General false positive ELISA reactions in visceral leishmaniasis. Implications for the use of enzyme immunoassay analyses in tropical Africa

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1. Introduction

Leishmaniasis is a neglected disease in tropical countries. Clinical and laboratory features may mimic autoimmune diseases and this can complicate the Leishmania diagnosis. Due to our previous investigation for false anti-CCP2 reactivity in Leishmania-infected subjects and our interest in immunity against the joint-specific collagen type II (CII) in rheumatoid arthritis (RA) we investigated the same cohort for anti-CCP antibodies.

We found elevated anti-CII reactivity in Leishmania-infected patients as compared to controls. When anti-CII OD values were compared with BSA-blocked control plates we found higher reactivity against BSA than in CII-coated plates in many Leishmania-infected patients. The percentage of such false positive anti-CII reactions increased with inflammatory activity, and was found in almost all Leishmania patients with highly active inflammatory disease, but was as low in Sudanese healthy controls as well as among Swedish RA patients. The correlation coefficients between false positive anti-CII and anti-CCP2 measured with a commercial ELISA were highest for patients with the most inflammatory disease but non-significant for Sudanese controls and Swedish RA patients, arguing that our findings may have general implications for ELISA measurements in leishmaniasis.

ELISA investigations in areas endemic for leishmaniasis might benefit from individual-specific control wells for each serum sample. This approach might also be applicable to other geographical areas or patient groups with high incidence of inflammatory and infectious diseases.

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investigations with control plates, and also this time showed higher reactivity in the control plates than in CII-coated plates. Intriguingly, almost all patients with highly active leishmaniasis showed such false reactivity. As our control plates in this recent experiment were only blocked, our results raise general concerns about ELISA measurement of autoantibodies in tropical Africa and perhaps also elsewhere where *Leishmania* infection or other highly inflammatory infections appear.

### 2. Methods

#### 2.1. Study subjects

The study was carried out at Tabarakalla rural hospital in Gedaref state in Northern Sudan, which it is endemic for *L. donovani*, and where the main *Leishmania* vector is *Phlebotomus orientalis* (Elshaﬁe *et al.*, 2007). A detailed clinical history and examination were obtained from each studied patient. Subjects were questioned about their ethnic, and geographic origin and were examined for clinical manifestations of VL and PKDL. A general clinical examination was conducted with particular reference to hepatomegaly, splenomegaly and lymph node involvement. All suspected patients were investigated for malaria; those with malaria infection were excluded. For diagnosis an inguinal lymph node aspiration was performed on those clinically suspected of having VL (i.e., all individuals with any of the following clinical findings: fever for >2 months, left upper quadrant pain, lymphadenopathy, splenomegaly, or wasting). All individuals with clinically suspected VL but with negative results on inguinal lymph node aspiration were subjected to bone marrow aspiration aiming to seek for *L. donovani* bodies for VL confirmation.

*Leishmania*-infected patients included 62 VL patients (20 females and 42 males; age median: 24 (26) years; range: 3–73), 44 PKDL patients (13 females and 31 males; age 23 (20) years; range: 3–27) and 49 healthy controls without any disease complaints from Tabarakalla village (26 females and 23 males; age 22 (20) years; range 3–45). Severely ill patients with VL admitted to the Tabarakalla hospital for hospitalization were classified as acute VL (*n* = 15). Patients not severely ill and treated as outpatients with daily injections of sodium stibogluconate were classified as sub-acute VL (*n* = 47).

Venous blood drawn from patients and healthy controls was separated by centrifugation and frozen in liquid nitrogen within 2 h of sampling and transported frozen in liquid nitrogen until analyzed in Uppsala. Data on 274 Swedish early RA patient sampled at the time of diagnosis that was made within 1 year after first symptoms, as previously described (Mullazehi *et al.*, 2007) and using exactly the same anti-CII ELISA are included for comparison.

Ethical approval for this study was obtained from the ethical committee from the Ministry of Health in Khartoum and Gedaref State, Sudan. Informed consent was obtained from all the adults who participated in the study. For younger children, consent was obtained from their parents. For the Swedish RA patients the ethical approval was obtained from the Ethical Committee atKarolinska Institutet, Stockholm. Ethical approval to conduct immune complex and autoantibody studies were obtained from the Ethical Board in Uppsala.

**Anti-CCP2 and anti-C1q-binding CIC ELISA measurements**

Anti-CCP2 was measured using the Immunoscan RA Mark 2 assay (Euro-Diagnostica, Malmö, Sweden). In accordance with manufacturer’s instructions 25 U/ml was used as a cut-off. The kit provides no standard points below 25 U/ml, and the ELISA standard curve was extended by serial dilution down to 3.13 AU/ml to yield quantitative data also in the low range. C1q-binding CIC were measured with ELISA as previously described (Åhlin *et al.*, 2015).

### 2.4. Statistics

Analyses with the Shapiro–Wilk test and probability (QQ) plots showed non-normal distribution of anti-CII levels among Sudanese controls, PKDL and VL patients, both for all data, as well as after exclusion of true anti-CII positive results. As we previously have shown anti-CII to be non-normally distributed also in RA patients (Mullazehi *et al.*, 2012) we used non-parametric tests. For the comparison between two groups, we used the non-parametric Mann–Whitney’s U test for unpaired samples. To estimate correlations between variables, we used the Spearman non-parametric test. *p* values < 0.05 were regarded as significant. All calculations were made by means of the software JMP 11 for Mac.

### 3. Results

#### 3.1. High anti-CII levels in *Leishmania*-infected patients

The median (mean) anti-CII levels among the Sudanese healthy controls were 16 (21) AU/ml. For the PKDL and VL patients the corresponding figures were 28 (43) and 48 (58) AU/ml respectively (Fig. 1). Levels were significantly higher among PKDL (*p* = 0.0004) and VL (*p* < 0.0001) patients as compared to Sudanese healthy controls. Using the cutoff previously defined for Sweden, 26% (13/49) of the Sudanese controls, 48% (21/44) of the PKDL patients and 65% (40/62) of the VL patients showed reactivity against anti-CII (Fig. 2).

The median (mean) values for Swedish RA patients was 12 (55), and 10% (27/274) showed anti-CII reactivity. As previously described (Mullazehi *et al.*, 2012) a small outlier subgroup of the Swedish RA patients showed very high levels of CII (9/274) patients with levels between 470 and 3522) not seen on our *Leishmania* cohort. The
proportions of CII positivity were also higher in subjects with Leishmania infection when compared to the Swedish RA patients (p < 0.0001 compared to both PKDL and VL patients; Fig. 1).

3.2. Leishmaniasis patients, especially acutely ill VL patients, exhibit false positive anti-CII reactivity in ELISA

After further evaluation of the anti-CII positive subjects with only blocked control plates we found that the majority of anti-CII positive Leishmania-infected subjects exhibited false positive reactivity. False positive anti-CII reactivity increased with increasing inflammatory activity in a step ladder fashion from Sudanese healthy controls to PKDL via subacute VL and finally to acute VL where almost all subjects (93% (14/15) show a false positive anti-CII reactivity. Among the Swedish RA patients 1.5% (4/274) exhibited false positive anti-CII reactivity, very similar to the Sudanese control subjects 2% (1/49). The distributions of negative, true positive and false positive anti-CII reactivity in the investigated groups using the Swedish cutoff are graphically depicted in Figs. 1 and 2. Also when using a higher cut-off based on the 95th percentile among the Sudanese controls (80.4 U/ml) we get a parallel relative increase in false reactivity when we move from Sudanese healthy controls (0/49, 0%) to PKDL (2/44, 4.5%), subacute VL (8/47, 17%) and acute VL (8/15, 53%; data not shown).

3.3. Non-related ELISA results correlate strongly in visceral leishmaniasis patients

To investigate whether the high degree of false positive anti-CII reactions especially in the VL group might be a universal finding in ELISA tests, we studied the correlation between the commercial ELISA in the different groups. As we hypothesized that especially false positive reactivities might show a high degree of correlation among patients with high inflammatory activity we first calculated correlation without excluding subjects with true positive results and thereafter re-calculated this correlation only for subjects with negative or false positive anti-CII results as shown in Fig. 3. The correlation coefficients increased after exclusion of true positive reactions for both Leishmania-infected groups, and the largest correlation coefficient was found for VL patients. Thus the false anti-CII reactivity showed the strongest correlation to results obtained from an unrelated commercial ELISA for VL patients, the group with the highest percentage of false positive anti-CII reactions, and the correlation coefficients increased when true positive anti-CII reactions had been excluded in PKDL and VL patients, both groups with inflammatory disease. For subjects not infected with Leishmania, Sudanese healthy controls and Swedish RA patients, there was no significant correlation between anti-CCP2 and anti-CII after exclusion of true positives. For the Sudanese subjects, this comparison could only be made for anti-CCP2 variation below the cutoff, as sera from the (false) anti-CCP2 positive Leishmania-infected subjects had been exhausted in the experiments described in (Åhlin et al., 2015).

3.4. No correlation between circulating immune complexes and false positive anti-CII reactivities among Leishmania-infected patients

As CIC levels are increased in Leishmania patients, and as CIC might be a cause of false positive reactions in ELISA we correlated CIC and anti-CII levels after exclusion of true positive anti-CII samples. For all Sudanese subjects (healthy controls, PKDL and VL patients) there was a significant correlation (r = 0.53, p < 0.0001, n = 123), whereas no significant correlation was seen for any of the groups investigated individually (controls: r = 0.26, p = 0.12, n = 37; PKDL: r = 0.36, p = 0.30, n = 35; all VL subjects: r = 0.26, p = 0.071, n = 51; subacute VL: r = 0.14, p = 0.36, n = 36, acute VL: r = 0.37, p = 0.22, n = 15). Due to lack

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**Fig. 1.** Anti-CII levels among the Sudanese and the Swedish subjects. The lower dashed horizontal line represents the 95th percentile cutoff value previously calculated for Swedish patients (29 AU/ml), the upper dashed horizontal line represents the 95th percentile calculated from Sudanese healthy controls and the solid lines represent the median levels of anti-CII for each group. All data are shown, and subdivided into negative (filled circles), true positive reactions with higher OD in CII wells than in BSA wells (triangles) and false positive reactions with higher OD in BSA wells than in CII wells (stars).
of serum, a number of Sudanese subjects were not investigated for CIC levels.

4. Discussion

In a previous report we defined that *Leishmania*-infected subjects from an endemic area in Sudan possess a false positive anti-CCP2 reactivities in ELISA, as the patients reacted equally well against ELISA wells with citrullinated peptides or with arginine-containing control peptides (Åhlin et al., 2015). In the current study we extend these studies to anti-CII, another RA-associated autoantibody. Also here *Leishmania*-infected patients react stronger to control wells. But as the control wells in our current study only are blocked with the same blocking agent as used in the CII-coated wells and this blocking solution is common to many widely used ELISA protocols, the findings might be valid for any ELISA using a simple blocking system, and raises general concerns about the detection of antibodies with ELISA in *Leishmania*-infected subjects and possibly also in other diseases with high inflammatory activity. Both in our previous report (Åhlin et al., 2015) and in our current study (Fig. 1), the false ELISA reactivities found in *Leishmania*-infected patients are found in the low positive range, whereas positive control groups with RA patients show strong and true positive reactions against anti-CCP2 and anti-CII, respectively. The findings might be applicable also to commercial ELISA systems as we show a high degree of correlation between anti-CCP2 and anti-CII especially in patients with VL, the most inflammatory form of leishmaniasis. None of the autoantibodies anti-CCP2 or anti-CII have any known association to *Leishmania* infection, and when we exclude the low number of true anti-CII positive reactivities and thus focus on the false reactive patients, the significant correlation between the two unrelated autoantibodies increase in both groups of *Leishmania*-infected patients. On the contrary, there is no such correlation between false anti-CII reactivity and anti-CCP2 in the control groups of Sudanese healthy individuals or Swedish RA patients. In fact, anti-CII and anti-CCP2 represent two clinically distinct phenotypes when investigated in the same RA cohort (Mullazehi et al., 2007; Rönnelid et al., 2005).

In our previous paper, the false positive anti-CCP2 reactivity was only marginally diminished after depletion of immune complexes (Åhlin et al., 2015), but we did not have enough samples left for parallel investigations for anti-CII. We anyhow think that the false anti-CII reactivity determined in *Leishmania*-infected subjects is probably not related to immune complexes, as none of the Sudanese subgroups showed any correlation between CIC and false anti-CII reactivity when analyzed separately. The fact that these variables correlated when all Sudanese subjects were analyzed together is probably a group effect. Both measures are increased in *Leishmania* infected patients as compared to in Sudanese controls, as we have shown previously for CIC (Åhlin et al., 2015) and in this paper for false positive anti-CII reactivity.

Our findings should be considered when handling the diagnostics of *Leishmania*-infected subjects in tropical areas like Sudan. Previous reports and our own experience (AE) state that the clinical presentation of *Leishmania* infection can be misclassified as autoimmune diseases.

Fig. 2. Proportions of negative, true positive and false positive anti-CII reactivities among the Sudanese and Swedish subjects. Reference range for positive reactivity is based on the cutoff based on the 95th percentile among Swedish healthy controls.
like autoimmune hepatitis or SLE, including autoantibodies like anti-nuclear antibodies (ANA), IgM rheumatoid factor, anti-DNA and anti-cardiolipin (Sakkas et al., 2008). In that clinical setting physicians might request autoantibody testing by ELISA to confirm the clinical suspicion of autoimmunity. If the requested ELISA present the kind of general false reactivity described in this report, misdiagnosis and wrong management in subjects infected with *L. donovani* infection may ensue.

Also previous papers have reported false ELSA antibody reactivity in tropical and developing areas, concerning malaria serology (Ghosh et al., 2001), anti-neutrophil cytoplasmic antibodies (ANCA) (Ghosh et al., 2008) and Chlamydia serology (Miyashita et al., 2008). In our paper we extend these findings to encompass two independent ELISA systems for measurement of autoantibodies not related to *Leishmania*-infected patients. We show that there is a strong correlation between the reactivities measured with these two ELISAs, especially when the few true anti-CII positive samples had been excluded, and thus that the finding of false reactivity is not dependent on specific autoantibody reactivities, but on the ELISA format per se.

Before performing this study we only had access to Swedish anti-CII data, and the definition of which samples should be subjected to a second round of comparison with only blocked wells was based on the reference range derived from Swedish controls. As the general anti-CII levels among healthy controls living in the Sudanese village of Tabarakalla turned out to be higher than among Swedish blood donors, we have calculated a second cutoff derived from Sudanese controls, and thereby we have defined individuals between the two cutoff lines as either true or false positive (Fig. 1). In this purely technical report including individuals from both countries we have chosen to keep the definition of positivity based on Swedish data, but as we describe in Section 3.2, the general findings are the same using a cutoff based on Tabarakalla controls (Figs. 1 and 2). In a clinically oriented paper, one should of course always interpret autoantibody data in relation to a control population that is clinically relevant to the study in question.

In our view, indirect ELISA or other solid phase immune assay tests for antibodies to be used in areas with *Leishmania* infection might be accompanied by simultaneous evaluation of reactivity in control wells without the target antigen. This is not currently the case, see e.g. (Abass et al., 2013). Very few commercial solid phase antibody systems use such individual control wells, to our knowledge the only exception being the anti-SA test for a subgroup of anti-citrullinated protein/peptide antibodies (ACPA) delivered by the company Euroimmun. An obvious drawback of this approach suggested by us is that the number of samples to be tested in each ELISA plate is diminished by 50% as half of the ELISA plates will be used for control wells.

As other reports have shown false reactivity in ELISA tests also in other tropical diseases (Ghosh et al., 2001, 2008; Miyashita et al.,...
such recommendations to use individual control wells might be extended to other infectious disease settings and also to other continents than Africa. One example is the false positive anti-CCP2 reactivity in hepatitis C-infected subjects in USA where the authors used only blocked ELISA plates as individual controls and reported that the binding was caused by nonspecific binding to plastic (Wener et al., 2004). The findings might not be limited to indirect antibody ELISA systems, as nonspecific binding to the matrix including e.g. blocking agent and plastic might appear also in direct ELISA and sandwich ELISA systems. These hypotheses need validation in further studies.

5. Conclusion

Our data imply that sera from *Leishmania*-infected subjects often react non-specifically in ELISA and we therefore suggest that individual-specific control wells should be included for antibody measurements in areas endemic for leishmaniasis. These suggestions might also be applicable to other geographical areas or patients groups with high incidence of inflammatory and infectious diseases.

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