PŮVODNÍ PRÁCE

Toxocara spp. seronegativity in Czech patients with early form of multiple sclerosis – clinically isolated syndrome

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ABSTRACT

Background: Helminth infections were reported to slightly modulate the host immune system response and decrease the risk of an autoimmune disorder, but on the other hand any infection may activate the immune system and trigger autoimmune reaction. In this study, we aimed to measure eosinophil levels and antibodies against Toxocara spp. in patients with clinically isolated syndrome (CIS) and multiple sclerosis (MS).
Methods: In total, 220 CIS patients and 62 MS patients were examined. Antibodies against Toxocara secretory/excretory antigens (TES) were measured with an ELISA method.
Results: A total of 1,983 measurements of eosinophil levels were performed in CIS patients, out of which 95 results in 21 different patients were above the upper normal limit of the laboratory, but it was mostly only a relative increase. Two patients showed eosinophil levels above 20 % but both of them suffered from severe allergy. None of the CIS patients had any clinical signs of parasitic infections and the serological tests for antibodies against Toxocara were all negative. In all MS patients, eosinophil levels were in normal range. Antibodies against TES were detected in only 1 out of 62 (1.6%) MS patients.
Conclusions: Based on our results it does not seem that Toxocara infection represents a potential trigger of MS. Nevertheless, our study indirectly confirms the hypothesis that parasitic infection may protect from autoimmunity.

KEYWORDS

multiple sclerosis – *Toxocara* – eosinophilia – clinically isolated syndrome

SOUHRN

Posová H., Hrušková Z., Havrdová E., Kolářová L.: Séronegativní *Toxocara spp.* u českých pacientů s časnou formou roztroušené sklerózy – klinicky izolovaný syndrom
Úvod: U infekcí parazitárními červy byla popsaná možnost lehkého ovlivnění imunitního systému a také snížení rizika autoimunitního onemocnění, ale na druhé straně také víme, že jakákoli infekce může imunitní systém aktivovat a být spouštěčem autoimunitního onemocnění. Cílem naší studie bylo sledování počtu eozinofilů a protilátek proti *Toxocara spp.* u pacientů s klinicky izolovaným syndromem (CIS) a roztroušenou sklerózou (RS).
Metody: Bylo vyšetřeno 220 pacientů s CIS a 62 s RS. Protilátky proti sekrečním/exkrečním antigenům *Toxocara* (TES) byly vyšetřeny metodou ELISA.
Výsledky: Celkově bylo u pacientů s CIS vyšetřeno 1 983 krevních obrazů s počty eozinofilů, ale pouze 95 výsledků u 21 různých pacientů bylo nad horním limitem normy naší laboratoře, většinou se jednalo pouze o relativní počty. U dvou pacientů bylo zvýšení významné (o více než 20 %), ale v obou případech se jednalo o alergiky. Žádný z pacientů s CIS neměl klinické příznaky parazitární infekce a sérologické testy na protilátky proti *Toxocara* byly negativní. U všech pacientů s RS byly hodnoty eozinofilů v rámci normálního rozmezí. Protilátky proti TES byly detekované pouze u jednoho z 62 (1,6 %) pacientů s RS.
Závěr: Naše výsledky nenasvědčují, že by *Toxocara* patřila mezi potencionální spouštěče RS. Na druhé straně naše studie nepřímo potvrdila hypotézy, že parazitární infekce mohou chránit před autoimunitou.

KLÍČOVÁ SLOVA

roztroušená skleróza – *Toxocara* – eozinofilie – klinicky izolovaný syndrom

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BACKGROUND

Multiple sclerosis is a chronic inflammatory CNS disease, which affects about 1 in 1000 individuals in the Western world [1]. In recent decades, the incidence of autoimmune diseases, multiple sclerosis (MS) among them, has substantially increased in developed countries. In general, genetic predisposition, immune dysregulation and environmental factors are believed to play a role in the etiopathogenesis of immune-mediated disorders, and particularly environmental changes are thought to be the major culprit of the observed increase [2]. Even though environmental factors influencing MS development have yet to be confirmed, the currently presumed

ones include sunlight (UV exposure or vitamin D deficiency), viral infections, hygiene (helminths), and cigarette smoking [3].
The so called hygiene hypothesis, as formulated by Strachan in 1989 [4] postulates that improved hygienic conditions and reduced exposure to microorganisms in economically developed countries resulted in increased immune reactivity, which supported the development of autoimmunity (and allergy) [2]. Helminths are known to bear the ability to slightly modulate the host immune response so that they could survive in the host body for a long time. By avoiding a strong activation of the immune system, helminthic infection may contribute to a lower risk of inflammatory disorders. Helminths have been noted to promote T helper 2 (Th2) and inhibit Th1/Th17 type of immune response, and, importantly, also shift the regulatory potential towards toleration.
A negative association between protozoan *Toxoplasma gondii* infection and the presence of multiple sclerosis has been previously observed [1]. In another study, parasite-infected MS patients showed a significantly lower number of exacerbations of the disease and a more favourable disease course when compared with uninfected MS patients [1].
Toxocariasis is a relatively common parasitic zoonosis, caused by nematodes of the genus *Toxocara*, the most important species for human being *Toxocara canis* and *Toxocara cati*. Seroprevalence of *Toxocara* in children is variable, depending on the region and its characteristics and can be as low as 1% (Spain) or as high as 67% (Argentina) [7]. In the Czech Republic, the seroprevalence of *Toxocara canis* ranged from 5.8% to 36.0% in various districts [8]. In our National Reference Laboratory, 555 out of 14,314 sera sent for examination in cases of clinical suspicion for *Toxocara* infection were positive (3.9%) between years 2006 and 2015 (*unpublished data*).
In this study, we aimed to measure eosinophil levels and antibodies against *Toxocara* spp. in patients with clinically isolated syndrome (CIS, a first episode of neurologic symptoms that lasts at least 24 hours and may or may not convert to multiple sclerosis) and in multiple sclerosis.

METHODS

Patient characteristics

The examinations were performed in patients with clinically isolated syndrome (CIS) and in patients with multiple sclerosis (MS).

CIS patients

The study population included 220 patients with CIS in which the evolution of clinical and MR imaging outcomes was investigated. Inclusion criteria were age between 18 and 55 years and enrolment into the study within 4 months of the first clinical event. Criteria included diagnostic MR imaging showing ≥ 2 T2-hyperintense lesions, an Expanded Disability Status Scale (EDSS) score of ≤ 3.5 and ≥ 2 oligoclonal bands in the CSF at the screening visit, before the start of treatment. Exclusion criteria were a second relapse before the baseline examination, missing or invalid clinical or MR imaging follow-up information after the baseline examination, and pregnancy.
Each study subject was treated with 3–5 g of methylprednisolone after the first symptom for 2–3 months before the study entry. At baseline, all patients received the same treatment, which included 30 μg of intramuscular interferon once a week. During the study, relapses were treated with 3–5 g of methylprednisolone Clinical visits were performed every 3 months, laboratory tests were assessed at diagnosis (before pulses of corticosteroids), at baseline (i.e. prior to the start of IFN treatment) and then every 6–12 months until month 48. Basic characteristics are displayed in Table 1.

Table 1. Demographic and clinical characteristics in patients with CIS over a 48-month period

(n = 220)	
Patient	**Data**
No. (%) female	145 (66)
Age at onset	y 28.7 ±7.9
Time to baseline	days 81.9 ± 23.7
EDSS at baseline	1.7 ±.7; (0.0–3.5)
EDSS at 48 months	1.8 ±0.9; (0.0–6.5)
112 CIS patients (53.3%) developed clinically definite MS (CDMS)	

The local ethics committee approved the study protocol, and each study subject gave written informed consent.

MS patients

This group consisted of 62 patients with MS (women/men 54/8), mean age 41.7 ± 7.98. A total of 32 patients were treated with interferon, 14 received fingolimod, 9 received natalizumab, 5 were on glatiramer acetate and 4 were treated with other therapy.

Blood collection and Laboratory assessments

Eosinophil levels were measured in routine clinical laboratory in General University Hospital in Prague. The serological examinations were performed with in-house enzyme-linked immunosorbent assay (ELISA) according to Uhlíková and Hübner [8] targeted on the detection of specific IgG antibodies to excretory-secretory *Toxocara* spp. antigens (TES) prepared according to de Savigny [9]. Immuno plates (Maxi Sorp, NUNC) were coated with 2.5 um TES in a carbonate buffer (pH 9.6) and left at 4 °C overnight. Next day, the plates were probed with serum diluted 1/200 and then with peroxidase-conjugated anti-human IgG (Jakson ImmunolResearch Laboratories, USA) diluted 1/60000. Binding reactions were visualized with o-phenylenediamine substrate (Sigma-Aldrich) containing H_2O_2 and stopped by $4NH_2SO_4$; the absorbance was measured at 490 nm (Dynatech MR 50000). In order to eliminate false positive results namely in immunosuppressed patients, all sera were tested also for presence of anti-*Trichinella* spp. *Taenia solium*, and *Echinococcus granulosus* antigens.

RESULTS

CIS patients

A total of 1983 measurements of eosinophil levels were performed in CIS patients, out of which 95 results in 21 different patients were above the upper normal limit of the laboratory. The median and mean eosinophil levels are displayed in Table 2. In most cases, only a relative increase (usually about 5% of total number of leukocytes) was noted, while the absolute levels were in normal range, and the eosinophil levels were often only temporarily increased. Only 2 patients showed eosinophil levels above 20%, but both of them suffered from severe allergy. One of these patients had high levels from the very beginning of the study (28.5%, absolute levels 1.8 x 10^9/l), the other patient had also consistently increased eosinophil levels that were, however, only borderline at entry (6.4%, 0.4 x 10^9/l). Overall, most of the patients (16/21) who had increased eosinophil levels were treated for allergy, the remaining 5 were not diagnosed with allergy but their eosinophil levels were only occasionally and only relatively increased. In general, none of the patients had any clinical signs of parasitic infections and the serological tests for anti-*Toxocara* antibodies as well as for other antigens (*Trichinella* spp., *T. solium*, *E. granulosus*) used were all negative.

Table 2. Absolute and relative eosinophil levels in CIS patients

	Relative (%)	Absolute (x10^9/l)
MAX	29.6	2.22
MEDIAN	1.5	0.1
MIN	0.2	0
Mean	2.179826	0.138987
Std. Dev.	2.149508	0.148541

MS patients

In MS patients, eosinophil levels were in normal range in all cases. Except for 1 out of 62 (1.6%), no anti-*Toxocara* antibodies were detected in any of the patient examined. The positive patient had only marginally increased relative eosinophil count (absolute levels were all in normal range). Her IgA levels were permanently low and IgG levels were on the lower normal range, with zero IgG4 and low IgG2 levels. She did not have any pets and did not display any clinical signs of parasitic infection.

DISCUSSION

Infection with helminth parasites may decrease the risk of developing an autoimmune disease, including multiple sclerosis. In this study, we examined the occurrence of anti-*Toxocara* antibodies in CIS and MS patients and found a very low number of positive patients. Even though direct comparison with other studies is difficult, the prevalence in this study seems to be lower than the previously reported seroprevalence of *Toxocara* in the Czech Republic [8], in keeping with the hypothesis that parasitic infection may protect from autoimmunity. Interestingly, results of a Norwegian MS case-control study of environmental factors confirmed potential protective effect of exposure to cats and/or dogs during childhood on MS, supporting the concept that the risk of autoimmune diseases like MS may increase with high hygienic standard. Nevertheless, as discussed in the paper, the pet owners may be more prone to parasitic infections, but they may also simply have more outdoor activities, leading to higher levels of vitamin D and thus reducing the risk of MS [10].

A different study from Iran investigated the prevalence of selected parasitic infections in patients with relapsing remitting MS and their family members and disclosed no differences between these two groups [11]. To our knowledge, there has been only one previous study [12] that investigated the prevalence of anti-*Toxocara* antibodies in MS (and also ankylosing spondylitis) and, contrary to the hygiene hypothesis, revealed that patients seropositive for *Toxocara* were almost six times more likely to suffer from MS than seronegative patients, even though this did not reach statistical significance (odds ratio 5.94 [95% confidence interval 0.64 to 55.53]) and there was no difference in seropositivity for *Toxocara* between MS patients and healthy controls.

A theory that *Toxocara* might play etiological role in the development of MS has been published before [13]. Based on the results of our previous study [14] in which *Toxocara canis* larvae in mice impaired the brain-blood barrier (accelerated the speed of migration to the host CNS), we originally hypothesized that the *Toxocara* infection may potentially trigger multiple sclerosis.

Infection, both acute and chronic, is known to activate the immune system response and it is conceivable that (presumably in genetic predisposed individuals, with a concurrent impairment of regulatory mechanisms, and possible role of other environmental factors) the immune system activation may lead to an initiation and/or flare of an autoimmune disease (as in the case of well-known association between *Staphylococcus aureus* and ANCA-associated vasculitis) [15]. In our study, however, the patients with newly developed autoimmune disease did not have increased eosinophil levels, and the presence or pathogenic role of parasitic infection was therefore unlikely, not only at the study entry, but also during the whole follow-up. Similarly, the very low prevalence of anti-*Toxocara* antibodies in our patients rather supports the concept of protective and not harmful effect of helminth infections. The classical and somewhat simplified hypothesis claimed that helminth infection promotes a Th2-polarized immune response, increasing the production of IL-4, IL-5 and IL-13 [1]. However, it is known that typical Th2-associted autoimmune diseases may also benefit from helminth infections, and therefore the augmentation of Th2 response as the primary beneficial mechanism behind helminth infections was doubted [1]. Previous studies described that helminth infection may be associated with the induction of both T and B regulatory cells and secretion of suppressive cytokines such as IL-10 and TGF-β, which may regulate the distorted immune response [16]. In a recent study, however, treatment of mice with a parasite (*Fasciola hepatica*) products attenuated the signs of experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. Interestingly, a significant decrease in Th1 and Th17 cells infiltration into brain was noted in this study and the protection against EAE was indepen-

dent of IL-4, IL-10, and Tregs while helminth-induced IL-5 and IL-33 played a key role in the protection against autoimmunity [17].

We are aware of the possible limitations of this study that include a relatively low number of patients and the lack of a control group. Furthermore, the low number of *Toxocara* seropositive patients did not enable us to perform further subgroup analyses and comparisons. Even though the immune response to parasitic infection may be impaired after immunosuppressive treatment, we do not expect this would explain low prevalence of anti-helminthic antibodies as the antibodies against bacteria, viruses or mycoses are present.

On the other hand, our study included well defined groups of patients followed up from the very beginning of neurological disorder and, given the paucity of information and the controversy of some of the available results as discussed in detail above, the results of the study added new pieces of knowledge into the complex field of yet not completely understood etiopathogenesis of MS.

We can conclude that based on the result of our study, parasitic infection does not seem as a potential trigger of MS because hardly any patients with CIS had increased eosinophil levels at the time of the first neurological symptoms. Our results indirectly confirm the hypothesis that parasitic infection may protect from an autoimmune disease even though this requires further confirmatory studies.

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