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A STUDY OF T CD4, CD8 AND B LYMPHOCYTES IN NARCOLEPTIC PATIENTS

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ABSTRACT - Narcolepsy is characterized by excessive daytime sleep and cataplexy. Little is known about the possible difference in pathophysiology between patients with or without cataplexy. *Objective*: To quantify T CD4, T CD8 and B lymphocytes in subgroups of patients with narcolepsy and the presence or absence of the HLA-DQB1*0602 allele between groups. *Method*: Our study was prospective and controlled (transversal) with 22 narcoleptic patients and 23 health control subjects. Patients underwent an all-night polysomnographic recording (PSG) and a multiple sleep latency Test (MSLT). The histocompatibility antigen allele (HLA-DQB1*0602), T CD4, CD8 and B lymphocytes were quantified in control subjects and in narcoleptics. *Results*: The HLA-DQB1*0602 allele was identified in 10 (62.5%) of our 16 cataplexic subjects and in 2 (33.3%) of the 6 patients without cataplexy (p=0.24). In control subjects, HLA-DQB1*0602 allele was identified in 5 (20%). A significant decrease in T CD4 and B lymphocytes was found in narcoleptic patients with recurrent cataplexy when compared with our patients without cataplexy. *Conclusion:* Autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis were associated with a decrease in sub-group of T CD4 and B lymphocytes. A drop in B lymphocytes count in reumathoid arthritis might, it is posited, be correlated to the presence of HLA-DRB1 allele along with an overall worsened outcome of the affliction. The theory of an increase in consumption of B lymphocytes over the maturation phase has likewise been put forward. Our study reinforces the view that narcolepsy should be considered from an immunological perspective.

KEY WORDS: narcolepsy, lymphocytes, immunology, HLA-DQB1*0602 allele.

Estudo dos linfócitos T CD4, CD8 e B em pacientes com narcolepsia

RESUMO - A narcolepsia é caracterizada por sonolência excessiva diurna e cataplexia. Pouco se sabe sobre as diferenças fisiopatológicas entre pacientes com e sem cataplexia. *Objetivo*: Quantificar os linfócitos T CD4, T CD8 e B e a presença do alelo HLA-DQB1*0602 nos subgrupos de pacientes com narcolepsia. *Método*: O estudo foi prospectivo e controlado (transversal) com 22 pacientes portadores de narcolepsia e 23 sujeitos controle. Os pacientes realizaram polissonografia (PSG) de noite inteira e teste de múltiplas latências do sono (TMLS). O alelo do antígeno de histocompatibilidade (HLA-DQB1*0602) e os linfócitos T CD4, T CD8 e B foram quantificados nos pacientes e sujeitos controle. *Resultados*: O alelo HLA-DQB1*0602 foi encontrado em 10 (62,5%) dos 16 pacientes com cataplexia e em 2 (33,3%) dos 6 pacientes sem cataplexia (p=0,24). Nos sujeitos controle, o alelo HLA-DQB1*0602 foi encontrado em 5 sujeitos (20%). Um aumento significativo de linfócitos T CD4 e uma diminuição de linfócitos B foi observado no grupo de pacientes com cataplexia freqüente quando comparado ao grupo de pacientes sem cataplexia. *Conclusão:* Doenças auto-imunes como lupus eritematoso sistêmico e artrite reumatóide têm sido associadas com diminuição de linfócitos T CD4 e B. Na artrite reumatóide, diminuição de linfócitos B e presença do alelo HLA-DRB1 tem sido associada a pior evolução. Para essa doença, a teoria de um maior consumo de linfócitos B em suas fases de maturação tem sido aventada. Os achados do nosso estudo reforçam a teoria imunológica da narcolepsia.

PALAVRAS-CHAVE: narcolepsia, linfócitos, imunologia, alelo HLA-DQB1*0602.

Narcolepsy is characterized by excessive daytime sleep and cataplexy. Sleep paralysis and hypnagogic hallucinations can be added to this clinical picture[1,2]. Narcolepsy involves an unknown physiopathology but with a known association with the HLA DQB1*06023 allele[3]. This would fortify the hypothe-

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sis of a genetic origin. Other pathophysiological hypotheses derive from the environment, infections and the immunological system.

Abnormalities in the neurotransmission of hypocretin were recently described in patients with narcolepsy[4]. Hypocretin is a neuropeptide synthesized by neurons whose cell bodies are located in the lateral hypothalamus. Their function is to maintain wakefulness. The association of hypocretin to dopaminergic pathways in frontal brain and noradrenergic pathways in the brain stem is well established. The smaller concentration of hypocretin in cerebral spinal fluid has only been characterized in narcoleptics with cataplexy[5]. In an immuno-hystochemical study, a reduction in the quantity of hypocretinergic cells of the lateral hypothalamus was observed, upon autopsy, in patients with cataplexy[6]. Immunological theory is based on the possible self-aggression of hypocretin cells in the hypothalamus. This self-aggression and subsequent loss in cells would result in a drop of hypocretin concentration which would in itself lead to narcolepsy[7]. As the HLA DQB1*0602 allele is prevalent in narcoleptics and as it is also associated with some known auto-immune diseases, the immnological theory therefore seems to gain impetus.

Patients with frequent outbursts of cataplexy highlight a greater prevalence of the HLA DQB1*0602 allele along with a marked diminution of hypocretin in the cerebral spinal fluid when compared with patients with absence of cataplexy. There is a lack of immunological studies in the literature which focus on patients with narcolepsy where the clinical and laboratory differences were assessed[8-11].

METHOD

The study was prospective and controlled (transversal) and was undertaken between November 2003 and February 2005 at the Sleep Disorders Institute, Department of Psychobiology, UNIFESP - EPM, São Paulo.

The study received the seal of approval from the Ethics and Research Committee of UNIFESP, identified by the registered code number 1139/03. All patients willingly agreed to participate in the study and signed the term of free consent.

Twenty three control subjects (14 male; age 35.2±14.6 years ±SE) without excessive daytime sleepiness and 22 narcoleptics (12 male; age 40.9±14.8 years ±SE) who attended the outpatients' unit for hypersomnolence were assessed. All narcoleptic patients were diagnosed according to International Classification of Sleep Disorders (ICS) criteria[2,12-14]. Patients were divided into 3 subgroups: one with frequent cataplexy (more than 1 attack per month, n=6), one with rare cataplexy (less than 1 attack per month, n=10) and the third without cataplexy (absence of attacks, n=6).

Peripherical blood samples were taken during morning fasting and were immediately sent for laboratory analysis for all subjects in the study. Identification of the presence of HLA DQB1*0602 allele in patients and in control subjects was also undertaken at this juncture. The study included a quantification of total T, T CD4, T CD8 and B-lymphocytes in 22 patients with narcolepsy as well as in our 23 control subjects.

Patients and control subjects who had an acute illness or displayed a type of any chronic illness in an active form were excluded from the study. Subjects who had been ingesting immunodepressants, stimulants or depressants and drugs or who were involved in alcohol or chemical substance abuse were also excluded from the study.

Polysomnography (PSG) – An all-night standard polysomnographic recording for all narcoleptic patients was undertaken at our sleep laboratory. On average, the recording began at 10 pm and was concluded at 6 am.

Sleep stages were analyzed according to Rechtschaffen and Kales[12]. Respiratory events were evaluated according to the recommendations of the 1999 Task Force of the American Academy of Sleep Medicine. Arousals and periodic movements of lower members were analysed according to the ASDA (1992) and AASM (1999) criteria respectively[13].

Multiple sleep latency test (MSLT) – Patients were woken up at 6 am following the all-night PSG. All electrodes of the previous night, except for EEG, EOG and EMG, were detached. The MSLT started at 8 am and, at 2-hour intervals, a total of 5 PSG recordings lasting 20 minutes each were undertaken. The rationale behind this approach was to quantify the average sleep latency and to evaluate the presence of REM sleep. The diagnosis of narcolepsy is advanced when two or more episodes of REM Sleep (SOREM) during the five naps have been observed. An average of the sleep latencies equal to or less than 10 minutes characterizes day time sleepiness. Excessive diurnal somnolence is viewed to be 5 minutes or less of sleep latency[1 4].

HLADQB1 0602 allele analysis* – HLADQB1*0602 allele analysis was carried out in the Genetic Laboratory attached to our Sleep Institute. The presence of DQB1*0602 was determined using the following procedure: The DNA was extracted from the white blood cells and the region where the DQB1*0602 allele is located was amplified by a polimerase chain reaction (PCR) using of DQBF (5'- CCCGCAGAGGATTTCGTGTT - 3') and DQBR (5'- AACTCCGCCCGGGTCCC - 3') primers.

These primers amplified DQB 1 *0602 and also the rare DQB1*0610, DQB1*0613 and DQB1*0614 alleles as a product of 218 basic pairs.

As an internal control for amplifications was used in the same PCR reaction. The following primers which only amplify exon 3 of the gene DRBI: EX3f (5'-TGCCAAGTGGAGCACCCAA - 3') and EX3r (5'- GCATCTTGCTCTGTGCAGAT - 3') were used. PCR analyses were performed using 35 cycles of 95ºC for 30 seconds, 35 cycles of 63ºC for 30 seconds and 35 cycles of 72ºC for 60 seconds.

Lymphocyte subset typing – Cells of the lymphocyte population were stained with antibodies directly labeled

Table 1. Demographic characteristics of subgroups of narcoleptic patients.

	Patients without cataplexy A	Patients with rare cataplexy B	Patients with frequent cataplexy C
Age (years±SE)	38.8±15.6	47±5.3	32±5.1
Sex			
M	2	3	1
F	4	7	5

Statistical analysis (t student test): AXB (sex p=0.80 and age p=0.11); BXC (sex p>0.50 and age p=0.69) and AXC (sex p>0.50 and age p=0.47).

Table 2. Polysomnographic data of our patients with narcolepsy.

Polysomnography	Patients without cataplexy (CA) n=6	Patients with rare cataplexy (CR) n=10	Patients with frequent cataplexy (CF) n=6	t student test p value
Sleep efficiency (%±SE)	88.95±1.8	71.5±16.3	83.7±14.6	p>0.05
Sleep latency (minutes±SE)	7.9±5.3 *	2.2±15	1.6±1.67 *	p=0.031
REM sleep latency (minutes±SE)	95.3±5.3	73.5±48	99.5±74	p>0.05
Stage1 (%±SE)	5.65±4.5	4.45±4.6	5.17±4.1	p>0.05
Stage 2 (%±SE)	59.15±9.2	61.45±6.8	56.7±6.1	p>0.05
Stages 3 and 4 (%±SE)	16.68±5.1	15.78±8.3	16±3.5	p>0.05
REM (%±SE)	19.46±4.6	19.28±5.8	21.9±6.3	p>0.05
Arousal (hour±SE)	19.3±28	10±7	12.2±20	p>0.05
Apnea-hypopnea index (AHI±SE)	6.3±1.1	5.7±4	7.4±4.8	p>0.05

Table 3. Multiple sleep latency test data of our patients with narcolepsy.

Multiple sleep latency test	Without cataplexy n=6	With rare cataplexy n=10	With frequent cataplexy n=6
Sleep latency (minutes±SE)	2.5±1.2	2.9±1.4	3.4±1.3
2 SOREM	5	7	2
3 SOREM	0	0	0
4 SOREM	0	0	2
5 SOREM	1	3	2

*Table 4. Comparison of the lymphocytes subgroups between patients with narcolepsy with and without HLA DQB1*0602 allele.*

Lymphocytes	Control subjects n=23	Narcoleptic patients without the HLA DQB1*0602 allele n=10	Narcoleptic patients with the HLA DQB1*0602 allele n=12	t student test p value
T	1524±433	1305.4±484	1718.25±597.2	p>0.05
T CD4	910.8±316	818.1±337	992.7±234	p>0.05
T CD8	480±180.7	408±185	689±514.1	p>0.05
B	285±125	235±98.6	324±152.6	p>0.05

Table 5. Comparison of lymphocytes between control subjects and groups of patients with narcolepsy.

Lymphocytes	Control subjects	Patients without cataplexy	Patients with rare cataplexy	Patients with frequent cataplexy	t student test p value
T	1524±433	1712.8±282	1458±811	1468.5±269	p>0.05
T CD4	910.8±316	1097.5±119.1*	850.5±378.1	834±175 *	*p=0.012
T CD8	480±180.7	572±222	566.7±587.9	543.8±239.5	p>0.05
B	285±125	379±130 **	270±140	212±86.2 **	**p=0.026

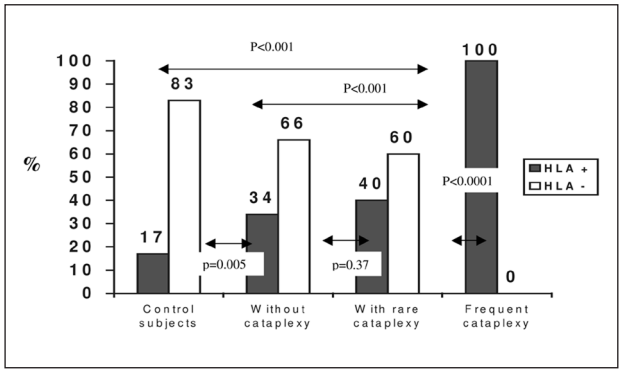

*Figure. Presence of HLA DQB1*0602 allele.*

with fluoroisothiocyanate (FITC) or phycoerythrine (PE) and analyzed by FACScan (Becton & Dickinson, Heidelberg, Germany). We used double staining with monoclonal antibodies (Dianova, Hamburg, Germany), CD4-PE conjugated IOT4, CD8-FITC conjugated IOT8, CD45-FITC conjugated IOL2, and CD29-FITC conjugated IOT29.

Statistical analysis – Variable distribution was verified using the Kolmogorov-Smirnov test with values presented as averages and standard deviation.

The Chi-squared test was used to test the homogeneity of groups of narcoleptic patients and control subjects. The Fischer test was used to test the homogeneity of subgroups of narcoleptic patients. The t student test for independent samples was used for comparison of subpopulations of lymphocytes and age between the groups. Statistical significance was attributed to $p \leq 0.05$.

RESULTS

The groups of narcoleptic patients and control subjects were similar regarding age and sex (sex p= 0.66; age p=0.20). Patients with narcolepsy were divided into three groups, as outlined in the introduction (Table 1). All of the patients answered the Epworth questionnaire with a maximum of 22 and a minimum of 9 points, with an average of 18.3±3.2. No difference in the sleepiness scale between the groups of patients could be noted.

The results of the PSG recordings of our patients are listed in Table 2. The sleep latency in patients with frequent cataplexy was smaller than in patients without cataplexy (Table 3).

The prevalence of the HLA DQB1*0602 allele was different between the control subjects and narcoleptic groups: p<0.001 for control subjects versus narcoleptics with frequent cataplexy, p<0.001 for control subjects versus patients with rare cataplectic attacks and p=0.005 between control subjects versus patients withouth cataplexy.

Additionally, a statistical difference in relation to the presence of the HLA allele was observed between the groups of patients: p<0.0001 for patients with rare cataplectic attacks versus patients with frequent cataplexy, p<0.001 between patients without cataplexy versus patients with frequent cataplexy (Figure). No difference was noted between patients with rare and absent cataplectic attacks p=0.37.

We failed however to note a statistical difference between the subgroups of lymphocytes in control subjects against narcoleptics or between the groups of narcoleptics themselves concerning the presence or not of the HLA DQB1*0602 allele (Table 4).

Differences between the subgroups of T CD4 and B lymphocytes subgroups were observed between narcoleptic patients without cataplexy and the group of patients with frequent cataplexy: p=0.012 for T CD4 and p=0.026 for B lymphocytes (Table 5).

DISCUSSION

There has been sound headway made in understanding the pathophysiology of narcolepsy. The greater prevalence of the HLA DQB1*0602 allele in patients with narcolepsy with cataplexy is well known[15]. The discovery of hypocretin, with a function of maintenance of wakefulness, and its reduction by cell loss in the lateral hypothalamus in patients with frequent cataplexy, is one possible explanation for daytime sleepiness[4].

Although lymphocytes have a key function regarding immunology mechanism, no specific alterations have been shown in patients with narcolepsy. Previous studies compared control-subject groups with narcoleptics and the presence of or lack of the HLA DQB1*0602 allele. None of the studies reviewed concentrated on subgroups of patients[7-10].

Our study showed that the prevalence of the HLA DQB1*0602 allele had increased in the narcoleptic population, being unanimous in patients affected by frequent cataplexy. This finding concurs with those of a previous study[16]. The quantification of T CD4 and CD8 and B lymphocytes failed to highlight a statistical difference between control subjects and patients with narcolepsy when the comparison was performed between patient groups with the presence of or absence of the HLA allele. However, the quantity of T CD4 and B lymphocytes cells was significatively reduced in the group of narcoleptic patients with frequent cataplexy when compared to patients without cataplexy (p=0.012 and p=0.026 respectively). One hypothesis to explain the above results is the observation of commorbidities associated with narcolepsy, such as a significant incidence of depression[17]. It is known that depression is associated with higher

plasma level of corticotrophines with an alteration of the cell immunological system[18]. Unfortunately, given that our patients were not systematically evaluated for depression, we are unable to verify, first-hand, this hypothesis.

Moreover, some autoimmune illnesses such as systemic lupus erythematosus and rheumatoid arthritis present a drop in T CD4 and B lymphocytes subgroups. The diminution of the population of lymphocytes B in rheumatoid arthritis can be correlated with the presence of the HLA DRB1 allele[19]. One hypothesis to explain these findings looks at the greater consumption of lymphocytes in some of the phases of cell maturation[20,21].

The association of the HLA DRB1 allele and reduction in T CD4 and B lymphocytes in patients with rheumatoid arthritis[19] and multiple sclerosis[21] can point to an immunological theory for narcolepsy. The HLA DRB1 allele are more prevalent in patients with a severe form of rheumatoid arthritis. Similarly our study highlighted a relatively larger quantity of CD4 and B lymphocytes in patients with frequent cataplexy compared with patients without cataplexy suggesting, as for the rheumatoid arthritis, a bimodal presentation of the disease.

Patients with multiple sclerosis presented with an infiltration of B lymphocytes in the brain. Patients with this disease also have a higher prevalence of the HLA DQB1*0602 allele[20]. Clinical and laboratory differences suggest a pathophysiological discrepancy between the subgroups of patients with narcolepsy.

Taking into consideration the aforementioned studies along with our results, we believe that a selective destruction of cells of the lateral hypothalamus via immunological aggression in an early phase of the illness, or prior to the onset of symptoms, could have occured. This may explain the symptoms of frequent cataplexy. Immunosuppressive therapy has been used in children and adolescents with early diagnosed narcolepsy. Initial results are likely to yield promising outcomes[21]. We posit that the results obtained in this work contribute to and reinforce the hypothesis of narcolepsy as an immunological disease.

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