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Serum antibodies reactive with non-human primate retroviruses identified in acute onset schizophrenia

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Schizophrenia is a pervasive neuropsychiatric disease of uncertain etiology. Previous studies have postulated that retroviruses may contribute to the etiology of some cases of schizophrenia. We examined the possible relationship between retroviral infection and schizophrenia by measuring antibodies to a number of different primate retroviruses in the sera of individuals undergoing their first hospitalization for this disease. Sera from patients with first onset schizophrenia and matched healthy controls were analyzed by immunoblot and enzyme linked immunosorbent assays using purified retrovirus antigens to identify and quantify antibodies reactive with retrovirus proteins. A significantly increased incidence of antibodies reactive to *gag* encoded proteins of Mason-Pfizer monkey virus (MPMV), baboon endogenous virus (BaEV) and simian retrovirus type 5 (SRV-5) was observed in the sera of schizophrenia patients compared to controls. The reactivity of the cases and controls displayed the greatest differences in terms of antibodies to the proteins of Mason-Pfizer monkey virus. Employing an algorithm of enzyme linked immunosorbent assay reactivity followed by immunoblot confirmation, we found that MPMV antibodies in 28.9% of the individuals with first episode schizophrenia patients as compared to 3.7% of the unaffected controls ($P < 0.009$, Fisher's Exact Test). These studies are consistent with the occurrence of retrovirus replication in some individuals who are undergoing their first episode of schizophrenia. *Journal of NeuroVirology* (2000) **6,** 492–497.

Keywords: schizophrenia; retrovirus; gag; protein; ELISA; immunoblot

Introduction

Schizophrenia is a chronic, severe and complex neuropsychiatric disease afflicting 0.2–0.5% of the population worldwide (Yolken and Torry, 1995). Although the cause of schizophrenia is unknown, epidemiological studies have identified both environmental and genetic risk factors for disease acquisition (Torrey and Yolken, 2000). Season of birth, birth in an urban area, household crowding, and perinatal complications have been identified as environmental risk factors for developing this disease (Torrey *et al*, 1997). From a genetic standpoint, familial incidence studies have demonstrated significantly increased risk of disease in first degree relatives of patients with schizophrenia compared to normal controls (Gottesman, 1991) and in monozygotic twin pairs compared to dizygotic pairs (Torrey, 1992).

It has recently been proposed that the replication of retroviruses and other retroelements within affected individuals may explain some of the clinical and epidemiological features of schizophrenia (Yolken *et al*, 2000; Deb-Rinker *et al*, 1999; Hart *et al*, 1999; Kim *et al*, 1999; O'Reilly and Singh, 1996). In general, retroviruses can exist in one of two forms, (i) an endogenous provirus integrated into and replicating simultaneously with genomic DNA and (ii) an exogenous form capable of existing separately from the host cell. The human genome contains multiple copies of human endo-

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genous retroviruses (HERVs), some of which are transcriptionally active in normal and malignant cells (Patience *et al*, 1997), cofactor activation and disease pathogenesis may occur many years after initial provirus integration.

To investigate the potential etiological role of human retroviruses in schizophrenia, we took advantage of previous observations that interspecies mammalian retroviruses share numerous serological epitopes, particularly between their *gag* encoded proteins (Thiry *et al*, 1985; Goudsmit *et al*, 1986; Montelaro *et al*, 1988; Maruyama *et al*, 1989; Egberink *et al*, 1990; Garry *et al*, 1990). We thus hypothesized that antibodies against an unidentified human retrovirus, if present in individuals with schizophrenia, would cross-react with proteins encoded by non-human retroviruses. To test our hypothesis, sera of individuals obtained during their first episode of schizophrenia were assayed for antibodies to mammalian retroviruses by enzyme-linked immunosorbent assay (ELISA) to quantify total virus antibody levels and by immunoblot assay to identify antibodies against specific virus proteins. Antibody levels measured in the individuals with first episode schizophrenia were compared to those measured in a matched group of unaffected control individuals.

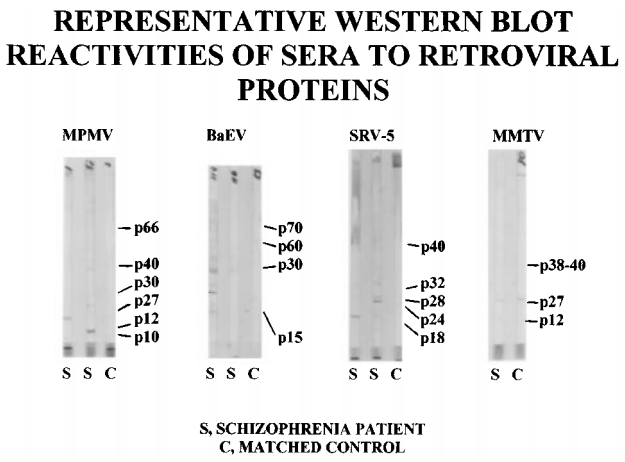

Figure 1 Representative immunoblot reactivities of schizophrenia and matched control sera. Retroviral proteins from MPMV, BaEV, SRV-5, and MMTV were resolved by SDS–PAGE, electroblotted to nitrocellulose, membrane strips reacted with sera from first-episode schizophrenia patients (S) or matched, healthy controls (C), and bound antibody detected with HRP-goat anti-human IgG antibody and TMB substrate. The positions of individual viral proteins are indicated on the right side of each strip reacted with the control serum. Note the presence of immunoreactive bands corresponding to MPMV p30, p27, and p12, BaEV p30 and p15, and SRV-5 p24 and p18 proteins in the selected sera from the individuals with schizophrenia.

Results

Retrovirus immunoblot reactivity of schizophrenia sera

Initial immunoblot assays were performed with a subset of 15 patients with first episode schizophrenia and 27 matched healthy controls. These samples were evaluated by immunoblot assay for antibodies to Mason-Pfizer monkey virus (MPMV), baboon endogenous virus (BaEV), simian retrovirus type 5 (SRV-5) and mouse mammary tumor virus (MMTV). Figure 1 shows representational immunoblot patterns of schizophrenia and control sera using these four viruses. Clear differences were seen in the reactivity profiles of sera from individuals with first episode schizophrenia compared to controls. Table 1 compiles the reactivity of sera with all *env* and *gag* encoded proteins identified on the immunoblot strips with polyclonal goat anti-virus serum. Statistically increased incidence of reactivity in the sera of individuals with schizophrenia as compared to control sera was observed with nine *gag* encoded proteins: MPMV p30, p27, p12 and p10, SRV-5 p28, p24 and p18, and BaEV p30 and p15. The greatest statistical differences between cases and controls were found in the rates of immunoreactivity to MPMV p27 and p12 proteins ($P<0.0005$).

In light of these initial findings, we performed more detailed analyses of antibodies to MPMV proteins. We employed both immunoassay and immunoblot measures to quantify antibody to MPMV proteins in the sera of 38 individuals with first episode schizophrenia and the 27 matched controls.

The results of immunoassay measurements for antibodies to MPMV are depicted in Figure 2. We found that, as a group, the individuals with first episode schizophrenia had increased levels of antibodies to MPMV as compared to the controls ($P<0.009$, Mann-Whitney *U*-Test). Definitive cut-off values for the determination of reactivity to MPMV in human sera have not been defined. We thus employed an arbitrary cut-off value of $A_{405}>=0.2$ and determined the number of sera in both groups which achieved this level of reactivity and which also reacted to at least two different bands to MPMV proteins on the immunoblot (Table 2). Using this algorithm, we found that 11 of 38 (28.9%) of individuals with first episode schizophrenia had antibodies to MPMV, while we found these antibodies in only one of 27 (3.7%) of the controls ($P<0.009$, Fisher's Exact Test, two-tailed).

Follow-up blood samples were also obtained from 10 of the individuals with schizophrenia approximately 1 year following their initial hospitalization. At this point in time the patients were taking neuroleptic medication and were clinically stable. As depicted in Table 3 four of these individuals were originally reactive to MPMV using the algorithm described above. Two of these

Table 1 Immunoblot assay reactivity of schizophrenia and control sera to retrovirus proteins.

Virus	*Virus protein (gene)*[a]	*% with band* *Cases* (*n*=15)	*Controls* (*n*=27)	*P value*[b]
MPMV	p40 (*gag*)	0.0	6.9	NS[c]
	p30 (*gag*)	46.7	10.3	$P<0.05$
	p27 (*gag*)	93.8	34.5	$P<0.0005$
	p12 (*gag*)	93.8	31.0	$P<0.0005$
	p10 (*gag*)	53.3	6.9	$P<0.02$
SRV-5	p40 (*gag*)	0.0	7.1	NS
	p32 (*gag*)	13.3	0.0	NS
	p28 (*gag*)	73.3	32.1	$P<0.02$
	p24 (*gag*)	26.7	0.0	$P<0.02$
	p22 (*gag*)	0.0	10.7	NS
	p18 (*gag*)	53.3	10.7	$P<0.02$
	p14 (*gag*)	6.7	0.0	NS
BaEV	gp180 (*env*)	6.7	0.0	NS
	gp70 (*env*)	0.0	6.9	NS
	p60 (*gag*)	0.0	17.2	NS
	p30 (*gag*)	60.0	13.8	$P<0.02$
	p15 (*gag*)	20.0	0.0	$P<0.05$
MMTV	p38–40 (*gag*)	13.3	7.1	NS
	p27 (*gag*)	48.7	14.3	NS
	p12 (*gag*)	0.0	3.6	NS

[a]Virus proteins were identified by comparison to immunoblot strips reacted with virus specific goat antisera and co-migration of molecular weight markers. Only virus proteins reactive with the sera tested are shown. Other proteins present on immunoblots but nonreactive were MPMV gp70 (*env*), p66 (*gag*) and p24 (*gag*), SRV-5 gp70 (*env*), BaEV p6 (*gag*) and MMTV gp52 (*env*). [b]Two-tailed Fisher's exact test. [c]NS, not significant ($P>0.05$).

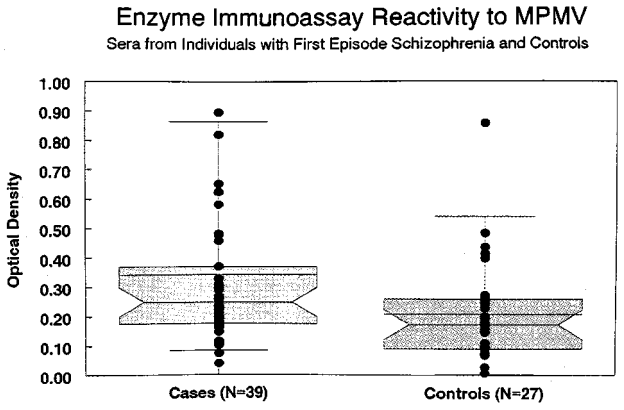

Figure 2 Comparison of schizophrenia case and matched control sera for MPMV ELISA reactivity. MPMV proteins were adsorbed to microtiter wells, reacted with sera from first-episode schizophrenia patients (cases) or matched, healthy controls, and bound antibody detected with HRP-goat anti-human IgG antibody and ABTS substrate, as described in the text. Optical density values (A_{405}) for individual samples from both groups are designated by solid circles. The bottom and top boundaries of the boxes indicate the 25th and 75th percentiles of the values. The bottom and top horizontal bars indicate the 5th and 95th percentiles of the values, respectively. The notch indicates the 95 confidence limits around the median.

patients (no. 2 and no. 9) showed evidence of reversion to seronegative status at follow-up. The additional two patients (no. 8 and no. 10) while still seroactive, had lower anti-MPMV optical density values at follow-up as compared to their values on their original hospital admission.

Discussion

The data presented in this study demonstrate that some individuals undergoing their first episode of schizophrenia have increased levels of antibodies directed at non-human primate retrovirus proteins, as compared to a matched control group. Immunoblot assays indicated that most of the antibodies were reactive with *gag* encoded gene products and that there was little evidence of reactivity to *env* encoded proteins. This specificity suggests that these antibodies probably did not arise from infection with primate viruses used for the assays, but rather from cross-reactive viral epitopes (Lee *et al*, 1984). Our findings are consistent with the results of Hart *et al* (1999) who reported a significantly increased percentage of sera with antibodies reactive to at least one HIV virus gag protein in individuals with schizophrenia as compared to unaffected controls. As in the case of our study, most of the anti-retroviral antibodies detected in their study were directed at *gag* encoded proteins. The analysis of additional viral peptides might further clarify the antigenic stimulus for the antibodies. In addition, use of standard reagents and the determination of specific cut-offs for reactivity would allow for the comparison of reactivity between different study populations.

The specific viruses or viral proteins which served as the antigenic stimulus for the antibodies to the *gag*-encoded proteins of MPMV and other non-human primates measured in the sera of some individuals with first episode schizophrenia is

Table 2 Comparison of schizophrenia and control sera by MPMV ELISA and immunoblot assay reactivities.

Parameter evaluated	*No. (%) reactive* *Cases* (*n*=38)	*Controls* (*n*=27)	P *value*[a]
ELISA reactive (arbitrary A_{405} cut-off[b])	25 (65.8%)	10 (37.0%)	$P<0.027$
ELISA+immunoblot reactive[c]	11 (28.9%)	1 (3.7%)	$P<0.009$

[a]Two-tailed Fisher's Exact Test. [b]MPMV ELISA with arbitrary cut-off A_{405}=0.2. [c]MPMV immunoblot with two or more bands.

Table 3 Correlation of MPMV antibodies with acute and residual phase schizophrenia.

Patient no.	*Initial sample* A_{405}[a]	# *Bands*[b]	*Inter.*[c]	*Follow-up sample* A_{405}[a]	# *Bands*[b]	*Inter.*[c]
1	0.300	0	Neg.	0.372	0	Neg.
2	0.225	2	Pos.	0.179	2	Neg.
3	0.308	1	Neg.	0.456	1	Neg.
4	0.101	0	Neg.	0.117	2	Neg.
5	0.164	1	Neg.	0.124	0	Neg.
6	0.107	3	Neg.	0.195	3	Neg.
7	0.231	1	Neg.	0.275	1	Neg.
8	1.839	2	Pos.	0.968	2	Pos.
9	0.895	2	Pos.	0.173	2	Neg.
10	0.235	2	Pos.	0.203	2	Pos.

[a]ELISA absorbence at 405 nm. [b]Number of immunoblot bands. [c]Interpretation of combined MPMV ELISA and immunoblot results; ELISA $A_{405} \geqslant 0.2$ and 2 or more immunoblot bands.

currently unknown. Similarly, since many of the individuals had symptoms of schizophrenia for several months prior to their first hospitalization and the obtaining of the first blood sample, the timing of the development of antibodies and the onset of symptoms could not be precisely defined. It is unlikely, however, that the development of antibodies was an artifact of hospitalization or exposure to medication since serum samples were collected early in the course of the patient's first hospitalization and the patients had not received antipsychotic medication prior to their initial hospitalization. Similarly the patients did not have a history of prior medical disease or substance abuse, rendering it unlikely that these factors contributed to their increased rate of seroreactivity to primate retroviruses measured in the individuals with recent onset schizophrenia. It is of note that the testing of follow-up samples obtained from two individuals who were seropositive to MPMV during their first hospitalization showed reversion to a seronegative status (Table 3). This finding indicates that the rate of MPMV seropositivity in schizophrenic patients may be higher during their first episode than later in the course of the disease.

Our data document that many individuals undergoing their first episode of schizophrenia have a significantly increased rate of antibodies directed at MPMV and related primate retroviruses. Our findings are consistent with recent findings of increased levels of retroviral sequences in the brains (Yolken *et al*, 1999), cerebrospinal fluids (Karlsson *et al*, 1999), and lymphocytes (Deb-Rinker *et al*, 1999) of individuals with schizophrenia. At this point, it is not known if the occurrence of increased levels of retroviral antibodies or nucleic acids in individuals with schizophrenia represents a cause or consequence of the disease state. Ongoing studies are directed at examining; (i) the seroprevalence of antibodies to other mammalian retroviruses in patients with first onset schizophrenia; (ii) the prevalence of antibodies to primate retroviruses in larger groups of geographically diverse individuals with schizophrenia and related neuropsychiatric diseases and controls; (iii) the sequential analysis of individuals with schizophrenia during different stages of their disease and undergoing different types of anti-psychotic therapy, and (iv) disease-associated interactions between retroviruses and other potential virus activating cofactors. The definition of a relationship between retroviral replication and schizophrenia may lead to an increased understanding of the etiopathogenesis of schizophrenia as well as to novel methods for the diagnosis and treatment of this devastating disease.

Materials and methods

Study populations

The case population consisted of a total 38 individuals with a first episode of schizophrenia, schizophreniform disorder, or schizoaffective disorder who were admitted to the Department of Psychiatry of the University of Heidelberg in 1998 or 1999. Clinical diagnoses were established by using the Structured Clinical Interview for DSM-IV.

The median age of the patients was 27 years (range 18–48 years). None of the patients had evidence of immunodeficiency or other immunological abnormalities on admission, nor did they have a history of medical or neurological disease or substance abuse. None of the patients had received neuroleptic medications prior to their admission to the hospital.

Control sera were obtained from 27 unaffected individuals matched for age, gender, ethnicity, geographic region, and socioeconomic status. The study was approved by the Ethical Committee of the University of Heidelberg.

Patients' samples were obtained by venipuncture at a mean of 16 ± 10.2 days following their first admission to the hospital. Serum was separated from whole blood shortly after collection and stored at -80°C until testing. All sera were tested and found negative for antibodies to HIV-1 and HTLV-I by enzyme immunoassay. Follow-up blood samples were available from 10 of the 38 patients. These samples were obtained a mean of 13.2 ± 1.4 months after the initial hospital admission. At this point in time all of the individuals were on treatment regimens and were clinically stable.

A subset of samples was subjected to the initial immunoblot analyses for the measurement of antibodies to MPMV, BaEV, SRV-5, and MMTV. This subset consisted of the first 15 patients admitted to the study for whom the diagnosis of schizophrenia or schizophreniform or schizoaffective disorder was confirmed. The entire set of 38 patients was subjected to the detailed enzyme immunoassay and immunoblot testing for antibodies to MPMV.

Retrovirus antigens

MPMV, BaEV and MMTV (strain 34-1R) were obtained from Quality Biotech (Camden, NJ, USA). SRV-5 was obtained from the Davis Primate Center (Davis, CA, USA). All viruses were purified from cell-free supernatants by the manufacturer by sucrose density gradient centrifugation and supplied as extracts in 1.0% Triton X-100.

Enzyme-linked immunosorbent assay

Triton X-100 extracted proteins were resuspended at 10 μg/ml in 50 mM sodium carbonate (pH 9.6) and added in 100 μl aliquots to individual wells of flat-bottom 96-well microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA, USA). Following incubation overnight at 4°, the wells were aspirated and blocked with 100 μl of 1% bovine serum albumin (BSA, Sigma, St. Louis, MO, USA) and 1% sucrose for 1 h at room temperature and washed three times with 1.5 mM imidazole, pH 7.4 (Sigma) containing 37.8 mM NaCl and 0.025% Tween 20 (PBST). Serum samples were diluted 1/100 in 50 mM phosphate buffered saline (PBS), pH 7.2 containing 0.3% Triton X-100 (Sigma), 0.2% Tween 80 (Sigma) and 1.0% BSA (MB buffer) and 100 μl/well incubated for 1 h at room temperature. The wells were washed three times with PBST and bound antibody was reacted with 100 μl of horseradish peroxidase (HRP)-conjugated goat anti-human IgG antibody (H+L, KPL, Gaithersburg, MD, USA) diluted 1/3,000 in MB buffer for 1 h at room temperature. The wells were again washed three times with PBST and 100 μl of peroxidase substrate 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (BioFX Laboratories, Inc., Randallstown, MD, USA) was added for 30 min. The reaction stopped with 100 μl of 1% sodium dodecyl sulfate (SDS) and the absorbance at 405 nm (A_{405}) quantified by an automated microtiter plate reader.

Immunoblot assay

Triton X-100 extracted proteins were resuspended at 10 μg/ml (MPMV, BaEV, MMTV) or 20 μg/ml (SRV-5) in 0.25 M Tris (hydroxymethyl)amino methane, pH 8.5 containing 1.1 M glycerol, 73 mM lithium dodecyl sulfate, 0.5 mM (ethylenedinitrilo)tetraacetic acid and 0.1% dithiothreitol (Invitrogen, Carlsbad, CA, USA), heated at 70°C for 10 min and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) on NuPAGE® 4–12% gradient Bis-Tris gels using 3-(N-morpholino)propanesulfonic acid running buffer (Invitrogen, Carlsbad, CA, USA). Resolved proteins were electrophoretically transferred to nitrocellulose membrane (0.45 μm, Schleicher and Schuell, Keene, NH, USA) in 25 mM Tris-HCl, pH 8.3, 0.192 M glycine and 20% methanol as described (Towbin *et al*, 1979), the membranes blocked with 5% non-fat dry milk in PBS and cut into 3 mm strips (0.3 or 0.6 μg virus protein per strip). Strips were incubated overnight at room temperature with constant agitation in 1.0 ml of serum samples diluted 1/100 in 5% milk-MB buffer, washed three times with 1.5 mM imidazole, pH 7.4 containing 37.8 mM NaCl and 0.025% Tween 20 and bound antibody reacted with 1.0 ml of HRP-goat anti-human IgG antibody (H+L, KPL) diluted 1/4,000 in 5% milk-MB buffer for 1 h at room temperature. Strips were again washed three times and developed with 1.0 ml of 3,3′,5,5′-tetramethylbenzidine dihydrochloride membrane substrate (BioFx Laboratories, Inc.). Virus proteins were identified by comparison to immunoblot strips reacted with virus specific goat polyclonal antisera and co-migration of prestained protein molecular weight markers (Invitrogen).

Statistical analyses

Differences in reactivity to retrovirus proteins between sera of patients with schizophrenia and matched controls were tested by Mann-Whitney *U*-test and Fisher's two-tailed Exact Test and considered significant at $P < 0.05$.

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