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Serum markers of rheumatoid arthritis in visceral leishmaniasis: Rheumatoid factor and anti-cyclic citrullinated peptide antibody

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Abstract

Rheumatoid factor (RF) has been described in visceral leishmaniasis (VL). However, there is no report of RF-isotype or other rheumatoid arthritis (RA) autoantibody in VL. This work investigated RF and anti-cyclic citrullinated peptide antibody (CCP-Ab) in sera from 35 inhabitants from a VL area: 15 from healthy persons (HIEA); 10 from VL patients (VL), and 10 from subjects cured of VL (CVL). The controls were represented by sera from 15 healthy individuals (HI) and from 10 RA patients from a VL free area. IgM-RF was investigated by immunoturbidimetry, while IgA-RF and CCP-Ab by enzyme-linked immunosorbent assay (ELISA). Increased RF-IgM production was found in 9 out of 10 sera from both VL and RA groups (median level 100 and 182 IU/ml respectively); in three out of CVL-sera (level 94 IU), and in only one HIEA-serum (level 58 IU). IgA-RF was only detected in RA-sera (5/10, 50%), while CCP-Ab was found in three VL and in four RA sera (median level 36.5 U and 161.5 U respectively). A strong correlation was observed between RF-IgM and VL in endemic area ($P < 0.0001$). We concluded that an increased IgM-RF production associated with sporadic and moderate CCP-Ab synthesis is an autoimmune characteristic of VL.

Keywords: Autoimmunity; CCP-Ab; RF-IgA; RF-IgM; Visceral leishmaniasis

1. Introduction

Rheumatoid factors are antibodies that react with IgG heavy chain epitopes in the interface of γ2–γ3 domains [1]. These autoantibodies are more frequently found in rheumatoid arthritis, but they may be observed in other autoimmune diseases, mainly Sjögren syndrome, and also in some infections caused by virus, bacteria or parasites [2].

Rheumatoid factor from RA patients differs from the IgM anti-IgG antibody that is found in infectious diseases. It reacts with high affinity with IgG epitopes due to the multiple somatic mutations in the V genes, and may be produced with different isotypes [1,3–5].

Rheumatoid factor is an important mediator of the immune response. As specific receptor of B cells, RF is able to present for T lymphocytes antigen in immune complexes with IgG antibodies. In the soluble form, it stabilizes the binding of low affinity IgG antibodies on the surface of pathogens, improves their opsonization, and also promotes the clearance of circulating immune complexes of IgG and antigen [6,7].

Rheumatoid arthritis is a systemic autoimmune disease of unknown etiology that may present articular as well as extra-articular clinical manifestations. RA patients may produce RF and other autoantibodies that are not essential to their diagnosis. Nevertheless, IgA-RF has been associated with extra-articular manifestations of RA, and CCP-Ab with bone erosion in this autoimmune disease [8–11].

In contrast to RA, parasitic rheumatism is a clinical syndrome largely unmentioned in medicine literature that is associated with parasite infection. It may be diagnosed through

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epidemiologic, clinical and laboratory criteria, which do not include autoantibody production [12].

Visceral leishmaniasis is an endemic parasitic disease in different geographic regions of the world, which has been associated with parasitic rheumatism [12,13]. A strong humoral immune response can be observed in VL, demonstrated by high production of antileishmanial antibody, circulating immune complexes, and an important polyclonal activation of B-lymphocytes that has been documented by synthesis of antibodies for self-antigens, mainly IgG, smooth muscle and nuclear proteins [14–16].

RF production in visceral leishmaniasis was first reported twenty years ago [14]. However, there are no detailed studies evaluating its frequency in different clinical groups from endemic areas, identifying its isotypes, or showing its association with other RA autoantibody in this parasitic disease.

The aim of this work was to investigate the presence of IgA and IgM rheumatoid factor, and of cyclic citrullinated peptide antibody in sera of individuals from an endemic area of visceral leishmaniasis, contributing to a better understanding of the autoimmunity findings previously described in human visceral leishmaniasis.

2. Materials and methods

2.1. Specimen

Thirty-five sera from individuals living in VL endemic areas in the Northeastern part of Brazil were evaluated. The donors were previously classified as healthy or infected by *L. chagasi* after a clinical evaluation, skin test of Montenegro and antileishmanial IgG serology, represented by enzyme-linked immunosorbent assay (ELISA) and indirect fluorescent antibody test (IFAT) with promastigote antigen [17,18]. Visceral leishmaniasis was assessed by clinical exam, positive serology and identification of *Leishmania* amastigote in bone marrow biopsy.

The following groups of sera from VL endemic area were evaluated: HIEA, formed by sera from 15 healthy individuals from endemic area that had negative Montenegro test and leishmanial serology; VL, represented by sera from 10 VL patients, presenting clinical manifestation and positive serology for visceral leishmaniasis. Sera from 10 individuals cured of VL after antimony therapy was also evaluated (CVL group). Two control groups from an area free of VL (Salvador, Bahia, Brazil) were used: HI, represented by 15 serum samples from healthy blood donors and RA, formed by sera from 10 RA patients from this same city, classified according to the American Rheumatism Association criteria [8]. This study was approved by a local ethical committee, and informed consent was obtained from all individuals.

2.2. Rheumatoid factor

Serum IgM rheumatoid factor was investigated by an immunoturbidimetry assay from BioSystems S.A (Costa Brava, Barcelona, Spain), using polystyrene latex particles coated with human IgG. The level of IgM rheumatoid factor was calculated from a reference curve from a WHO standard.

Serum IgA-RF production was tested by indirect ELISA. In brief, micro-wells of flat bottom polystyrene plates (Nunc Maxsorp, Denmark) were incubated (18 h, 4 °C) with 100 μl of carbonate coating buffer (50 mM, pH 9.6) containing 500 ng of purified human IgG, which was isolated from a pool of sera from healthy individuals through anion-exchange chromatography using DEAE-Sephacel column. The free sites of the solid phase were blocked with PBS-BSA, a medium represented by 1% bovine serum albumin (BSA) in phosphate buffered-saline (PBS, 150 mM NaCl containing 10 mM phosphate buffer, pH 7.4). For the immune reaction with RF-IgA, the sera were diluted at 1/101 in this medium containing 0.05% Tween 20 (PBS-BSA/T) and incubated for 1 h at 25 °C into the IgG sensitized wells, using 100 μl/well. The wells were washed three times with PBS containing 0.05% Tween 20 (PBS-T) and incubated with 100 μl of goat IgG anti-human IgA alkaline phosphatase conjugate (Sigma, St. Louis Mo., USA), diluted at 1/25,000 in PBS-BSA/T in the same condition above (1 h, 25 °C). After, the wells were washed, and incubated with a substrate of *p*-nitrophenyl phosphate (PNP) in diethanolamine buffer containing $MgCl_2$ for 30 min, at 37 °C. The enzymatic reactions were stopped with 100 mM EDTA and measured at 405–600 nm in a Diamedix BP-12 ELISA. The level of IgA-RF was expressed as Absorbance (A).

2.3. Anti-cyclic citrullinated peptide antibody

Anti-cyclic citrullinated peptide IgG antibodies (CCP-Ab) were investigated by indirect ELISA using the QUANTA Lite™ CCP2 and CCP3 IgG ELISA (INOVA Diagnostics, Inc, San Diego, USA). All sera were tested diluted at 1/101, and their levels of CCP antibody were calculated using a low positive control serum as recommended by the manufacturer. A CCP-Ab level $\geq$20 IU was positive, using the following criteria: low (20–39 U), moderate (40–59 U) or strongly positive ($\geq$60 U).

2.4. Statistical analysis

The abnormal production of both IgM and IgA-RF in the different clinical groups was evaluated measuring the increase of their levels, using as reference their statistically calculated cut-offs from 15 sera from HI group (IgM-RF = 45 IU/ml and IgA-RF = 0.079 A respectively) [19]. The concentration of IgM-RF, IgA-RF and CCP-Ab was expressed as median. The non-parametric tests of Kruskal–Wallis and Mann–Whitney were used to compare the medians of RF titer of the different groups. The Chi-square test evaluated the association between RF and VL. The level of significance set at $P < 0.05$. The GraphPad 4.0 software was used in the statistical analysis.

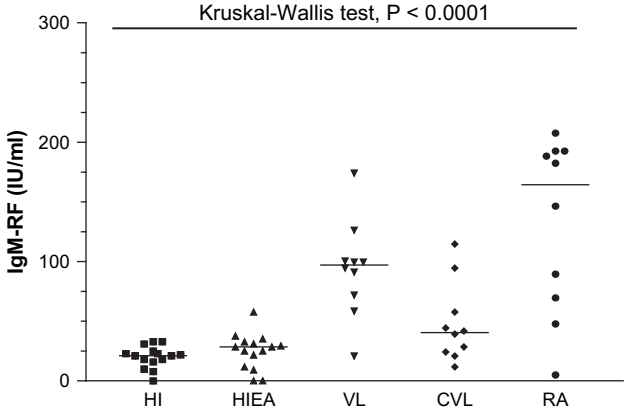

Fig. 1. IgM-RF seropositivity in healthy control individuals (HI and HIEA), VL patients, and subjects cured of VL and RA patients. Horizontal bars represent IgM-RF median level.

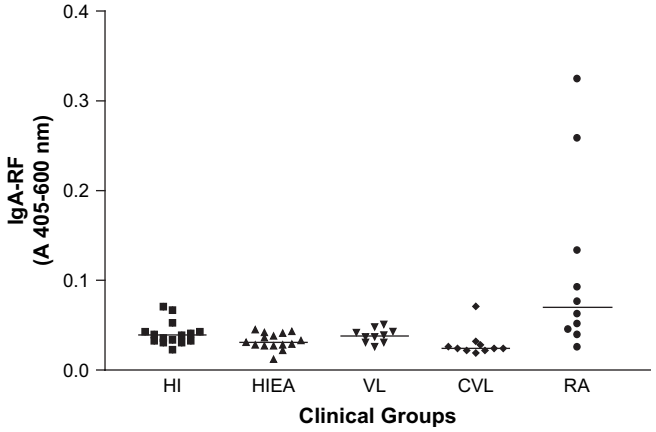

Fig. 2. IgA-RF seropositivity in healthy control individuals (HI and HIEA), VL patients, and subjects cured of VL and RA patients. Horizontal bars represent the median level of IgA-RF.

3. Results

3.1. Rheumatoid factor seropositivity

The levels of IgM rheumatoid factor were different in the groups studied (Kruskal–Wallis test, $P < 0.0001$). The median level of IgM-RF in the healthy control groups HI and HIEA was 21 and 28 IU/ml respectively, while an increased production of IgM-RF above the cutoff of 45 IU/ml was verified for only one individual from the HIEA group. Nine out of 10 (90%) VL patients and three out of 10 (30%) CVL subjects presented a high level of IgM-RF (100 IU/ml and 94 IU/ml respectively). In RA group, a high level of IgM-RF was found in 9 out of 10 (90%, 182 IU/ml) patients (Fig. 1).

There was no difference between the levels of IgM-RF of VL and RA groups, but VL patients presented the highest concentration of RF-IgM when compared with CVL group (Mann–Whitney test, $P = 0.0185$). A strong association between IgM-RF and VL was observed when were evaluated the groups from endemic area of visceral leishmaniasis (chi-square test, $P < 0.0001$).

Positive IgA-RF (Absorbance > 0.079 A) was only found in sera from RA patients (5/10, 50%), presenting this group a median absorbance of 0.134 (Fig. 2).

3.2. CCP-Ab

Anti-CCP IgG antibody was found in three VL sera (median level = 36.5 U, range 24–81 U) and in four serum samples from RA group (median level = 161.5 U, range 92–270 U). There was no positive CCP-Ab reactive serum in HI, HIEA or CVL group.

4. Discussion

Human visceral leishmaniasis is associated with important IgM-RF production, and in minor frequency, CCP-Ab synthesis. This immune response of autoantibody decreases after successful treatment with antimony and is not observed in healthy individuals living in VL endemic areas.

In contrast with rheumatoid arthritis, the immune response of IgM-RF in visceral leishmaniasis is not shared by IgA-RF, showing that the production of RF in VL does not exhibit isotype switch. Such a finding may suggest the involvement in this autoimmune response of $CD5^{+}$B-1 B cells that are committed to produce multispecific autoantibodies, including IgM-RF of low affinity, after a weak T-cell interaction [20]. However, it is known that RF-B lymphocytes can capture immune complex and efficiently present its processed antigenic peptides for T lymphocytes [6]. Thus, RF-B cells could play an important role in the altered immune response verified in VL, inducing a strong autoantibody immune response and an exuberant synthesis of antileishmanial antibodies. On the other hand, the interaction of IgM-RF with IgG antibodies linked to *Leishmania* amastigote could produce larger sized immune aggregates, improving parasite opsonization through Fcγ receptors on macrophage surface, and spreading *Leishmania* infection. Such a possibility is supported by the finding that antileishmanial IgG antibodies are involved in both *Leishmania* infection and IL-10 production in vitro, as demonstrated in an experimental model represented by VL serum, axenic amastigote and cultured macrophage [21].

The finding of anti-CCP antibody in VL patients was unexpected. These antibodies are thought to have high diagnostic specificity for rheumatoid arthritis, and are now used as good markers of early RA and laboratory tools to evaluate RA evolution [22]. Their absence in sera from CVL group suggests that CCP-Ab may be associated with active VL, and together with IgM-RF, they disappear after successful treatment. Thus, CCP-Ab production could be caused by citrullination of host proteins during *Leishmania* infection and would represent a new aspect of VL immunopathogenesis. However, the participation of hypergammaglobulinemia, circulating immune complex, or polyreactive antibodies in the CCP-Ab reactivity observed in VL patients should be investigated.

In conclusion, an increased IgM-RF production, occasionally accomplished of moderate CCP-Ab seropositivity, is an

autoimmune finding in VL that deserves future studies to elucidate their possible involvement in VL parasitic rheumatism and immunopathogenesis.

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