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Anti-*Anisakis* sp. antibodies in serum of patients with sepsis and their relationship with γδ T cells and disease severity

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ABSTRACT

Immunosuppression in sepsis reduces both αβ and γδ T cell subsets. *Anisakis* sp. is a parasitic nematode with a high prevalence in Spain. Previous contact with the parasite is related to a decrease in γδ T cells. Anti-*Anisakis* antibodies were measured and related to αβ and γδ T cells in 114 septic patients versus 97 healthy controls. Significant differences were seen with respect to the groups with severe sepsis and septic shock where lower anti-*Anisakis* levels were observed. A similar decrease appeared in the case of specific IgM with significant differences between the groups of control/uncomplicated sepsis versus severe sepsis and septic shock. These differences were also apparent in the case of specific IgA. The lowest IgE levels were detected in the septic shock group. Anti-*Anisakis* IgG levels significantly increased in septic shock groups compared with the controls. We observed positive correlations among anti-*Anisakis* IgA levels and all γδ T cell subsets. There were negative correlations among IgA levels and APACHE and SOFA indices. Greater contact with the parasite (IgG) was directly related with septic shock, inflammation and markers of sepsis severity. A lack of protection in the mucosa (IgA and γδ T cells) was associated with the disease severity.

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1. Introduction

Increasing evidence supports a central role for immunosuppression in sepsis. The initial immune response in sepsis is a hyperinflammatory state (cytokine storm), but it rapidly progresses to a hypoinflammatory response (immunosuppression) (Hotchkiss et al., 2013). This immune dysfunction may lead to increased host susceptibility to secondary opportunistic infections (Otto et al., 2011) or to reactivation of latent infections (Luyt et al., 2007; Limaye et al., 2008).

T cells are classified as αβ and γδ T cells depending on the type of T cell receptor (TCR) expressed on their surface. While αβ T cells are the most frequent in peripheral blood, γδ T cells represent only 5–10%. However, γδ T cells represent more than 50% of T cells in mucous membranes and are very common among intraepithelial T lymphocytes. γδ T cells have characteristics common to both innate and adaptive immune cells, and they are considered to be a first line of defence against common microorganisms (Chien et al., 2014). We have recently demonstrated that immunosuppression in sepsis reduces both αβ and γδ T cell subsets, although γδ T cells showed the largest decrease and this reduction became more intense when the septic condition became more severe. Similarly, mortality was associated with a significant decrease in γδ T cells (Andreu-Ballester et al., 2013). We have also demonstrated a greater expression of IgE against the opportunistic agent *Encephalitozoon cuniculi* in septic patients which was, interestingly, related to a decrease in αβ and γδ T cells in peripheral blood (Andreu-Ballester et al., 2014).*Anisakis* sp. is a parasitic nematode that cause anisakiosis (Kassai et al., 1988) and whose seroprevalence is highly variable among different Spanish regions, with rates that range from 0.43% (Valiñas et al., 2001) to 22.1% (del Rey Moreno et al., 2006). In spite of the elevated rates of fish consumption in Spain, positivity is more prevalent among subjects who frequently consumed fresh and partially cooked fish than among consumers of frozen, boiled or baked fish (Puente et al., 2008). In a previous study (data not shown) we analysed the levels of anti-*Anisakis* antibodies in the serum of healthy persons. That study showed that previous contact with the parasite was related to a decrease in all γδ T cell subsets (Zamora et al., 2017).

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The aim of the present study was to analyse the levels of anti-*Anisakis* antibodies in the serum of septic patients for comparison with healthy persons as well as to determine whether the specific Ig levels were related to αβ and γδ T cells and the severity of the disease.

2. Materials and methods

2.1. Study population

In this retrospective study, sera from 114 patients who met the criteria for sepsis in our previous study were analysed (Andreu-Ballester et al., 2013). All patients were admitted to the Emergency Department of the Arnau de Vilanova Hospital and Intensive Care Unit of the Dr. Peset Aleixandre Hospital, both in Valencia (Spain). Sepsis was defined according to internationally established criteria, as well as its different stages: sepsis without organ failure and with organ failure (severe sepsis) (Levy et al., 2003; Dellinger et al., 2004). In addition, patients had to meet the following requirements: they were not suffering from immunodeficiency or autoimmune diseases, they had not been vaccinated in the last 6 months, and they were not undergoing immunosuppressive therapy. The control group (97 subjects) was recruited from relatives of patients admitted to hospital who were not relatives of the septic patients. They were required to have the same characteristics as the patients in addition to not suffering from any acute infectious diseases. Both patient and control groups were informed in writing of the study objectives and gave their authorization to participate in the study. In the case of patients with a significantly altered state of awareness, consent was obtained from their relatives. The Research and Ethics Committee of Arnau de Vilanova Hospital approved the study.

2.2. Methods of blood sample analysis

Whole blood was stained using direct immunofluorescence with the following monoclonal antibodies: CD45, CD4, CD8, CD56, CD2, CD3, CD19, TCRαβ and TCRγδ. Monoclonal antibodies were conjugated with FITC, phycoerythrin (PE), phycoerythrin-Texas red (ECD), and R-phycoerythrin-cyanine 5 (PC5).

The γδ T lymphocyte populations were analysed with PC5 conjugated anti-human γδ TCR (Beckman Coulter, Miami, USA (clone: IMMU510)). The αβ T lymphocyte populations were analysed with PC5 conjugated anti-human αβ TCR (Beckman Coulter (clone: IP26A)). Fluorescence analysis was performed using a Beckman-Coulter multiparameter flow cytometry analyzer, Cytomics FC 500, Florida (USA) and later analysed with CXP Software. Minimums of 30,000 events were measured.

The level of C-reactive protein (CRP) was measured in the sera of patients with a heterogeneous enzymatic sandwich immunoassay with an end-point immunofluorescence reading method (Vitros Chemistry Products®, New Jersey, USA).

The erythrocyte sedimentation rate (ESR) was measured in TEST 1 (Alifax, Padovo, Italy) using quantitative capillary photometry-based technology.

Procalcitonin was measured with an immunoassay "ECLIA" (electrochemiluminescence immunoassay), automated in a Cobas e601 analyser (Roche Diagnostics, Basel, Switzerland).

Lactate was measured with an ABL800 FLEX (Radiometer Copenhagen, Denmark) blood gas analyser by direct potentiometric electrolyte analysis.

2.3. Anisakis sp. antigen and determination of specific antibodies

L3s of *Anisakis* were extracted from blue whiting (*Micromesistius poutassou*) and were homogenised following sonication and extraction in PBS as previously described (García-Palacios et al., 1996).

ELISA plates (Costar, Corning, NY, USA) were sensitised by the addition of 10 μg mL^{-1} of larval antigen. Human sera at 1/100 in PBS-Tween containing 0.1% BSA were added and incubated. Horseradish peroxidase (HRP) conjugated goat anti-human Igs, (IgM, IgG or IgA; Biosource International, Camarillo, CA, USA) were used (Daschner et al., 2002; Gutiérrez and Cuéllar, 2002).

For the IgE determination test, sera were added at a 1/2 dilution. A murine monoclonal antibody against an epsilon human IgE chain (IgG1κ, E21A11, INGENASA, Madrid, Spain) was added and incubated, followed by a HRP conjugated goat anti-mouse IgG1 (gamma) (Life Technologies, Grand Island, NY, USA) (Gutiérrez and Cuéllar, 2002; Martínez de Velasco et al., 2006).

The anti-*Anisakis* antibody levels were measured in septic patients at the time of admission without any previous treatment.

In order to carry out the comparison of the quantitative variable mean values, subjects were divided into two groups according to their anti-*Anisakis* antibody levels. Values were considered positive if they were higher than the mean of the O.D. of 211 studied sera obtained for each Ig plus one respective S.D.

2.4. Statistical analysis

When normality was assumed (Kolmogorov–Smirnov test), *t* or ANOVA tests were used to compare the quantitative variable mean values. When the hypothesis of normality for the quantitative variable was not accepted, the Mann–Whitney *U* test was used. Correlation studies using Pearson's Correlation Coefficient were performed to compare Ig levels and T cell subsets.

The frequencies of cell subsets did not show a normal distribution. When values were transformed into Napierian logarithms, they came to the normal distribution with the exception of the B cells ($P = 0.40$). Normal values of the population were calculated using the equation: Exponent (mean Napierian logarithm ± 1.96 * S.D. Napierian logarithm) (Table 1). $P < 0.05$ was considered significant. Data were analysed using the statistical software SPSS, version 19 (SPSS Inc., Chicago, USA).

3. Results

Tables 2 and 3 show the characteristics of patients with sepsis. There were no differences between the mean ages of the septic patients (65.5 ± 20.9) and the control group (63.1 ± 21.2) ($P = 0.413$). Sixty-three out of 97 healthy controls were male (64.9%) and 34 were female (35.1%) versus 67 (58.8%) and 47 (41.2%), respectively, in the group of patients with sepsis.

Table 1
Normal values and reference range[a] of T cell subsets/mm^3.

T cell subset	Mean	Median	S.D.	Reference range
CD3+	1487.3	1482.0	447.3	720.5–3070.1
CD3+CD4+	927.0	927.2	519.6	408.2–2105.2
CD3+CD8+	583.8	580.0	586.8	236.2–1443.2
CD3+CD56+	223.1	232.1	158.8	49.3–1008.5
CD3+ αβ	1363.6	1336.2	457.4	651.7–2853.5
CD3+CD4+ αβ	880.3	904.6	545.2	375.1–2065.8
CD3+CD8+ αβ	432.8	455.1	797.1	137.2–1365.7
CD3+CD56+ αβ	44.6	50.0	262.8	4.3–453.0
CD3+ γδ	45.6	45.2	603.2	7.0–297.8
$CD3+CD4^{-}CD8^{-}$ γδ	31.7	35.0	769.8	4.3–234.0
CD3+CD8+ γδ	11.2	10.6	899.0	1.3–90.6
CD3+CD56+ γδ	5.4	6.4	59.1	0.3–85.1
CD19+	181.3	206.2	7.1	46.2–710.5

[a] Reference range of T and B cells in healthy adult subjects ($n = 157$) were from a previous study (Andreu-Ballester et al., 2012).

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