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RNA From Borna Disease Virus in Patients With Schizophrenia, Schizoaffective Patients, and in Their Biological Relatives

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Numerous interactions of the immune system with the central nervous system have been described recently. Mood and psychotic disorders, such as severe depression and schizophrenia, are both heterogeneous disorders regarding clinical symptomatology, the acuity of symptoms, the clinical course, the treatment response, and probably also the etiology. Detection of p24 RNA from Borna disease virus (BDV) by the reverse transcriptase polymerase chain reaction in patients with schizophrenia, schizoaffective disorder, and in their biological relatives was evaluated. The subjects were 27 schizophrenic and schizoaffective patients, 27 healthy controls, 20 relatives without psychiatric disease, and 24 relatives with mood disorder, who attended the Psychiatric Ambulatory of Londrina State University, Paraná, Brazil. The subjects were interviewed by structured diagnostic criteria categorized according to the *Diagnostic and Statistical Manual of Mental Disorders-IV*, axis I, (SCID-IV). The mean duration of illness in schizophrenic and schizoaffective patients was 15.341 ± 1.494 years and the median age at onset was 22.4 ± 7.371 years. There were no significant differences in gender ($P = 0.297$), age ($P = 0.99$), albumin ($P = 0.26$), and body mass index (kg/m^2) ($p = 0.28$), among patients, controls, and relatives. Patients and biological relatives had significantly higher positive p24 RNA BDV detection than controls ($P = 0.04$); however, the clinical significance of BDV remains to be clarified. J. Clin. Lab. Anal. 22:314–320, 2008.

Key words: Borna disease virus; schizophrenic; schizoaffective disorder

INTRODUCTION

Schizophrenia is a highly complex and pervasive neuropsychiatric disorder of uncertain etiology (1). Current data from family, twin, and adoption studies, as well as from epidemiological surveys, suggest that the etiopathogenesis involves the interplay of complex polygenic influences and environmental risk factors operating on brain maturational processes. Among the environmental factors, winter–spring births, perinatal infections, household crowding, upbringing in urban areas, and pet ownership support the concept that schizophrenia could be triggered by infectious agents affecting the brains of genetically susceptible individuals (2–6).

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Teicher et al. (7) hypothesize that stress affects brain development via the activation of multiple pillars of the stress response. Viruses are possible infectious agents in chronic nervous system diseases of unknown etiology because of their potential for neurotropism and latency (8).

Viral infections are suspected to play a role in the etiology of schizophrenia. A Finnish cohort study suggested that childhood central nervous system (CNS) viral infections are associated with fivefold increased chances of developing schizophrenia in adulthood (9).

Recently, an involvement of retroviruses in the pathogenesis of schizophrenia has been hypothesized (6,10–12). It is well established that human retroviruses, such as human immunodeficiency virus (HIV) and human T-cell leukemia virus, can replicate in cells of the CNS, thereby causing neurological and psychiatric symptoms in some infected individuals (13,14).

The predilection of the virus for the limbic-hypothalamic region and production in some animals of a syndrome somewhat resembling human affective disorders by the finding of viral antibodies in psychiatric patients suggested the possibility that Borna disease virus (BDV) may be involved in some human affective disorders (15).

Evidence to support a role for viral infection in the pathogenesis of neurodevelopmental disorders was neonatal Borna virus infection in Lewis' rats (16). Data demonstrate that neonatal BDV brain infection of rats can be a valuable animal model system for studying the relationship between abnormal brain development and resultant behavioral deficits. Werner-Keišs et al. (17) have demonstrated that the expression profiles of BDV-glycoprotein, BDV-nucleoprotein, and their mRNAs are significantly different indicating that BDV-glycoprotein expression is regulated in vivo in experimentally infected Lewis rats. Pletnikov et al. (18) describe BDV is tropic for limbic and cerebellar circuitry. Infection causes a spectrum of behavioral deficits depending on the host's age, immune status, CNS maturity, and genetics (16). Dittrich et al. (19) found specific learning deficiencies together with subtle behavioral alterations, suggesting that BDV causes certain modulations of high integrative brain functions that are only detectable under experimental conditions.

BDV is a neurotropic, negative and single-stranded enveloped RNA virus that persistently infects the CNS of many animal species and may cause behavioral disturbances reminiscent of autism, schizophrenia, and mood disorders (18,20) of various domestic animal species. It replicates in neurons and astrocytes without inducing cytopathic effects (21). Infection causes disturbances in behavior and cognitive functions, but can also lead to a fatal neurologic disease. Bode et al. (22) reported that the proportion of BDV antibody carriers was higher than 30% among patients with major depression. In addition, they also detected a high rate of viral RNA in peripheral blood mononuclear cells (PBMC) derived from psychiatric patients at a high rate by reverse transcriptase-polymerase chain reaction (RT-PCR) (23).

Studies involving plasma and serum for BDV protein detection using immunological assay have been published and raised controversial interpretations (24–26).

Despite controversy about potential association with human neuropsychiatry illnesses, BDV affords an intriguing model for the study of these illnesses. Neonatal BDV-infected rats display neurodevelopment, physiologic, and neurobehavioral abnormalities that closely parallel some of the main features associated with several human mental disorders (27). Further proof came from the discovery that peripheral blood monocytes carry viral antigens: the first data on different viral genomic transcripts in such patients' cells as well as sequence data of transcripts. Both viral markers seem to coincide with acute episodes of mood disorders, thus pointing to a new human virus infection possibly threatening mental health (23).

BDV could be isolated from the brain tissue of a schizophrenic patient (28); another study reported of viral nucleic acid in both schizophrenic patients and mental health workers (29); still another one also verified the same virus in psychiatric patients (essentially schizophrenia and affective disorders) and family member (30), BDV footprints were also found in patients with mood disorders using PCR- and protein enzyme-linked immunosorbent assay methods (31).

Patients with mood and psychotic disorder had higher positive BDV (33.3%) detection in the peripheral blood cells than in controls (13.3%) (32). Between 3 and 45% of schizophrenic patients were reported to have BDV serum antibodies compared with 0–5% of controls without psychiatric disorders (33).

Some evidence of possible involvement of BDV infection in the pathogenesis of human mental disorders has been demonstrated by several groups of researchers. BDV causes changes in brain function resulting in the disturbance of movement and behavior and may be associated with human psychiatric disorders (31,32,34,35).

The predilection of the virus for the limbic-hypothalamic region and production in some animals of a syndrome somewhat resembling human affective disorders suggested the possibility that BDV may be involved in some human affective disorders (34).

BDV may be a virus-induced immunopathologic disease of the CNS, with changes in levels of several

cytokine (36). Elevation in cytokines such as IL-6, interleukin-1β (IL-1β), and tumor necrosis factor-α (TNFα) may play a mechanistic role in the association between prenatal exposure to infection and schizophrenia. During infection and ischemia, these cytokines are elevated in the CNS, representing two neurodevelopmental insults that are associated with increased risk of schizophrenia (37).

In this study, the aim was to detect p24 RNA BDV in patients with schizophrenia, schizoaffective disorder, as well as their biological relatives, and healthy controls.

MATERIALS

Subjects

The subjects were schizophrenic and schizoaffective adult outpatients of the Psychiatric Ambulatory of Londrina State University (UEL), Paraná, Brazil. The patients were in remission, in chronic treatment with typical antipsychotic (haloperidol) and atypical antipsychotic agents (olanzapine, clozapine, risperidone, ziprasidone). There were 27 schizophrenic and schizoaffective patients, 27 healthy volunteer controls, 20 first-degree relatives without psychiatric disease, and 24 first-degree relatives with mood disorder. Subjects younger than 18 years old and older than 55 years old were excluded from all groups.

Subjects were required to be in good health conditions, defined as the absence of chronic diseases that affect the immune system such as HIV infection, hepatitis B virus, and hepatitis C virus infections. Also, to be included in the study the subjects were required not to take immunosuppressive drugs, not to be abusers of alcohol or other dependence substances, and to have been free from acute infectious or inflammatory reactions for at least two weeks before the study.

The schizophrenic and schizoaffective outpatients were interviewed by structured diagnostic criteria categorized according to the criteria of the fourth edition of the American Psychiatric Association's *Diagnostic and Statistical Manual of Mental Disorders*, DSM-IV, Axis I, (SCID–IV) (American Psychiatric Association, 1994) translated into Portuguese (38). The schizophrenic patients were classified as paranoid type ($n = 12$), disorganized type ($n = 5$), residual type ($n = 4$), and schizoaffective disorder ($n = 6$).

The 29 healthy volunteers, who were recruited from the community, were free of any serious medical illnesses, and had never taken psychotropic drugs or presented current or past psychiatric disorders as determined by their reported history during the clinical interview and by structured diagnostic criteria categorized according to the DSM-IV, Axis I. Two controls were excluded from the study after a blood test detected hepatitis virus C infection, a chronic illness known to affect the immune system. The 46 biological relatives were volunteers, aged from 18 to 55 years. They were interviewed by the structured interview (DSM-IV, Axis I, and SCID-IV). One first-degree relative was excluded from the study after a blood test detected HIV infection.

The research was approved by the Human Ethics Research Committee of Londrina State University. After patients, controls, and biological relatives had given written informed consent, a sample of peripheral blood was drawn.

Demographic characteristics (age, sex, marital status, occupational level), number of cigarette packets smoked in a lifetime, expressed as cigarette/years/packet, clinical data such as the onset of the disease, the body mass index (BMI expressed in kg/m^2), and laboratorial (serum level of albumin) were collected from all the subjects enrolled in the study.

Detection of BDV p24 RNA in Human Peripheral Blood Cells

Cells were isolated from 10 ml of blood peripheral blood sample collected with EDTA anticoagulant. The samples were coded by number and were randomly analyzed, without separation based on the individual's category. The application of RT-nested PCR was chosen to guarantee the sensitivity for the detection of phosphoprotein (p24) gene of BDV in peripheral white blood cells, since the concentration of the nucleic acid specific to BVD in the sample could be reduced.

Analysis of BDV p24 RNA From Human PBMC

The total RNA was extracted from peripheral white blood cells with TRizol (Invitrogen, Grand Island, NY) according to the manufacturer's instructions.

The complementary DNA (cDNA) Sequences

The RNA was re-suspended in 20 µl of sterile water treated with diethylpyrocarbonate (Invitrogen). The cDNA sequences were amplified using RT-PCR reaction of reverse transcriptase for 6 µl RNA, using specific outer antisense primer and a first strand cDNA synthesis kit (Perkin Elmer Gene AmpRNA PCR kit-Part number N808-0017; Perkin Elmer, Branchburg, NJ).

A reverse transcription was carried out with 50 pmol of the specific type outer antisense primer using cloned murine leukemia virus reverse transcriptase (Perkin Elmer).

The BDV-specific primers (GenBank accession number: NC 001607) were used for amplification: BDV1 (5′-TGACCCAACCAGTAGACCA-3′), BDV2 (5′-GT CCCATTCATCCGTTGTC-3′), BDV3 (5́-TCAGACC

TABLE 1. Demographic Characteristics of Healthy Controls, Schizophrenic Patients, Relatives Without Mental Disorder, and Relatives With Mood Disorder, From Londrina, Southern Brazil

	Controls ($n = 27$)	Patients ($n = 27$)	Relatives without mental disorder ($n = 20$)	Relatives with mood disorder ($n = 24$)	Analysis		
Characteristics	*n* (%)	*n* (%)	*n* (%)	*n* (%)	χ^2	df	*P*-value
Gender							
Male	14 (51.9)	16 (59.3)	9 (45.0)	8 (33.3)	3.67	3	0.290
Female	13 (48.1)	11 (40.7)	11 (55.0)	16 (66.7)			
Marital status[a]							
Unmarried	6 (22.2)	16 (59.3)	4 (20.0)	6 (25.0)	11.44	3	0.009
Married	20 (74.1)	11 (40.7)	15 (75.0)	18 (75.0)			
Occupational							
Impairment	0 (0.0)	26 (96.3)	0 (0.0)	4 (16.7)	77.00	3	<0.001
No impairment	27 (100.0)	1 (3.7)	20 (100.0)	20 (83.3)			
	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	*F*	df	*P*-value
Age (years)	38.8 $\pm$ 10.3	38.1 $\pm$ 10.3	3880 $\pm$ 10.7	38.4 $\pm$ 10.0	0.028	3	0.990

[a]Missing one marital status χ^2, χ^2 test; df, degree of freedom; *F*, ANOVA *n*, number of samples; %, percentage; SD, standard deviation.

CAGACCAGCGAA-3′), BDV4 (5′-AGCTGGGGAT AAATGCGCG-3′) according to Kishi et al. (39). The reaction conditions for both PCR rounds were the same (20 mM Tris HCL pH 8.4, 50 mM KCL, 1.5 mM $MgCL_2$, 200 μM dNTP, and 1.25 unit of Taq polymerase), and consisted of an initial denaturizing step of 94°C for 5 min followed by 40 cycles of 94, 60, and 72°C for 1 min each and a final extension of 72°C for 10 min on thermocycling (PCR sprint ThermoHybaid from Biosystems, Ashford, Middlesex, UK). The second PCR was mostly in the same conditions as the first PCR, but the annealing temperature was 57°C. To analyze primer specificity, the samples were sequenced and compared with data in the National Center for Biotechnology Information (NCBI) NIH database. The analysis demonstrated that the amplified fragment was compatible with a sequence of BDV in the GenBank, under accession NC 001607.

Detection of PCR Products

PCR products of 392 base pairs were revealed by a 10% acrylamide gel silver staining eletrophoresis. The PCR products were purified and directly sequenced using DYEnamicTM ET dye terminator cycle sequencing kit (Amersham Pharmacia Biotech, Piscataway, NJ) in a MegaBaceTM sequencer (Amersham Pharmacia Biotech). Amplified nucleotide sequences were analyzed by comparison with the GenBank BDV sequence database.

Statistical Analysis

Groups were compared according to demographic and clinical variables by using the χ^2 test (2) or Fisher test, analysis of variance (ANOVA), and Kruskal–Wallis test. The results are reported as means and $\pm$standard deviation (SD) or median and range. Differences between groups were considered statistically significant when $P < 0.05$. The significance of the differences among patients, controls, relatives without mental disorder, and relatives with mood disorder was analyzed by ANOVA if the samples were normally distributed and the variances in the samples were homogeneous, or Kruskal–Wallis if the results showed the variances in the samples were not homogeneous. The χ^2 test (2) was used to evaluate the frequency of BDV detection in the study groups. Odds ratio (OR) and 95% confidence intervals (CI 95%) were calculated for comparison between reference groups.

RESULTS

Demographic Data

The groups were compared in relation to age, sex, marital status, and occupational level (Table 1). There were no group differences in age or gender. The group differed in occupational impairment and marital status. Patients were more unmarried than in controls, and relatives ($P = 0.009$). Occupational impairment was significantly higher in patients than in controls and relatives ($P < 0.0001$).

Clinical and Laboratorial Characteristics

The four groups were compared according to clinical variables. The mean duration of illness was 15.341 years (SD $\pm$ 9.90) and the median age at onset was 22.4 years (SD $\pm$ 7.371). A higher percentage of patients had attempted suicide ($n = 8$) in comparison with the controls ($n = 0$), the relatives without mental disorder

($n = 0$), and the relatives with mood disorder ($n = 4$) (Fisher test 14.32, df = 3, $P = 0.002$).

Data showed there were no significant differences between groups in BMI (kg/m^2). Patients were 26.18 SD $\pm$ 5.31 kg/m^2, controls were 26.8 SD $\pm$ 4.6 kg/m^2, relatives without mental disorder 24.0 SD $\pm$ 5.2 kg/m^2, relatives with mood disorder 25.58 SD $\pm$ kg/m^2 ($A = 1.2$, df = 3, $P = 0.28$, ANOVA test). The serum levels of albumin were not significantly different in the four groups. In the patients the levels were 4.2 $\pm$ 0.46 g/dL, among the controls the levels were 4.15 $\pm$ 0.35 g/dL, in the relatives without mental disorder, 4.1 $\pm$ 0.33 g/dL, and among the relatives with mood disorder, the levels were 3.96 $\pm$ 0.49 g/dL ($A = 1.3$,df = 3, $P = 0.26$, ANOVA test).

The schizophrenic patients were classified as paranoid type ($n = 12$), disorganized type ($n = 5$), residual type ($n = 4$), and schizoaffective disorder ($n = 6$) (Table 2).

The groups were compared according to frequency of BDV-RNA detection (Table 3). The frequency of p24 RNA BDV detection was significantly higher in patients [OR = 4.6; CI 95%: 1.25–16.97] and relatives without mental disorders [OR = 5.75; CI 95%: 1.45–22.78] than in healthy controls ($\chi^2 = 7.84$, $P = 0.049$, df = 3).

DISCUSSION

These findings are the first ones to demonstrate that the patients were chronically ill (duration of disease 15.3 years), had earlier disease onset (22.4 years), presented more suicide attempts, more hospitalizations, had a worse occupational history, and lower frequency of marriage. Poor prognostic features include insidious onset, long duration of symptoms, and history of psychiatric problems, affective blunting, obsessive–compulsive symptoms, poor premorbid personality, poor work history, celibacy, and young age of onset (40).

In this study, p24 RNA for BDV was detected in patients with schizophrenia or schizoaffective disorder (44.4%); relatives without mental disorders (50.0%); relatives with mood disorders (37.5%); healthy controls (14.8%). Studies of BDV were reported to be positive in 12.1% of schizophrenic patients, 12.1% of family members, 11.5% of mood disorder patients, and 2.9 % of healthy controls (30).

The high occurrence of BDV infection in this population studied should be discussed. First, all the subjects were recruited in Londrina, a town with approximately 500,000 inhabitants, located in south of Brazil, where the majority of population-based study was migrants from the rural region, which has a large area of fertile pasture lands that supports a very extensive herd of cattle.

Originally Borna disease has been described as a natural infection of horses in Germany (41). Now it is also known in sheep (42), cats (43), and domestic fowl (44). Though natural infection has not been reported in primates, subhuman primates can be infected experimentally (45,46). Antibodies to BDV proteins have been found in patients with neuropsychiatric disorders (35,47,22), suggesting that BDV or a related agent may be pathogenic in humans.

A disease of horses in Germany, resembling that what is now called Borna disease, has been described as early as 1660. BDV (started to be named around 1970) infects vertebrate animal species and can cause CNS disorders, thus leading to an association with psychiatric disorders. This virus and its genome were finally characterized by Briese et al. (48) in 1994.

The possibility of contamination of the sample during the laboratory procedures may not have occurred, because the detection of p24 BDV RNA from the PBMC was done by RT-PCR and it was performed according to the international security's instructions. In

TABLE 2. The DSM-IV Categorization of the Patients

	BDV positive		BDV negative	
Schizophrenia types	12n	%	15n	%
Paranoid type (n12)	2	17.0	10	83.0
Schizoaffective ($n = 6$)	4	67.0	2	33.0
Disorganized ($n = 5$)	2	40.0	3	60.0
Residual ($n = 4$)	4	100.0	0	0.0

TABLE 3. The Frequency of p24 RNA of BDV Detection in Healthy Controls, Schizophrenic Patients, Relatives Without Mental Disorder, and Relatives With Mood Disorder, From Londrina, Southern Brazil

	BDV positive		BDV negative			
Group	*n*	%	*n*	%	Odds ratio	95% CI
Controls ($n = 27$)	4	14.8	23	85.2	1.00	Reference
Patients ($n = 27$)	12	44.4	15	55.6	4.60	1.25–16.97
Relatives without mental disorder ($n = 20$)	10	50.0	10	50.0	5.75	1.45–22.78
Relatives with mood disorder ($n = 24$)	9	37.5	15	62.5	3.45	0.90–13.25

BDV, Borna disease vírus; $\chi^2 = 7.84$; df = 3; *P*-value = 0.0493 *n*, number of samples; %, percentage; CI, confidence interval.

addition, all the samples were analyzed in duplicate. Although products of RT-PCR are usually detected in agarose gels, in this study they were discovered in acrylamide gels, which are more sensitive to perform a detailed analysis. Some times, the amplification was not visible in agarose, but was detected in acrylamide gels.

Recently, Porombka et al. (49) proposed a rapid method for gene expression analysis of BDV in neurons and astrocytes using laser microdissection and real-time RT-PCR, which provides an effective tool for the analysis of cell-specific viral transcription efficiency and allows elucidating virus–host interactions and virus persistence mechanisms in the CNS.

Viral infection with BDV could enter in the heterogeneous list of environmental factors that may increase the risk of adult psychosis, influencing the susceptibility genes impact upon the molecular biology of the synapse. The genes may all converge functionally upon schizophrenia risk via an influence upon synaptic plasticity and the development and stabilization of cortical microcircuitry (50).

Mental illnesses usually arise from multiple interacting factors, such as genes, gene expression, viruses, toxins, nutrition, birth injury, and personal experiences (51). Although the BDV infection is not an alone predisposing factor by itself; the viruses had some impact on the way that the brain cells were functioning. It is important to highlight their ability to invade the cells and to produce changes in the genetic material. Both the positive association of the HLA-B*15 in patients with schizophrenia, schizoaffective disorder, and in their biological relative, and the negative association of HLA-B*35 in relatives without mental disorders could be interpreted with the increased risk or protection of developing the disease in response to infections, and other stressors during critical stages of brain development (52).

The increased serum interleukin-6 (IL-6) concentration in patients with schizophrenia, schizoaffective disorder, and in relatives with mood disorder than in relatives without mental disorder and controls (53) may constitute an activation of the inflammatory response system that could be related to a viral infection.

These findings suggest some involvement of the contribution of BDV in the pathogenesis of patients with schizophrenia, schizoaffective disorder, and in their biological relatives.

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