Molecular Psychiatry (1999) 4, 33–38

ORIGINAL RESEARCH ARTICLE

High seroprevalence of Borna virus infection in schizophrenic patients, family members and mental health workers in Taiwan

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Borna disease virus (BDV), a negative-strand RNA virus, has been reported to be associated with severe psychiatric disorders. The association is mainly based on the findings that patients with schizophrenia and depression have a higher seroprevalence rate of BDV-specific antibodies than controls. In addition, psychiatric patients were also found to have a higher detection rate of BDV transcripts in their blood than controls. By using an improved Western blot analysis, we first demonstrated that Chinese schizophrenic patients from Taiwan also have a higher seroprevalence of BDV-specific antibodies than controls (12.1% *vs* 2.9%, $P < 0.001$), providing support to the positive association between BDV and psychiatric disorders in our population. Because of the contagious nature of viral infection, we further examined patients' family members and mental health workers, who have close contact with patients. We found that both groups also have a higher seroprevalence of BDV-specific antibodies, 12.1% and 9.8%, respectively, than controls. This finding provides some evidence for a possible human-to-human transmission of Borna disease virus. Our finding needs further independent verification from other research groups and the clinical relevance of this preliminary observation deserves further study.

Keywords: Borna disease virus; schizophrenia; prevalence; infection; pathology

Introduction

Schizophrenia is a serious, chronic debilitating mental disorder affecting about 1% of the general population;[1] its etiology remains elusive despite several decades of intensive study. Previous genetic epidemiological studies have indicated genetic components in its etiology.[2] However, the concordance rate of schizophrenia in monozygotic twins is approximately 50% on average, suggesting that other non-genetic factors are also important contributors to its pathology. Environmental factors such as viral infection have been suggested as a possible etiology of schizophrenia.[3–6] Recent studies have provided evidence to indicate that Borna disease virus (BDV) may play an important role in the pathogenesis of schizophrenia.[7–10]

Borna disease virus is a neurotropic, enveloped, negative-stranded RNA virus, which was first recognized as the infectious agent of an horse encephalitis endemic in eastern Germany. Several groups of researchers have demonstrated that patients with neuropsychiatric disorders have a higher rate of serum antibodies against BDV-specific nucleoprotein (40 kD, p40) and/or phosphoprotein (24 kD, p24),[11–15] suggesting that human neuropsychiatric disorders may be associated with BDV infection.[16] Further studies also revealed that psychiatric patients have a higher detection rate of BDV transcripts from peripheral blood[17,18] or postmortem brain tissue than controls,[19,20] adding further support to the important role of BDV infection in the pathogenesis of human psychiatric disorders. Nevertheless, several research groups presented contradictory data showing no association between BDV and psychiatric disorders. Richt *et al* reported that they failed to detect BDV transcripts in peripheral blood cells from psychiatric patients.[21] Similarly, Kubo *et al* found no association of BDV infection and psychiatric disorders in the Japanese population while investigating the BDV-specific antibodies and BDV transcripts among psychiatric patients and normal controls.[22] Lieb *et al* also reported that they were unable to detect BDV-specific RNA in blood from psychiatric patients from

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Received 31 March 1998; revised and accepted 17 September 1998

Germany.[23] Moreover, the presence of BDV transcripts in normal human brain tissue has been reported, indicating that BDV can latently infect normal brain tissue.[24,25] Hence, the association of BDV infection and human psychiatric disorders needs further clarification.

The reservoir and route of transmission of human BDV infection are still not very clear. Animal studies suggested that BDV is likely to be transmitted through saliva and nasal secretions, and the olfactory nerve might be the main entrance to the body.[26] In addition, BDV may also be transmitted through the hematogenous route, since BDV has been isolated from peripheral blood.[27] Takahashi *et al* reported higher seroprevalence of BDV infection in blood donors living near thoroughbred horse farms in Hokkaido, Japan.[28] A similar high seropositive rate of BDV infection has been observed among veterinarians, laboratory technicians, and farm workers who were exposed to BDV-infected ostriches in Israel.[29] These studies indicate the possible animal-to-human transmission of BDV. In this regard, we were interested to know whether schizophrenic patients' family members, such as parents and siblings, as well as mental health workers who have long-term close contact with patients, would have a higher seropositive rate of BDV infection than the general population.

The purpose of this study was first to investigate the BDV seroprevalence rate in Taiwanese people and to elucidate its possible association with schizophrenia using a case-control approach. Second, it was to investigate the BDV seroprevalence rate among patients' family members and mental health workers, who have close contact with mental patients.

Materials and methods

Subjects

Taiwanese schizophrenic patients fulfilling the diagnostic criteria of chronic schizophrenia according to the DSM-IV (American Psychiatric Association) were recruited from two long-stay mental hospitals located in northern Taiwan. The clinical diagnosis of these patients was evaluated by three senior psychiatrists (F-C Wei, F-J Koong, H-C Liu) with consensus. The patient group consisted of 182 males and 132 females, with a mean age of 40 years. Most of the patients had the residual type of schizophrenia ($n = 125$), the other clinical diagnoses included paranoid type ($n = 54$), disorganized type ($n = 79$) and undifferentiated type ($n = 56$). The non-psychiatric control group consisted of 169 anonymous blood donors, and 108 unscreened outpatients from a local community hospital in Taipei City, including 132 males and 145 females. The mean age of the control group was 35 years. Furthermore, one asymptomatic parent or sibling from each unrelated schizophrenic family was randomly selected for immunoassay as a family member group. The family member group comprised 70 males and 62 females, with a mean age of 45 years. In addition, 82 mental health workers, including seven psychiatrists, 59 nurses, six social workers, eight occupational therapists, and two clinical psychologists, were recruited for immunoassay with informed consent as a mental health worker group. This group consisted of 14 males and 68 females with a mean age of 38 years. Blood samples were taken from each subject with EDTA as anticoagulant, and plasma was isolated for immunoblot assay.

Generation of expression constructs

The IMPACT^{TM}I: one-step protein purification system (New England Biolabs, MA, USA) was used for expression and purification of recombinant BDV p40 and p24 proteins. Full-length cDNA of BDV p40 and p24 were generated using reverse transcription-polymerase chain reaction (RT-PCR). Total RNAs isolated from Madine Darby canine kidney (MDCK) cells persistently infected with BDV were used as templates. The primers used for RT-PCR were : 5′-tgaacacacgcat atgccacccaagagacgc-3′ (forward) and 5′-aaagcacccgggg tttagaccagtcacacc-3′ (reverse) for p40; 5′-tcaggaggctcat atggcaacgcgaccatcg-3′ (forward) and 5′-aaagcacccgggt ggtatgatgtcccattc-3′ (reverse) for p24. The cDNAs were ligated into prokaryotic expression vector pCYB2 by *Nde*I and *Sma*I sites and transformed into *E. coli* BL21. The authenticity of the expression constructs was verified by sequencing reactions.

Expression and purification of BDV p40 and p24 proteins

Recombinant proteins were induced, purified with chitin affinity chromatography, and cleaved from fusion proteins according to the manufacturer's instructions. In brief, the gene encoding BDEV p40 and p24 proteins was inserted into the cloning site of pCYB2 vector to create a fusion between the C-terminus of BDV p40 (and p24) and the N-terminus of intein. The gene encoding a small chitin-binding domain has been added to the C-terminus of the intein for affinity purification. When the crude cell extracts of the induced *E. coli* expression system are passed through a chitin column, the fusion protein binds to the chitin column while all other contaminants are washed through the column. The fusion is then induced to undergo an intein-mediated self-cleavage on the column by overnight incubation at 4°C in the presence of DTT. The BDV p40 (and p24) is released while the intein-chitin binding domain fusion partner remains bound to the column. The purified recombinant BDV p40 and p24 recombinant proteins have two extra amino acids (proline and glycine) at their C-terminus after cleavage from the fusion protein. The antigenicity of these recombinant proteins was verified by the monoclonal antibodies (kind gifts from Dr Ikuta).

Western blot analysis

Ten micrograms each of chitin affinity purified recombinant BDV p40 and p24 proteins were separated in 15% SDS-PAGE, and transferred onto a polyvinylidene difluoride membrane (PVDF; Millipore, Bedford, MA, USA) using Mini-Trans-Blot Cell (Bio-Rad, Hercules, CA, USA). Ten-fold dilution of patients' plasma was applied to the membrane using Mini-Protean II Multi-

screen Apparatus (Bio-Rad). Immunoreactions of anti-BDV antibodies to recombinant BDV antigens were detected by ProtoBlot II AP system (Promega, Madison, WI, USA) according to the manufacturer's instructions. The specificity of anti-BDV antibodies from positive plasma was examined by competition assay.

Competition assay
Approximately 10^{10} of MDCK cells with BDV persistent infection were pelleted, and resuspended in 1 ml lysis buffer (100 mM Tris-HCl, pH 9.0; 100 mM NaCl, 0.5% Triton X-100) containing protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany). Anti-BDV positive plasma was incubated with three volumes of cell lysates at 4°C overnight, then cleared by centrifugation. Western blot analysis of the preabsorbed plasma was performed as described. MDCK cells without BDV infection were used as negative controls.

Results

Isolation of recombinant BDV-specific proteins
We first isolated the recombinant BDV-specific nucleoprotein (40 kD, p40) and phosphoprotein (24 kD, p24) using a prokaryotic expression system, IMPACT I (Intein Mediated Purification with an Affinity Chitin-binding Tag, New England Biolabs) as described in the Methods section (Figure 1). The purified recombinant BDV p40 and p24 recombinant proteins have two extra amino acids (proline and glycine) at their C-terminus after cleavage from the fusion protein. The purified proteins were used as antigens in western blot analysis for detection of BDV-reactive antibodies from patients' plasma. The specificity of the positive sera that recognize p40 and p24 was verified by competition assay as described in the Methods section. The results are shown in Figure 2.

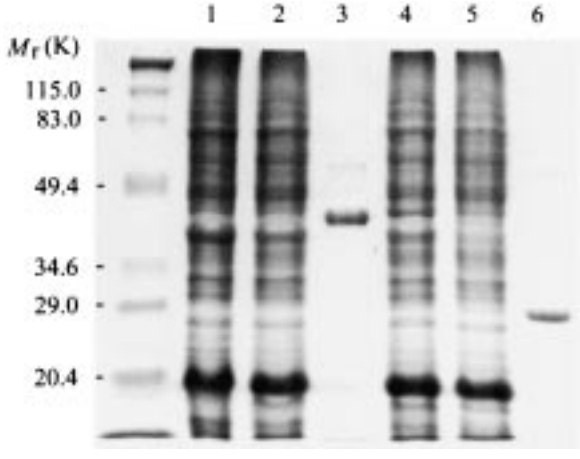

Figure 1 Expression and purification of the recombinant BDV p40 and p24 proteins from *E. coli* using the IMPACT I system. Lane 1: uninduced cell extract from *E. coli* transfected with p40 expression plasmid pCYB2. Lane 2: induced cell extract with BDV p40. Lane 3: eluted purified BDV p40 protein after DTT-induced cleavage reaction. Lane 4: uninduced cell extract from *E. coli* transfected with BDV p24 expression plasmid pCYB2. Lane 5: induced cell extract with BDV p24. Lane 6: eluted purified BDV p24 protein after DTT-induced cleavage reaction.

BDV seroprevalence in schizophrenic patients
We examined 314 schizophrenic patients and 274 non-psychotic controls. Of the 314 schizophrenic patients assayed, 35 were positive to p40, and three were positive to p24, the seroprevalence was 12.1%. In contrast, of 274 non-psychiatric controls studied, eight were positive to p40; none was positive to p24, the seroprevalence was 2.9%. The BDV seroprevalence in schizophrenic patients was significantly higher than that in controls (odds ratio: 4.63; 95% confidence interval: 2.29–9.52) (Table 1).

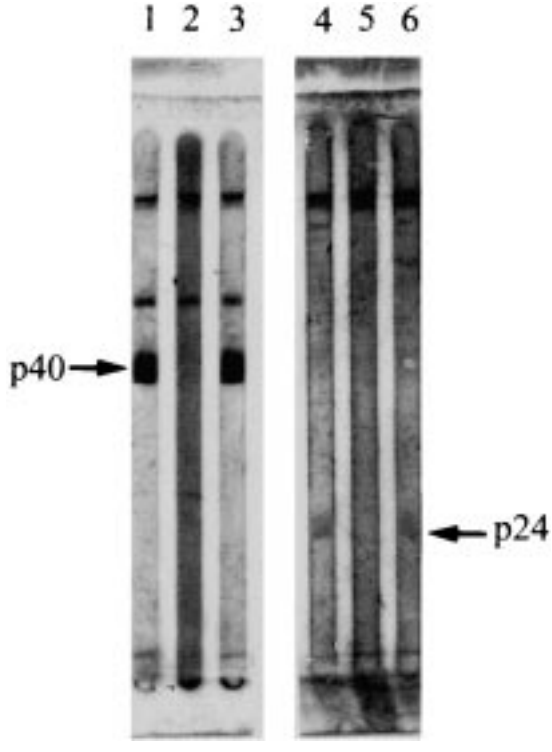

Figure 2 Immunoblot analysis of anti-BDV specific antibodies in plasma from patients. Lane 1: positive plasma against recombinant BDV p40 protein at 1:10 dilution. Lane 2: positive plasma preincubated with cell extracts of MDCK cell lines persistently infected with BDV. The absence of the immunoreaction demonstrates the specificity of the positive plasma with anti-BDV p40 antibodies. Lane 3: positive plasma preincubated with cell extracts of MDCK cell lines without BDV infection, which serves as a negative control. Lane 4: positive plasma against recombinant BDV p24 protein at 1:10 dilution. Lane 5: positive plasma preincubated with cell extracts of MDCK cell lines persistently infected with BDV. The absence of immunoreaction demonstrates the specificity of the positive plasma with anti-BDV p24 antibodies. Lane 6: positive plasma preincubated with cell extracts of MDCK cell lines without BDV infection, which serves as negative control.

Table 1 Seropositive rate of BDV-specific antibodies in schizophrenic patients, unaffected family members, mental health workers and non-psychiatric controls

	Anti-BDV positive	*Anti-BDV negative*	*Prevalence (%)*	*OR (95% CI)*
Schizophrenia	38	276	12.1	4.63 (2.25–9.52)
Family members	16	116	12.1	4.64 (2.06–10.43)
Mental health workers	8	74	9.8	3.64 (1.40–9.46)
Controls	8	269	2.9	1

OR: odds ratio.
CI: confidence interval.

BDV seroprevalence in asymptomatic family members of patients

In a total of 132 asymptomatic unrelated family members studied, we identified 13 people who were positive to p40, two were positive to p24, and one was positive to both p40 and p24. The seroprevalence was 12.1%, significantly higher than that in controls (odds ratio: 4.64; 95% confidence interval: 2.06–10.43) (Table 1).

BDV seroprevalence in mental health workers

We further assayed plasma collected from mental health workers, including clinical psychiatrists, psychiatric nurses, social workers, occupational therapists, and clinical psychologists, who have long-term close contact with mental patients. Of 82 subjects studied, eight were positive to p40, none was reactive to p24. The seroprevalence rate was 9.8%, which was also significantly higher than that in the general population (odds ratio: 3.64; 95% confidence interval: 1.40–9.46) (Table 1).

Discussion

In this study, we demonstrate a positive association between BDV infection and schizophrenia in Chinese patients from Taiwan. Chinese schizophrenic patients were found to have a significantly higher seroprevalence rate of anti-BDV antibodies than the general population. Our results are consistent with most of the other seroepidemiological reports detecting a higher seroprevalence rate of BDV among psychiatric patients, providing further support to the premise that BDV infection may play an important role in the pathogenesis of human mental disorders. Our study, to our knowledge, is the first to demonstrate the presence of anti-BDV antibodies in the Chinese population, which also adds further evidence to BDV being more worldwide distributed rather than geographically restricted.

In this study, 70 out of 805 subjects were seropositive to BDV antigens. The majority of the positive sera (64 of 70, 91.4%) recognized BDV p40 antigen, while only 5 of 70 (7.1%) recognized BDV p24 antigen, and only one serum recognized both BDV p40 and p24 antigens. These results are consistent with the studies of Fu *et al*[13] Sauder *et al*[15] and Bode,[16] but discrepant from the study by Waltrip *et al*,[7] who reported more p24 seropositivity than p40 positivity. The reason for the discrepant results is unknown; one of the possible explanations is that our antigens are prokaryotic recombinant proteins, while other groups used native proteins. In addition, we also found low titers of BDV antibodies in our positive plasma specimens, which are also consistent with the findings from other BDV serological studies in the literature.[12–16] Previous studies showed that the low dilution of sera from psychiatric patients may generate false positive reactions in BDV serological study due to non-specific binding of some autoantibodies or other unknown factors.[30,31] To increase the specificity of the Western blot analysis in detecting the BDV antibodies in our study, we use a novel prokaryotic expression system (IMPACT) to generate and to purify recombinant BDV antigens for Western blot analysis as described in the Methods section. The chitin affinity-purification of expressed fusion proteins and the intein-mediated self-cleavage of BDV fusion protein, induced by incubating with dithiothreitol (DTT) at low temperature, facilitate the production of recombinant BDV nucleoprotein and phosphoprotein in an easy and efficient way. The purified recombinant BDV-specific p40 and p24 antigens would provide better specificity in Western blot analysis, because they contain only two extra amino acids at their C-terminus, and are supposed to be superior to the whole cell extracts prepared from BDV-infected cells or BDV cDNA-transfected cells, which show more background reactions in Western blot analysis. The specificity of the anti-BDV antibodies was further verified by the competition assay using MDCK cell lines persistently infected with BDV. Thus, the development of this novel Western blot analysis system should facilitate further clinical and epidemiological studies of BDV infection.

Previous serological studies of BDV did not show specific association of seropositivity with distinct human neuropsychiatric disorders. Positive anti-BDV sera were found in patients with affective disorders, schizophrenia, organic mental disorders, neurotic disorders, and even in patients with HIV infection.[32] In fact, BDV was found to persist inside the limbic system, a brain area involved in the control of human mood, behavior and cognitive function.[33] Hence, it is likely that patients with BDV infection may have various neuropsychiatric symptoms. Although in this study we investigated the BDV seroprevalence among schizophrenic patients, it is likely that positive anti-BDV sera may be present in patients with other neuropsychiatric diagnoses. Further studies are needed on this issue.

The most intriguing finding in this study is that, we not only identified higher than general population seropositive rates of anti-BDV antibodies among schizophrenic patients in our population, but also revealed

similarly higher seropositive rates of anti-BDV antibodies among asymptomatic first degree relatives of schizophrenic patients and mental health workers. This finding in our study is comparable to the data from Takahashi *et al*[28] and Weisman *et al*,[29] who found a higher BDV seropositive rate in people living in the neighborhood of horse farms, and in workers exposed to BDV infected animals, respectively. These results suggested not only the possible animal-to-human transmission of BDV, but also the likely human-to-human transmission of BDV as indicated in our study. Nevertheless, the clinical relevance of these findings is still not clear, because the causal-effect relationship of BDV infection with human psychiatric disorders is not yet well established. It would be interesting and also important to closely follow those asymptomatic family members and mental health workers with positive BDV serological reaction identified in our study.

The seroimmunological studies of BDV infection should be regarded with caution, because the positive reaction that we detected may be due to cross-reaction of anti-BD-like virus antibodies. Hence, it is important to embark on molecular biological studies to detect the BDV-specific RNA, and to isolate BDV from patients to further support the association in our population. Currently, we have been able to detect BDV transcripts from the white blood cells from patients (unpublished data), which should be useful for further study of the association between BDV and human psychiatric disorders in our population.

In conclusion, we report a positive association between BDV or BD-like virus infection and schizophrenia in the Chinese population from Taiwan. In addition, we find that both family members of schizophrenic patients and mental health workers also have higher seropositive rates of anti-BDV or anti-BDV-like virus antibodies than the general population. This observation needs independent verification from other research groups, and the clinical relevance of these findings needs further exploration.

Acknowledgements

The authors are grateful to Dr Kazuyoshi Ikuta and Dr Takaaki Nakaya from Hokkaido University, Japan, for their invaluable assistance and help in this study.

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