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BRIEF REPORT

CIGB-814, an altered peptide ligand derived from human heat-shock protein 60, decreases anti-cyclic citrullinated peptides antibodies in patients with rheumatoid arthritis

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Abstract

Rheumatoid arthritis (RA) is a chronic T cell-mediated autoimmune disease. Serum autoantibodies against cyclic citrullinated peptides (anti-CCP) are significant markers for diagnosis and prognosis of this disease. Induction of immune tolerance as therapeutic approach for RA constitutes a current research focal point. In this sense, we carried out a phase I clinical trial in RA patients with a new therapeutic candidate (called CIGB-814); which induced mechanisms associated with restoration of peripheral tolerance in preclinical studies. CIGB 814 is an altered peptide ligand (APL), derived from a CD4+ T cell epitope of human heat-shock protein 60 (HSP60), an autoantigen involved in the pathogenesis of RA. Twenty patients with moderate disease activity were included in this open label trial. Sequential dose-escalation of 1, 2.5 and 5 mg of CIGB-814 was studied. Consecutive groups of six, five, and nine patients received a subcutaneous dose weekly of the peptide during the first month and one dose monthly during the next 5 months. The peptide was well tolerated and reduced disease activity. Here, we reported the quantification of anti-CCP antibodies during the treatment with this APL and in the follow-up stage. Anti-CCP antibodies were quantified in the plasma from patients by a commercial enzyme immunoassay at baseline (T0) and at weeks 28 and 48. Results showed that CIGB-814 induced a significant reduction of anti-CCP antibodies. In addition, this decrease correlated with clinical improvement in patients assessed by Disease Activity Score in 28 joints (DAS28) criteria. These findings reinforce the therapeutic potential of CIGB-814.

Keywords Anti-CCP · APL · CIGB-814 · HSP60 · Rheumatoid arthritis

Introduction

The crucial role of T cells in the pathogenesis of RA is well established [1]. B lymphocytes play a critical role too in the pathogenesis of this disease. They are the source of the rheumatoid factors (RF) and anti-citrullinated protein antibodies, which contribute to immune complex formation and complement activation in the joints [2].

Citrullination may be a consequence of abnormal proteins metabolism occurring at inflammation sites. Exposure to environmental agents that trigger the disease and NETosis of neutrophils represent the main sources of citrullinated autoantigens [3]. Determination of anti-CCP levels during the therapy, may give some evidences about effectiveness of the treatment.

Biologic therapy is an alternative for patients not responding to disease-modifying antirheumatic drugs (DMARDs). However, many patients have an inadequate response to such therapies [4]. Also, this therapy remains insufficient in 40–50% of patients with RA [5].

There are some limited data on the effects of oral DMARD on anti-CCP antibodies and very few data on the effects of biological agents on these antibodies [6, 7]. Other studies have indicated that responsiveness to rituximab therapy is better in RA patients who are anti-CCP-positive, suggesting a possible relationship between the pathogenic capacity of anti-citrullinated protein antibodies (ACPA) and the efficacy of rituximab [8].

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In an interesting study, Wunderlich et al. reported that DMARDs targeting the adaptive immune response such as abatacept and rituximab significantly lowered anti-CCP2 IgG levels, while cytokine inhibitors and methotrexate had no significant effects on anti-CCP2 IgG levels. In addition, only rituximab lowered total IgG level [9].

Induction of peripheral tolerance constitutes an attractive therapy for RA. This strategy is based on the use of autoantigens or epitope derived from these, such as APLs [10, 11]. Selection of a specific autoantigen is a crucial point for APLs design. Studies on the immunopathogenesis of autoimmune arthritis in the rat adjuvant arthritis (AA) model of RA as well as observations in patients with RA and juvenile idiopathic arthritis have unraveled immunoregulatory attributes of self-Hsp65-directed immunity [12].

In addition, HSP60 has been successfully used in the induction of tolerance in autoimmune arthritis models [13].

We previously design a novel APL from human HSP60, using bioinformatics tools. This APL (called previously APL-1 and here CIGB-814) increases the frequency of regulatory T cells (Treg) and inhibits significantly IL-17 in experimental assays [14, 15]. Also, CIGB-814 reduced the course of arthritis in two animal models [14, 16].

Recently, a phase I clinical trial was concluded, in which the safety and pharmacokinetics of CIGB-814 were evaluated [17, 18].

The primary goal of this work was to quantify anti-CCP antibodies in the plasma from patients, during the clinical trial. In addition, this study explores a possible correlation between anti-CCP antibodies levels and the clinical improvement of patients.

Patients and methods

Study design

Study design was according to scale sequential doses of CIGB-814: 1 mg, 2.5 mg, and 5 mg. Patients received a subcutaneous dose weekly of the peptide during the first month and a monthly dose during the next 5 months. Patients were followed for 5 months after the last dose of the study.

The restriction for using DMARDs, glucocorticoids, and nonsteroidal anti-inflammatory drugs (NSAIDs) was extended from the washout period, including the therapy phase and up to 3 weeks after the last CIGB-814 dose. DMARDs and NSAIDs could be administered if disease flares, according to the physician's criteria. Otherwise, only analgesics were permitted [18].

This clinical trial was registered under number RPCEC00000238 at the Cuban Registry of Clinical Trials (www.registroclinico.sld.cu).

Patient samples

Twenty patients with moderate disease activity ($3.2 < \text{DAS28} < 5.1$) were enrolled in this study.

Active disease was defined using DAS28-erythrocytes sedimentation rate (ESR) (DAS28-ERS). All patients had radiological damages in dominant hand, quantified through Simplified Rheumatoid Arthritis Magnetic Resonance Imaging Score (SAMIS). In addition, all patients had a moderated disability, measured by HAQ-CU (Cuban adaptation of the Health Assessment Questionnaire-Disability Index) and Short Form 36 Health Survey (SF-36) questionnaire [18].

Plasma samples were obtained before treatment (T0, baseline) and at weeks 28, 36, and 48. Anti-CCP antibodies concentrations were assessed by ELISA (CCPlus®, Immunoscan, Euro Diagnostica AB, Sweden).

Patients with antibody levels above or equal to 25 U/mL were considered positive (anti-CCP+); while patients with values below this threshold were considered negative (anti-CCP−).

Statistical analysis

Data were analyzed using GraphPad Prism version 6.00 (GraphPad Sofware, San Diego CA, USA). Data were examined for normality and equal variance with Kolmogorov-Smirnov and Bartlett's tests, respectively. The differences between groups for clinical activity were analyzed with ordinary one-way ANOVA with Tukey's multiple comparisons test. Antibodies concentrations samples were examined by unpaired t test. P values less than 0.05 were considered statistically significant. Spearman's rank correlations were used to examine associations between antibodies concentrations and clinical activity.

Results

Baseline characteristics

Patients were predominantly women (85%) with moderate disease activity (100%) and a median duration of the disease of 8.5 years (Table 1). Three patients dropped the study. One patient withdrew voluntarily and another one stopped due to allergy to paracetamol, both before therapy was completed. Another patient dropped voluntarily during follow-up stage.

CIGB-814 caused significant reduction of anti-CCP antibodies in RA patients

Anti-CCP in the plasma from patients was quantified by a commercial ELISA. This ELISA quantified antibodies against four antigens: Vimentin, Collagen type II, Fibrinogen and α-enolase. The proportion of patients who were considered anti-

Table 1 Baseline characteristics of patient

CIGB-814 doses	1 mg ($n = 6$)	2.5 mg ($n = 5$)	5 mg ($n = 9$)
Age (years)*	52.0 ± 9.30	44.4 ± 6.27	50.9 ± 8.52
Gender (% female)	100.00	100.00	66.66
Duration of RA (years)	12.70 ± 5.68	6.00 ± 3.94	7.11 ± 8.95
Anti-CCP antibody† (% positive)	60.00	60.00	62.50
RF‡ (% positive)	40.00	33.33	62.50
Methotrexate (% patients)	100.00	100.00	100.00
dose (mg/week)	11.25	10.00	11.67
Concomitant cDMARDs for RA (% patients)			
Hydroxychloroquine	66.66	40.00	33.33
Sulfasalazine	16.66	20.00	22.22
Prednisone use (% patients)	100.00	100.00	100.00
ESR (mm/h)	24.60 ± 27.62	38.60 ± 19.83	40.50 ± 20.21
DAS28-ESR	4.34 ± 0.55	4.37 ± 0.64	4.62 ± 0.42

*Data reported as mean values ± SD unless otherwise indicated

† Anti-CCP antibody positivity (> upper limit of normal (ULN) = 25 U/mL)

‡ RF positivity (> ULN = 18 U/mL)

Anti-CCP, anti-cyclic citrullinated peptide; *cDMARDs*, conventional disease-modifying antirheumatic drugs; *DAS28-ESR*, Disease Activity Score for 28-joint counts based on the ESR; *ESR*, erythrocyte sedimentation rate; *RF*, rheumatoid factor

CCP positive was 61%. In this study, we show the results from 18 patients who completed the clinical trial.

Therapy induced a significant decrease in the concentration of anti-CCP antibodies at the end of the treatment and during the follow-up phase, compared to T0 (Fig. 1). Similar results were found for patients classified as anti-CCP−.

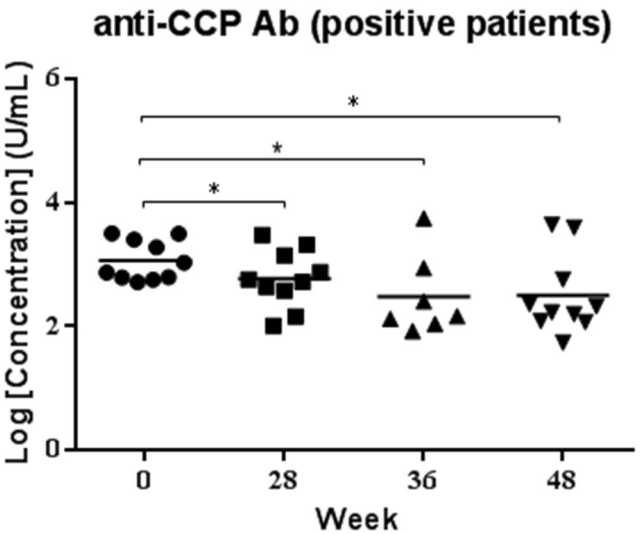

Fig. 1 Therapy with CIGB-814 caused significant reduction of anti-CCP antibodies in RA patients. Concentrations of antibodies in plasma were measured by specific ELISA. Plasma samples were obtained before treatment (T0, baseline) and at weeks 28, 36, and 48. Log concentration of anti-CCP is expressed as mean and were analyzed using unpaired *t* test (*$P < 0.05$). Patients with antibody levels above 25 U/mL were considered positive (anti-CCP+)

In addition, CIGB-814 did not modify RF (type IgM/IgG) levels during therapy or in the follow-up phase (results not showed).

CIGB-814 caused a decrease of DAS28-ESR scores and correlated with reduction of anti-CCP antibodies in RA patients

All patients (anti-CCP+ and anti-CCP−) showed a decrease of DAS28-ERS scores, during the treatment and in the follow-up stage (Fig. 2).

Clinical improvement of patients correlated with the reduction of anti-CCP antibodies induced by CIGB-814, at the end of the follow-up stage (Fig. 3).

Discussion

Phase I clinical trial with CIGB-814 showed that treatment was well tolerated at all doses [18].

Although RA is mediated by the activation of T cell clones, with TH1 and TH17 phenotypes, antibodies such as RF and anti-CCP contribute to disease pathogenesis.

Presence of anti-CCP is associated with a rapid progressive evolution of RA, with an early development of erosive lesions, increased activity of the disease and disability, compared to anti-CCP negative patients [19].

Here, we quantified anti-CCP antibodies in the plasma from patients, at the start and end of the therapy with CIGB-

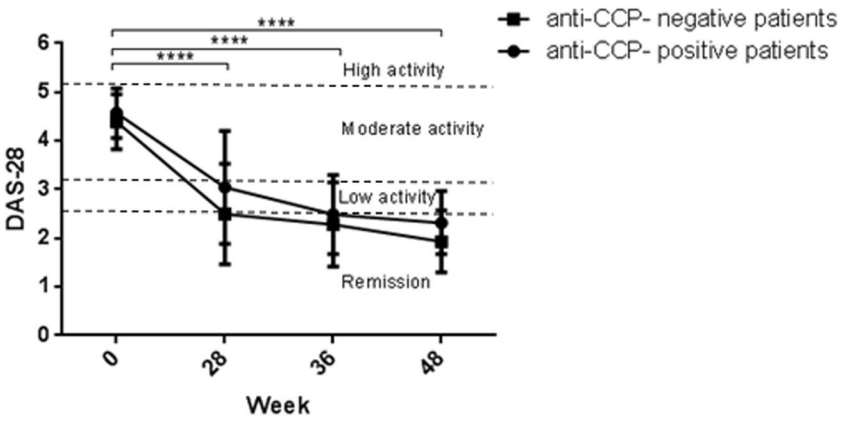

Fig. 2 CIGB-814 caused a decrease of DAS28-ESR scores, during the treatment and in the follow-up stage in RA patients (positive or negative anti-CCP). All patients began the treatment with moderate disease activity. Rates of remission (defined as DAS28-ESR of < 2.6) at weeks: 28, 36, and 48 are showed. Values are the mean + standard deviation and were analyzed using ANOVA and Tukey's post-test (***$P < 0.001$)

814 as well as in the follow-up stage. Patients included in this study had moderate disease activity. All patients had received two or more DMARDs before enrolment. However, these previous therapies did not decrease anti-CCP levels in patients.

Therapy with CIGB-814 induced a significant reduction of anti-CCP antibodies at the end of the treatment and during the follow-up phase. In addition, RF remains constant throughout the treatment. These results reinforce the therapeutic potential of CIGB-814 and suggest that CIGB-814 could have an effect on the plasma cells secreting these antibodies. CIGB-814 induces Treg in several experimental systems [14–16]. Treg have different action mechanisms, which may be dependent on cell contact mediated by granzyme A and B or perforins, through which they can induce apoptosis on effector T cells and other cells such as B lymphocytes producing anti-CCP antibodies [20].

Another possibility is that CIGB-814 influences on citrullination process. In RA, exposure to the environmental agents that trigger the disease and NETosis are main sources for protein citrullination [3].

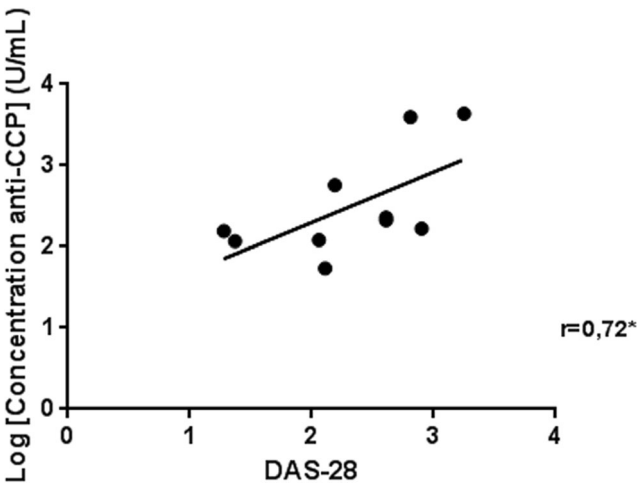

Fig. 3 Correlation between anti-CCP antibodies levels and DAS28-ESR scores, at the end of the study. Data were transformed to log (Y). The analysis was performed using a Spearman correlation test. (*) means correlation ($P < 0.05$)

In this sense, some authors have found that HSP60 affects the activity of neutrophils and NETosis. Osterloh et al. reported HSP60 directly enhances phagocytic activity of neutrophils, this event may be associated with NETosis [21]. Dapunt et al. demonstrated that bacterial HSP GroEL from *Staphylococcus epidermidis* biofilms induced DNA release and promoted NETosis [22].

In addition, we identified NETosis as main biological route associated with action mechanism of CIGB-814 in an ex vivo study with neutrophils isolated from patients with RA, using proteomics tools (unpublished results). Currently, a new study using more sensitive proteomics tools to confirm these results is in progress.

Inflammatory cytokines such as IL-17A and TNF-α induce NETosis in RA neutrophils [23]. IL-17 has widespread inflammatory effects on the joint, orchestrates bone and cartilage damages and induces recruitment of proinflammatory mediators to the synovium [24].

CIGB-814 significantly decreases IL-17 in patients treated with 2.5 mg. Reduction of IL-17 was in correspondence with the clinical response of patients [18].

All patients had a clinical improvement during the treatment and in the follow-up stage and this improvement correlated with the reduction of anti-CCP antibodies induced by CIGB-814. Here, is possible to think that this correlation was due to the inclusion of MTX at the end of the treatment (week 28). Although the molecular mechanism of MTX has been studied exhaustively, this does not associate with a reduction of anti-CCP antibodies [25].

CIGB-814 reduced disability, even though it is generally accepted that 2 years of treatment is required to demonstrate prevention of disability. MRI analysis corroborated the results obtained in the clinical evaluation. No new areas of bone erosion were found during the study. In addition, there was a reduction of edema and synovitis in the studied hand of patients [18].

On the other hand, therapy with CIGB-814 decreases the levels of IFNγ- and IL-17 in patients with RA [18]. Clinical trials in RA patients treated with different biological therapies suggest key roles for TNF-α, IL-6, and IL-17 in the regulation

of ACPA [6]. The fact that CIGB-814 decreases inflammatory background (pathogenic antibodies and proinflammatory cytokines) can promote activation of Treg. Additionally, our peptide induces proliferation of Treg cells in mononuclear cells from synovial fluid and peripheral blood of RA patients [14], which have suppressive capacity [15]. Consequently, these evidences support the therapeutic effect of CIGB-814 and its effective use in the control of inflammation and induction of peripheral tolerance in patients with RA.

Conclusion

Therapy with CIGB-814 induced a significant reduction of anti-CCP antibodies. This decrease correlated with clinical improvement in patients. These results indicate a therapeutic potentiality of this peptide and support further investigations of this candidate drug for treatment of RA.

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Compliance with ethical standards

Disclosures None.

Ethical approval The Ethics and Scientifics Committees at each study site approved the protocol: National Reference Center for Rheumatic Disease, Center for Genetic Engineering and Biotechnology, and Cuban Regulatory Authority (reference number 05-017-12-B).

Informed consent All subjects provided theirs written informed consent. Patients were recruited from the National Reference Center for Rheumatic Diseases, Havana. This clinical trial was registered under number RPCEC00000238 at the Cuban Registry of Clinical Trials (www.registroclinico.sld.cu).

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