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Retro-inverso peptide analogues of *Trypanosoma cruzi* B13 protein epitopes fail to be recognized by human sera and peripheral blood mononuclear cells

L.K. Iwai[a,b], M.A. Duranti[a], L.C.J. Abel[a], M.A. Juliano[b], J. Kalil[a,1], L. Juliano[b], E. Cunha-Neto[a,*]

[a]*Laboratory of Immunology, Heart Institute (InCor), and Division of Allergy and Immunopathology, Department of Medicine, University of São Paulo School of Medicine, São Paulo, Brazil*
[b]*Department of Biophysics, Federal University of São Paulo, São Paulo, Brazil*

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Abstract

Retro inverso (RI) analogues of antigenic synthetic peptides, which are made of D-amino acids with a reversed sequence, may mimic the side chain conformation of natural all-L peptides. RI analogues were cross-reactively recognized by antibodies and CD4+ T cells reactive against natural all-L synthetic peptides or native proteins in animal models. Since peptides containing D-amino acids are highly resistant to proteolytic digestion, cross-reactive RI analogues may be ideal for in vivo administration to humans as synthetic peptide vaccines or immunomodulators. B13 is an immunodominant tandemly repetitive protein from *Trypanosoma cruzi*, a protozoan parasite that is the causative antigen of Chagas' disease. In order to test whether RI peptides can be recognized by human antibody and T cells, we synthesized two all-L peptides containing the immunodominant B (S12) and T (S15.7) cell epitopes of B13 protein from *T. cruzi* and their retro (R, made of all-L amino acids with reversed sequence), inverso (I, made of all-D amino acids) and RI analogues. Recognition of peptides S12, S12-R, S12-I and S12-RI by anti-B13 antibodies in sera from *T. cruzi*-infected patients was tested in competitive ELISA assay with recombinant B13 protein as the solid phase antigen. Peptides S15.7 and its topological analogues were tested at the 10–50 μM range in proliferation assays on peripheral blood mononuclear cells (PBMC) from S15.7-responder individuals. The median percentage inhibition of B13 ELISA for peptide S12 was 94%, while those of the RI analogue or the other topological analogues were below 12%. While peptide S15.7 was recognized by PBMC from all subjects tested, none recognized the RI analogue of the S15.7 T cell epitope. Our results indicate that cross-reactivity with natural epitopes is not an universal property of RI analogues. This may limit the general applicability of the use of cross-reactive RI analogues as human vaccines and immunotherapeutic agents.

1. Introduction

Immunization with synthetic peptides has been widely used for the induction of protective anti-microbial immune responses or epitope-specific tolerance in animal models [37] and offer significant promise for use in human diseases [42,51]. Vaccines based on synthetic peptides offer several advantages over conventional vaccines derived from infectious material: i. they are chemically well defined and stable at room temperature; ii. their production costs can be lower and iii. their administration may be associated with less adverse effects [5]. Synthetic peptides usually carry a single epitope, and peptide vaccines that focus the immune response toward defined epitopes could be useful in chronic infectious diseases, where T cells directed against the immunodominant epitopes might have been inactivated and T cells specific for cryptic epitopes might still be active [49,52]. Furthermore, synthetic peptide epitopes can be engineered to present neoepitopes or alter the hierarchy of dominant/cryptic T cell epitopes, bypassing recognition escape mechanisms [16,19]. In experimental autoimmune diseases, the administration of synthetic peptides including the pathogenic epitope by alternative routes can specifically decrease or change the character of the response to pathogenic epitopes without interfering with the immune response as a whole, protecting the individual

* Corresponding author. Tel.: +5511-30667060/30826619; fax: +5511-30829350.
E-mail address: edecunha@usp.br (E. Cunha-Neto).
[1]JK is an International Scholar at the Howard Hughes Medical Institute.

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against subsequent challenge with the pathogenic peptide [27] or even down-modulating an ongoing immunopathological response [20,31].

Since peptides made of L-amino acids are highly susceptible to degradation by proteolytic enzymes [18], in vivo administration is troublesome, and a large amount of peptide is often necessary to obtain substantial effects. The oral administration of 200 μg peptide/mouse was necessary for induction of peptide-specific tolerance [45], which is the equivalent by weight of 700 mg for a 70-kg adult human. The SPf-66 polypeptide, a candidate synthetic peptide-based malaria vaccine, elicited specific antibody responses in human volunteers after three subcutaneous injections of 2 mg [39], but failed to exert anti-malarial protection in subsequent clinical trials. In order to overcome this problem, intense work has focused over the last years on the synthesis of peptide analogues in the search for mimics with enhanced activity and biological half-lives [23]. Peptides made of D-amino acids, known to be very poor substrates for proteases [18], were tested for their higher in vivo stability. D-amino acid polymers could elicit immune responses; however, these immune responses were not cross-reactive with their L-amino acid polymer analogues [29,30,36]. Retro-inverso-peptides (also called all-D-retro or retroenantio), which are synthesized by replacing L-amino acids with D-amino acids and reversing the sequence direction, were first described ca. 20 years ago [21]. Such peptide analogues are able to maintain the side chain topology of the parent peptide, since the resulting side chain disposition is very similar in both analogues [21,24].

Over the last decade, it was shown that anti-native protein or peptide antigen antibodies generated in experimental animals could cross-reactively recognize their RI analogues [5,8,23,38,54]. The RI analogue of histone H3 (130–135) could elicit antibodies that cross reacted with the native sequence [11,34] and RI analogues of the immunodominant epitope of the VP1 protein (141–159) of the foot-and-mouth disease virus (FMDV) induced greater and longer-lasting antibody titers than its parent peptide in rabbits and guinea pigs [9,38], and could induce protection in swine associated to anti-virus particle antibodies [40].

At the T cell level, in murine models, the RI analogue of the murine IgH $\gamma 2a^b$ (435–447) peptide was recognized by an I-A^d restricted murine T cell clone elicited by peptide $\gamma 2a^b$ (435–447), although stimulatory concentrations were 300-fold higher than those needed for the parent peptide [4]. Additionally, RI peptide analogues of T helper cell epitopes VP1 (103–115) from poliovirus, and murine IgH $\gamma 2a^b$ (435–446) were found to bind weakly to murine MHC class II molecules and to induce murine T cells to proliferate in vitro in the presence of the cognate peptide [35]. The oral administration of the RI analogue of encephalitogenic peptide (87–99) from myelin basic protein (MBP) induced tolerance towards the induction of experimental autoimmune encephalomyelitis (EAE) by the parent peptide [33].

Although several reports showed cross-reactive recognition of RI peptides by antibody and T cells from animal models, little is known about recognition of RI peptides by humans. There is a single report on recognition of RI analogues by human antibodies—sera from systemic lupus erythematosus (SLE) and Sjögren's syndrome could recognize natural and RI analogues of peptides Ro52 (277–291), Ro60 (304–324) and histone H3 (30–45) [6], and there is no information on crossreactive recognition of RI analogues of peptide epitopes by human T cells.

Here we tested the recognition of retro-inverso analogues of immunodominant B and T epitopes of B13 protein from the protozoan *Trypanosoma cruzi* by Chagas disease patients and normal individuals. Chronic Chagas' disease is caused by the protozoan parasite *T. cruzi* and is endemic in Latin America. The immunodominant recombinant antigen B13 is a tandemly repeated domain of the *T. cruzi* 140/116 kDa antigen located on the membrane surface of infective trypomastigotes. B13 antigen is cross-reactively recognized with cardiac myosin—the major heart protein—by antibodies and heart-infiltrating T cells from Chagas' disease patients, which may contribute to the pathogenesis of the inflammatory heart disease occurring in 30% of infected patients [12,13]. The recombinant B13 antigen is recognized by sera from 98% of patients with Chagas' disease [22] and the 12mer peptide S12 (PFGQAAAGDKPS-NH_2), spanning one repeat unit of the B13 protein encompasses the immunodominant epitope (FGQAAAGDK-NH_2) [12–17]. Further, it was observed that B13 and its peptides are recognized by T cells in a MHC class II-restricted fashion, and that PBMC from HLA-DQ7-positive *T. cruzi*-infected or normal individuals proliferate in response to B13 protein [1,14,15]. Among normal individuals, the immunodominant T cell epitope is the 15mer peptide S15.7 [28]. In this paper, we tested the recognition of S12 and its topological analogues by serum antibody with a competitive ELISA assay, and the T cell recognition of S15.7 and its analogues in PBMC proliferation assays.

2. Materials and methods

2.1. Samples

Heparinized venous blood samples were obtained from Chagas' disease patients, seropositive to *T. cruzi*, followed at Instituto do Coração, São Paulo. Samples were centrifuged and the plasma fraction was used in antibody detection assays. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation ($d = 1.077$) from heparinized peripheral blood of *T. cruzi* sero-negative, normal volunteers, positive for HLA-DQ7 (HLA-DQA1*0501/DQB1*0301) previously tested for PBMC reactivity to B13 peptide S15.7. Sample collection procedures were cleared by the Committee on Ethics of the São Paulo University Hospital.

2.2. Peptide synthesis

Peptides S12 (PFGQAAAGDKPS-NH_2), S15.7 (KPSLFGQAAAGDKLS-NH_2) and their corresponding retro, inverso and retro inverso analogues were synthesized by solid phase technology using 9-fluorenylmethoxycarbonil (Fmoc) strategy [3] with the automated benchtop simultaneous multiple solid-phase peptide synthesizer PSSM8 (Shimadzu, Japan). Fmoc protected amino acids and TGR resin were purchased from Novabiochem (USA). All the peptides were cleaved from the resins by treatment with King's reagent (80% TFA, 2.5% triisopropylsilane, 2.5% ethaneithiol, 5.0% anisol, 5.0% water and 5.0% phenol) [32]. Retro peptides S12-R (SPKDGAAAQGFP-NH_2) and S15.7-R (SLKDGAAAQFGLSPK-NH_2) were synthesized by reversing the order of the amino acid sequence. Inverso peptides S12-I (pfgqaaagdkps-NH_2) and S15.7-I (kpslfgqaaagdkls-NH_2) were synthesized by using D-amino acids instead of L-amino acids, maintaining the original order of the amino acid sequence. Retro-inverso peptides S12-RI (spkdgaaaqgfp-NH_2) and S15.7-RI (slkdgaaaqgflspk-NH_2) were synthesized by performing both modifications. The normal and retro inverso modification analogues display a similar orientation of their side chains, while the resulting peptide backbone was reversed [11], the CO-NH bonds are NH-CO in the R and RI analogues. As the C terminus of the native peptide is amidated, the N termini were not acetylated, so that the -NH_2 group was maintained at both ends. Resulting peptides were analyzed by reverse phase high performance liquid chromatography RP-HPLC (Shimadzu, Japan) on a C18 column (Shimadzu, Japan) using a 5–95% acetonitrile gradient in 0.1% TFA. Peptides' quality was assessed by Maldi-Tof Mass Spectrometry on a TofSpec-E instrument (Micromass, UK) using an α-cyano-4 hidroxycinmamic acid matrix and an acceleration and detection voltage at 20 kV and 15 kV respectively.

2.3. Antibody assay

Competitive inhibition of B13 ELISA was performed essentially as described [12]. Briefly, appropriate dilutions of serum samples (yielding OD's in the 0.3–0.8 range) from *T. cruzi* B13 sero-positive Brazilian patients, pre-adsorbed with 8 μg/ml *Escherichia coli* lysate to reduce background, were pre-incubated with the synthetic peptides (200 mM) overnight at 4°C [17]. Each serum/peptide mixture was then incubated 1 h at 37°C in triplicate wells of a Corning polypropylene 96-well microtiter plate previously sensitized with 20 ng/well of recombinant B13 protein [22]. The reaction was developed using an anti-human IgG-peroxidase conjugate with o-phenylenediamine as chromogenic substrate. The absorbance was subsequently measured at 490 nm. All sera and peptides were tested simultaneously. The antigenic activity for each serum/peptide combination was scored as the percentage inhibition of binding in B13 ELISA test, measured according to the following equation, where the non-related peptide (pNR) sequence NKSAKQFSLHIMDSQPDGS-NH_2, derived from human T-cell receptor Vα chain (TCR Vα13) was used as a control peptide:

$$(OD_{(serum+pNR)}) - (OD_{(serum+test\ peptide)})/$$
$$(OD_{(serum+pNR)}) \times 100$$

The values of the percentage inhibition were then plotted for each peptide/serum combination. The median percentage of inhibition was calculated from inhibition data from all six sera for a given peptide and is expressed as median (min-max).

2.4. T cell proliferation assays

PBMC were incubated in Dulbecco's modified Eagle's medium (DMEM-GIBCO, Grand Island, NY) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μg/ml gentamicin, 10 mM HEPES buffer and 10% normal human serum (complete medium). In the proliferation assay, cell cultures from nine S15.7 positive subjects were carried out in triplicate in round-bottom 96-well microtiter culture plates (10^5 cells/well; final volume 0.2 ml) with each S15.7 or analogue peptide at 10, 25 and 50 μM, or B13 protein (5 μg/ml); phytohaemagglutinin (PHA) 5 μg/ml (Sigma, USA) was used as a positive control, and complete medium as a negative control. Plates were incubated in 5% CO_2 at 37°C for 5 days and cultures were pulsed with 1 μCi/well (^{3}H)-thymidine (Amersham, Australia) for the final 18 h. (^{3}H)-thymidine incorporation was determined with a Betaplate beta counter (Wallac, USA). Data are represented as mean cpm of triplicate cultures and the stimulation index (SI) defined as mean cpm experimental with antigen/mean cpm of culture medium control. SI values $\geq$ 2.5 were considered positive.

2.5. Statistical analysis

Statistical comparison of percent inhibition or stimulation index values for each peptide was performed with the non-parametric Mann-Whitney's rank sum test.

2.6. HLA class II typing

DNA was extracted alternatively by DTAB/CTAB or salting out methods [41]. Briefly, buffy-coat from 4 ml of blood was mixed with DTAB 12% solution (DTAB 12%, NaCl 2.25 M, Tris pH 8.6 150 mM, EDTA 75 mM), vigorously mixed and incubated for 5 min at 68°C. After the addition of 2 volumes of chloroform, samples were vigorously mixed and centrifuged for 2 min at 10000 g to separate into three phases. The upper phase was transferred to a new tube containing 2 volumes of CTAB 0.5% (CTAB 0.5%, NaCl 0.04 M), DNA precipitated and washed further

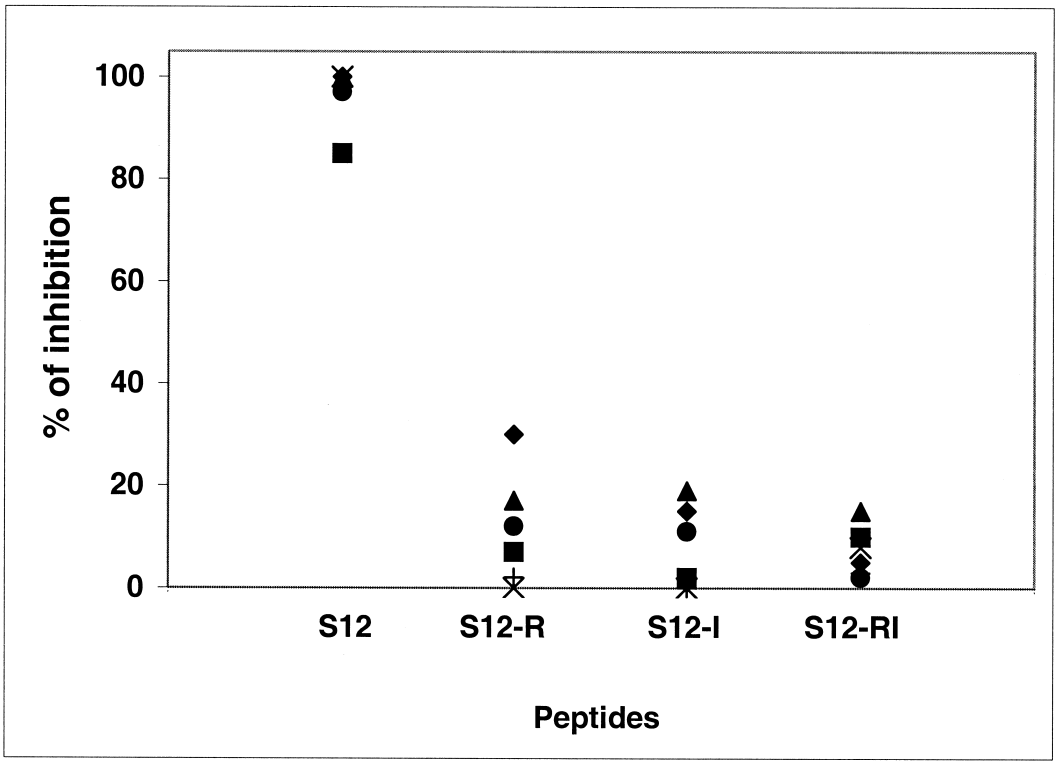

Fig. 1. Percentage of inhibition of competitive ELISA assay of S12 peptide and its retro, inverso and retro-inverso analogues. Peptides at 100 μM were tested against plasma samples from 6 different Chagasic patients at 1:500–1:6000 dilutions.

with ethanol 99.5%, precipitated, washed with ethanol 70% and centrifuged at 12000 g. DR typing was performed by low resolution PCR-SSP [7]. DQA1 and DQB1 typing were performed by PCR-SSO using generic primers for exon-2 amplification [41], as follows:

DQA1 forward: 5′-ATGGTGTAAACTTGTACCAGT-3′

reverse: 5′-TTGGTAGCAGCGGTAGAGTTG-3′

DQB1 forward: 5′-CATGTGCTACTTCACCAACGG-3′

reverse: 5′-CTGGTAGTTGTGTCTGCACAC-3′.

PCR conditions were 200 ng of DNA, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin, 0.2 mM dNTP, 20 pmoles of each primer, 2 U Taq DNA polymerase (Cenbiot, RS, Brazil), in 55 μl total volume. Samples were cycled 30 times for 1′ 94°C, 1′ 55°C, 1′ 72°C. A 10% aliquot of the PCR products was checked on 1.2% agarose gel electrophoresis with φX digested with *Hae*III as a size marker. Two-5 μl of the amplified product was denatured in 160 μl NaOH 0.4N/25 mM EDTA for 5 min at room temperature. Replicate filters were prepared by loading 30 μl of the denatured sample onto a Hybridot manifold apparatus (BRL, Gaithersburg, USA). The membranes were dried for 2 h at 80°C. 20 pmol of each oligonucleotide probe were labeled with 40 μCi (γ^{32}P) ATP using T_4 polynucleotide kinase. Blots were pre-hybridized with 10 ml of 6× SSPE, 5× Denhardt's solution, 0.5% SDS and 100 μg/ml of SSDNA for 4 h at 54°C and hybridized overnight with the added probe at the same temperature. Filters were washed twice in 2× SSC/0.1% SDS at room temperature, and once in TMAC solution (3M TMAC, 50 mM Tris, 2 mM EDTA, 0.1% SDS, Denhardt 5×) for 45 min at 59°C. Membranes washed in 2× SSC for a few minutes at room temperature before exposure to X-ray film. Each blot was re-hybridized 2–3 times. To remove the probe, blots were washed 3 times in (95°C) TE 10–10 (Tris pH 7.6/10 mM, EDTA 10 mM).

3. Results

3.1. Antibody recognition of S12 peptide and its topological analogues

The antigenic activity of B13 peptide S12 and its analogues was evaluated by measuring, for each peptide, the percent of inhibition of binding of the anti-B13 antibodies present in sera from *T. cruzi* infected patients to recombinant protein B13 in the solid-phase. The results obtained with six sera from chagasic patients are reported in Fig. 1. The median percentage of inhibition for peptides S12 and S12-RI was 99% (min-max 85–100%) and 6.5% (2–15%), respectively. The median percentages of inhibition for the retro-(S12-R) and inverso-(S12-I) analogues were also low,

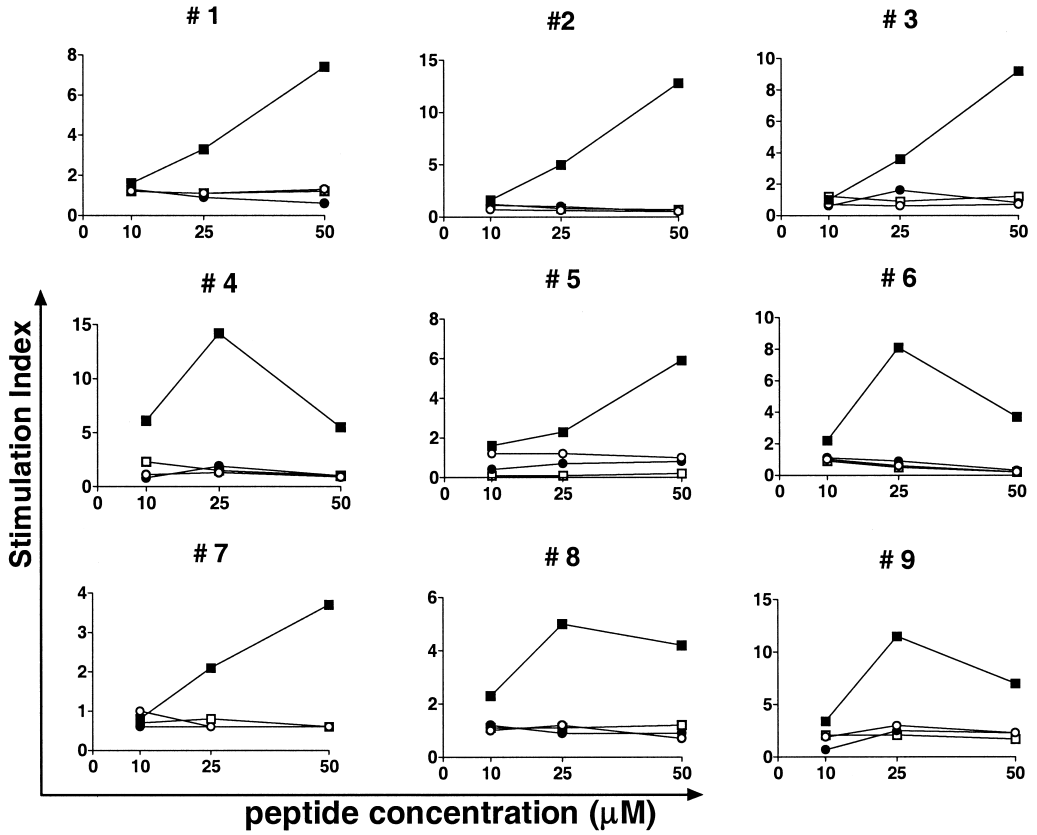

Fig. 2. PBMC proliferative response to B13 peptide S15.7 and its retro, inverso and retro-inverso analogues. PBMC (10^5 cells/well) were stimulated with peptides at 10, 25, and 50μM as described. Each graph represents the SI values of a single individual. (■ = S15.7, ○ = S15.7-R, ● = S15.7-I and □ = S15.7-RI)

9.5% (0–30%) and 6.5% (0–19%). Two of the 6 sera displayed the highest percentages of inhibition—in the 15–30% range—for all non-natural topological analogues S12-R, S12-I, S12-RI, while the other four sera displayed minimal inhibition values for those peptides.

3.2. T cell recognition of peptide S15.7

We tested the proliferation of PBMC from individuals previously typed for HLA-DQ7-positive and known to be S15.7-responder against peptides S15.7, S15.7-R, S15.7-I and S15.7-RI (Fig. 2). There was a dose-dependent increase in the stimulation indexes and in the number of reactive subjects against peptide S15.7, as follows: at 10 μM, average SI = 2.3, 2/9 responders; at 25 μM, average SI = 6.1, 7/9 responders; at 50 μM, average SI = 6.6, 9/9 responders. Stimulation indexes with S15.7 peptide were significantly higher at 50 or 25 μM than at 10 μM ($P = 0.002$ and $P = 0.01$, respectively). While 9/9 individuals recognized the native S15.7 peptide at 50 μM, none recognized the RI analogue, even at the highest peptide concentrations ($P < 0.001$). The retro and inverso analogues of S15.7 were recognized by PBMC from a single individual, albeit with low stimulation indexes, at 25 μM.

4. Discussion

While anti-B13 antibodies from all *T. cruzi*-infected patients' sera reacted with peptide S12 from B13 protein, the S12-RI and the other topological analogues failed to be recognized (Fig. 1). It is relatively easy to imagine that peptides S12 (PFGQAAAGDKPS-NH_2), and S12-R (SPKDGAAAQGFP-NH_2) could not bind to the same set of cross-reactive antibodies, since their side chain disposition is clearly distinct [6]. D-amino acid analogues such as S12-I infrequently cross-react with its parent peptide, for a similar reason [6]. However, given the predicted ability of RI peptides to maintain the side chain disposition [9,11,21,23,53,54], it is more difficult to explain the failure of S12-RI to be recognized by anti-B13 antibodies, in face of the numerous examples of cross-reactive recognition of parent and RI analogues by antibodies [5,8,9,11,21,23,38,53,54]. Our group described that the antigenicity of peptides containing the immunodominant B13 epitope correlated with the ability to form secondary left-hand helical structures as assessed by circular dichroism (CD) and nuclear magnetic resonance (NMR) studies [17]. CD and ^{1}H NMR spectroscopy studies of parent and RI peptides from FMDV VP1 protein (141–159) showed that while the parent peptide could form a left hand α-helix in the presence of trifluoroethanol—a solvent

which dielectric constant is similar to the one present inside or at the protein surface [17]—its RI analogue tended to form an opposite right hand α-helix [10,43]. This was predictable since RI analogues of a given sequence display switched ϕ, ψ angles (ψ, ϕ) [25]. Likewise, a possible three-dimensional structural difference in the side chain disposition of the peptides in α-helix could hypothetically explain the lack of recognition of S12-RI by anti-B13 antibodies. Studies are in progress to approach this question. It must be said, however, that most of the reports showing crossreactive recognition of RI analogues and native peptides used protocols involving immunization with RI peptide in animal species, while the present study pertains the testing of serum antibodies preformed against the native peptide/protein in an immune response against a pathogen, immunization protocols that may not be directly comparable. It thus cannot be excluded that the different antigenicity of RI analogues may be due to differences in type of immunization and/or animal species.

The dose-response curve of peptide S15.7 on PBMC proliferation assays from HLA-DQ7-positive subjects shows that its interaction with MHC and/or T cell receptor is of low affinity. It has been shown that peptides encompassing the T cell epitope of B13 bind to the MHC class II molecule HLA-DQ7 with low affinity, at the micromolar range [2]. Peptide S15.7-RI failed to be recognized even at high concentrations. The fact that a single individual out of the 9 tested could recognize the other topological analogues, S15.7-R and S15.7-I, in the absence of S15.RI recognition, may indicate the existence of T cell clones with non S15.7 cross-reactive specificity, as discussed above for anti-S12 antibodies. Our negative results are in contrast with recent reports that RI analogues from antigenic peptides could bind to MHC class II—albeit with lower affinity—as well as being recognized by murine T cells [4,35]. It has been shown that retro inverso analogues of T cell epitope peptides of snake toxin α (24–36), hen eggwhite lysozime (103–121), ovalbumin (323–339) and of λ repressor (12–26) did not bind detectably to I-E^d and I-A^d molecules [26]. The lower affinity of RI peptides for MHC molecules could potentially explain the higher concentrations necessary for T cell stimulation by most RI analogues reported to date [4,35]. It cannot be excluded that, in case the affinity of S15.7 parent peptide binding to MHC were higher, the S15.7 RI analogue could have ability to stimulate a T cell response at high doses.

The HLA-DQ7 molecule is known to preferentially bind peptides with small side chain residues like alanine, glycine and serine [44,50], as is the case with peptide S15.7. These authors suggested that hydrogen bonds formed between the floor of the antigen-binding groove of the HLA-DQ molecule and the peptide backbone, rather than side chains, stabilize the peptide:HLA-DQ interaction [44]. The murine molecule I-A^d is homologous to human HLA-DQ and, like HLA-DQ7, displays a preference for binding peptides rich in small side chain residues [47,48]. X-Ray crystallography of peptides ovalbumin (323–339) and influenza haemagglutinin (126–138) bound to I-A^d molecule indicate that all but one of the 15 hydrogen bond interactions to the I-A^d molecule were with the peptide backbone, involving residues conserved among I-A and HLA-DQ molecules [47]. For that matter, comparison by molecular modeling of H-$2K^b$ complexed to VSV-8 (N52–59) peptide or its retro inverso analogue showed that the RI analogue established less hydrogen bonds to the MHC molecule than the parent peptide [46]. Thus, it is likely that in S15.7-RI, the interconversion of backbone carboxy and amide groups of the peptide backbone also leads to a reduced number of hydrogen bonds to the antigen binding groove and failure of T cell presentation.

Our results showed that not all antigenic epitopes possess cross-reactive RI peptides. The ability to preserve antigenic cross-reactivity may rely on structural features of the peptide and its ligands, along with the nature of the immunization—whether natural or pathogen-induced. B cell epitopes that are recognized in a secondary structure, which cannot be mimicked by RI analogues, may be predicted not to have cross-reactive RI epitopes. Conversely, T cell epitopes which rely on peptide backbone, rather than side chains, for major hydrogen bonding to MHC, and have low binding affinities to MHC, may also have inefficient RI analogues due to unstable/low affinity peptide:MHC complex formation. Thus, the application of cross-reactive retro-inverso analogues for vaccinal or immunomodulatory peptides appears to be limited by variables that may depend on sequence, immunization protocol, or host species, and must be tested empirically on a case-to-case basis. Furthermore, given the inherent variability of the immune response—including immunoglobulins and T cell antigen receptors—and the genetically heterogeneous human population, one would have to screen a significant number of individuals to ensure that a RI analogue immunogen is as effective as the parent peptide for in vivo prophylactic/therapeutic administration.

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