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Lymphocyte Function in Autism and Rett Syndrome

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Abstract
Peripheral blood lymphocytes from 17 patients with autism were separated on a Ficoll-Hypaque density gradient. Patients had normal numbers of T and B cells and T cell subsets. Although CD4:CD8 ratios were normal for the whole group (2.09 ± 0.97), 6 patients had elevated ratios (>2.2) and 5 had decreased ratios (<1.5). Mitogen-induced proliferation (concanavalin-A and phytohemagglutinin) was normal as was the autologous mixed lymphocyte reaction for the whole group. There was an abnormally increased percentage of DR+ (activated) T lymphocytes in 11 patients. With increasing age percentage of DR+ lymphocytes decreased. No patient had interleukin-2 (IL-2) receptor+ cells. Similar investigations performed on blood samples from 8 girls with Rett syndrome produced normal results. 11 of 17 autistic patients had an abnormally increased percentage of DR+ but not IL-2 receptor+ lymphocytes suggesting 'incomplete' activation, a finding which is seen in autoimmune diseases. The decrease in activated cells with increasing age suggests that there may be an autoimmune process which is more active earlier in life in a subset of autistics.

Key Words
Autism
Lymphocytes
Neuroimmunology
Rett syndrome

Introduction

Autism is a syndrome characterized by social and communicative deficits of early onset accompanied by abnormal behaviors. There are many biomedical causes underlying autistic symptomatology [1]. However, in the majority of autistic patients no clear etiology has been ascertained.

Immune system abnormalities have been associated with autism. These have included inhibition of macrophage migration in response to human myelin basic protein [2], reduced mitogen-induced lymphocyte blastogenesis [3, 4], decreased numbers of T lymphocytes with altered ratios of helper to suppressor T cells [4], decreased helper T cell and B cell numbers [5], deficiency of suppressor-inducer T cells [6], decreased natural killer cell activity [7], and the demonstration of circulating antibodies to serotonin receptors [8]. In another study of 14 patients with autism, 8 had an abnormal lymphocyte proliferative response to mitogens and to autologous lymphocytes and monocytes [9].

This study was undertaken in an attempt to help to better define immune system abnormalities in autism.

For comparison, girls with Rett syndrome were also investigated. In Rett syndrome, following normal early development, there is cognitive and functional decline frequently associated with autistic symptomatology [10, 11]. It is possible that cellular immune system abnormali-

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ties may underlie or be associated with the neurologic regression seen in Rett syndrome. It appears that a thorough immune system investigation of Rett syndromes has not been previously undertaken.

Materials and Methods

Blood samples were obtained from a total of 17 patients with autism. There were 16 males and 1 female. The age range was from 8 to 23 years with a mean age of 17. The diagnosis of autism conformed to the DSM-III-R criteria. There were no identified biomedical causes of autism in any of the studied population. Parental signed consent was obtained prior to phlebotomy. This study was approved by ethics review committees. Simultaneously drawn blood samples from healthy young adults served as controls for the lymphocyte stimulation studies.

Blood samples were obtained from 8 Rett syndrome girls with an age range of 2–15 years and a mean age of 8. The heparinized blood samples were obtained at 8 different geographic areas in the United States and courier-delivered to the laboratory for analysis. In all cases, a blood sample from a healthy young adult accompanied the Rett syndrome sample, in the same package, to control for handling and shipping differences. In all cases, blood samples were received and processed less than 24 h from the time when they were drawn.

Lymphocyte Proliferation

Peripheral blood lymphocytes (PBL) were isolated on a Ficoll-Hypaque density gradient [12]. After washing, 200 µl of cells were plated at a concentration of 5×10^5 cells/ml in 96-well microplates in RPMI-1640 medium with 10% fetal calf serum and *L*-glutamine. Triplicate wells were cultured in the presence or absence of phytohemagglutinin (PHA; 1 and 10 µg/ml), and concanavalin A (Con A; 5 and 50 µg/ml) at 37°C in 5% CO_2. After 78 h, 1 µCi/well of ^{3}H-thymidine was added and cells were cultured for a further 18 h prior to harvesting with a MASH apparatus (Microbiological Associates, Walkersville, Md., USA) and counted using a Beckman liquid scintillation counter (No. 3801). Mean absolute counts of triplicate cultures were determined. A stimulation index was calculated using the following formula: mean stimulated count/mean unstimulated count.

Autologous mixed lymphocyte reactions (AMLR) were carried out between 10^5 responder PBL and 10^5 irradiated (3,000 rad) stimulator PBL in a volume of 200 µl. These were cultured in complete medium at 37°C in 5% CO_2 for 7 days. ^{3}H-thymidine was added 18 h prior to harvesting. Proliferation was measured as above.

Lymphocyte Surface Phenotype

PBL cell surface phenotype was determined by indirect immunofluorescence. Briefly, 10^6 isolated PBL were incubated with saturating amounts of murine monoclonal antibody. After washing, cells were then incubated with FITC-conjugated goat anti-mouse, isotype-specific immunoglobulin. The percentage of fluorescent-positive cells was determined from a 2-parameter analysis of at least 10^4 lymphocytes on a gated lymphocyte population. The green fluorescence intensity was detected at 488 nm with a laser power of 500 mW using a Coulter Epics V flow cytometer (Coulter Electronics, Hialeah, Fla., USA).

Murine monoclonal antibodies recognizing CD3 (pan T cell marker), CD4 (helper/inducer T cell marker), CD8 (suppressor/cytotoxic T cell marker), CD20 (pan B cell marker) and anti-HLA DR marker were obtained from Coulter Immunology, while CD25 [anti-interleukin-2 (IL-2) receptor] was obtained from Becton-Dickinson (Mississauga, Canada).

Lymphocyte surface phenotype normative data from the Hospital for Sick Children immunology laboratories were used for comparison. In addition, blood samples from 12 healthy young adults were analyzed for lymphocyte surface phenotypes.

Statistics

Student's t test was used for statistical analysis. Values are shown as mean ± standard deviation (SD).

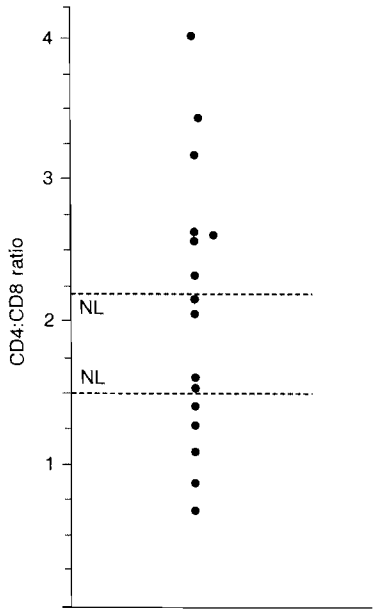

Fig. 1. CD4:CD8 ratio in patients with autism. Normative values (NL) are indicated. Only 4 values are within normal limits. The CD4:CD8 distributions cluster into those >2 and <2.

Results

Lymphocyte Surface Phenotype

Autistics had normal numbers and percentages of T and B cells and CD4+ and CD8+ cell subsets. However, the CD4:CD8 ratios were normal in only 4 autistics (fig. 1) while in 6 patients elevated ratios were seen (>2.2) and 5 patients had decreased ratios (<1.5). Although the mean value of 2.09 ± 0.97 was within normal limits, there was a clustering of the CD4:CD8 ratios into two general groups: those >2 and <2.

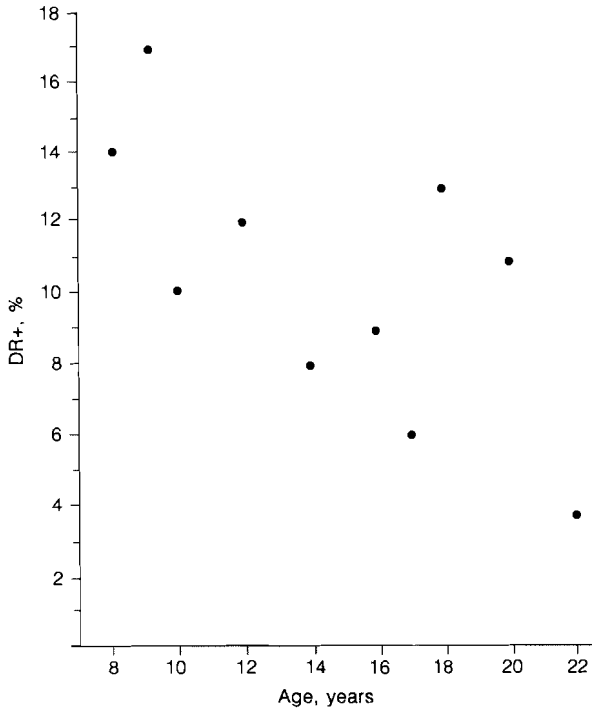

Fig. 2. DR+ percentage plotted against age for those patients with autism who had DR+ lymphocytes. The DR+ percentages are all elevated (normal <2%). There is a decrease in DR+ percentage with age.

There was an increased number of DR+ (activated) T cells in patients with autism (normal <2%; fig. 2). For the group as a whole, the percentage of DR+ T lymphocytes was 7.73 ± 5.75. Within the DR+ group (11 patients) there was a decrease in percentage of DR+ cells with increasing age (fig. 2). No patient demonstrated any IL-2-receptor-positive T lymphocytes.

Lymphocyte surface phenotype results were normal in the Rett syndrome patients and in the studied controls.

Lymphocyte Proliferative Response

Mitogen-induced proliferation to both Con A (5 and 50 μg/ml) and PHA (1 and 10 μg/ml) in patients with autism did not differ from controls or from patients with Rett syndrome.

The AMLR was within normal limits when the group was examined as a whole. However, there was a significantly decreased AMLR (8,630 ± 3,045; c.p.m. ± SD) for the 5 patients with CD4:CD8 <1.5 as compared to the 9 patients with a normal or elevated CD4:CD8 (15,213 ± 7,700; $p < 0.05$). In contrast, when patients were divided by numbers of DR+ (activated) T cells into normal numbers of DR+ T cells and increased number of DR+ T cells, there was no difference in AMLR (12,887 ± 4,351 vs. 12,863 ± 8,181). The AMLR response in the Rett syndrome patients did not differ from controls.

There was no correlation between any of the results and medication intake (one patient was taking each of the following medications: carbamazepine, haloperidol, methylphenidate and pimozide; two were taking thioridazine).

Discussion

Autism is a clinically defined syndrome characterized by social and communicative deficits accompanied by abnormal behaviors. Although there are many biomedical causes of autism, the majority of affected patients do not have a defined etiology. It is possible that an autoimmune process may be pathogenically involved in a subset of autistics as previous studies have demonstrated a variety of immune abnormalities in autism. To better define these abnormalities we examined circulating lymphocyte numbers and function. In contrast to previous reports which demonstrated decreased total T and CD4+ lymphocytes [4, 5], we found normal numbers of T cells and T cell subsets (both CD4+ and CD8+ cells). This discrepancy may be the result of different methods since we used rosetting and complement depletion. In our study we demonstrated a normal mean CD4:CD8 ratio for the overall population but only 4 patients actually had CD4:CD8 within normal limits. Six patients had increased CD4:CD8 ratios (>2.2) while 5 had a decreased CD4:CD8 ratio (<1.5). This again differs from the previously mentioned study which demonstrated an overall decreased CD4:CD8 ratio [4]. Interestingly, in multiple sclerosis, a neurologic disease which may have an autoimmune basis, patients can be divided into different groups according to different CD4:CD8 ratios [13]. In contrast to a previous report of decreased B cell numbers [5], we did not find any abnormalities in B cell function or number.

To further dissect immunoregulation, we examined the proliferative responses of patients' lymphocytes to mitogens and autologous lymphocytes. We found normal responses to both PHA and Con A in all patients. Our results do not confirm previous reports of decreased blastogenesis in autism [3, 7, 9]. However, in the previous studies, the individuals studied were significantly younger and had autism for a comparatively shorter period of

time. In our study, the age range was 8–23 years with a mean of 17. The age ranges in the previous reports were 2–12 years with a mean of 7 [3], 3–32 years with a mean of 11 [7], and 7–14 years [9].

In agreement with a previous report [9], we found a statistically significant decreased responsiveness to self antigens (AMLR) in a subset of patients (5 patients with CD4:CD8 ratios <1.5). These patients had a decreased CD4:CD8 ratio as a result of either an increased percentage of CD8+ cells, a decreased number of CD4+ cells or a combination of these factors. This relationship of a decreased CD4:CD8 ratio and a decreased AMLR was previously demonstrated in kidney transplant patients [14]. This is not surprising as it has been demonstrated that in normal individuals, CD4+ cells are the main proliferating cell in the AMLR [reviewed in ref. 15] and therefore a decreased number of CD4+ cells would result in a decreased AMLR. In contrast, there was no relationship between the percentage of DR+ (activated) T lymphocytes and responsiveness in the AMLR. This was unexpected since previous studies have shown that many autoimmune diseases, which are characterized by the presence of activated T lymphocytes, have abnormally decreased AMLR [16–18] although multiple sclerosis patients have abnormally elevated AMLR [19].

We examined patients for evidence of in vivo activation of T lymphocytes. We found normal numbers of IL-2-receptor-positive cells but an increased number of T lymphocytes bearing surface class II major histocompatibility (MHC) antigen (DR+ cells). This may be surprising as both IL-2 receptor and class II MHC molecules are acquired after activation of T cells. However, a similar discrepancy of early (IL-2 receptor) and late activation antigens (DR or VLA-1) has been seen in autoimmune diseases such as rheumatoid arthritis or juvenile rheumatoid arthritis [20, 21]. This may reflect a time dependence of activation, with many late-activated cells present in autoimmune diseases as compared to in vitro activated cells, or may reflect distinct subsets of cells which are capable of becoming activated. Again, a similarity to multiple sclerosis is seen, as late-activation but not early-activation antigens have been demonstrated in multiple sclerosis [22]. For unknown reasons, the presence of late-activated T cells appears to be a common finding in autoimmune diseases. To our knowledge evidence of T cell activation in autism, as demonstrated by the presence of IL-2 receptor or DR+ T cells, has not been previously investigated.

The percentage of DR+ T lymphocytes decreased with increasing age. This result suggests the possibility of a much more active immune system process early in life, in a subset of autistics, which with aging progressively becomes more quiescent. Frequently, autistic symptomatology appears and progresses within the first 3 years of life [1]. The age range of our studied group was considerably older, 8–23 years. Thus it is possible that our results indicate a pathogenically active immune system abnormality early in life that may be resolving with age. However, the sequelae of the damage to the central nervous system is lifelong.

The fact that only a subset of our autistic patients had DR+ lymphocytes is not surprising. Autism is a syndrome caused by various etiologies [1]. If autoimmune processes were to play a pathogenic role in causing autistic symptomatology, this would be in only a subset of autistics. None of our studied patients had an identified biomedical cause for their autism.

It has been previously shown that 30–40% of children with autism have hyperserotonemia [reviewed in ref. 1]. Serotonin has multiple effects including in vitro immunomodulating properties [23, 24]. Therefore, hyperserotonemia as seen in a percentage of autistic children may potantially alter the in vivo immune function of these patients. However, the primary in vitro effect of serotonin on PBL is to inhibit lectin-induced proliferation [24], an observation which we did not make in our autistic population. Serotonin serum levels were not determined in this study.

We did not detect cellular immune system abnormalities in Rett syndrome.

In summary, our results, in addition to those of other authors, suggest that autism in a subset of patients may have an autoimmune basis. This autoimmune process may be apparent only at a young age. This hypothesis is supported by our finding of fewer activated T cells with increasing age and by increased immune system abnormalities reported by others who have studied younger populations. Further studies are necessary to confirm these hypotheses.

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