aseptic collection of semen by masturbation into a universal collector, with the use of neither lubricants nor water. Within 4 h after ejaculation, the semen specimens were submitted to the following protocol: they were diluted 1:2 in PBS and then centrifuged at 800 g for 30 min. The supernatant constituted the seminal plasma that was stored at -80 ℃. The frozen samples were sent to the GIMAP team, Saint Etienne, France, for analysis.

Cell culture

The HEC-1A cell line was used for mimicking the female genital tract. It was cultured in Dulbecco's minimal essential medium (DMEM-F12 medium, Cambrex BioScience, Verviers, Belgium) to which were added 2% fetal bovine serum (FBS) and 1% solution containing penicillin, streptomycin and amphotericin B (Sigma-Aldrich, St. Louis, MO, United States). The experiments of stimulation were performed on 96-well culture plates (BD Falcon, Franklin Lakes, NJ, United States) seeded with cells cultured for 2 d with a final density of 100000 cells/well.

Measurement of the secretion of CCL20 by HEC-1A cells

HIV- or HIV+ seminal plasmas diluted 1:10 in culture medium were added to the HEC-1A cells. Recombinant human interleukin 1 beta (IL-1β) (Peprotech, Neuilly-Sur-Seine, France) at the concentration 25 ng/mL was used as positive control, as previously reported[7,10]. Culture medium DMEM-F12 served as negative control. After an overnight incubation, the CCL20 production

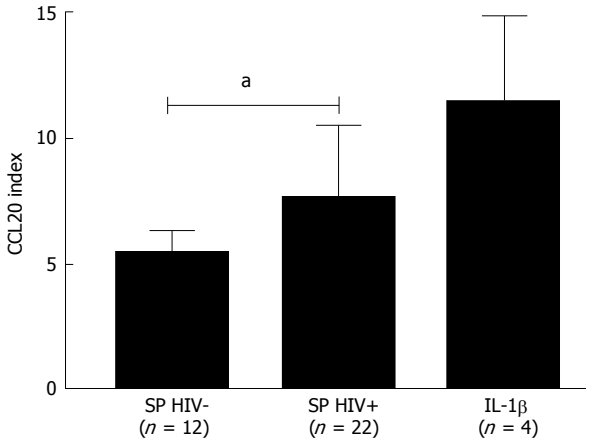

Figure 1 The Chemokine (C-C motif) ligand 20 production by HEC-1A cells exposed to seminal plasma specimens from human immunodeficiency virus-seronegative- or human immunodeficiency virus-seropositive+ subjects as expressed by comparison to untreated cells [Chemokine (C-C motif) ligand 20 index]. Interleukin-1 beta (IL-1β) was used as positive control. [a]*P* < 0.05. SP: Seminal plasma; HIV: Human immunodeficiency virus-seronegative.

was measured in the supernatants of HEC-1A cells by using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Quantikine, R and D Systems, Abingdon, United Kingdom) as recommended by the manufacturer. Each assay was performed in duplicate. The results were expressed as relative CCL20 index corresponding to the ratio between the amount of CCL20 produced by the specimen and the negative control (culture medium only).

Viral load in seminal plasma

The HIV-Monitor kit (Roche Diagnostic Systems, Branchburg, NJ, United States) was used to quantify the HIV RNA in seminal plasma samples from HIV+ subjects. The detection lower limit was of 50 copies/mL. The RNA was extracted from the specimens using a modified silica protocol (QIAmp RNA viral kit; Qiagen, Chatsworth, CA, United States).

Measurement of lactoferrin in seminal plasma

Measurement of lactoferrin in seminal plasma specimens was performed by ELISA technique. A standard curve was prepared by using different concentrations of lactoferrin from human milk (Sigma-Aldrich). Seminal plasma samples (diluted 1:10 in PBS) were distributed into wells at the concentration of 100 μL per well and incubated at 37 ℃ for 1 h. Albumin from chicken egg whites (Sigma-Aldrich) was used for blocking. Lactoferrin was detected with rabbit anti-human lactoferrin antibodies (L3262; Sigma-Aldrich) incubated for 1 h at room temperature followed by peroxidase conjugated anti-rabbit antibodies (Sigma-Aldrich). After several washes, o-phenylenediamine was used as substrate and optical densities at 492 nm were measured. Each assay was performed in duplicate.

Measurement of total protein in seminal plasma

The Bradford technique was used for the measurement of the total protein content[11]. Seminal plasma was diluted 1:75 in PBS and distributed in triplicate in microplate wells under a volume of 150 μL per well in addition to the same volume of Bradford reagent (Sigma-Aldrich). Bovine serum albumin was used to perform the standard curve. The reading was performed by spectrophotometry at 590 nm.

Fractionation of seminal plasma by ion exchange chromatography

Specimens of seminal plasma from HIV- subjects were diluted 1:10 in 50 mmol/L NaCl pH 7.4 (buffer A) and applied onto a column of affinity (Hitrap Q FF, GE Healthcare Life sciences, Velizy-Villacoublay, France) equilibrated at room temperature with the same buffer. A discontinuous gradient was used for the elution of seminal plasma proteins by using six different concentrations (5%, 10%, 20%, 30%, 40%, 50%) of a buffer containing 500 mmol/L NaCl (buffer B). Buffer B was passed through the column at a flow rate of 0.5 mL/min using the HPLC AKTA purifier system (GE Healthcare Life sciences). The successive fractions were tested for their capability to induce the secretion of CCL20 by HEC-1A cells as well as for lactoferrin and total protein content as described above.

Statistical analysis

The data expressed in experimental units are presented as mean ± SD. Statistical analyses were performed using the GraphPad Prism software (San Diego, CA, United States). The Mann-Whitney test was used to compare two means. Correlations were analyzed using the Spearman's *r* test. *P* values < 0.05 were considered as statistically significant.

RESULTS

Seminal plasma promotes the induction of secretion of CCL20 by HEC-1A cells

The secretion of CCL20 was measured by ELISA in the supernatants of HEC-1A cells incubated for 17 h with culture medium DMEM-F12 only (negative control), IL-1β (25 ng/mL, positive control) or each of 34 seminal plasma specimens (22 from HIV+ subjects and 12 from HIV- subjects) diluted 1:10 in DMEM-F12. The CCL20 stimulation was expressed in number of times its production increased in comparison to untreated cells (CCL20 index). IL-1β used as positive control was responsible for an increase of 11.36 ± 3.36 times in the production of CCL20. The mean production of CCL20 by HEC-1A in presence of seminal plasma from HIV- and HIV+ subjects was increased by respectively 5.38 ± 0.91 and 7.57 ± 3.26 times with comparison to untreated cells (negative control). The difference between the two groups was statistically significant ($P < 0.05$ by Mann-Whitney test) (Figure 1).

CCL20 production by HEC-1A cells correlated with lactoferrin in seminal plasma

Using the same 34 specimens of seminal plasma (12

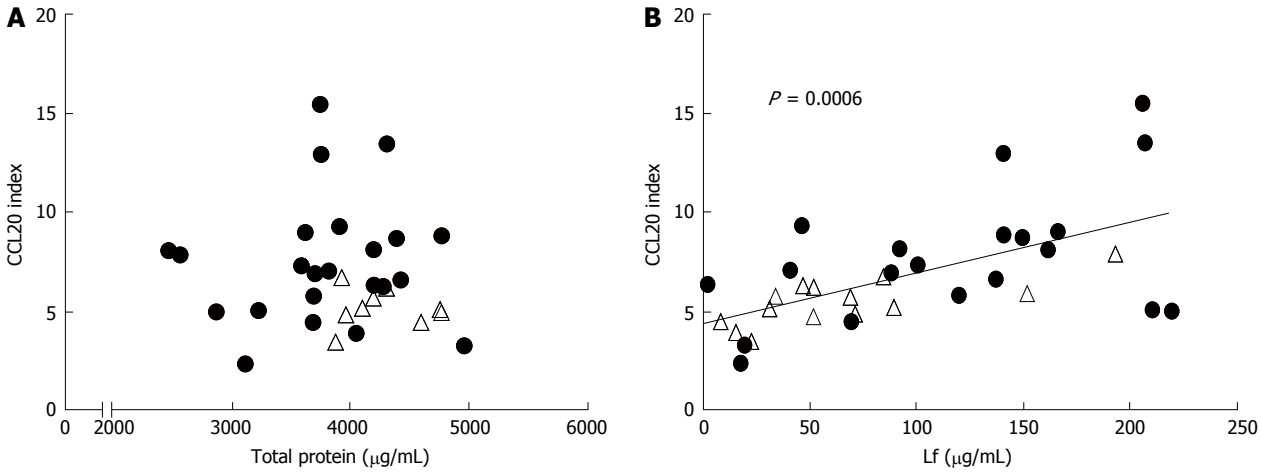

Figure 2 Correlation between total protein (A) or lactoferrin concentrations (B) and Chemokine (C-C motif) ligand 20 production by HEC-1A cells stimulated with seminal plasma. Seminal plasma from HIV-negative (open triangles) or HIV-positive subjects (closed circles). Lf: Lactoferrin; HIV: Human immunodeficiency virus-seronegative. CCL20: Chemokine (C-C motif) ligand 20.

from HIV- and 22 from HIV+ subjects), no correlation was observed between the concentration of total proteins in seminal plasma and their ability to stimulate the secretion of CCL20 by HEC-1 cells (Figure 2A). In contrast, the ability to produce CCL20 by HEC-1A cells positively correlated to the concentration of lactoferrin in the seminal plasma samples (r coefficient = 0.56; CI: 0.26-0.76; $P < 0.001$ by the Spearman's r test) (Figure 2B).

Seminal plasma fractions with the highest concentrations of lactoferrin were responsible for the greatest stimulation of CCL20 by HEC-1A cells

In order to verify whether lactoferrin present in seminal plasma was responsible for the production of CCL20 by HEC-1A cells, the proteins from a pool of seminal plasma specimens from 12 HIV- subjects were fractionated by ion exchange chromatography. Each fraction was then tested for its ability to stimulate the production of CCL20 and for its concentration of lactoferrin and total proteins (Figure 3).

As shown in Figure 3B, the amount of CCL20 produced by HEC-1A cells was closely related to the concentration of lactoferrin present in the plasma fraction ($r = 0.8942$, CI: 0.7773-0.9514, $P < 0.0001$ by the Spearman's r test). Fractions with the greatest concentration of lactoferrin (fractions 1, 3-5, 7-9, 10-13 in Figure 3B) corresponded to those exhibiting the highest capacity for inducing the production of CCL20 by HEC-1A cells.

Correlation between viral load in seminal plasma from HIV+ subjects and its ability to stimulate the production of CCL20 by HEC-1A cells

From the 22 seminal plasma specimens from subjects tested seropositive for HIV, the 5 samples exhibiting a detectable viral load (> 50 copies/mL) increased the production of CCL20 by a factor of 10.3 ± 4.2 times as compared to the negative control whereas the 17 samples with undetectable viral load (< 50 copies/mL) stimulated the production of CCL20 by a factor of 6.7 ± 2.6 times with reference to the negative control. A trend was observed between the 2 groups but the difference was not statistically significant due to the small size of effectives (Figure 4).

DISCUSSION

Heterosexual route is the most common way for HIV transmission resulting in a significant increase of HIV-infected women in recent years[12,13]. Women are more susceptible to HIV transmission, notably because of the large size of genital mucosa that is exposed to semen and also because semen contains higher concentrations of virus than vaginal fluid[14].

Seminal plasma confers a survival advantage to spermatozoids within the relative hostile environment of the female genital tract[15]. However, more recent studies have shown that seminal plasma is able to provide to vaginal mucosa a set of signaling molecules that are capable of interacting with epithelial cells of the female reproductive tract, these interactions triggering molecular and cellular changes that resemble an inflammatory response[16].

Signaling molecules present in seminal plasma may increase the secretion of chemokines and cause vascular changes that lead to the recruitment and activation of macrophages, granulocytes and DCs[17] including LCs[10]. LCs present in the vaginal mucosa are known as "Trojan horse" that facilitate the passage of HIV through the vaginal mucosa and present them to the CD4+ cells[18]. CCL20 is the main chemokine involved in the recruitment of LCs and its production by epithelial cells could be related to an increased risk of HIV infection. In this way, Li *et al*[19] demonstrated that the reduction of CCL20 secretion by epithelial cells treated with glycerol monolaurate, a vaginal microbicide, prevented the mucosal transmission of Simian Immunodeficiency Virus. These data are an additional argument for the determining role of CCL20 in the contamination process by HIV.

In this study, we found that seminal plasma was able

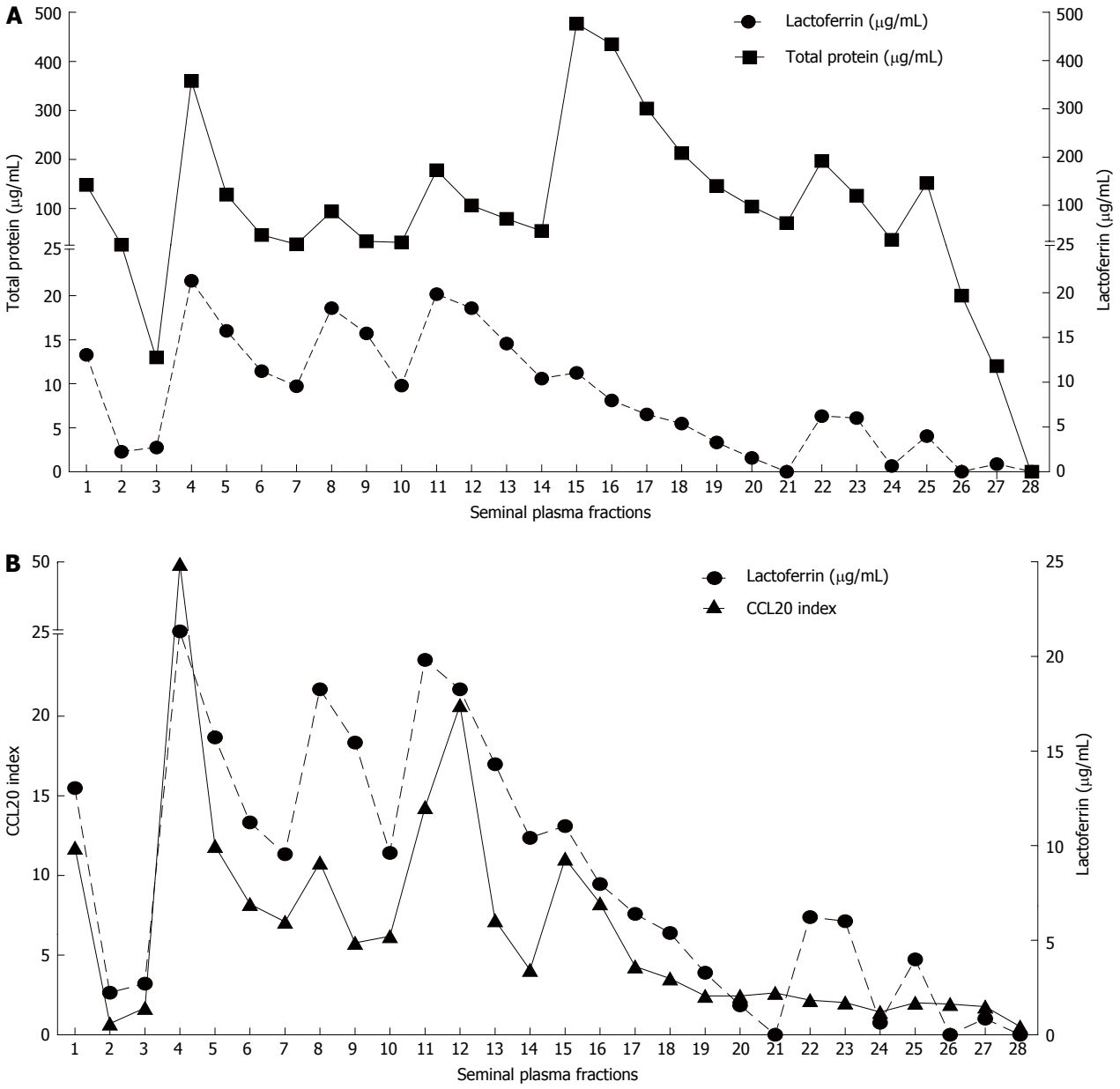

Figure 3 Fractionation by ion exchange chromatography of pooled seminal plasma specimens from 12 human immunodeficiency virus-seronegative subjects. A: Total protein (upper curve) and lactoferrin (lower curve) concentrations, expressed in μg/mL, in each fraction; B: Correlation between the CCL20 index after stimulation of HEC-1A cells by each fraction (closed squares) and its concentration in lactoferrin expressed in μg/mL (closed circles).

to stimulate the production of CCL20 by HEC-1A, with a statistically significant advantage for that originated from HIV+ patients as compared to HIV- subjects. These results confirm those previously published by our team[10] that showed a higher increase in the production of CCL20 by the SiHa cell line derived from vaginal epithelium when stimulated with seminal plasma from HIV+ subjects, however without statistical significance. Sharkey *et al*[17] also showed that human seminal plasma is capable of interacting with cervical and vaginal tissues for inducing the production of proinflammatory cytokines.

Cremel *et al*[7] demonstrated that vaginal epithelial cells increased the secretion of CCL20 in response to stimulation by proinflammatory cytokine IL-1β. The present study shows that seminal plasma from HIV+ and HIV- subjects produces similar effects on the cells lining the endocervical monostratified (HEC-1A), suggesting that seminal plasma contains components able to generate a response, even if not specific, in the female genital mucosa, mediated by CCL20 secretion.

One of the potential candidates that could stimulate CCL20 secretion by female genital epithelial cells is lactoferrin, a globular glycoprotein of the transferrin family with a molecular mass of 80 kDa and present in large amounts in various secretions. Lactoferrin is considered as an important element of nonspecific humoral immunity and was shown to exhibit a protective effect, particularly against HIV because of its interference with the viral gp 120 and Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin receptor

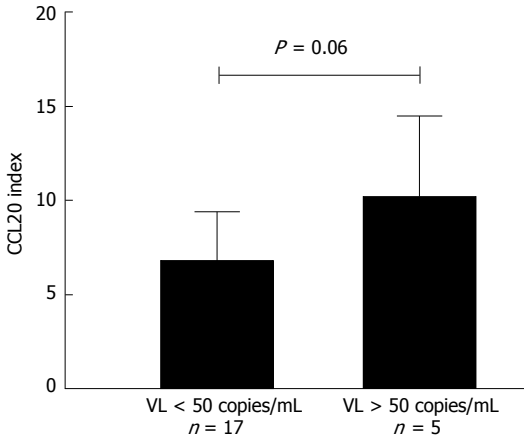

Figure 4 The Chemokine (C-C motif) ligand 20 production by HEC-1A cells treated with seminal plasma from human immunodeficiency virus-positive subjects with either detectable viral load > 50 copies/mL or undetectable viral load (< 50 copies/mL). CCL20: Chemokine (C-C motif) ligand 20; VL: Viral load.

receptor[20,21]. In contrast, other studies have reported that some peptides from the cleavage of this protein by elastase or proteinase type Ⅲ enzymes exhibit a strong pro-inflammatory activity in different mucosae[22,23]. In this study, we found a positive correlation between the concentration of lactoferrin in seminal plasma and the production of CCL20 stimulated by HEC-1A, even if it cannot be excluded that an additional factor could contribute to this activation. Another finding of our study that suggests the participation of lactoferrin or its cleavage products as activating factors of increased secretion of CCL20 by genital mucosal cells is the result obtained after chromatography fractionation performed on a pool of seminal plasma samples from HIV- subjects (the volume of seminal plasma was not enough to perform the same experiments with seminal plasma samples from HIV+ subjects). Indeed, the fractions that were the most efficient for CCL20 secretion stimulation were those containing the highest concentration of lactoferrin. The fact that the lactoferrin activity was distributed in discontinuous pattern along the chromatogram (Figure 3B) could be explained by the tetrameric conformation of the protein that can correspond to associations of different molecular masses, and also by the contribution of degradation products of lactoferrin after enzymatic digestion, which were shown to exhibit a strong pro-inflammatory effect[22,23].

Interestingly, as shown in Figure 4 for the subgroup of HIV+ subjects, the specimens exhibiting high viral loads were shown to stimulate more efficiently the production of CCL20 (although the difference did not reach statistical significance due to the small size of effectives); this finding is an additional evidence for the existence of a correlation between the HIV load of seminal fractions and their ability to promote CCL20 stimulation. Viral shedding in seminal plasma was recently shown to be closely related to the presence of high levels of pro-inflammatory cytokines, including granulocyte colony stimulating factor, tumor necrosis factor-alpha, interferon-gamma and IL-10[24]. In the light of the above discussion regarding lactoferrin, it can be hypothesized that the amount of pro-inflammatory components derived from this protein may be higher in HIV+ than in HIV- subjects, and notably in those with high seminal HIV load.

All these data argue in favor of a significant role of lactoferrin or its degradation products on CCL20 secretion by female genital mucosa. Despite the need of complementary studies for confirming these findings, our results are indicative of the role of some of these proteins in HIV transmission through the female epithelium tract and suggest that they must be taken into consideration for the prevention of HIV heterosexual contamination process. From a clinical point of view, it would be useful to identify the molecules implicated in this facilitation in order to develop intra-vaginal products capable of neutralizing their activity.

COMMENTS

Background

Sexual transmission of human immunodeficiency virus type 1 (HIV-1) accounts for 60% to 90% of new infections, especially in developing countries. During male-to-female transmission, in the absence of breaches in the genital mucosa, the epithelium crossing by HIV could occur through the recruitment of immune cells with migratory properties, such as macrophages, lymphocytes or Langerhans cells.

Research frontiers

The Chemokine (C-C motif) ligand 20 (CCL20) secretion by human vaginal epithelial cells has been shown to be enhanced in the presence of semen resulting in chemoattraction of Langerhans cells that are permissive to HIV infection, but the compound(s) involved in this stimulation is (are) not yet characterized.

Innovations and breakthroughs

In the present study, seminal plasma was shown to promote the induction of secretion of CCL20 by monolayers of endocervical epithelium cells (HEC-1A cell line). The effect was significantly higher with seminal plasma from HIV seropositive than HIV seropositive subjects. CCL20 production by HEC-1A cells correlated with the concentration of lactoferrin in seminal plasma. After fractionation of seminal plasma, those with the highest concentrations of lactoferrin were responsible for the greatest stimulation of CCL20 by HEC-1A cells. In conclusion, lactoferrin present in seminal plasma correlated with an increased production of CCL20 by HEC-1A cells and therefore could facilitate HIV entry through the genital mucosa.

Applications

Lactoferrin itself or, more likely, some of its degradation products could facilitate HIV entry through the recruitment of immune cells. It would be interesting to characterize precisely the molecules involved in this phenomenon in order to evaluate if they may constitute a target for antiviral protection.

Terminology

The CCL20 is an important immune effector molecule that is able to attract immature immune cells. Lactoferrin is a globular glycoprotein of the transferrin family that is present in large amounts in various secretions, including seminal plasma; it is considered as an important element of nonspecific humoral immunity.

Peer review

The current article described the seminal plasma/ lactoferrin affects the CCL20 production by HEC-1 cells. It is an interesting and important topic.

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MINIREVIEWS

Role of histo-blood group antigens in primate enteric calicivirus infections

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Abstract

Human noroviruses (NoV) are associated with large proportion of non-bacterial diarrhea outbreaks together with > 50% of food-associated diarrheas. The function of histo-blood group antigens (HBGAs) in pathogenesis of virus infection was implicated. Until recently however, due to lack of a robust animal and *in vitro* models of human NoV infection, only the partial knowledge concerning the virus pathogenesis (receptor, co-receptor and target cell) and absence of viable vaccine candidates were the frequently referenced attributes of this acute diarrheal illness. Recently, a novel group of enteric caliciviruses (CV) of rhesus macaque host origin was discovered and described. The new genus within the family Caliciviridae was identified: Rhesus Enteric CV, *i.e.*, "Recovirus" (ReCV). ReCVs are genetically and biologically close relatives of human NoVs, exhibit similar genetic and biological features and are capable of being propagated in cell culture. ReCVs cause symptomatic disease (diarrhea and fever) in experimentally inoculated macaques. Formulation and evaluation of efficient NoV vaccine might take several years. As suggested by recent studies, inhibition of HBGAs or HBGA-based antivirals could meanwhile be exploited as vaccine alternatives. The purpose of this minireview is to provide the guidance in respect to newly available primate model of enteric CV infection and its similarities with human NoV in utilizing the HBGAs as potential virus co-receptors to indirectly address the unresolved questions of NoV pathogenesis and immunity.

Key words: Calicivirus; Norovirus; Recovirus; Rhesus macaque; Macaca mulatta; Enteric infection

Core tip: To inform academic community and clinical practice, this short review summarizes existing hypothesis and evidence regarding the relationship between histo-blood group antigens and propensity of enteric caliciviruses to cause infection in primate species.

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INTRODUCTION

Identification of the ABO blood groups was pioneered in 1900's, independently, by Landsteiner[1] and Jansky[2]. In the 1970's, it was described that ABO antigens are associated with sugar moieties, specifically, with N-acetylgalactosamine (type A) and galactose (type B)[3]. Glycoproteins expressed on human red blood cells (RBC) represent A, B and H antigens with H functioning as the precursor for the A, B and O epitopes. It was demonstrated that histo-blood group antigens (HBGAs) including ABO and/or Lewis play a role in pathogenesis of certain infectious diseases including human norovirus (NoV) and *Helicobacter pylori*. The *ABO* genes are in humans positioned on chromosome 9[4,5]. According to molecular comparison of chromosome 9 with genomic DNA of other primate

species, analogous loci are present in rhesus macaque's (Macaca mulatta) chromosome 15[6]. Complex polymorphisms of human HBGA relationships concerning the secretor, *Lewis* and *ABO* gene families including their phenotypic (fucosyl- and glycosyl-transferase mediated) characteristics were extensively reviewed elsewhere[7]. Briefly, these genetic polymorphisms directly determine susceptibility or resistance to HBGA-recognizing pathogens including several groups of enteric caliciviruses (CVs).

Enteric CVs and human NoVs in particular are worldwide annually responsible for significant morbidity and mortality in young children[8]. In the Unites States alone, approximately 23 million cases of acute diarrhea are attributed to NoV infections each year[9,10]. No robust animal or cell culture model existed until recently to mimic the complex NoV genetics and pathogenesis including the host HBGA interactions with the virus. With discovery and characterization of human NoV's close relatives "rhesus enteric caliciviruses (ReCV)" the prototype of which is Tulane virus (TV), such studies became feasible[11-15]. As recent studies indicate that ReCVs can infect humans, likely by utilizing the primate HBGAs as co-receptors for virus entry[13,16], it is of interest to continue to elucidate the role of these molecules in enteric calicivirus infections.

CALICIVIRUS HOST-SPECIFIC RECEPTOR RECOGNITION PATTERNS

Recent *in vitro* experiments with synthetic glycoconjugates with relevance as CV receptors have revealed at least three distinct patterns of virus recognition[17]. While human NoV, ReCV/TV and bovine NoV all utilize HBGAs; other CVs such as feline CV and porcine sapovirus utilize sialic acid *via* N- and O-linked glycoproteins, respectively; and murine NoV uses sialic acid in a strain-dependent manner[17,18]. These distinct patterns of receptor recognition in different hosts have profound implications in virus pathogenesis, as for example porcine sapovirus-compatible O-linked glycoproteins are expressed alongside enteric goblet cells as well as in organs including liver, heart and cerebrum[17].

RELATIONSHIP BETWEEN PRIMATE HBGAs AND ENTERIC CVs

The ABO and Lewis blood groups were described in human and non-human primates (NHP). The specific antigens of these blood groups have been implicated in human NoV and ReCV infections as the virus attachment factors[13,19-21]. First study that suggested such interactions was based on results generated with anti-A/B antibody-mediated hemagglutination inhibition assays[22]. The anti-A and anti-B antibodies are also linked with transplantation immunity. These antibodies are triggered during the early life by exposure to environmental antigenic stimuli including those induced by common viruses. Hence, immunity induced by these stimuli is referred to as "communal immunity". The RBCs of 13 different species were tested for their capacity to bind with NoV antigens but only the human and chimpanzee cells showed reactivity[22]. This is consistent with notion that only humans and anthropoid apes but not other primate species express ABH antigens on their RBCs[23,24]. Non-anthropoid primates including rhesus monkeys secrete HBGAs into mucosal fluids[24,25]. Such an inherent difference between the rhesus and homo species is thought to be due to evolutionary pressures that asserted themselves during last 5 millions years to alleles encoding the blood groups[5]. From these and other studies, evidence suggests that *ABO* gene polymorphism in primates was generated more through the process of convergent evolution[26]. Notwithstanding, there still are shared features between the human and NHP blood group antigens including the capability of human HBGAs to recognize ReCVs and capability of rhesus HBGAs to recognize human NoVs[13]. Saliva analysis can be used to determine the specificity of ABO antigens in rhesus macaques and other monkey species.

CONSIDERATIONS FOR PRE-CLINICAL STUDIES WITH PATHOGENS THAT UTILIZE HBGAs

All primates including humans, apes and monkeys are secreting HBGAs into mucosal fluids-depending on their secretor phenotype. Since biomedical research with anthropoid apes is due to understandable ethical constraints severely restricted, bulk of the pre-clinical research is currently conducted with other animal models, from which the best available human-like alternative is rhesus macaque (Macaca mulatta). Knowledge about the individual animal HBGAs is therefore required. An assumption that HBGA profile of randomly selected group of research macaques will reflect the free-ranging population is inaccurate due to selective importation and breeding in captivity of animals from different parts of the world. As most of the captive research rhesus macaques in the United States are of Indian origin, they predominantly belong to HBGA type B, oppose to free-ranging macaques from South-East Asia and China that possess more polymorphic distribution of their HBGAs with significant proportions of type A, B, AB and O[13,24].

HBGA phenotyping of 500 rhesus monkeys of the Tulane colony revealed majority of animals being type B. Although this result is consistent with some historical studies conducted in 1970's[27], results conducted with free-ranging macaques in Thailand showed more polymorphic distribution[24]. Unpublished results conducted by our group at Tulane indicate differences between the Indian *vs* Chinese origin rhesus macaques: The Chinese macaques appear to have more human-like distribution (14% type A, 65% B, 11% O and 10% AB) than Indian macaques (97%

type B) (Farkas T personal communication).

An important distinction between the rhesus and human enteric caliciviruses is the capability of rhesus caliciviruses to be propagated *in vitro*[10]. A hypothesis that both rhesus and human enteric caliciviruses utilize HBGAs as the *in vivo* cell entry receptors/co-receptors could therefore be addressed with HBGA-defined, experimentally challenged macaques. Consideration would have to be given to a pre-screening stage of experiment when animals, free of virus-specific antibodies and defined in respect to their ABO and Lewis blood groups, are identified from the larger pool of candidates.

ARE HBGAs PRIMARY OR SECONDARY DETERMINANTS OF ENTERIC CV INFECTION IN PRIMATES?

It was proposed that human NoVs bind with carbohydrate moieties of the ABH and Lewis antigens when these are secreted into biological fluids and that such binding is associated with productive infection and illness[27-29]. In fact, inhibition of HBGA binding was suggested as an antiviral strategy for treatment of NoV infection[30]. Secretor or non-secretor phenotype depends on complex polymorphisms of *ABO, FUT2* and *FUT3* loci[7,21]. Few recent studies however, demonstrated that not only secretors but also non-secretors might get infected, suggesting that no strong correlation exists between the NoV infections and HBGA specificities of their hosts[31,32]. Another study with NoV virus-like particles also suggested that binding of NoV antigens to intestinal epithelial cells takes place regardless of HBGA expression on the surface of these cells[33]. An explanation that not all of these studies did take into account the HBGA binding properties of NoVs involved[21] seems unsatisfactory, not fully addressing the controversy. In order to corroborate the link between susceptibility to enteric calicivirus infection and its host ABO/Lewis phenotype, a challenge experiment with a well-defined NHP surrogate of NoV infection might need to be considered.

As enteric calicivirus infections were demonstrated in ABO type B (secretor) macaques of the Tulane colony regardless of their Lewis antigen characteristics, studies with type A and O macaques could provide further clues regarding the susceptibility or resistance of a particular phenotype to infection. In addition, retrospective HBGA analysis of recently reported Bangladeshi patients, infected with rhesus enteric calicivirus (TV strain), should also be informative[16].

Latest structural analysis of GI human NoVs revealed critical extension of the P domain loop region that appears to be responsible for binding of the GI.7 NoV with non-secretor HBGAs[34]. The "extended P domain loop" is not present on other GI NoVs that are known to bind with secretor HBGAs. These findings are for the first time directly addressing the controversies in respect to relationship between human NoV infectivity and its host HBGA properties. Nevertheless, these remarkable structural analysis data will need to be corroborated by epidemiological investigations and experiments with illness-prone models of human NoV infection. Because of its human-like characteristics, ReCV/TV model might be the one suitable for such purpose.

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AIM AND SCOPE

World Journal of Virology (*World J Virol*, *WJV*, online ISSN 2220-3249, DOI: 10.5501) is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

WJV covers topics concerning arboviral infections, bronchiolitis, central nervous system viral diseases, DNA virus infections, encephalitis, eye infections, fatigue syndrome, hepatitis, meningitis, opportunistic infections, pneumonia, RNA virus infections, sexually transmitted diseases, skin diseases, slow virus diseases, tumor virus infections, viremia, zoonoses, and virology-related traditional medicine, and integrated Chinese and Western medicine. Priority publication will be given to articles concerning diagnosis and treatment of viral diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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ORIGINAL ARTICLE

Nuclear factor κB represses the expression of latent membrane protein 1 in Epstein-Barr virus transformed cells

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Abstract

AIM: To investigate the role of nuclear factor κB (NF-κB) in the regulation of Epstein-Barr virus (EBV) latent membrane protein 1 (LMP1) in EBV transformed cells.

METHODS: LMP1 expression was examined in EBV transformed human B lymphocytes with modulation of NF-κB activity.

RESULTS: EBV infection is associated with several human cancers. EBV LMP1 is required for efficient transformation of adult primary B cells *in vitro*, and is expressed in several pathogenic stages of EBV-associated cancers. Regulation of EBV LMP1 involves both viral and cellular factors. LMP1 activates NF-κB signaling pathway that is a part of the EBV transformation program. However, the relation between NF-κB and LMP1 expression is not well established yet. In this report, we found that blocking the NF-κB activity by Inhibitor of κB stimulated LMP1 expression, while the overexpression of NF-κB repressed LMP1 expression in EBV-transformed IB4 cells. In addition, LMP1 repressed its own promoter activities in reporter assays, and the repression was associated with the activation of NF-κB. Moreover, NF-κB alone is sufficient to repress LMP1 promoter activities.

CONCLUSION: Our data suggest LMP1 may repress its own expression through NF-κB in EBV transformed cells and shed a light on LMP1 regulation during EBV transformation.

Key words: Nuclear factor κB; Epstein-Barr virus; Latent membrane protein 1; Latency; Transformation

Core tip: We find a classical feedback inhibition of Epstein Barr virus (EBV) Latent membrane protein 1 (LMP1) and nuclear factor κB (NF-κB): LMP1 activates NF-κB, and NF-κB inhibits LMP1 expression. The regulatory loop may benefit EBV transformation processes.

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INTRODUCTION

Epstein-Barr virus (EBV) is a human-herpesvirus that infects most humans without causing an obvious disease. However, EBV is associated with nasopharyngeal carcinoma, Hodgkin's lymphoma, Burkitt's lymphoma, post-transplantation lymphoproliferative diseases, and central nervous system lymphoma in certain healthy and

immune-compromised hosts[1-3].

EBV transforms adult primary B cells into continually growing lymphoblastoid cell lines (LCLs) and concomitantly establishes type Ⅲ latency *in vitro*[1]. Nine viral proteins are expressed, including six nuclear proteins (EBNA1, -2, -3A, -3B, -3C, and -LP) and three integral latent membrane proteins (LMP1, -2A, and -2B) plus EBV-encoded RNAs[3,4].

EBV LMP1 is an integral membrane protein and acts as a constitutively active, receptor-like molecule[5]. LMP1 is required for the efficient transformation of primary B cells *in vitro*[6-8]. Also, LMP1 is able to transform rodent fibroblasts[9-11]. In addition, LMP1 seems to be a central effector of altered cell growth, survival, adhesive, invasive, and antiviral potential in EBV transformed cells[12-18].

The nuclear factor κB (NF-κB) molecule plays a pivotal role in regulating a variety of biological processes, such as immunity, cell survival, and proliferation[19,20]. The NF-κB pathway can be regulated by many stimuli, and its activity is tightly controlled to ensure a transient response to infection or other stimuli. NF-κB transcription factors are homodimers or heterodimers of REL homology domain proteins p50, p52, RelA, RelB, or cREL[19,20]. LMP1 activates both canonical and noncanonical NF-κB pathways by the use of cellular signaling proteins, such as tumor necrosis factor-receptor-associated factors[21]. The functional importance of NF-κB is exemplified by the fact that the blockade of NF-κB triggers apoptosis of EBV-transformed lymphoblastoid cells[22].

Previously, there are several conflicting reports about the relation between NF-κB and LMP1[23-25]. During our study on NF-κB and LMP1, we found that NF-κB is likely to be the negative regulator for the LMP1 expression in EBV-transformed cells. LMP1 may negatively regulate its own expression through NF-κB, which is a classical feedback loop. Our results may provide an insight in NF-κB's role in viral transformation and add the complexity of viral gene regulation and their relation to transformation.

MATERIALS AND METHODS

Plasmids, antibodies, and reporter assays

Expression plasmids of LMP1 and its signaling defective mutant, LMP-DM, were described previously[26]. The NF-κB expression plasmids (p65 and p50), and NF-κB reporter construct with two consensus recognition sites were gifts from Albert Baldwin. pCMV-beta-gal and renilla luciferase expression plasmids were purchased from Clontech. CD4 expression plasmid was provided by Dr. Jenney Ting. LMP1 promoter reporter construct, LMP-ISRE-luc and LMP-GAS-luc were described previously[27]. LMP1 (CS1-4) antibody was purchased from Dako. Glyceraldehyde-3-phosphate dehydrogenase (0411) and inhibitor of κB (IκB) (SC-371) antibodies were purchased from Santa Cruz Biotechnology. Tubulin antibody was purchased from Sigma (T6557). The luciferase and beta-galactosidase assays were performed by standard methods[27,28].

Cell culture, transfection, and inducible expression of IκBα

The IB4 cell line was an EBV-transformed B cell line with type Ⅲ latency and were maintained in RPMI 1640 plus 10% fetal bovine serum (FBS). 293T cells were human fibroblast cell line. The cells were maintained in Dulbecco's Modified Eagle's Medium plus 10% FBS. The inducible IκB expression IB4 line was the gift from Cahir-McFarland *et al*[22] and were maintained in RPMI160 + 10% FBS plus 1 μg/mL tetracycline. For the induction of IκBα, cells were washed three times with RPMI without tetracycline and suspended in the media with or without tetracycline at a concentration of 10^5 cells per milliliter as described[22]. Cells were analyzed within 24 h after the initial inductions. The Electroporation (320 V; 925 μF) was used for the transfection of IB4 cells as described previously[11,29-31]. For transfection of 293T cells, the attractene transfection reagent (Qiagen) was used following manufacturers recommendations.

Isolation of CD4 positive cells

Enrichment for CD-4-positive cells was performed with the use of anti-CD-4-antibody conjugated to magnetic beads according to the manufacturer's recommendation (Dynal, Inc.). IB4 cells were transfected with CD-4 expression and other plasmids. One day after the transfection, the cells were used for isolation of CD-4-positive cells with the use of Dynabeads CD4 (Dynal, Inc.) The transfected cells were incubated with Dynabeads CD4 at 72 μL of beads/10^7 cells for 15-30 min at room temperature with gentle rotation. CD4-positive cells were isolated by placing the test tube in a magnetic separation device (Dynal magnet). The supernatant were discarded while the CD4-positive cells were attached to the beads. The CD4-cells-attached beads were washed 3 times in phosphate-buffered saline plus 2% FBS. The isolated cells were used to prepare cell lysates immediately. Total time for isolation was approximately 30-40 min. No tetracycline was used in the process.

Western blot analysis with enhanced chemiluminescence

Separation of proteins on sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out following standard protocol. After the proteins were transferred to a nitrocellulose or Immobilon membrane, the membrane was blocked with 5% nonfat dry milk in TBST (50 mmol/L Tris-HCl pH 7.5, 200 mmol/L NaCl, 0.05% Tween-20) at room temperature for 10 min. It was then washed briefly with TBST, and incubated with the primary antibody in 5% milk in TBST for 1 h at room temperature, or overnight at 4 ℃. After washing with TBST three times (10 min each), the membrane was incubated with the secondary antibody at room temperature for 1 h. It was then washed three times with TBST, treated with

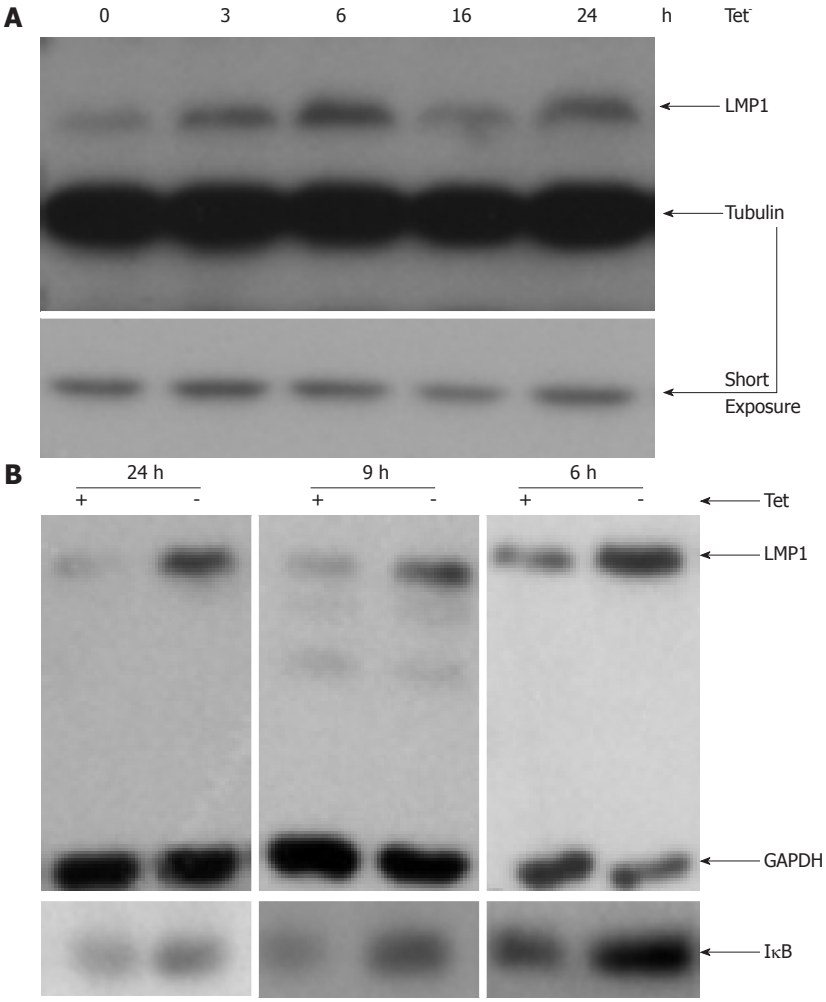

Figure 1 Blockage of nuclear factor-κB increases the expression of latent membrane protein 1 in Epstein-Barr virus-transformed cells. Inducible IκB-expression IB4 line were washed three times with fresh RPMI1640 medium, and re-suspended in tetracycline (Tet) plus or minus medium. Cells were isolated at indicated time, and used for Western blot analysis with indicated antibodies. The expression of latent membrane protein 1 (LMP1) was shown in (A) and the expression of IκB was shown in (B). GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

enhanced chemiluminescence detection reagents, and exposed to Kodak XAR-5 film.

Statistical analysis

Student *t* tests between groups of data were carried out with the use of Excell 2013. The statistically significant differences between the indicated samples were assessed by *P* values.

RESULTS

Inhibition of NF-κB increased the expression of LMP1

To determine the role of NF-κB in LMP1 expression in EBV-transformed cells, we used an EBV-transformed LCL (IB4 cell line) in which expression of a degradation-resistant mutant IκBa that was regulated by tetracycline (Tet)[22]. We found that LMP1 was induced upon Tet removal (Figure 1). Moreover, the potential correlation between the induction of IκB and the expression of LMP1 was examined. Within six hours after culture in media lacking tetracycline (Tet-media), IκBa was induced to stable levels (Figure 1; Panel B), however at three hours post removal of Tet, the IκBa induction was not consistently detected (data not shown). Increase in IκBa expression was also detected during 6-24 h after induction. The endogenous LMP1 was associated with Tet-removal and IκBα inductions (Figure 1). Therefore, LMP1 is increased upon IκBa induction in IB4 cells.

Overexpression of NF-κB decreased the expression of LMP1

Next, we tested whether the activation of NF-κB itself would affect the expression of endogenous LMP1 in EBV transformed cells. NF-κB can be activated by many stimuli, however, the specificity of the treatment might vary significantly. Therefore, we chose to use the ectopic expression of NF-κB, or p65 and p50 simultaneous, in IB4 cells. The reason to choose IB4 as it is a parental line for the inducible IκB line. IB4 cells were transfected with the expression plasmids p65 and p50 at 1:1 ratio, and the transfected cells were enriched by CD4 selection (see "Materials and Methods" for detail). No tetracycline was

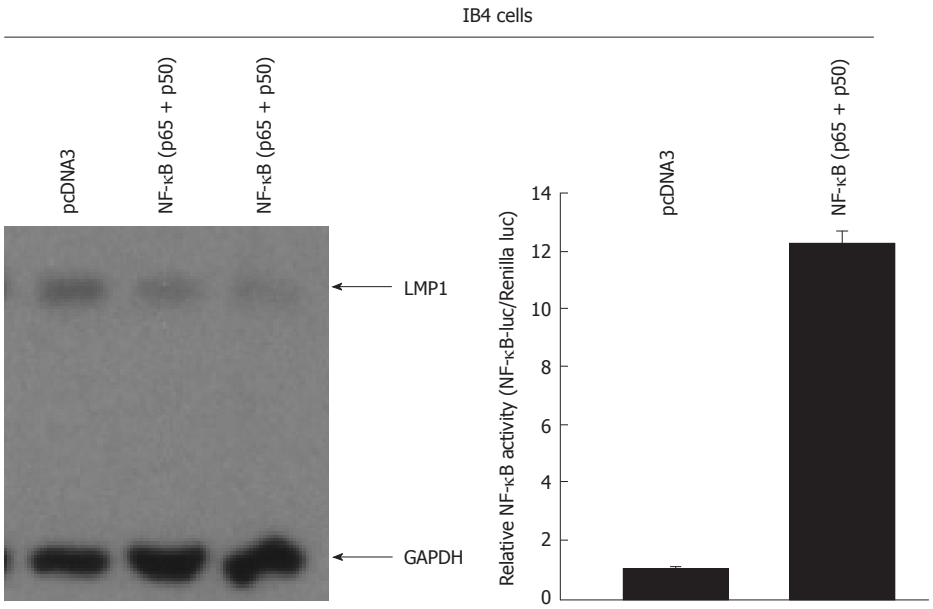

Figure 2 Overexpression of nuclear factor-κB decreases the expression of latent membrane protein 1 in Epstein-Barr virus-transformed cells. IB4 cells were transfected with CD4 expression plasmid along with pcDNA3 (vector) or nuclear factor κB (NF-κB) expression plasmids (p50 and p65 expression plasmids at 1:1 ratio). One day after the transfection, the transfected cells were enriched using the CD4 magnetic beads, and the cell lysates were used for Western blot analysis. The identity of the proteins is as shown. The right panel: IB4 cells were transfected with the indicated plasmid as shown at the top along with NF-κB specific reporter construct and Renilla luciferase reporter plasmid. One day later, luciferase and Renilla luciferase activities were measured. Relative promoter reporter activities are shown. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; LMP1: Latent membrane protein-1.

involved as plain IB4 cells are used. Western blot analyses were used for detection of the expression of LMP1 in enriched transfected cells. The NF-κB activity was increased and LMP1 was reduced in the NF-κB-transfected IB4 cells (Figure 2). Therefore, the endogenous LMP1 is reduced upon NF-κB activation in EBV-transformed cells.

LMP1 represses its own promoter activity

It is well established that there is one functional NF-κB recognition site in LMP1 promoter and NF-κB binds to the site in the LMP1 promoter[23-25]. In addition, LMP1 activates NF-κB pathway through at least two independent domains[32]. We examined if LMP1 could repress the LMP1 promoter reporter constructs (Figure 3A). LMP-DM is an expression plasmid that has mutations in two functional domains of LMP1 for NF-κB activation[26]. The promoter reporter constructs and LMP1 or LMP-DM expression plasmid were co-transfected into 293T cells and the reporter activities were measured. As shown in Figure 3B, LMP1 was able to repress the LMP1 promoter reporter constructs. However, LMP-DM failed to repress the same reporter constructs, which correlated with that data that LMP-DM failed to activate NF-κB pathway (Figure 3C). Therefore, LMP1 represses its own promoter reporter constructs.

NF-κB represses LMP1 promoter reporter construct

Next, we tested whether the activation of NF-κB alone would affect activities of the LMP1 promoter reporters. The promoter reporter construct and NF-κB (p65 + p50) expression plasmids were co-transfected into 293T cells and the reporter activities were measured. As shown in Figure 4, NF-κB activation alone was able to repress the LMP1 promoter reporter construct. The NF-κB-specific reporter construct was activated by the co-transfection of p65 and p50 expression plasmids, suggesting the NF-κB was functional (Figure 4B).

DISCUSSION

Both viral, such as EBNA2 and EBNA-LP, and cellular factors, such as IRF7, RBP-jκ, PU.1, and STAT are involved in the regulation of LMP1 in various EBV latencies and transformation processes[33-36]. LMP1 needs to be tightly regulated during viral transformation processes because LMP1 itself is a perplex protein with multiple functions, such as proliferative and anti-proliferative activities. The higher amounts of LMP1 may convert itself from a proliferative function to an anti-proliferative one[37].

Activation of NF-κB and the consequence of the action during viral transformation have been established clearly. NF-κB is required for the maintaining of the growth phenotypes of the transformed cells, and NF-κB seems to be responsible for most of the cellular changes during the transformation[38]. However, the relation between the NF-κB and LMP1 expression is somewhat unclear. While it is clear that there is a NF-κB recognition site in LMP1 promoter and NF-κB is able to physically bind to the site, the exact function of NF-κB on LMP1 expression is in debate[23-25]. During our research on LMP1 and other cellular factor interactions, we find that: (1) inhibition of NF-κB enhances the endogenous LMP1

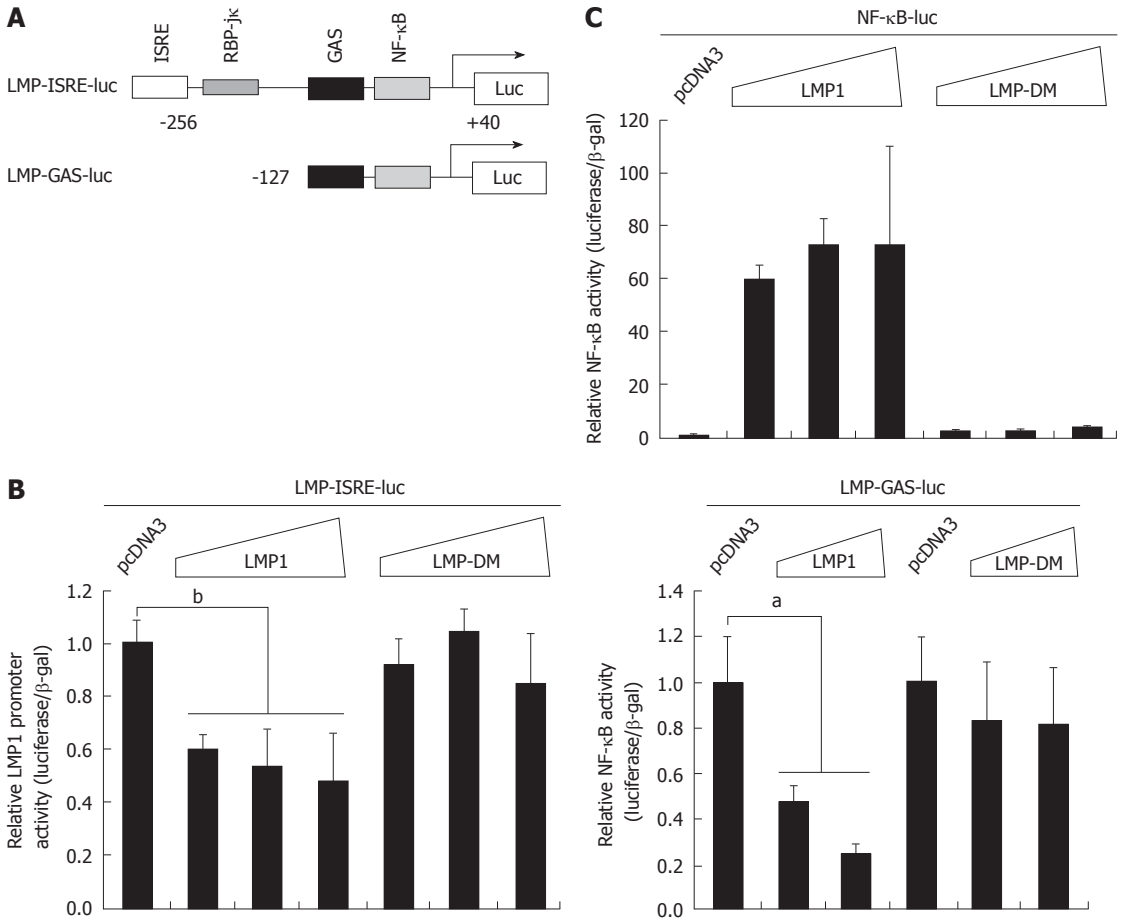

Figure 3 Latent membrane protein 1 negatively regulates its own promoter activity. A: Schematic diagram of Epstein-Barr virus (EBV) latent membrane protein-1 (LMP1) promoter reporter constructs. RNA start site is shown. The drawing is not to scale; B: 293T cells were transfected with LMP1 promoter reporter construct and expression plasmid (0.01, 0.05, and 0.1 μg) as shown at the top. The LMP-DM has mutations in the critical domains in LMP1 for signaling. Cell lysates were used for the luciferase and β-galactosidase assays. Relative promoter reporter activities (luciferase/β-galactosidase) are shown. The results represented an average of triplicate transfections; Standard error bars are shown. Results from one representative experiment are of shown. The statistically significant difference between the indicated samples is denoted as $^{a}P < 0.05$; $^{b}P < 0.01$; C: 293T cells were transfected with the indicated plasmid as shown at the top. Nuclear factor κB (NF-κB) specific reporter construct was used. One day later, luciferase and β-galactosidase activities were measured. Relative promoter reporter activities are shown.

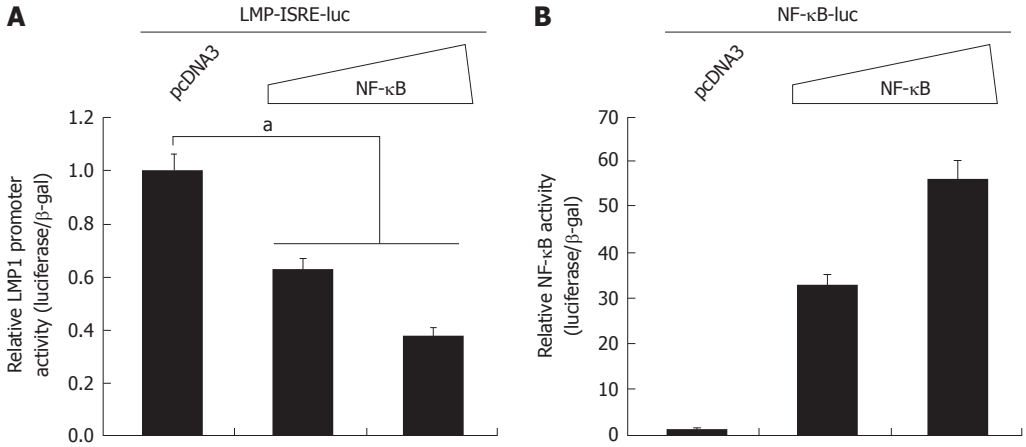

Figure 4 Nuclear factor κB represses latent membrane protein 1 promoter activity. A: 293T cells were transfected with latent membrane protein-1 (LMP1) promoter reporter construct and nuclear factor κB (NF-κB) expression plasmid (0.05 and 0.1 μg; p65 and p50 at 1:1 ratio) as shown at the top. Cell lysates were used for the luciferase and β-galactosidase assays one day later. Relative promoter reporter activities (luciferase/β-galactosidase) are shown. Standard error bars are shown. The statistically significant difference between the indicated samples is denoted as $^{a}P < 0.05$; B: 293T cells were transfected with the indicated plasmid as shown at the top. NF-κB specific reporter construct was used. One day later, luciferase and β-galactosidase activities were measured. Relative promoter reporter activities are shown.

expression, and activation of NF-κB leads to reduced expression of endogenous LMP1 in EBV transformed cells (Figures 1 and 2); (2) LMP1 repressed the LMP1-promoter reporter construct activities, and the repression was correlated with NF-κB activities (Figure 3); and finally (3) NF-κB itself repressed the LMP1-promoter activities (Figure 4). From all the results, it seems that NF-κB is a negative regulator of LMP1 in type Ⅲ latency or EBV transformed cells. Interestingly, when we used NF-κB inhibiting drug (BAY11-7082) for the LCL and found that the effects on LMP1 expression were not obvious (unpublished observations). We reasoned the side effects of chemical NF-κB inhibitor might influence the end results. Because our experimental approaches were based "solely" on NF-κB activation/inactivation, the results about NF-κB activation and LMP1 expression might be more reliable than a chemical activator and inhibitor of the NF-κB. Moreover, IB4 is a cell line transformed by EBV *in vitro* and has been used extensively in EBV research[22,39-43]. The results based on the IB4 cells might be more comparable to others researches. Of note that induction of IκB in terms of time course and LMP1 expression seems to be slightly different from the previous report[22]. We think the discrepancies might be due to the growth condition for the cells in various laboratories and passages numbers of the cells might be slightly different. Interestingly, we have found that the detection of the IκB induction by IκB antibody was more sensitive than the FLAG antibody (data not shown).

As we mentioned above, there are several conflicting reports about LMP1 and NF-κB[23-25]. It is hard to reconcile with all the conflicting reports. We think the endogenous LMP1 levels, genetic differences in cell line used, the presence of other viral factors, type of assays, and promoter construct differences may all collectively caused the two quite different conclusions. We have used lines with high endogenous or ectopic LMP1 expression, therefore, the results may be most suitably extrapolated to type Ⅲ latency or EBV transformed cells *in vitro*. Because high LMP1 is detrimental for growth, the observed negative effects might make sense in the LMP1-high situations. Furthermore, NF-κB might have dual roles in various backgrounds. Our results are in line with one previous report[24].

Because the response activated by NF-κB is so potent, tight regulation of the NF-κB activity is needed. There are many mechanisms for NF-κB signaling to be terminated to prevent potential tissue pathology due to prolonged expression of inflammatory mediators[44]. Fortunately, many of the NF-κB target genes encode inhibitors of the signaling pathways, which allow the inflamed tissues to reset to normal function once the danger has passed[44]. LMP1-mediated NF-κB activation seems to be the major mediator affecting cell expression programs in EBV-infected cells. The negative regulation of LMP1 expression by NF-κB would offer EBV a feedback inhibition to fine adjust NF-κB activity.

As NF-κB, LMP1 molecule *per se* is also need to be tightly regulated as it has both pro- and anti-proliferative effects[37]. The negative roles of NF-κB in regulation of LMP1 may offer EBV a feedback inhibition for its own LMP1 expression. The feedback loop between NF-κB and LMP1 might be important for the control of NF-κB as well as LMP1 activities, and eventually EBV transformation as a whole.

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COMMENTS

Background

Epstein-Barr virus (EBV) is a human herpesvirus with increasing medical significances. EBV is associated with many cancers and able to transform adult primary B cells into continually growing lymphoblastoid cell lines. EBV Latent membrane protein-1 (LMP1) is an integral membrane protein and acts as a constitutively active, receptor-like molecule. LMP1 is required for the efficient transformation of human primary B cells *in vitro* and possibly *in vivo*. In addition, LMP1 seems to be a central effector of altered cell growth, survival, adhesive, invasive, and antiviral potential in EBV transformed cells. The nuclear factor κB (NF-κB) molecule plays a pivotal role in regulating a variety of biological processes, such as immunity, cell survival, and proliferation. The NF-κB pathway can be regulated by many stimuli, and its activity is tightly controlled to ensure a transient response to infection or other stimuli. The functional importance of NF-κB is exemplified by the fact that the blockade of NF-κB triggers apoptosis of EBV-transformed cells.

Research frontiers

The relation between EBV and NF-κB, especially LMP1 and NF-κB, has been extensively studied. Other than the involvement in the EBV transformation, it is apparent that NF-κB regulates LMP1 expression *via* a well conserved binding site in LMP1 promoter region, however how the end results of the regulation is confusing. There are several conflicting reports about the relation between NF-κB and LMP1.

Innovations and breakthroughs

During the authors study on NF-κB and LMP1, The authors found that NF-κB is likely to be the negative regulator for the LMP1 expression at least in EBV-transformed cells. LMP1 may negatively regulate its own expression through NF-κB, which is a classical feedback loop.

Applications

The authors results provide an insight in NF-κB's role in viral transformation and add the complexity of viral gene regulation and their relation to transformation. This will be useful to determine the therapeutic potential and benefit of drugs that targeting NF-κB and/or LMP1 in EBV-associated cancers.

Terminology

Both EBV LMP1 and NF-κB are proteins involved in the process called viral transformation in which normal cells are converted into cancerous ones by a virus. Such a mechanism is crucial in viral pathogenesis. Non-surprisingly, EBV LMP1 and NF-κB has a classical regulatory loop.

Peer review

The study design and paper writing are OK. The methods provided in this study are correct.

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ORIGINAL ARTICLE

Substrate specificity of avian influenza H5N1 neuraminidase

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Abstract

AIM: To characterise neuraminidase (NA) substrate specificity of avian influenza H5N1 strains from humans and birds comparing to seasonal influenza virus.

METHODS: Avian influenza H5N1 strains from humans and birds were recruited for characterising their NA substrate specificity by using a modified commercial fluorescence Amplex Red assay. This method can identify the preference of α2,6-linked sialic acid or α2,3-linked sialic acid. Moreover, to avoid the bias of input virus, reverse genetic virus using NA gene from human isolated H5N1 were generated and used to compare with the seasonal influenza virus. Lastly, the substrate specificity profile was further confirmed by high-performance liquid chromatography (HPLC) analysis of the enzymatic product.

RESULTS: The H5N1 NA showed higher activity on α2,3-linked sialic acid than α2,6-linked ($P < 0.0001$). To compare the NA activity between the H5N1 and seasonal influenza viruses, reverse genetic viruses carrying the NA of H5N1 viruses and NA from a seasonal H3N2 virus was generated. In these reverse genetic viruses, the NA activity of the H5N1 showed markedly higher activity against α2,3-linked sialic acid than that of the H3N2 virus, whereas the activities on α2,6-linkage were comparable. Interestingly, NA from an H5N1 human isolate that was previously shown to have heamagglutinin (HA) with dual specificity showed reduced activity on α2,3-linkage. To confirm the substrate specificity profile, HPLC analytic of enzymatic product was performed. Similar to Amplex red assay, H5N1 virus showed abundant preference on α2,3-linked sialic acid.

CONCLUSION: H5N1 virus maintains the avian specific NA and NA changes may be needed to accompany changes in HA receptor preference for the viral adaptation to humans.

Key words: H5N1 avian influenza virus; Neuraminidase; Sialic acid; Adaptation; Substrate preference

Core tip: We analyzed neuraminidase (NA) substrate specificity of avian influenza H5N1 strains from humans and birds using a modified fluorescence assay, and the substrate specificity profile was further confirmed by

high-performance liquid chromatography analysis of the enzymatic product. The H5N1 NA showed higher activity on α2,3-linkage. Interestingly, NA from an H5N1 human isolate that was previously shown to have heamagglutinin (HA) with dual specificity showed reduced activity on α2,3-linkage. These suggest that the H5N1 virus maintains the avian specific NA activity and that changes in the NA may be needed to compensate for changes in the HA specificity for the viral adaptation to human hosts.

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INTRODUCTION

Neuraminidase (NA) is a tetrameric type Ⅱ transmembrane glycoprotein on the envelope of influenza virus. NA molecule consists of three domains: globular head, stalk and transmembrane domains[1-3]. The function of NA is to cleave terminally bound sialic acid on carbohydrate chains of glycans on cell surface and viral envelope in order to release newly budded virions from host cells[2]. If the function of NA is impaired, sialyl residues on the surface of virus particles and infected cells will be bound by heamagglutinin (HA), which leads to virus aggregation at the cell surface preventing the dissemination of infection[4,5].

HA of avian and human influenza viruses bind preferentially to α2,3- and α2,6-linked sialic acid, respectively. This difference is believed to play an important role in the interspecies barrier of influenza transmission between avian species and human. A change in the receptor preference is required for emergence of a new pandemic strain from avian influenza viruses[6]. HA and NA counteract each other, and their activities need to be balanced for the efficient viral replication and respiratory- droplet transmission[4,7]. NA activities on α2,3- and α2,6-linked sialic acid have been previously characterized for some avian and seasonal influenza viruses[8-10]. NA of N2 subtype from human and avian influenza viruses had been studied for substrate specificity[8,9]. Avian and early human isolated N2 showed much more activity on α2,3-linked sialic acid than α2,6-linked. However, late human N2 isolation trended toward increase substrate specificity for α2,6-linked while maintaining the α2,3-linked activity. The N1 substrate specificity had also been studied[10]. Similar to N2 activity, N1 isolated from avian hosts showed much higher activity on α2,3- than α2,6-linked substrate, while human viruses showed reduced activity to α2,3- and increased activity to α2,6-linked sialic acid. From these finding we can conclude that human isolated NA shows the increased substrate specificity on α2,6-linked,which is found in human respiratory tract, while maintaining specificity on α2,3-linked sialic acid[8,9]. Because α2,3-linked sialic acid is expressed on the intestines of aquatic birds which is believed to be the primordial reservoir for all subtype of influenza A virus[11]. Occasionally viruses are transmitted to other host species and introduce avian viral gene to non-avian hosts like human. This situation can lead to severe outbreaks or pandemics[11]. Moreover, a recent study showed that replacing *NA* gene of North American triple reassortant swine influenza virus with that of 2009 pandemic H1N1 virus altered the enzymatic activity and led to an enhanced efficiency of respiratory-droplet transmission in ferrets[7]. Therefore, the monitoring of NA activity on substrate specificity is needed.

Highly pathogenic H5N1 avian influenza virus is causing a wide-spread epidemic in poultry with occasional transmission to humans and poses a serious pandemic threat. While receptor preference of H5N1 HA has been extensively studied[12-14], data on their NA substrate specificity are scarce. We therefore characterised NA activity of H5N1 viruses in comparison to NA of a seasonal influenza virus.

MATERIALS AND METHODS

Cell and virus culture

Madin-Darby canine kidney (MDCK) cells were maintained in minimum essential medium (MEM) with 10% fetal bovine serum (FBS) in the present of Gentamicin, Penicillin G and Fungizone. 293T cell were maintained in Dulbecco's modified Eagle medium supplemented with 10% FBS, antibiotics and antifungal. Viruses used in this study are shown in Table 1. Viruses were cultured in MDCK cells in MEM without phenol red to avoid the interference with the fluorescent assay[15,16].

Generation of reverse genetic virus

Reverse genetic viruses were generated by DNA transfection as described by Hoffmann *et al*[17]. The *NA* genes were extracted from A/Thailand/KAN-1A/2004, A/Thailand/676/2005, A/Thailand/3(SP-83)/2004 and seasonal influenza virus, A/Thailand/AW10/2010 (H3N2), respectively and cloned into pHW2000. Then, 1 μg of pHW2000 expressing NA-DNA was transfected into the co-cultured of MDCK and 293 T cell in Opti-MEM (Gibco, United States) with the other seven genomic segments of A/Puerto Rico/8/34(H1N1) in the presence of TransLT according to the manufacturer's instructions. Thirty hours post transfection, fresh Opti-MEM containing TPCK-trypsin was added to the cells at the final concentration 0.5 μg/mL in the cell suspension. The HA titer of the NA reverse genetic virus was determined by Hemagglutination test.

NA Amplex Red® assay

NA activity was assayed using Amplex Red® assay following the instruction provided by the manufacturer (Molecular Probe, Inc.). This assay utilizes Amplex Red to detect H_2O_2 generated by oxidation of desialylated galactose which is the end product of neuraminidase action. In the presence of horseradish peroxidase, H_2O_2 reacts with 1:1

Table 1 Virus strains and sources

Virus	Subtype	Passage	Source
A/Thailand/KAN-1A/2004	H5N1	MDCK8	Human
A/Thailand/676/2005	H5N1	MDCK8	Human
A/Thailand/3(SP-83)/2004	H5N1	MDCK8	Human
A/Openbill stork/Thailand/VSMU-4-NSA/2004	H5N1	MDCK4	Avian
A/Openbill stork/Thailand/VSMU-5-NSA/2004	H5N1	MDCK4	Avian
A/Chicken/Bangkok/VS-MU-1/2006	H5N1	MDCK4	Avian
A/Chicken/Thailand/BF2037/2007	H5N1	MDCK4	Avian
A/Openbill stork/Thailand (Nakhonsawon)/VSMU-32/2005	H5N1	MDCK4	Avian

MDCK: Madin-Darby canine kidney.

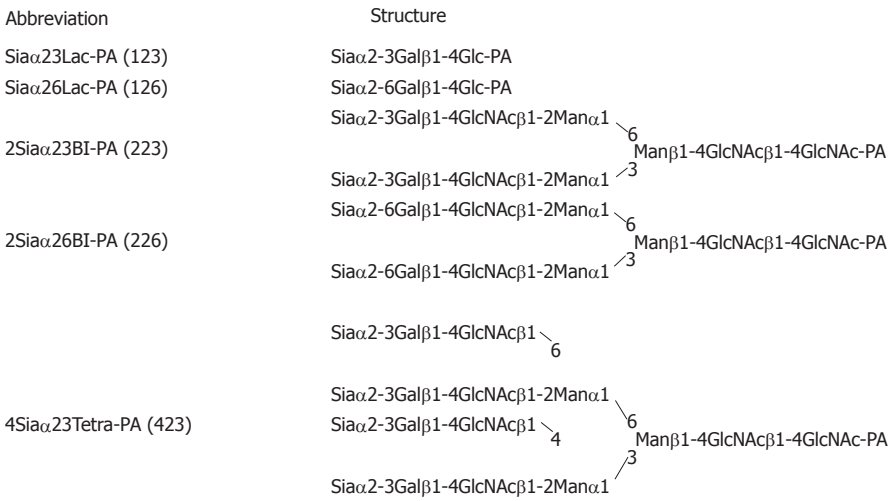

Figure 1 Pyridylamino oligosaccharide that used for high-performance liquid chromatography neuraminidase assay.

stoichimetry with Amplex Red reagent, then, generates Resorufin, the red-fluorescent oxidation product, which is detected at 640 nm. The method had been modified in order to study the substrate specificity by using 2 types of glycopolymer instead of fetuin. The substrates which were applied for this assay was Neu5Acα2,3LacNAcb-p-Aminophenyl (pAP) and Neu5Acα2,6LacNAcb-pAP which contained α2,3-linked sialic acid and α2,6-linked sialic acid, respectively[12,18]. Briefly, 10 μL of 64 HA unit of virus was mix with 10 μL of Amplex red reaction mixture in the present of 0.5 μg of either Neu5Acα2,3LacNAcb-pAP or Neu5Acα2,6LacNAcb-pAP for virus and 2 μg of each for reverse genetic virus. The NA activity on each substrate was detected at 640 nm after incubation at 37 ℃ for 110 min. Percentage of fluorescence correlated to NA activity of each virus was subtract with mock and plotted and analysed by using GraphPad Prism version 4.0 for windows (GraphPad software, San Diego, California; http://www.graphpad.com). Mean ± SEM from triplicate experiments were calculated for NA activity. One-way Anova were used to determine *P*-value for the significant difference between viruses. A *P*-value of $\leqslant$ 0.05 was considered significant.

NA assay by high-performance liquid chromatography

To confirm the NA activity by Amplex Red® assay, NA was determined the activity by using high-performance liquid chromatography (HPLC) as previously described[19]. Viruses was incubated at 37 ℃ for 2 h with buffer and 10 pmole of each Neu5Acα2,3- or Neu5Acα2,6-pyridylamino (PA)-glycopolymer shown in Figure 1. Twenty-five microlitres of saturated $NaHCO_3$ were added, then heated at 100 ℃ for 10 min to inactivate virus. The reaction then concentrated by using CentriVap (Labconco, United States) prior analysis with HPLC which was performed on a Shodex NH2P-50 4E column (4.6 mm × 250 mm) at a flow rate of 0.6 mL/min. PA-glycopolymers were detected by fluorescence (excitation wavelength, 310 nm; emission wavelength, 380 nm). Two eluents were used, A and B. Eluent A was acetonitorile: water:acetic acid (930:70:3, v/v/v) adjusted to pH 7.0 with aqueous ammonia; Eluent B was acetonitorile:water: acetic acid (200:800:3, v/v/v) adjusted to pH 7.0 with aqueous ammonia. The column was equilibrated with Eluent A:Eluent B (95:5). After injecting Eluent A:Eluent B (86:14) in 3 min, to Eluent A:Eluent B (73:27) in 17 min, to Eluent A:Eluent B (52:48) in 59 min, and then to Eluent A:Eluent B (25:75) in 6 min.

RESULTS

NA substrate specificity of H5N1 viruses from humans and animals

The substrate specificity NA from H5N1 using two syn-

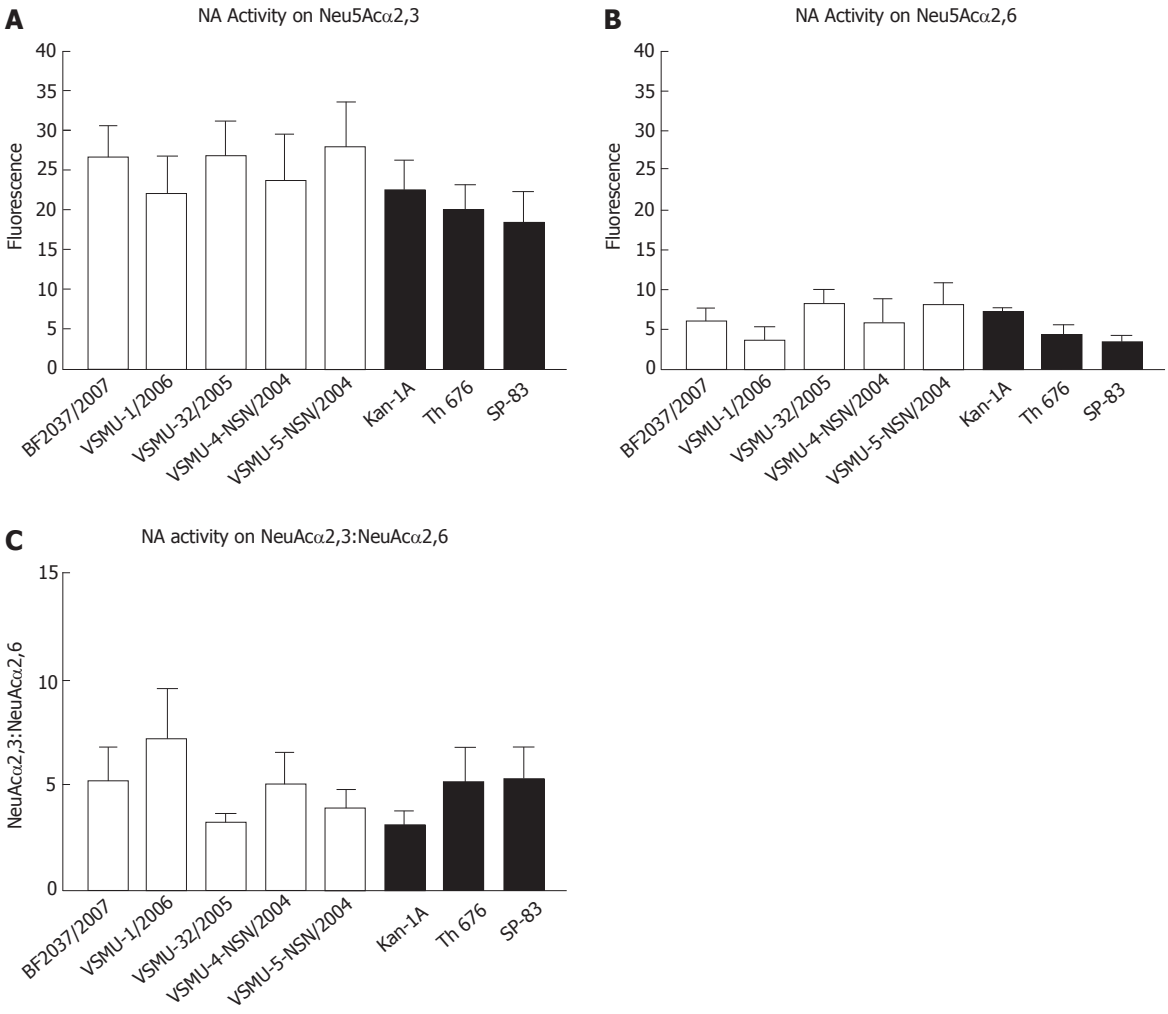

Figure 2 The N1 activity on substrate specificity by Amplex Red® assay. Substrate-specific neuraminidase activity of H5N1 avian influenza viruses isolated from animal (white bars) and human (black bars) was measured by a modified Amplex Red® assay. The α2,3-linked sialoside specific activity was measured using Neu5Acα2,3LacNAcb-pAP as substrate (A), whereas the α2,6-linked sialoside specific activity was measured using Neu5Acα2,6LacNAcb-pAP (B). The fluorescence related to neuraminidase activity from triplicate experiments had been shown as mean ± SEM. In order to show substrate preference, ratios between the α2,3- and α2,6-specific activity are shown (C).

thetic glycopolymers as substrate in Amplex Red® fluorescence assay was shown in Figure 2. The NA activity was 10-30 fluorescence unit, average 23.55 ± 1.489 (mean ± SEM), on α2,3-linked sialosides (Figure 2A) and 5-10 fluorescence unit, average 6.133 ± 0.667 (mean ± SEM) on α2,6-linked sialosides (Figure 2B). Comparing between the two substrates, H5N1 isolates from humans and animals showed higher activity on α2,3-linked sialic acid than α2,6-linked ($P < 0.0001$) with the ratio of activity on α2,3-linked sialosides to the activity on α2,6-linked sialosides of 4.685 ± 0.2092 (mean ± SEM) (Figure 2C).

Moreover, the NA activity of the reverse genetic viruses on 2,3- and 2,6-linked sialosides were shown in Figure 3A and 3B, respectively. While the NA from the H3N2 virus showed low activity on both α2,3- and α2,6-linkage, the reverse genetic viruses with the NA from the H5N1 viruses showed markedly higher activity on α2,3-linkage than on α2,6-linkage giving a high α2,3- to α2,6-ratio with *P*-value = 0.0249 (Figure 3C).

NA substrate specificity by HPLC analysis

To confirm the NA specificity profile, another assay using PA-glycopolymers and HPLC analysis of the enzymatic products was performed on a human isolate of the H5N1 virus (KAN-1) (Table 2). In concordance with the Amplex Red® assay, the H5N1 NA showed robust activity on α2,3-linked glycopolymers and undetectable activity on α2,6-linked glycopolymers. The two assays thus together conclusively showed that NA activity of the H5N1 virus had a α2,3-linkage preference.

DISCUSSION

Although, thiobarbituric acid method is the gold standard to detect NA activity, it is time-consuming and sensitive to interference by complex culture media[20]. Moreover, these methods use NANA, 4-methylumbellifery or fetuin as the substrate, which could not distinguish the substrate specificity because fetuin contained both α2,3-linked

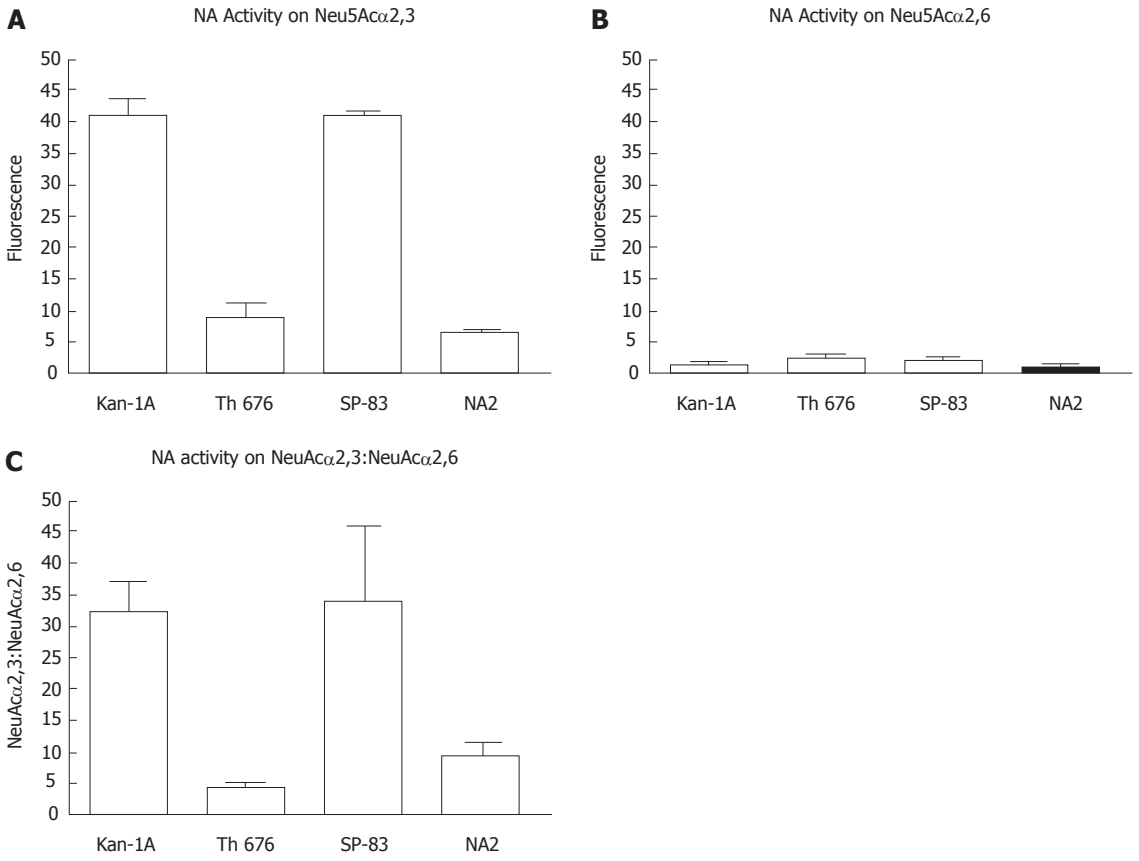

Figure 3 The neuraminidase activity on substrate specificity of reverse genetic virus by using Amplex Red® assay. The neuraminidase (NA) activity on α2,3- and α2,6-sialosides was shown in term of mean ± SEM from individual triplicate experiments on (A) and (B), respectively. The ratios of α2,3- and α2,6-substrate specific NA activity are shown on (C).

Table 2 Neuraminidase activity measured by high-performance liquid chromatography analysis

Linkage type	Siaαx Lac-PA	DiSiaαx BI-PA		4Siaαx Tetra-PA
	Digested	One sialic digested	Two sialic digested	Completely digested
α2,3	100	3.5%	96.5%	100%
α2,6	0	0	0	ND

ND: Not done.

and α2,6-linked sialosides. In order to detect the NA substrate specificity, there are several proposing methods to differentiate the substrate specificity, *i.e.,* BODIPY-labeled substrate, glycan array and library screening format[10,21-23]. These methods required modification and purification on neuraminidase which is not the original forms of neuraminidase from influenza virus[21,22]. To avoid the modification on influenza neuraminidase, the commercial Amplex Red® assay was modified by changing the substrate. In this assay all viruses had to be cultured in phenol red free medium to avoid the interference of fluorescent assay as previously described[15]. Similar to Amplex Red® assay, NA activity by HPLC also use the fluorescent labeled substrates and can detect the NA activity from the virus directly. Moreover, HPLC can also separate the size of digested substrates[19] which reflect to NA activity whether it completely or partially digest substrates with more than one sialic acid molecules.

Not only alpha-linkage that affect the substrate specificity, but the sialygalactoside; the basic form of sialic acid also effected the substrate specificity because their variations between species[24]. There are 3 forms of sialic acid, N-acetylneuraminic acid (Neu5Ac), N-glycolylneuraminic acid (Neu5Gc) and deamineuraminic acid[21,24,25]. Several studies showed that most of human viruses prefer to cleave Neu5Ac, which is predominantly expressed on human upper respiratory tract and is the most abundant sialic form in nature[21,24-26]. Therefore, this study used the Neu5Acα2,3- and Neu5Acα2,6-linkage which are the majority form of sialic acid in human on for substrate specificity.

The NA substrate specificities of this study is in agreement with previously published data showing predominant NA activity on α2,3-linked sialosides in avian viruses[8,21,22]. However, the input virus in the NA assay of this study

was normalized by their HA unit, a difference in HA activity may bias the amount of the input virus. To avoid this problem, reverse genetic viruses carrying NA from either human isolated H5N1 or a seasonal influenza virus (H3N2) with the rest of the genome including HA from PR8 strain was generated. Therefore, the viral input can be reliably normalized and the NA activity between the H5N1 NA and the H3N2 NA could be reliably compared.

The general patterns of NA substrate preference of avian and seasonal influenza viruses are in agreement with previously published data[10,27-29]. The similar sialic acid preference between HA and NA of the same group of viruses suggests that compatibility between HA and NA is important for optimal viral infection. On the other hand, NA function is not only to release progeny virions from producer cells but also to help virions penetrate mucus layer of respiratory mucosa[24,30]. This may explain why seasonal influenza viruses maintain α2,3-linkage specific NA activity despite their HA specificity to only α2,6-linked sialic acid. Human mucin is rich in α2,3-linked sialic acid, and NA activity against this type of sialic acid may be required for virions to reach target cells underneath the mucus layer[31]. The high α2,3-linkage specific NA activity of H5N1 avian influenza virus may help the virus penetrate the mucus layer and enhance the viral infection in humans.

Although the change in NA substrate preference does not seem to be a prerequisite for emergence of a pandemic virus, the NA substrate preference of H3N2 seasonal influenza virus and the H5N1 isolate with dual-specific HA (A/Thailand/Th676/2005, which was previously shown to have a dual specific HA conferred by two mutations at position 129 and 134[12]) suggested that the adaptation by decreasing α2,3-specific activity may help balance the HA adaptation toward human receptor specificity. The balance between HA and NA play a crucial role in the viral fitness and the emergence of pandemic virus[23,32]. The NA mutations, A138S, E259D, N325T and A343T, were observed. These mutations were located near either framework or active site of the NA[9,33-35]. Therefore, the adaptation of NA function may be resulting from either each or combination of these mutations.

COMMENTS

Background

Neuraminidase (NA) is a glycoprotein on the envelope of influenza virus. NA cleaves viral receptor on the cell surface in order to release virions from host cells. If the NA function is impaired, virions will aggregate on the cell surface hindering the dissemination of infection. NA activities on bird-type and human-type receptor substrate have been previously characterised for some avian and seasonal influenza viruses. It can be concluded that NA from human viruses shows the increased substrate specificity on human-type sialic acid, which is found in human respiratory tract, while maintaining specificity on bird-type sialic acid. Occasionally viruses are transmitted to other host species and introduce avian viral gene to non-avian hosts like human and this can lead to severe outbreaks or pandemics. Therefore, the monitoring of NA activity on substrate specificity is required.

Research frontiers

Highly pathogenic H5N1 avian influenza virus is causing a wide-spread epidemic in poultry with occasional transmission to humans and poses a serious pandemic threat. The authors therefore characterised NA activity of H5N1 viruses in comparison to NA of a seasonal influenza virus.

Innovations and breakthroughs

In order to study the NA substrate specificity, there are several proposing methods to differentiate the substrate specificity which required modification and purification on neuraminidase which is not the original forms of neuraminidase from influenza virus. To avoid the modification on influenza neuraminidase, the commercial Amplex Red® assay was modified by changing the substrate, instead. Similarly, HPLC used fluorescent labelled substrates and can detect the NA activity from the virus directly. The authors' data showed that H5N1 avian influenza isolates from both humans and birds maintained the NA activity profile with preference for bird-type receptor, except for a human isolates that was previously shown to have HA with dual specificity. This H5N1 virus showed reduced activity on bird-type substrate suggesting a requirement for compatibility with its HA that gained binding to human-type receptor. A138S, E259D, N325T and A343T mutations were found in the NA of this virus.

Applications

This study suggests that NA substrate specificity must be monitored for assessing the risk of cross-species transmission.

Terminology

α2,3-linked sialic acid or bird type substrate is the sialic acid that is mostly found in avian gastrointestinal and respiratory tract while α2,6-linked sialic acid is abundant in human respiratory tract.

Peer review

The authors have performed a good study, the manuscript is interesting.

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