Abstract. Background/Aim: The etiology of autism spectrum disorders (ASD) still eludes investigators. Several viral infections have been associated with ASD etiopathogenesis but few studies have ever focused on the role of HHV-6 and HHV-8, two members of the herpesviridae family. The aim of the present study was to evaluate seropositivity rate and levels of antibodies to HHV6 and HHV-8 in children with ASD compared to controls. Patients and Methods: We measured and compared seropositivity rate and levels of antibodies to HHV6 and HHV-8 in 30 children with ASD (14 with autistic disorder and 16 with non-autistic disorder ASD) and in 28 healthy controls of the same age. Results: Seropositivity rate and levels of the two antibodies were similar in cases and controls. Seropositivity rate and levels of antibodies were not correlated with disease severity in children with ASD. Conclusion: Levels and seropositivity rate of antibodies to HHV-6 and HHV-8 do not differ between children with ASD and controls.

Autism spectrum disorders (ASD) are neuropsychiatric disorders characterized by significantly abnormal or deficient social interaction, communication abilities and language and considerable narrow pattern of activities and interests (1). Symptoms start before three years of age with evidence of abnormal cognitive functioning, learning, attention, and sensory processing (2). Autism spectrum disorders can be associated with numerous comorbidities such as mental retardation, epilepsy, aggression and self-hurting behavior. Behavioral interventions and psychopharmacological treatment are now the mainstay of treatment of ASD (3). One of the most interesting aspects of pervasive disorders is the significant change in terms of epidemiology reported in the past two decades. The incidence of ASD has dramatically increased in recent years and it now affects 1 out of 88 newborns (4). There is a well-defined higher prevalence in boys, namely 1:51 in boys vs. 1:252 in girls (4). However, it remains to be established whether these findings represent a real epidemic of ASD (5), a simple effect of improved diagnostic means, or a combination of both. The exact etiology of ASD is unknown, although specific interactions between genetic and environmental factors have been implicated in the condition (6). A family study provides significant evidence in favor of the genetic component of susceptibility to ASD. In fact, an estimated heritability of 37% has been reported for ASD (7).

Approximately 10% of patients with ASD are reported to be affected by syndromic autism, due to known genetic disorders such as Mendelian diseases or monogenic disorders [e.g. fragile X syndrome, type 1 neurofibromatosis, tuberous sclerosis, Down syndrome, etc. (8)], extended chromosomal re-arrangements, specific prenatal teratological agents, or exposure to drugs, such as thalidomide, misoprostol, and valproic acid (9-13). The remaining 90% of patients suffer from non-syndromic or idiopathic autism. In these cases, it is thought that the etiology lies in the interaction of multiple genes with one or more environmental factors (6, 14).

Altered neurodevelopment during the first and second trimester of prenatal life has been recognized as the underlying neuropathological cause of ASD in most patients (15). On the contrary, Rogers depicts autism as a disorder with gradual onset whose core etiopathogenetic mechanisms may develop during the first two to three years of life (16).

Among the environmental factors thought to be involved in the genesis of ASD, it has been proposed that infections, especially viral infections, may be able to trigger the disorder (17, 18). Vaccinations have also been implicated in ASD
etiology, but this theory has been refuted (19). Several reports have linked viral infections to ASD, mainly herpesviridae [herpes simplex (HSV), cytomegalovirus (CMV) (10), varicella zoster (VZV) (13, 20-30)], mumps (31), influenza (32), lymphocytic choriomeningitis (33) and polyomaviruses (34, 35).

The aim of our study was to evaluate the seropositivity rate and antibody titers against Human Herpes Virus-6 and Human Herpes Virus-8 in a cohort of children with ASD compared to a cohort of healthy same-age controls.

**Patients and Methods**

**Patients.** Patients admitted as in- or out-patients to the Child and Adolescent Neuropsychiatry Unit at the Second University of Naples and to the Department of Pediatrics of the Federico II University of Naples, Italy, were recruited between January 2010 and June 2011. The children’s parents/legal guardians supplied consent forms and the Ethics Committee of the Federico II University of Naples approved the study (protocol number: 85/09). Inclusion criteria for cases were diagnosis of ASD according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth edition, Text Revision (DSM-IV-TR) (1) and informed consent signed by parents/guardians; the only exclusion criterion was the inability to sign an informed consent form.

Controls were enrolled at the Division of Pediatric Surgery where they were admitted for minor surgical treatments (e.g. phimosis, hernia, cryptorchidism, vesicoureteral reflux,hydrocele testis, etc.). They underwent an interview to rule out possible ASD and, if such a disorder was identified, the affected children were excluded from the study.

To authenticate the diagnosis of autism, cases were administered the Autism Diagnostic Interview, revised version (ADI-R) (23), and the Autism Diagnostic Observation Schedule (ADOS)-Generic (38). The Childhood Autism Rating Scales (CARS) (37) and the Autism Diagnostic Interview, revised version (ADI-R) (36), the Autism Diagnostic Interview, revised version (ADI-R) (36), the
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**Viologous tests.** IgG antibodies against HHV-6 and HHV-8 were detected using an enzyme-linked immunosorbent assay (ELISA) technique (indirect ELISA for HHV-6 IgG and indirect ELISA for KSHV/HHV-8 IgG; Advanced Biotechnologies Inc. Columbia, MD, USA). In detail, to detect specific antibodies, 8-well strips were coated with optimal amounts of solubilized KSHV/HHV-8 whole-virus extracts or HHV-6 antigen. Three incubation steps were needed: a) tested human samples were added to the microtiter wells and any specific antibodies to HHV-6 or HHV-8 present in the sample bind to the antigen-coated plates, thereby forming immunological complexes. After the incubation, the strips were washed to remove unbound sample components; b) anti-human IgGs conjugated to horseradish peroxidase (HRP) were added to the wells and during incubation combined with the antibody-antigen complexes already present in the wells. After incubation, the wells were washed to remove unbound conjugate; c) HRP substrate tetramethylbenzidine was added to the wells. During incubation, enzyme-mediated cleavage of the substrate resulted in color change. A stop reagent was added, after which, the wells with positive samples changed color with an intensity proportional to the levels of antibodies, which was measured spectrophotometrically. To obtain a suitable cut-off value, we averaged the readings of three negative control wells and multiplied the result by 3 (HHV-8) or 2 (HHV-6), according to the instructions provided by the manufacturer.

We calculated the optical density (O.D.) ratios by dividing the reading of each sample well by the cutoff value. O.D. ratios ≤0.75 correspond to negative samples, O.D. ratios ≥1.00 correspond to positive samples, while O.D. ratios between 0.75-1 are considered borderline.

**Statistical analysis.** For quantitative variables, the Kolmogorov-Smirnov test was used to check for Gaussian distribution. In this case, data are given as mean±standard deviation (SD) and the Student’s t-test for unpaired variables was applied for comparisons. In cases of non-Gaussian distribution, data are reported as the median and interquartile range (IQR) and the Mann-Whitney U-test was used for comparisons. To compare quantitative data among more than two groups [controls, autistic disorder (AD), and non-AD ASD], comparisons were performed using the ANOVA or Kruskal-Wallis test, in case of Gaussian or non-Gaussian distribution respectively. As age may significantly impact on antibody titer, a generalized linear model (GLM) was used to correct antibody titer for age and sex. The \( \chi^2 \) test with Yates correction (or Fisher’s exact test where appropriate) was used for categorical variables. Spearman’s rho test was used for correlations between continuous variables. A p-value of 5% or less in the two-sided test was considered statistically significant. Logistic regression analysis was used to correct seropositivity rate for age and sex. All statistical analyses were carried out using the Statistical Package for the Social Sciences, version 18.0 (SPSS Inc. Chicago, IL, USA).

**Results**

We enrolled 58 children in the study, 30 with ASD (14 with AD and 16 with non-AD ASD) and 28 controls. None of the controls was found to be affected by a neuropsychiatric disorder. The median age was 5.83 years for cases (IQR=4.16-8.42 years) and 5.88 for controls (IQR=3.16-8.40 years) \( (p=0.798) \). Males outnumbered females, both among cases (26/30, 86.7%) and controls (25/28, 89.3%, \( p=0.999 \)).

We assessed the rate of seropositivity for the two human herpesviruses (HHVs) in cases and controls. As shown in Table I, the rate of seropositivity was similar in cases and controls \( (p=0.999 \text{ both for HHV-6 and HHV-8}) \). Logistic regression analysis showed that neither age nor gender affected the seropositivity status in relation to the two antibodies. We measured and compared O.D. ratios for HHV-6 and HHV-8 in cases and controls. Respective OD ratios are reported in Table II. Although O.D. ratios cannot be considered strictly titers, the data obtained are indicative of antibody concentration. As shown in Table II, the OD ratios of antibodies against the two HHVs did not differ between cases and controls. The GLM revealed that neither age nor gender affected the level of the two antibodies.

We also evaluated the levels and seropositivity rate of the two antibodies in three specific categories, children with AD, children with non-AD ASD and controls. As shown in Table II, neither the seropositivity rate for the two viruses nor antibody titers for HHV-8 differed significantly among the
three groups, whereas antibody titers for HHV-6 differed significantly between children with AD and those with non-AD ASD. At multivariate analysis (GLM), no variable was independently associated with anti-HHV-6 OD. In detail, p-values for age, sex and AD condition vs. non-AD ASD condition were 0.704, 0.095, 0.358, respectively. Therefore, neither antibody level nor seropositivity rate was associated with ASD status.

Discussion

Viruses of the herpesviridae family have several characteristics that make them an intriguing topic of study and research. In fact, they employ a plethora of mechanisms to circumvent clearance by the host immune system. They are able to cause persistent infection, and are characterized by frequent reactivations during the host’s life. According to Stack et al. (39), HHVs are able to activate immune inhibitory pathways that control autoimmunity and limit excessive responses to harmless antigen in mucosal surfaces. In this way, they can antagonize T-cell responses both during acute infection and during persistence in mucosal tissues by dampening anti-viral immunity and promoting virus survival and dissemination.

HHVs have also been implicated in several disorders, especially in autoimmune diseases, because they are known to act as triggers of immune deregulation. For instance, similar events are thought to be involved in the genesis of multiple sclerosis (40, 41), rheumatoid arthritis (42), autoimmune thrombocytopenia (43) and systemic lupus erythematosus (44, 45). Many theories, ranging from direct cytolysis of target tissue cells to such immunological processes as antigen mimicry and polyclonal lymphocyte activation have been invoked to explain the correlation between HHV infections and these autoimmune disorders. However, the exact mechanisms involved continue to elude investigators.

ASD is widely described as being a disorder of immune deregulation (18, 46-50) that, in an early or late stage of brain development, causes neurological damage in one or more areas of the brain. Numerous studies focused on virus infection, as a specific co-factor in the triggering of this deranged immune response in ASD pathogenesis (17, 18, 31, 33, 34, 51-54). Several members of herpesviridae family, i.e., HSV (20, 55-58), CMV (13, 21-30, 59, 60) and VZV (61), have been reported to be associated with ASD, based on clinical studies or case series. Indeed, HHVs can trigger the production of a variety of pro-inflammatory cytokines during the acute phase of infection (62-64), as demonstrated by high levels of interferon in brain tissue during HSV-induced encephalitis (65). Despite its high prevalence, studies of HHV-6 in ASD are scarce (66-68). Yet, this virus is closely associated with a complex immune deregulation condition called drug-induced hypersensitivity syndrome (69-71). This disorder is an infectious disease due to HHV-6, an allergic systemic reaction to medicines and a complex event of deregulation of the immune system (69).
Although exanthema subitum is the most common manifestation of HHV-6 infection, this virus is known as a neurotropic virus (72-74) and can result in prolonged, recurrent seizures and meningitis in a minority of children. It can also cause encephalitis in otherwise healthy children. These children exhibit an altered level of consciousness, seizures, psychosis, or cranial nerve deficits (75-77), and a case of developmental regression ensuing HHV-6 encephalitis has been described (78). Bozola et al. reported three cases of HHV-6 meningoencephalitis in previously healthy children followed-up for 10 years. Two of the patients presented invalidating sequelae (79). In detail, one patient developed speech disturbance and the other persistent hemiplegia and bilateral visual deficit. The authors concluded that HHV-6 meningoencephalitis could be associated with a wide range of clinical outcomes, from long-term neurological sequelae to a benign post-infectious clinical course (79). Binstock hypothesized that several ASD subgroups could derive from intra-monocyte chronic infections with pathogens such as measles virus, CMV or HHV-6 (80).

Two studies that evaluated the prevalence or titer of antibodies to HHV-6 in serum of patients with ASD vs. controls (66, 68), did not show significant differences between the two groups. Results for serological association between HHV-6 and brain autoantibodies were conflicting. In the first study, Singh et al. found higher rates of antibodies to basic myelin protein and neuron-axon filament protein in ASD (84% of ASD cases with serum positive for HHV-6-IgG were also positive for auto-antibodies to myelin basic protein and 72% of HHV-6-IgG-positive were also positive for anti-neuron–axon filament protein) (66). On the contrary, the second article showed no serological association for HHV-6 and several brain auto-antibodies (beta-amyloid protein, brain stem; cerebral cortex; cerebellum; caudate nucleus; 2',3'-cyclic nucleotide phosphohydrolase; galactocerebroside; glial filament acidic protein; hippocampus; myelin basic protein; neuron-axon filament protein; S100 protein) (68).

Similarly to these studies, we found a comparable prevalence and levels of antibodies to HHV-6 between cases and controls. Moreover, we first looked for an association between rate of positivity or antibody levels and severity of the diseases assessed with severity scales. We found no difference between more severe and milder forms of ASD. In fact, neither the seropositivity rate nor antibody titers for HHV-6 differed significantly between children with AD and those with non-AD ASD. In fact, the finding of higher levels of antibodies to HHV-6 observed at univariate analysis in non-AD ASD versus AD was not confirmed at multivariate analysis. Taken together, these results do not definitively rule out a role of HHV-6 in ASD pathogenesis because we cannot exclude that a genetic and environmental predisposition may make these children more vulnerable to the virus (18). However, our data fail to provide any evidence of such an association.

No study has yet assessed whether HHV-8 is associated with ASD. No study evaluated its possible role in the onset of ASD, although it belongs to the herpesviridae family and shares with the other members of this family specific immunopathogenetic mechanisms regarding persistence and triggering of immune disorders. In particular, a study on the contribution of innate immunity to immune surveillance of this virus revealed substantial alterations of the Natural Killer cell receptor repertoire in healthy HHV-8 carriers and these observations are consistent with distinct immune evasion mechanisms that allow HHV-8 to elude Natural Killer cell responses in a stepwise fashion, first at the early stages of infection to facilitate the maintenance of viral latency, and later to promote tumor cell growth (81). Our study demonstrates that HHV-8 exposure is minimal among children regardless of their health status. This prevalence (1 out of 58 children) reflects data on HHV-8 prevalence (82, 83) which is reported to increase with age (84, 85).

In conclusion, our study shows that the rate of previous infections with HHV-8 is minimal in both healthy children and those with ASD and that the rate of HHV-6 exposure is not dissimilar between these children, nor it is related to the severity of ASD. New strategies, including the simultaneous study of co-factors implicated in ASD (18) and the use of animal models of ASD are needed to elucidate the role (if any) of HHV-6 infection in the pathogenesis of ASD.

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References


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