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Multiple sclerosis and anti-*Plasmodium falciparum* innate immune response

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Abstract

Several epidemiological investigations conducted in Sardinia, insular Italy, indicate that the strong selective pressure of malaria along the centuries may have concurred to the elevated genetic MS-risk in this region. To test such hypothesis in an experimental setting, we have compared the immune response to *P. falciparum* (the causative agent of malaria) in Sardinian MS patients relative to their ethnic healthy controls and control MS patients of different ethnicity. To this purpose, the *P. falciparum*-driven peripheral mononuclear cell proliferation, the production of pro-inflammatory cytokines of the innate immunity such as TNF-α, IL-6 and IL-12 and the ability to inhibit the parasite growth have been tested in relation to HLA-DR alleles and TNF promoter polymorphisms known of being associated to MS.

We found that *P. falciparum*-induced proliferation, cytokine production and parasite killing are significantly augmented in Sardinian MS patients as compared to controls ($p<0.01$). Additionally, a correlation is found with genes associated to Sardinian MS, namely the TNF_{-376A} promoter polymorphism and the class II HLA-DRB1*0405 allele. In conclusion, we have found evidences that some genetic traits formerly selected to confer a protective responses to *P. falciparum* now partially contribute to the elevated MS susceptibility amongst Sardinians.

Keywords: Multiple sclerosis; Malaria; *Plasmodium falciparum*; TNF; HLA

1. Introduction

It is known that malaria exerts a strong selective pressure which can result in an enrichment of alleles that may increase the susceptibility to other diseases. Since the first "malaria hypothesis" (Haldane, 1949), several haemoglobinopathies (Modiano et al., 2001), thalassaemias (Flint et al., 1986; Allen et al., 1997), HLA and TNF polymorphisms (Hill et al., 1991; Knight et al., 1999; McGuire et al., 1999) as well as red-cell enzyme deficiencies (Ruwende et al., 1995) have been considered efficient genetic weapons to protect many populations from severe forms of *P. falciparum* malaria. Some examples have been clearly documented in the population of Sardinia, insular Italy, which include the selection of alleles determining the glucose-6-phosphate dehydrogenase deficiency, also known as favism, and the β-thalassaemia in response to the heavy environmental burden of the century-lasting malaria (Siniscalco et al., 1961; Brown, 1981).

Multiple sclerosis (MS) is an immune-mediated disease of the central myelin with a putative (auto)immune-mediated pathogenesis and unknown aetiology (Hohlfeld and Wekerle, 2004). A number of predisposing genes are believed to act in concert with precipitating environmental events to determine MS appearance, although there is no consensus as to how many

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and which of either one is the causal factor(s) (Lincoln et al., 2005). The geographical distribution of MS describes areas of high (northern Europe), medium (Mediterranean basin) and low prevalence rates (Africa; Pugliatti et al., 2002). However, the prevalence of MS has raised in Sardinia during the last four decades resulting in one of the highest worldwide despite its Mediterranean location (Pugliatti et al., 2001).

Several studies conducted on red cells, HLA, mitochondrial and Y chromosome DNA gene-frequency clearly indicate that Sardinians have a different phylogeny from Europeans, including mainland Italians, along with a high value of consanguinity due to their geographical isolation (reviewed in Sotgiu et al., 2004).

In 1951, the WHO-coordinated Sardinian campaign eradicated malaria from the island (Brown, 1998). Perhaps not coincidentally, and certainly not fully justified by an improved diagnostic accuracy, a 3-fold increase of MS incidence has been recorded in Sardinia starting from 1960 (Rosati et al., 1986; Pugliatti et al., 2005). This "MS epidemics" represents a rare event and still an unsolved enigma. However, because 50 years is too short a span for a substantial Sardinians' genetic change, an environmental modification becomes a likely determinant (Sotgiu et al., 2003; Pugliatti et al., 2006).

Compared to other Caucasians living in the same Mediterranean area, Sardinians have had among the highest mortality rate for malaria (Kaneko et al., 2000; Brown, 1998). Curiously, the "Sardinian" MS-associated HLA haplotype has the highest odds ratios in the same former highest-rate malarial areas (Bitti et al., 2001) and the frequency of some TNF polymorphisms, associated with both MS and malaria (Knight et al., 1999; McGuire et al., 1999; Fernandez-Arquero et al., 1999), are 10-fold elevated amongst Sardinians as compared to any neighbor populations (Wirz et al., 2004).

Despite these circumstantially good suggestions, a clear-cut experimental association between MS and malaria has never been demonstrated to date. With the present study, we aim at disclosing evidences of an abnormal immunological response to *P. falciparum* in Sardinian MS patients (sMS) as compared to their ethnic unrelated healthy controls (sHC) and MS patients from continental Italy (iMS). Monocytes and lymphocytes of the peripheral blood (MNC) were used to this purpose as they are protagonists of the immune response against *P. falciparum* and of the pathogenesis of MS (Lassmann et al., 2001; Deininger et al., 2002; Hendriks et al., 2005).

2. Materials and methods

2.1. Patients and controls

Twenty-eight patients of Sardinian ancestry (18 females and 10 males, mean age 29±5 years) with definite MS (Poser et al., 1983) (sMS) and 28 age and sex-matched ethnic healthy controls (sHC, mean age 28±2) were recruited for this study. An additional series of 16 age and sex-matched MS patients (11 females and 5 males, mean age 31±4) of mainland Italian phylogeny (iMS) were also selected as control of sMS. All donors gave a written consent to participate to this study.

2.2. HLA-DR and TNF-promoter genotyping

Polymorphic DRB1 gene and dot blot analysis with oligonucleotide probes were carried out according to published methods (Marrosu et al., 1997). SNPs of the TNF promoter region were determined as previously described (Wirz et al., 2004).

2.3. P. falciparum cultures and antigen preparation

P. falciparum clone 3D7A (Walliker et al., 1987) were maintained *in vitro* according to the method described by Trager and Jensen (1976). Parasites were grown in O^+ red blood cells in RPMI 1640 plus hypoxanthine 50 μg/ml, supplemented with 10% defibrinated human plasma at 37 °C, in a 2% O_2 and 5% CO_2 atmosphere. The culture medium was refreshed daily and the parasitaemia monitored using thin blood smears (Giemsa stain). *P. falciparum* trophozoites were used for antigen preparation. To obtain parasite synchronization, 8–10% parasitaemia cultures were centrifuged at 1500 rpm. The pellet was resuspended in incomplete RPMI medium supplemented with 0.75% Gelatine (Merck) to make an overall 10% parasite suspension, and incubated at 37 °C for 45 min. Top layer was washed in phosphate-buffered saline (PBS). Morphological analysis of Giemsa-stained slides indicated that resulting cultures typically contained >80% trophozoite stages. Parasitized erythrocytes were lysed with 0.05% saponin solution in 1×PBS (Wallach, 1982), pelleted and flash frozen in liquid nitrogen. The pellet of *P. falciparum* was repeatedly (six times) sonicated on ice for 30 s, Bandelin Sonopuls, Berlin. A lysate composed of a mixture of *P. falciparum* antigens was obtained.

2.4. P. falciparum-driven MNC proliferation test

Peripheral blood was collected in EDTA-containing tubes. Mononuclear cells (MNC) were isolated by centrifugation on a discontinuous density gradient (Lymphoprep; 1.077 g/ml; Nycomed, Oslo, Norway). The optimal concentration of *Plasmodium* to be used in the MNC proliferation experiments was preliminarily determined with the use of a dose-dependent assay, as follows: 2×10^5 MNC/well from 4 MS patients and 4 HC were plated in triplicate on a microtiter plate alone or in the presence of either 10 μg/ml LPS, or *P. falciparum* lysate at concentrations of 0.1, 1 and 10 *Plasmodium*/MNC. After 48 h, cell cultures were harvested, transferred in another 96-well plate and incubated with BrDU. After an additional 3 h culture a proliferation assays was performed, in triplicate, following instructions from the manufacturer (Amersham Biosciences). Optical density (OD) was measured using an ELISA reader. According with the manufacturer indications, OD value of 1 is equivalent to 20 [^{3}H]-thymidine cpm$\times10^{-3}$ in a proliferation test of 24 h duration and 500 L929 cell/well concentration.

At the highest *P. falciparum* concentration (10 *Plasmodia*/MNC) the proliferative response was the lowest, possibly due to a toxic effect. The concentration of 1 *Plasmodium*/MNC gave the highest response and was therefore used for the remaining tests. MS patients and controls were all rhesus+ to exclude an