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The Heavy Chain Variable Segment Gene Repertoire in Chronic Chagas′ Heart Disease[1]

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Patients chronically infected with *Trypanosoma cruzi* develop chronic Chagas′ heart disease (cChHD). Their Ab response is suspected to be involved in the cardiac pathogenesis. Reactivity of serum Abs from these patients has been extensively studied but little is known about the diversity of the in vivo IgG repertoire. We analyzed 125 variable H chain (VH) genes and compared it to repertoires from healthy individuals, and patients with autoimmune processes and other infections. VH were from plasma cells isolated from heart tissue of three cChHD patients and from a Fab combinatorial library derived from bone marrow of another cChHD patient. The role of the parasite in shaping the Ab repertoire was assessed analyzing VH genes before and after panning against *T. cruzi* Ag. Among recovered VH genes, a significantly increased representation of VH4 was observed. Plasma cells at the site of cardiac infiltration showed an increased VH1 usage. CDR3 lengths were similar to the ones found in the healthy repertoire and significantly shorter than in other infections. VH derived from anti-*T. cruzi* Fab and plasma cells showed a higher proportion of hypermutated genes, 46.9% and 43.75%, respectively, vs 30.9% of the cChHD patient repertoire, pointing to the role of parasite Ags in the shaping of the humoral response in Chagas′ disease. No histological evidence of germinal center-like structures was observed in heart tissue. In accordance, VH analysis of heart plasmocytes revealed no evidence of clonal B cell expansion, suggesting that they migrated into heart tissue from secondary lymphoid organs. *The Journal of Immunology,* 2009, 183: 8015–8025.

Chronic Chagas′ heart disease (cChHD),[3] caused by the protozoan parasite *Trypanosoma cruzi*, is a slowly evolving inflammatory cardiomyopathy that may lead to severe cardiac dilatation, congestive heart failure, and death (1, 2). Histological examination reveals mononuclear cell infiltration, myocytic damage and fibrosis (3). Parasite cells and/or parasite nests are rarely detected in heart tissue sections of chronically infected individuals, although different PCR amplification techniques reveal the presence of parasite DNA (4). The scarcity of parasites in heart, as well as a lack of correlation between detection of parasite nests and inflammation, suggested that autoimmune processes could be involved in the pathogenesis of cChHD (5, 6). It has been postulated, that an autoimmune response could be initiated by T cells activated by *T. cruzi* Ags that resemble self-Ags (5, 6). Alternatively, the parasite may cause heart tissue damage leading to the exposition of cryptic host Ags and subsequently to the induction of inflammation (5, 6).

Abs specific for several immunodominant *T. cruzi* Ags (7, 8), and Abs reacting with both parasite Ags and self-peptides have been characterized in cChHD (5, 9, 10). Among the latter, those binding and activating cardiac receptors, particularly the β1 adrenergic receptor, have also proven to be pathogenic in animal models (11, 12). They act as partial cardiac receptor agonists and are capable of inducing conduction block on isolated rabbit hearts (13). Analogous or equivalent Abs occur in patients with idiopathic dilated cardiomyopathy (IDC) (see Refs. 14–18, clinical and histological comparisons between cChHD and IDC have been described in Refs. 14 and 16, respectively), as well as in inappropriate sinus tachycardia (19). That the mentioned chronic diseases, both infectious and idiopathic, share closely related epitopes within the primary structure of the β1 adrenergic receptor and have such a circumscribed cardiac phenotype is a biological mystery (14, 19, 20). Interestingly, several B cell-mediated autoimmune diseases, such as myasthenia gravis (21) and Graves′ disease (22), as well as T cell mediated ones, such as insulin-resistant diabetes (23) also present Abs to hormonal receptors. In these cases, as well as in cChHD and IDC, the Abs exert their pathogenic effects by interacting as agonists or antagonists with the respective receptors.

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[3] Abbreviations used in this paper: cChHD, chronic Chagas′ heart disease; IDC, idiopathic dilated cardiomyopathy; DS, denaturing solution; GC, germinal center; FDC, follicular dendritic cells; scFv, single chain variable fragment; VH, variable heavy chain; CDRH, complementary determining regions of heavy genes; FR, framework regions; PBL, peripheral blood lymphocytes.

Analysis of the Ig variable (V) gene usage in patients with the mentioned autoimmune diseases has uncovered differences in their Ig V repertoire compared with healthy controls, even though none of these differences can be considered major (24). In addition, analysis of gene usage in target organs and tissues of patients with these autoimmune diseases have demonstrated that there is a highly compartmentalized clonal expansion of B cells driven by a limited number of Ags (24–27). Up to date, no attempt has been made to analyze the Ab repertoire of patients with cChHD, nor to characterize the Ig V gene usage of plasmocytes from severe inflammatory infiltrates of heart lesions that could include non-canonical germinal center (GC)-like structures (25, 26).

The aim of this study was to analyze the V gene repertoire of patients with cChHD to determine whether usage of particular gene families or individual genes for the creation of the general Ab repertoire is skewed in patients with cChHD compared to healthy subjects and to patients with autoimmune diseases or other chronic infections.

We report a partial analysis of the human H chain Ab repertoire of patients with cChHD. First, a micromanipulation technique was used to amplify sequence and analyze H chain genes from B lymphocytes from cardiac inflammatory lesions of three different cChHD patients, and second, the VH of recombinant IgG from a patient with cChHD from a combinatorial Fab library, was analyzed before and after selection on *T. cruzi* epimastigote extracts. Selection was performed using an original screening procedure based on the removal of *T. cruzi* bound Fab by serum from the donor patient.

Our results suggest that gene usage investigation contributes to enrich the overall picture of the chronic human infection with *T. cruzi*. The V gene repertoire in Chagas′ disease presents evidences of Ag driven maturation and certain differences to the repertoire of healthy controls, albeit not major. Results from panning of the human recombinant Fab library point to the role of the parasite in shaping of the Ab repertoire in these patients. Moreover, analysis of the V_H repertoire from heart tissue provided no evidence as to the existence of organ-specific B cell expansion. It is noteworthy, that these results open the way to analyze the specificity of B cells present in the inflammatory infiltrate of heart tissue. This fact is of relevance, not only for studies concerning pathogenesis of cChHD but also for investigations involved in the pathogenesis of IDC and other idiopathic cardiomyopathies.

Materials and Methods

Patients

Fourteen patients with cChHD who came from the same endemic region located in the center and northwest of Argentina were evaluated at the Cardiology Unit of the Hospital Fernandez in Buenos Aires. They underwent a complete clinical and cardiologic examination, an electrocardiogram at rest, an exercise stress test, a 24-h electrocardiogram Holter monitoring, and a B-mode echocardiogram. Ventricular arrythmias and sinus node dysfunction were diagnosed as reported previously (28). After clinical evaluation, blood samples were drawn, and sera were obtained and coded. At the laboratory, ELISA and Western blots against *T. cruzi* Ags were performed. According to these results patient 4, suffering from severe sinus node dysfunction and right bundle branch hemiblock, and one of the patients with the highest levels of anti-*T. cruzi* Abs and high levels of functionally active anti-cardiac receptor Abs was selected to be the bone marrow donor to construct the Fab library.

Endomyocardial biopsies from two other patients suffering from cChHD (patient 1 and patient 2) and sections of right ventricle from another cChHD patient (patient 3) who was subjected to heart transplantation were analyzed. These patients were further characterized (29) as described by Schijman et al. (16).

The study protocol complied with the Helsinki Declaration and was approved by the Committee for Ethical and Legal aspects of Research (CELAR) of the Institute for Genetic Engineering and Molecular Biology, Buenos Aires, Argentina.

Preparation and staining of heart tissue sections

Sections were prepared from human cardiac tissue from patients 1, 2, and 3. The heart tissue samples were shock frozen, and 8-μm cryosections were prepared. Frozen sections were fixed with acetone and stored at −70°C until used. To determine the degree of inflammation, consecutive sections of human heart tissue were single labeled with mouse mAbs specific for B cells (anti-CD20; Dakopatts), T cells (anti-CD3, anti-CD4, and anti-CD8; Dakopatts), follicular dendritic cells (Wue-2; Greiner), macrophages (anti-CD14 and anti-MAC-3; Dakopatts), and for plasma cells (anti-Wue-1, Greiner). They were detected by alkaline phosphatase-anti-alkaline phosphatase complex (Dakopatts) followed by incubation with the fuchsin substrate (Dakopatts).

Proliferating cells were identified using the rabbit anti-human Ki-67 Ab (Dakopatts), which recognized a nuclear Ag presented in dividing cells only. A biotinlylated donkey anti-rabbit Ab was used as secondary Ab followed by extravidin-alkaline phosphatase (Sigma-Aldrich) and fuchsin substrate (Dakopatts). The nuclei of the cells were counterstained with Papanicolau hematoxylin.

Microdissection of cells from tissue sections

Single cells or groups of cells from the hearts of patients 1, 2, and 3 were dissected using a 600-fold magnification and a hydraulic micromanipulator (Narishige). The micromanipulation process was performed on a Diaphot 300 inverted microscope (Nikon). Single cells, groups of cells (2–5), or small areas of tissue were cut from stained sections using a sharp tip attached to a micromanipulator, prepared from GD-1 glass capillaries (1 mm × 90 mm) on a vertical micropipette puller, PB-7 (Narishige) according to supplier's instructions. Pipettes of 20–50 μm in diameter were prepared, polished on a grinding machine, and attached to silicon tubing (1 mm diameter), which was in turn attached to a manual piston pump (Cell Tram Oil; Eppendorf). Excised cells were suctioned into the pipette and transferred into a 0.5-ml tube. Isolated cells were digested in 0.7 mg/ml proteinase K (Boehringer Ingelheim) for 1 h at 50°C. Digested cells were immediately subjected to DNA amplification or stored at −20°C until use.

Cloning of heavy and light chains directly from isolated B lymphocytes from cChHD patients

Whole DNA preparations made from microdissected single or group of plasma cells were used to amplify the VH region genes by using the following specific primers: VH mix (VH1 5′-CAGGTGCAGCTGGTG CAGTCTGG-3′, VH2 5′-CAGATCACCTTGAAGGAGTCTGG-3′, VH3 5′-GAGGTGCAGCTGGTGSAGTCTGG-3′, VH4 5′-CAGGTGCAGCT GCAGGAGTCGGG-3′, VH5 5′-CAGGTGCAGCTACAGCAGTGGGG-3′, VH6 5′-TACAGCTGCAGCAGTCAGGTCCAGG-3′), JH mix, internal (JH1 5′-TGAGGAGACGGTGACCAGGGTGCC-3′, JH3 5′-TGA AGAGACGGTGACCATTGTCCC-3′, JH4 5′-TGAGGAGACGGT GACCAGGGTTCC-3′, JH6 5′-TGAGGAGACGGTGACCGTGGTCCC-3′), and JH uni external (5′-CTCACCTGAGGAGACGGTGACC-3′). VH mix and JH mix internal were synthesized by TIP MOLBIOL (Berlin, Germany) and JH uni external by Eurogentec. All PCR reactions were performed in a Trioblock thermocycler (Biometra).

Obtention of RNA and cDNA from bone marrow of a cChHD patient

By sternal punction of patient 4, 5-ml bone marrow was obtained and immediately added to a 50-ml tube containing denaturing solution (DS = 8.4 M guanidine thiocyanate, 0.7% trisodium citrate, and 0.5% sarcosyl in diethyl pyrocarbonate-treated water, adding immediately 100 μl of β-mercaptoethanol to each 14 ml of DS before use). To each 10 ml of the sample (bone marrow + DS), 1 ml of 3 M sodium acetate (pH 4), 10 ml of acidic phenol, and 2 ml of chloroform/isoamylic alcohol (24:1) were added, and the mixture was very well vortexed. After 15 min of keeping on ice, the samples were centrifuged 30 min at 10,000 × g, and the supernatants were carefully aspirated and put into clean RNase-free Corex tubes. One volume isopropanol was added and left 10 min at room temperature. After a 20 min centrifugation at 10,000 × g and washing with 75% ethanol, the pellet was resuspended in 200 μl of RNase-free water. RNA concentration was quantified with UV spectrometer (140 μg of total RNA were obtained) and its quality was assessed on an agarose gel, confirming the integrity of rRNA. To obtain cDNA, first strand synthesis using reverse transcriptase standard procedure (Life Technologies) was performed according to manufacturers' instructions. The cDNA was UV quantified and stored at −20°C.

Table I. *Clinical features of patients 1 (heart biopsy samples), 2 (heart biopsy samples), 3 (heart tissue sections), and 4 (bone marrow donor)*[a]

Patient	Age (years)	Sex	Serology for *T. cruzi*	Clinical Evaluation	Analyzed Sample
1	48	M	Positive	Myocarditis active, mild, focal	Heart biopsy (6 VH sequences)
2	58	M	Positive	Myocarditis active, moderate	Heart biopsy (4 VH sequences)
3	48	F	Positive	Myocarditis active, severe	Heart right ventricle, tissue sections (6 VH sequences)
4	48	F	Positive	Severe sinus node dysfunction and right bundle branch hemiblock	Bone marrow (Fab library)

[a] Clinical evaluation was performed according to Chiale et al. (28) and Schijman et al. (16).

PCR for the amplification of VH and VL regions of immunoglobulins from bone marrow RNA

Variable regions amplifications corresponding to heavy chains, κ and λ L chains were performed separately using specific primers according to a method described (30). A PerkinElmer Thermocycler was used for amplifications.

Library ligation and electroporation

Digestions of 20 μg of pComb3X vector and 20 μg of purified Fabκ and Fabλ fragments were performed with *Sfi*I (New England Biolabs). For library ligation, 1.4 μg of vector was combined with 1.4 μg of digested Fabκ or Fabλ fragment. For library transformation, 300 μl of electrocompetent *Escherichia coli* XL1-Blue F′ bacteria were electroporated with 5 μl of the ligation reaction. Three electroporations for Fabκ and three for Fabλ were conducted.

Fab-PCR

Individual clones after library transformation and panning were picked and introduced into a PCR tube containing 10 μl of nuclease-free water. Forward primer ompseq (5′-AAGACAGCTATCGCGATTGCAG-3′) and reverse primer gback (5′-GCCCCCTTATTAGCGTTTGCCATC-3′) were used to amplify the ~1600 bp Fab fragment. PCR products were separated on a 2% agarose gel.

Panning against T. cruzi *epimastigote lysate*

Phage bearing Fab fragments on their surface were selected by panning on Ag-coated wells. Microtiter wells (Nunc Immunoplates) were coated with 20 μg/ml of *T. cruzi* epimastigote lysate and blocked with 1% BSA-PBS-0.01%Tween 20 (PBT). Polyethylene glycol-precipitated phage were added (50 μl/well) and incubated for 1 h at 37°C. Wells were washed 5 times (in the three subsequent panning rounds washing were increased up to 15 washes) with PBS-Tween 20. For elution, the serum of patient 4 in a 1/100 dilution was added and the microtiter plate was incubated for an additional 1 h. The eluted phage were then allowed to infect *E. coli* XL1 BlueF′ cells to amplify selected phage and submitted to three further rounds of panning.

Fab-ELISA

To determine enrichment in specific anti-*T. cruzi* Fab bearing phage, Fab-ELISA was performed with the phage present in the supernatants in the culture medium. For reactivity measurement of individual clones, Fab periplasmic extracts (see below) were used at a dilution of 1/10. In brief, 96-microwell plates were coated with *T. cruzi* epimastigote lysate as described above, using irrelevant Ags as negative controls and blocked for 1 h at 37°C with PBT. Culture supernatants or periplasmic Fab extract were incubated for 1 h and binding was determined by revealing with anti-human $(Fab)_2$ fragment conjugated to peroxidase (Pierce) in a 1/5000 dilution in PBT followed by incubation with 100 μl of TMB substrate (3,3′,5,5′-tetramethylbenzidine dihydrocholoride 3 mg/ml in 0.1 M citrate buffer; 7.2% DMSO; 1,8% glycerol) per well. The enzymatic reaction was stopped after 10 min by adding 50 μl of HCl 1 N and the optical densities were read at 450 nm with an automated plate reader (Molecular Devices). Cut-off ratios were determined as mean OD plus 2σ of reactivity for periplasmic extracts expressing Fab fragments that were not panned against *T. cruzi* Ags. As a control, a periplasmic extract of untransformed bacteria was used.

Obtention of Fab periplasmic extracts

To obtain soluble recombinant Fab not fused to phage pIII gene, clones presenting 1600 bp-full length Fab insert (as determined by Fab-PCR) were transformed into *E. coli* non-suppressor strain Top10F′. For Fab expression, an adapted protocol from Skerra and Pluckthun (31) was used.

Sequence analysis

Nucleic acid sequencing was conducted in Amersham Biosciences on a Megabace 1000 (Molecular Dynamics) and in the Center of Applied Genomics (Centro de Genomica Aplicada, CeGA, University of Buenos Aires, Argentina) on a Megabace 500 using template amplification with primers ompseq, gback (see above), and leadVH (5′-GCTGCCCAAC CAGCCATGGCC-3′) for sequencing with an adaption of the Templiphi protocol (Amersham Biosciences, standard operating procedure/protocol by Amir Ghadiri/production sequencing group, September 5, 2001). Variable region sequences of H and L chains were analyzed with IgBlast program (National Library of Medicine, National Institutes of Health; www.ncbi.nlm.nihg.gov/blast/igblast) and the International ImMunoGeneTics (IMGT)/V-QUEST alignment tool at IMGT database (32). Mutations were identified comparing each sequence with germline sequences and were defined on the basis of nucleotide changes in the VH or VL segment. Replacement (R) and silent (S) mutations were determined in framework regions (FRs) and complementary determining regions (CDRs). The probability that an excess or scarcity of R mutations in VH CDR or FR occurred by chance was calculated by a multinomial distribution model (33). All sequences are available from GenBank (http://www.ncbi.nlm.nih.gov/GenBank) under the following accession numbers EU549720–EU549735 for the 16 VH genes obtained from the biopsy plasma cells and EU619960–EU620068 for the 109 analyzed VH genes from the Fab library.

Statistical analysis

To determine significant differences in distribution between healthy peripheral repertoire and cChHD-derived sequences, the χ^2 test was used. $p < 0.05$ was considered significant. If significant, each single degree of freedom was examined for significant contribution to the total. To analyze the CDR3 length, the Mann-Whitney *U* test was used.

Results

VH repertoire of plasma cells from heart tissue of patients with Chagas′ heart disease

In a majority of patients with chronic Chagas′ disease, the heart is its major target organ (1, 3). These patients display high anti-*T. cruzi* Ab levels together with Ab specificities against heart surface structures that have been linked to the pathogenesis of Chagas′ heart disease (10). However, there is no information about Abs secreted by plasma cells at the sites of heart lesions.

Previous work showed that micromanipulation can be used to microdissect and analyze V gene usage of plasma cells from splenic GC, in systemic lupus erythematosus patients (33) and in GC-like structures detected within nonlymphoid target tissues in rheumatoid arthritis (24, 26), myasthenia gravis (21), Sjogren's syndrome, and primary biliary cirrhosis (24). In our case, heart sections of three cChHD patients were studied (Table I). Individual plasma cells or small groups of plasma cells identified by positive anti-Wue-1 reactivity were microdissected. The DNA from these cells was prepared, amplified, and sequenced to allow analysis of their Ig V(D)J rearrangements. The histological environment of plasma cells was evaluated by immunohistochemistry as described in *Materials and Methods*. The search for evidences of GC-like

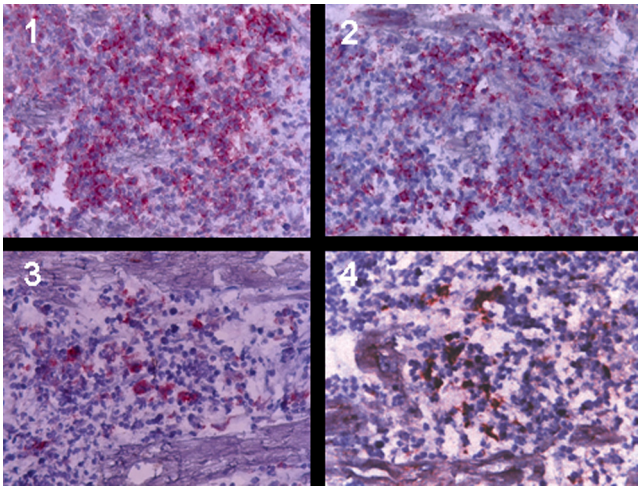

FIGURE 1. Cellular composition of an inflammatory infiltrate of heart tissue of patient 3. Consecutive sections were labeled with specific Abs for: 1) $CD4^+$ T cells; 2) $CD8^+$ T cells; 3) macrophages; 4) plasma cells (anti-Wue1). Magnification, ×100. The nuclei were counterstained with Papanicolau hematoxylin. Positive cells were revealed with new fuchsin (red color).

structures was performed using markers for follicular dendritic cells (FDC) and CD20 B cells. Moreover, Abs against the nuclear Ag Ki-67 were used to assess the presence of proliferating cells in sites presenting strong signs of inflammation.

In the case of patient 1, histological analysis of heart sections obtained from biopsy samples, revealed that the structure of myocardial tissue was not damaged (Fig. 1; Ref. 29). Few T cells, macrophages, and plasma cells were spread throughout the tissue. Neither CD20 B cells nor FDC were observed. Clinically, the whole picture of the inflammation site was diagnosed as mild (see Table I) (16, 29). A previously reported search for *T. cruzi* DNA remnants in the same heart sections detected, by means of PCR, *T. cruzi* genomic and kinetoplast DNA (29). Moreover, "in situ hybridization", using a kinetoplast-derived DNA probe, allowed localization of *T. cruzi* amastigotes in only one of the tissue sections of this patient (29).

Single plasma cells were identified and dissected, allowing the amplification of six functional VH. One of them belonged to the VH1 family (IGHV1–2), three to VH3 (IGHV3–30, −30–3 and −53) and two to the VH4 family (IGHV4–30-4 and −59) (Table II). These VH sequences were derived from JH4 and JH6 segments. CDR3H lengths varied between 24 and 66 bp (mean 47.4 bp) representing 8 to 22 aa residues, with an average of 13 ± 6 aa residues.

Comparison of the nucleotide sequences of the amplified VHs with the corresponding germline showed an average of 21.9 nucleotide changes per VH. Analysis of the mutational pattern of the VH genes showed an increase of somatic mutations in CDRs in comparison to FRs in two out of six amplified VH genes (IGHV4–31 and IGHV3–64, Tables II and III). Amino acid sequence alignments of CDRs to the closest germlines of these two VH genes are shown in Table III. The alignment of the amino acid sequence of VH4–59 variant with the germline showed replacements in the three FRs and CDRs 1 and 2, such as the aspartic in position 26 and basic amino acids, arginine and histidine in CDR1H. Changes in FR3H were notably increased (supplemental table).[4]

[4] The online version of this article contains supplemental material.

In marked difference with heart sections from patient 1, the histological analysis of heart tissue sections from patient 2 showed signs of active inflammation. Clusters of up to 30 mononuclear cells, mainly composed by T cells were observed disrupting myocyte arrangements (Fig. 1 in Ref. 29). Macrophages and plasma cells were in close vicinity to T cells and, as in patient 1, neither CD20 B cells nor FDC were observed. In these heart sections, no parasites were detected; however, several sections presented evidence of *T. cruzi* genomic and/or kinetoplast DNA (29). Groups of 2 to 5 plasma cells were selected for microdissection. From their DNA four VH, three of them of the VH1 family (IGHV1–2 and two IGHV1–69-01) and one from the VH4 family (IGHV4–2), were obtained (Table II). All of them were functional, but both amplified VH1–69 genes were different.

About usage of the JH gene segments, two VHs were derived from JH6, and the other two from JH4 and JH5. CDR3H lengths varied between 45 bp and 57 bp (mean 49.5), 15–19 aa residues (average 16 ± 2 aa residues). Compared with patient 1, a higher frequency of somatic mutations was observed for patient 2, on average 34.2 ± 6 changes/VH. In three out of four amplified VH (IGHV1–69-1, IGHV1–2, and IGHV4–4), somatic mutations preferentially accumulated in CDRs, as it is shown in Table II and Table III. Amino acid sequence alignments of CDRs to the closest germinal lines of these three VH genes are shown in Table III. Two out of four VH sequences from patient 2 were derived from IGHV1–69-01, both presented similar number of mutations as compared with the VH germline gene, but with different distribution. While similar number of replacements were found in FRs, one of the sequences (EU549726) presented no mutation in CDR1H but six mutations in CDR2H, whereas the other one, VH (EU549728) presented the opposite, seven replacements in CDR1H and only one in CDR2H (Table III and supplemental table). It was noteworthy, that a metionine was found in both CDRs (supplemental table).

The heart tissue from patient 3 showed severe inflammation. Labeling with specific markers for different mononuclear cells showed that the infiltrates were composed of 80% of T cells, approximately half of them were positive for CD8 and the other half for CD4 (Fig. 1). These cells were in close association with multiple macrophages and plasma cells. In a few sections, groups of up to 100 plasma cells were detected. Moreover, isolated plasma cells were observed dispersed throughout the tissue. CD20 B cells and FDC were not detected. Furthermore, the absence of proliferating B cells was confirmed by the negative results of labeling with anti-Ki-67 Abs. No parasite cells or nests were found, but as in patient 2, the sections showed evidence of kinetoplastid DNA (29). After microdissection, eight VH genes were amplified, six of them were functional. Their sequence revealed that one belonged to the VH1 family (IGHV1–69-01), four to VH3 (IGHV3–11, −30, −48, −74) and one to the VH4 family (IGHV4–59) (Table II). JH4 and JH6 were the two JH gene segments represented among these amplified genes, a similar result to that obtained for patient 1. CDR3H lengths varied between 30 and 51 bp (mean 42 bp) representing 10–17 aa residues, on average 14 ± 3 aa residues. The CDR3H mean length of patient 3 was similar to that of patient 1, but shorter than that of patient 2. As in previous cases, VH sequences showed a high frequency of somatic mutations, ranging between 10 and 42, on average 25 ± 10 mutations/VH. But only two out of six VH sequences showed a positive selection for R mutations in CDRs. Both of them belong to VH3 family (IGHV3–74 and IGHV3–11; Table II). Amino acid sequence alignment of CDRs to the corresponding germlines of these two VH is shown in Table III. The amino acid sequence of the VH1–69-01 variant from this patient showed changes with respect to the

Table II. *Mutational analysis of the VH genes isolated from the heart biopsy samples of patients 1 and 2 and from heart tissue sections of patient 3*

Patient	Percentage of Identity to Germline	VH Family	JH Segment	No. of Somatic Mutations	FR R/S	CDR R/S	CDRH3 Length (bp)
1	89	V4–31*02	J4*02	33	1.9	9	39
	71	V3–64*04	J4*02	11	0.3	6	ND
	91.5	V4–59*01	J6*03	24	2.2	1.7	30
	95	V3–7*01	J4*02	14	0.8	1.5	33
	95.5	V3–30*04	J6*02	14	6	1.3	72
	97.2	V1–2*02	J6*03	6	2	1	63
2	89	V1–69*01	J4*02	30	0.7	1.2	51
	92	V1–2*02	J6*02	22	2.3	5	54
	91.3	V1–69*01	J5*02	11	1.7	4	63
	86.5	V4–4*02	J6*02	39	1.1	3.7	54
3	91	V1–69*01	J6*02	26	0.9	2	36
	96.5	V3–30*01	J4*02	11	2	1.5	54
	92	V4–59*01	J6*03	23	1	2	51
	95	V3–74*02	J6*02	14	5	3	57
	94.8	V3–11*03	J4*02	13	2.5	5	42
	88	V3–48*03	J4*01	35	3.3	2	45

germline gene in both CDR1H and CDR2H, but accumulated them mainly in FR3H. On the contrary, the amino acid sequences derived from VH3–30-15 and VH4–59-01 germlines showed lower number of replacements with respect to the germline sequence, that were almost equally distributed along the whole variable region (supplemental table).

It was noteworthy that all sixteen VH genes from biopsy samples had a different rearranged CDR3H; no evidence of an expanding B lymphocyte clone was detected.

Considering the sequence of all amplified VHs from heart sections of these three patients, sixteen VH genes showed deviation of more than 2% from the corresponding germline genes. Using the multinomial distribution model equation for the presence of Ag selection, seven out of sixteen VH genes (43.75%) showed evidences of Ag-driven selection.

The fact that sequences derived from the same VH germline genes were amplified from different patients, allowed us to compare their amino acid sequences. The two sequences derived from VH1–69-01 genes from patient 2 presented more mutations in FR1H than the one from patient 3, whereas the three of them showed a highly mutated FR3H. Sequence EU549726 from patient 2 and that of patient 3 presented a high number of replacements in their CDR2H (supplemental table). On the other hand, analysis of the IGHV1–2-2 sequences from patient 1 and 2, showed for the latter a higher number of replacements by polar amino acids in both CDRs (supplemental table).

VH sequences derived from VH3–30-15 genes from patients 1 and 3, both showed low number of amino acid replacements throughout all VH regions. However, analysis of sequences derived from VH4–59 showed that the variant of patient 3 presented less number of replacements than the one from patient 1 (supplemental table). Most interestingly, both VH4–59 variants presented similar replacements by aspartic, arginine, and histidine in their CDR1H (supplemental table).

The detailed analysis of the different VH and the fact that all sixteen VHs from the biopsy samples had different rearranged CDR3Hs demonstrated the different origin of each Ab. No evidence of an expanding B lymphocyte clone was obtained.

VH repertoire of Fab derived from a recombinant Ab library

To study the direct influence of *T. cruzi* in the shaping of the Ab repertoire, another approach was used. An Ig Fab library derived from bone marrow mRNA of a donor cChHD patient (patient 4 in Table I) was constructed. Variable and constant regions of H and L chains were amplified by PCR, and cloned into pComb3X as described in *Materials and Methods*. The complete Fab library contained approximately 1×10^8 cfu per μg of transformed DNA.

Table III. *Amino acid sequence alignment of CDRs to the corresponding closest germinal line*[a]

	Sequence	CDR1 (27–38)	CDR2 (56–65)	CDR3 (104–118)
Patient 1	**M99683 VH4–31*02**	GGSISSGGYYWS	IYYS...GST	
	EU549720 E1	GASVTNGVYYWT	-N--...---	EGRGIPPEFDYW
	M77299 VH3–64*04	GFTF....SSYA	ISSN..GGST	
	EU549721 E2	EVQL....VESG	G-G....-R-	ND
Patient 2	**X62106 VH1–2*02**	GYTF....TGYY	INPN..SGGT	
	EU549727 E8	----....SDN-	---K..----	CA**G**FHKAGGYD**N**RMWGMD**L**W
	L22582 VH1–69*01	GGTF....SSYA	IIPI..FGTA	
	EU549728 E9	ADI-....MN-G	----..---P	CARRPKRCSGSGS**P**YNECWFD**L**W
	X92232 VH4–4*02	GGSIS...SSNW	IYHS...GST	
	EU549729 E10	--TMR...-GT-	VSD-...-V-	CAREARIGSGELRDY**AL**DVW
Patient 3	**Z17392 VH3–74*02**	GFTF....SSYW	INSD..GSST	
	EU549734 E14	---L....GN--	-KT-..--N-	C**V**RDSYDRSDS**N**GKYYGMDVW
	X92287 VH3–11*03	GFTF....SDYY	ISSS..SSYT	
	EU549735 E15	--I-....G---	--T-..-I--	CARGLC**T**S**S**SCY**S**DYW

[a] Hypermutated VH genes from plasma cells of heart tissues are shown. Sequences E1 and E2 derived from patient 1; E8, E9, and E10 from patient 2; and E14 and E15 from patient 3. Amino acids that are identical to the germline sequence are symbolized by dashes. Mutated amino acids are indicated in capital letter for CDR1/2 and in capital bold letter for CDR3.

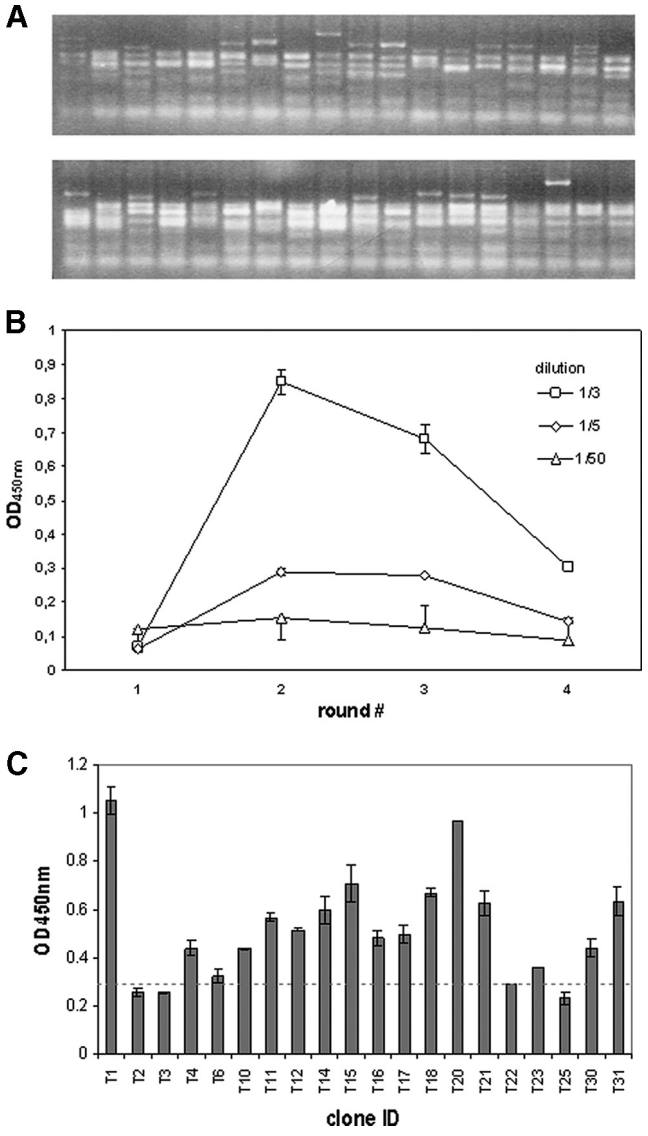

FIGURE 2. DNA fingerprint of human recombinant Ig library and anti-*T. cruzi* Fab. *A*, DNA from Ab library was amplified by PCR and digested with the restriction enzyme *Bst*OI (analyzed clones: 36). *B*, Enrichment of specific anti-*T. cruzi* phage during panning measured by polyclonal phage-ELISA. *C*, Anti-*T. cruzi* reactivity of individual Fab selected from the third round of panning against *T. cruzi* epimastigote lysate.

Forty eight clones randomly chosen from Fabκ library and 48 randomly chosen from the Fabλ library were analyzed for full-length insert by colony-PCR. Seventy three positive PCRs were obtained showing full-length insert. *Bst*OI restriction analysis showed generalized insert diversity (Fig. 2*A*) that was further confirmed by sequencing. Since these 73 recombinants were selected randomly, they constitute a representation of the repertoire of the infected patient. Accordingly, the set of VH composed by these 73 recombinants will be referred to as the VH of the patient repertoire (also cChHD patient repertoire).

Anti-T. cruzi Fab

Thereafter, anti-*T. cruzi* Fab were selected from the phage display library by successive rounds of panning. Because elution with glycine pH 2.2 showed no specific enrichment (data not shown), we selected Ag binding clones by competition with serum of the cChHD patient 4 (Table I). The phage repertoire was panned in four successive selections for binders. The phage input for each round of panning was in the range of 10^{11}-10^{12}. The output was in the range of 5×10^3 to 10^4 for the first round, and reached 10^7 in the third. Enrichment in anti-*T. cruzi* phage was followed by phage ELISA. An OD of 0.06 was measured for the first round of panning, that rose to 0.8 by the third round (Fig. 2B). Subsequently, 52 anti-*T. cruzi* Fab were selected. From all of them, only 36 (70%) presented a full length insert and showed different *Bst*OI restriction patterns. Twenty full length clones from round 3, which showed the highest reactivity in phage-ELISA, were expressed as soluble Fab. From these, 16 out of 20 recognized the *T. cruzi* epimastigote Ag on ELISA (Fig. 2C) with OD above 0.3, and presented no reactivity to Ags from other sources.

Table IV. *Distribution of VH families*

	Healthy Adult PBL Repertoire[a]	cChHD Repertoire	Anti-*T. cruzi* Fab	cChHD Heart Plasma Cells
VH1	63 (15.0%)	5 (6.8%)	2 (5.6%)	5 (31.3%)[b,c]
VH2	10 (2.4%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
VH3	232 (55.1)	36 (49.3%)	15 (41.7%)	7 (43.7%)
VH4	91 (21.6)	23 (31.5%)	16 (44.4%)[b,d]	4 (25%)
VH5	12 (2.9%)	6 (8.2%)[d]	3 (8.3%)	0 (0.0%)
VH6	8 (1.9%)	1 (1.4%)	0 (0.0%)	0 (0.0%)
VH7	5 (1.2%)	2 (2.7%)	0 (0.0%)	0 (0.0%)
Total	421	73	36	16

[a] The healthy human adult PBL repertoire was obtained from Brezinschek et al. (35).
[b] Significant differences when compared with the cChHD library ($p < 0.05$).
[c] Significant differences between anti-*T. cruzi* Fab and cChHD heart plasma cells ($p < 0.05$).
[d] Significant differences when compared with the healthy PBL repertoire ($p < 0.01$).

VH gene usage

Analysis of the variable regions of H chains of the 73 phage representing the patient repertoire, and the 36 anti-*T. cruzi* phage selected after panning were characterized, and compared with the distribution of VH families described for the peripheral blood lymphocytes (PBL) of healthy individuals by Brezinschek et al. (35, 36). In the patient repertoire, representatives of all VH families were found with exception of VH2 (Table IV, Fig. 3). VH3 was used more frequently, followed by VH4, VH5, VH1, VH7, and VH6 (Table IV, Fig. 3). In comparison to the repertoire of healthy individuals, VH usage in the patient repertoire showed a decrease in VH1 and VH3, a slight increase in VH4 and VH7 and a significantly higher representation of the VH5 family (8.2% vs 2.9%, $p < 0.01$). In comparison to the patient repertoire, selection on *T. cruzi* Ag induced a change in the representation of the VH families

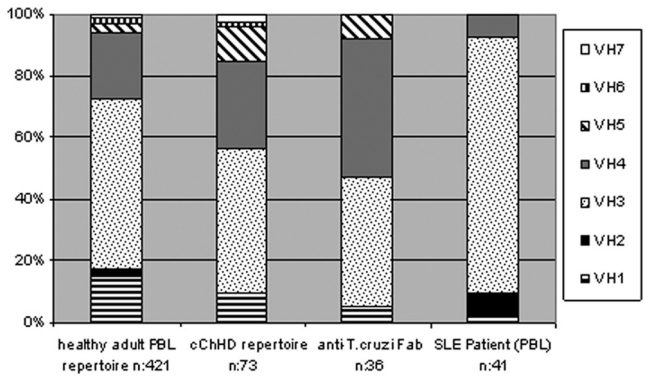

FIGURE 3. VH gene usage. Comparison of VH usage among healthy human adult PBL repertoire (obtained from Brezinschek et al. 35), cChHD repertoire, anti-*T. cruzi* Fab and systemic lupus erythematosus patient PBL repertoire (obtained from Dorner et al. 37).

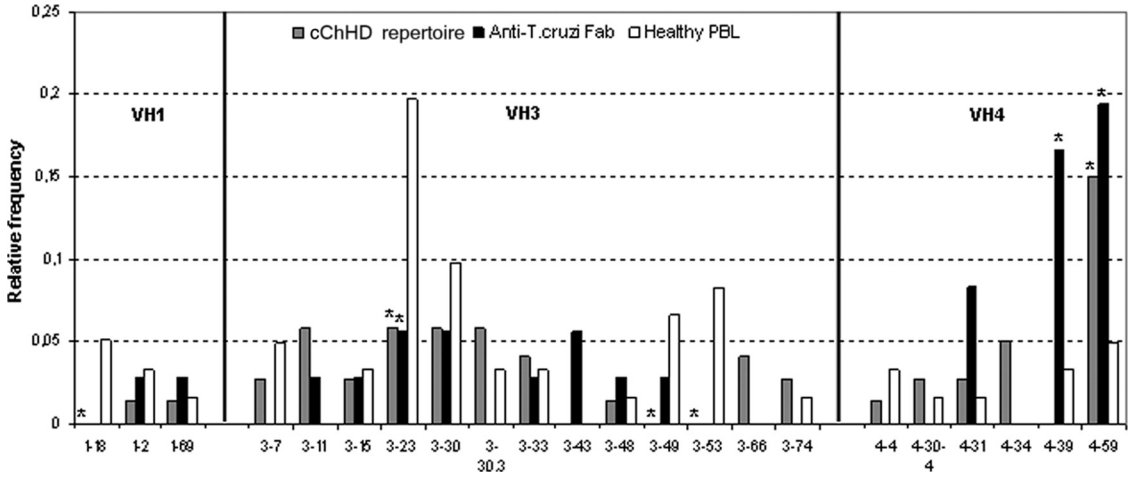

FIGURE 4. Most frequently employed VH gene segments. Comparison of the cChHD repertoire and the anti-*T. cruzi* Fab with the healthy PBL repertoire according to data in Brezinschek et al. (35). *, Significant difference with the healthy repertoire frequency ($p < 0.05$).

(Table IV, Fig. 3). A slight decrease in VH1 as well as VH3 representation was evident, although not significant. However, selection on parasite Ags significantly increased the representation of the VH4 family among the anti-*T. cruzi* V_H genes (Table IV and Fig. 3). This increase in VH4 representation was the most notable difference to the VH gene family distribution of healthy individuals, and to the patient repertoire (Fig. 3).

In Fig. 4, the most frequently used VH germline genes of healthy individuals were compared with the germline gene distribution of VH from the cChHD patient repertoire and the anti-*T. cruzi* Fab. None of both sets of VH included any member of the IGVH-1–8. Notably, the patient repertoire showed a statistically significant under-representation of the VH3–23 gene, accompanied by a significant lack of VH3–49 and VH3–53 genes (Fig. 4). A decrease of the VH3–30 representation was also evident (Fig. 4). With respect to the VH4 family, the main feature of the patient repertoire was an increased representation of VH4–30-4, VH4–34 and a significant increase in the number of VH4–59 genes. This repertoire had four different genes of the VH4–34 subset (5.5%). After panning on parasite extract, the germline representation changed. A relative increase in the representation of VH1–2, VH1–69, VH3–43, VH3–48, and VH4–31 genes was observed, together with the identification of VH3–49 genes. Notably, panning excluded the representation of VH4–34 genes, whereas significant increases in the representations of VH4–39 and VH4–59 were observed (Fig. 4). It was of interest for our study that, 13 out of 36 anti-*T. cruzi* selected Fab were composed by VH4 germline genes.

CDR3 composition and length

Analysis of the JH segment distribution showed significant differences between the healthy and the cChHD patient repertoire, with JH1 being found more frequently in the latter, an increase that is accompanied by a significant decrease in the use of the JH6 segment (Table V). The patient repertoire also presented an increase in the representation of JH2, JH3, and JH5 gene segments, whereas JH4 showed a slightly decreased representation as compared with the human healthy repertoire. After panning, there was a significant drop in the representation of the JH3 segment as compared with the patient repertoire. Panning also decreased to some extent the representation of the JH2, while JH4 and JH5 presented a slight increase. As in the cChHD patient repertoire, JH1 was significantly over-represented and JH6 under-represented (Table V).

With respect to DH segment usage, no significant differences between the healthy repertoire, the cChHD patient repertoire and the anti-*T. cruzi* VH were observed.

Relative frequencies of CDR3 lengths are shown in Fig. 5. Abs derived from cChHD patients (patient repertoire, anti-*T. cruzi* Fab and VH from plasma cells of heart tissues) presented a mean nucleotide length of 38.0 ± 10.2, 35.2 ± 10.2, and 42.2 ± 12.6, respectively, which did not differ significantly from the healthy one (41.2 ± 2 nt). However, CDR3s were significantly shorter than those reported for active immune responses against different infectious agents (50.4 ± 0.9 nt, $p < 0.01$) (38).

Somatic hypermutation analysis

Analyzed VH genes were considered mutated using a 98% germline homology cut off as described by Kienle et al. (39) and Arons et al. (40). All analyzed VH genes were mutated, with the exception of three VH genes from the patient repertoire that presented 98% homology to the corresponding germline genes. In accordance, the germline homology rate ranged from 79% to 98% (median, 88%) for the patient repertoire, and 76% to 96% with a median homology of 87.3% in the set of VH from *T. cruzi* selected phage.

A total of 109 VH gene sequences were analyzed using the multinomial distribution model equation for the presence of Ag selection, as assessed by conservation of the FR sequence and/or excess of R mutations in CDR (see *Materials and Methods* and Ref. 34). The rearranged VH genes derived from Fab

Table V. *Composition of CDR3: distribution of JH segments*[a]

	Healthy Adult PBL Repertoire	cChHD Repertoire	Anti-*T. cruzi* Fab	cChHD Heart Cells
JH1	1 (0.7%)	7 (9.6%)[b]	5 (13.8%)[b]	0 (0%)
JH2	5 (3.5%)	6 (8.2%)	2 (5.6%)	0 (0%)
JH3	9 (6.3%)	8 (11%)	0 (0%)[c]	0 (0%)
JH4	75 (52.1%)	33 (45.2%)	21 (58.3%)	7 (43.75%)
JH5	15 (10.4%)	12 (16.4%)	6 (16.7%)	1 (6.25%)
JH6	39 (27.1%)	7 (9.6%)[b]	2 (5.6%)[b,d]	8 (50.0%)[c]
Total	144	73	36	16

[a] The healthy human adult PBL repertoire was obtained from Brezinschek et al. (35).
[b] Significant differences when compared with the healthy PBL repertoire ($p < 0.05$).
[c] Significant differences when compared with the library.
[d] Significant differences between anti-*T. cruzi* Fab and cChHD heart cells.

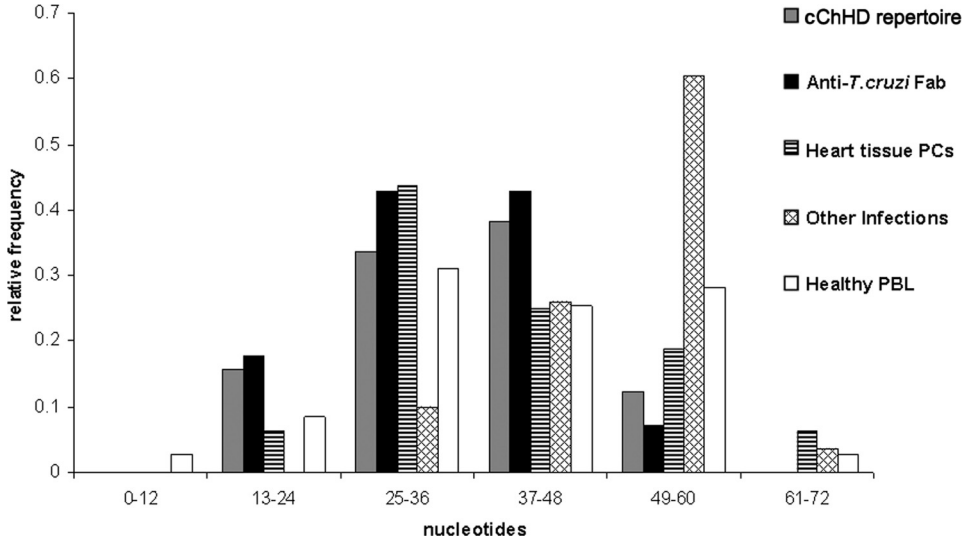

FIGURE 5. Distribution of CDR3 length. Comparison between H chain genes obtained from the cChHD repertoire, anti-*T. cruzi* Fab and heart tissue plasma cells vs the healthy repertoire (35) and various infections derived Ab sequences (38).

representing the patient repertoire and those from the anti-*T. cruzi* selected phage were highly mutated. It is noteworthy, that VH genes from the anti-*T. cruzi* Fab include more mutations than those from the cChHD patient repertoire, namely 15 out of 32 VH, approximately 47%, showed evidences of Ag-driven selection against slightly more than 30% in the cChHD repertoire, 21 out of 65 VH. For example, amino acid sequence alignments of members of VH3–43-02 gene family, that were only apparent after panning, showed that these VH concentrated amino acid changes to the germline gene, within CDR1 and 2 (supplemental table). Analysis of VH from anti-*T. cruzi* Fab from the members of the VH4–39 family showed a similar trend compared with the germline gene and to the only member of the VH4–39 gene family from the repertoire (supplemental table). In addition, for this gene family, extensive amino acid changes were also evident in the three FR (supplemental table). Amino acid changes through out the VH were also observed for the VH4–59 variants of the cChHD patient repertoire as well as in the VH4–59 variants of anti-*T. cruzi* Fab. It was of interest for this study that in the cChHD repertoire, two VH4–59 gene sequences, EU619973 and EU619998 (supplemental table), that shared the same CDR3H sequence were characterized. These clonally related VH, that differed in 4 aa within the CDR1H and had the same CDR2H sequence, probably recognized the same Ag. The fact that these and other Fab were not included within the set of anti-*T. cruzi* Fab, does not necessarily mean they do not recognize parasite Ags. Anti-*T. cruzi* Fab reacting with stage-specific amastigote (intracellular form) and trypomastigote (blood stream form) Ags may have been excluded in panning experiments that use parasite insect stage Ags (epimastigote form).

Amino acid sequence alignments of CDRs to the closest germline are shown in Table VI for 12 hypermutated anti-*T. cruzi* VH genes.

Integrative analysis

An integrative analysis allowed to align and compare the different VH amino acid sequences of the same families from three of the four patients. Patients 1 and 2 and the patient repertoire of patient 4 showed VH1–2 genes. In comparison to the germline sequence, amino acid changes were evident in CDR1 and CDR2 for patients 2 and 4. In addition, the latter presented a high number of changes in FR3. The less mutated VH1–2 gene was that of patient 1. Patients 1 and 3 and the repertoire of patient 4 presented VH3–30 genes. Low number of amino acid changes with respect to the germline were detected in this case, one or two per CDR, with the major number of changes concentrating in FR3 (supplemental table). A similar trend was observed for the VH4–59 genes of patient 1, patient 3, the repertoire of patient 4 and the anti-*T. cruzi* Fab (supplemental table). The less mutated amino acid sequences with respect to the germline were two VH from the anti-*T. cruzi* Fab, EU620035 and EU 620063 (supplemental table).

Discussion

Functional autoantibody specificities against cardiac receptors have been described in Chagas′ heart disease. In several autoimmune diseases, such as myasthenia gravis, Graves′ disease and insulin-resistant diabetes, Abs against hormonal receptors play a major pathogenic role, suggesting that common autoimmune processes linked chronic infections caused by *T. cruzi* and the mentioned autoimmune pathologies (14). To determine whether usage of particular gene families or individual genes for the creation of the general Ab repertoire was skewed in patients with cChHD compared with healthy subjects and to patients with autoimmune diseases or other chronic infections, we undertook the study of the VH repertoire of patients with cChHD in two settings: 1) plasma cells present in heart tissue of three patients; and 2) bone marrow of a fourth patient.

Indeed, previous histological characterization of heart tissue from patients with cChHD evidenced signs of a permanently active inflammatory process, with inflammatory sites composed by T and B cells and macrophages (3, 16) accompanied by parasite persistence (14). It was hypothesized that Chagas′ heart inflammatory landscapes could include GC-like structures. However, the analysis of heart samples from the three cChHD patients (patients 1, 2, and 3, Table I) covering different ranges of inflammatory infiltrations, from mild to severe myocarditis (Fig. 1) did not confirm this hypothesis. Although heart plasma cells were closely associated with T cells and macrophages, GC-like structures were not observed. Neither CD20 B cells nor follicular dendritic cells, which are essential for the development of the microenvironment of GC, were detected.

To assess whether the cells from the inflammatory sites of heart tissue from patient 3 were in a proliferative state, sections were stained with the anti-human Ki-67 Ab. The absence of staining suggested that infiltrating inflammatory cells, including

Table VI. *Amino acid sequence alignment of CDRs to the corresponding closest germinal line*[a]

	CDR1	CDR2	CDR3
M99651 VH3–9*01	GFTFDDYAMH	ISWNSGSIGYADSVKG	
EU620036 T-10	--------I-	-------VA-------	DITSTVDGAARSRFDS
AB019439 VH3–33*01	GFTF....SSYG	IWYD..GSNK	
EU620037 T-14	--S-....DN-V	--N-..-KT-	CAKDISEDCSSITCQGLPVYYL
Z27506 VH1–69*02	GGTF....SSYT	IIPI..LGIA	
EU620040 T-20	----....D---	-V-L..---K	CRTYRPGSQHYFDSW
M99660 VH3–23*01	GFTF....SSYA	ISGS..GGST	
EU620041 T-21	---I....TK--	VLND..V-D-	ND
EU620042 T-23	----....T---	AGSG...V-R	CAKFRGQLLESYFFDSW
X92259 VH4–39*03	GGSIS..SSSYY	IYYS...GST	
EU620044 T-31	---SY..T----	V---...RR-	LYVRDDSDYDIIDWLSW
X92287 VH3–11*03	GFTF....SDYY	ISSS..SSYT	
EU620046 T-4	--K-....----	YI-D..--RH	IIVRDIVFRGVGSTARLD#YW
X92297 VH4–39*04	APSA....SSYY	IYYS...GST	
EU620048 T-12	VAPSS..NN--F	--H-...-TI	STVRDTPPYHFPLDDW
Z18901 VH3–43*02	GFTF....DDYA	ISGD..GGST	
EU620054 T-35	--R-....-S-P	-T--..--R-	CAKEVGTYGVSWYNGLDHW
L06615 VH3–30*04	GFTF....SSYA	ISYD..GSNK	
EU620055 T-37	--D-....-DF-	T-F-..-NIR	CARDFIGGSPDYFDHW
X05715 VH4–39*02	GGSIS..SSSYY	IYYS...GST	
EU620056 T-38	-D---..N-P--	V--T...-R-	CARLLGGVGET##
M99652 VH3–11*01	GFTF....SDYY	ISSS..GSTI	
EU620062 T-45	----....--S-	SYI-TT----	CARVRAARGATYYYYYMDVW
L10094 VH4–39*01	GGSIS..SSSYY	IYYS...GST	
EU620067 T-51	---V-..N-N--	--VT...-T-	CARHAEDATGDDSVFGVVLPFDKW

[a] Hypermutated VH genes from selected anti-*T. cruzi* Fab are shown. Amino acids that are identical to the germline sequence are symbolized by dashes. Mutated amino acids are indicated in capital letter for CDR1/2. The only deletion is symbolized by point.

plasmocytes, rather migrated into heart tissue from secondary lymphoid organs than proliferated and expanded in situ. The fact that none of the three patients presented GC-like structures in the studied heart specimens further supported this assumption. So far, no proofs exist that in heart of patients with Chagas′ disease a local, heart or parasite, Ag driven cell response is generated. Nevertheless, the presence of parasite cells and rests may have been an appropriate stimulus to attract and subsequently seed the observed inflammatory processes (3, 16, 29, 41). This assumption was supported by the analysis of VH gene sequences isolated from infiltrating plasmocytes. Comparison of these VH sequences to the germline genes, showed that the majority were highly mutated. Within these VH, somatic mutations were not randomly distributed, nucleotide exchanges accumulated preferentially in the CDR (supplemental table). In turn, this finding suggested that plasmocytes from heart tissue lesions were derived from long term memory cells, or from B cells selected for their affinity, that migrated into heart tissue from secondary lymphoid organs. Accordingly, comparison of the V(D)J rearrangements of the amplified VH genes also revealed that none of the plasma cells were clonally related. The fact that the rest of *T. cruzi* were found near plasma cells suggested that *T. cruzi* Ags may have been the primary targets of these Abs (29).

To search for direct evidences of the role of the parasite in shaping the humoral response of a patient with cChHD, a bone marrow derived human recombinant Ab library was constructed and panned against parasite Ags. As described for VH from heart plasmocytes, VH from anti-*T. cruzi* Fab showed an accumulation of R mutations in CDR, indicating that these Abs underwent an Ag driven selection process. Around 47% of the VH from Fab selected by panning on *T. cruzi,* 15 out of 32, were hypermutated, against approximately 30% of the VH, 21 out of 65, from the Fab that represent the patient repertoire. Comparison of the VH repertoire of patient 4 with that of healthy individuals, and patients with autoimmune diseases revealed certain differences that cannot be considered major. Families VH1 and VH3 were less represented than in the healthy repertoire, whereas VH4 and VH5 were increased (Fig. 3). Panning on parasite Ags caused a slight decrease in the representation of the VH1 and VH3 gene families and a significant increase in the representation of the VH4 gene family (Fig. 3). However, the VH distribution of heart plasmocytes neither resembled the VH distribution of the patient repertoire nor that of the anti-*T. cruzi* Fab. In the heart samples of the three patients, at least a member of the VH1 family was found, always accompanied by at least one member of the VH4 family, surprisingly three VH1 genes were found in the heart repertoire of patient 2. In patients 1 and 3, the VH3 family was the most represented VH family, as in the case of the healthy repertoire. At this time, no explanation for this differential distribution of the VH1 genes in heart tissue of cChHD is evident. The confirmation of this tendency awaits further analysis of the VH repertoires of heart tissue from other patients. Despite differences in the VH gene distribution, the high proportion of hypermutated VH of plasmocytes from heart sections, in which the rest of the parasites were identified, seem to be an additional although yet indirect, evidence of the importance of parasite in selecting the Ig repertoire of patients with cChHD. Molecular cloning and expression of single chain recombinant Abs encompassing VH and VL derived from heart plasmocytes, currently underway in our laboratory, will further confirm or discard this assumption.

When VH genes from cChHD patients were compared with VH of IgG from patients with other infections, the main difference was noted in the CDR3 length. CDR3 of H chains from cChHD patients resemble those of the human healthy repertoire but was shorter than CDR3 reported for other human infections (50.4 ± 0.9 nt, $p < 0.01$; Fig. 5), particularly compared with the HIV infection repertoire (38). However, it was noteworthy that VH from heart tissues presented a bias toward longer CDR3, as shown by a higher proportion of CDR3 with 49–60 nt (18.2%)

vs 12% and 7% in the library and in anti-*T. cruzi* phage, respectively (Fig. 5).

Concerning the D segment usage, it was similar to that of the healthy repertoire. Nevertheless, JH segment usage seemed somewhat different. The JH4 segment was present as in the healthy repertoire; however, JH1 was more and JH6 less frequently found in cChHD Fab (Table V). On the other hand, JH segment distribution in H chain genes obtained from heart tissues resembled the distribution of the healthy repertoire with the exception of JH6 that was over-represented (Table V).

To our knowledge, this is the first study of VH usage in a human protozoan infection. Previous studies were devoted mostly to the healthy repertoire (35), autoimmune disorders (21–23, 25, 26, 33, 37, 42, 43), and viral infections (reviewed in Ref. 38).

VH genes amplified from the heart of cChHD patients, as well as anti-*T. cruzi* Abs selected from a phage display library, presented a high degree of somatic mutations. Indeed, between 43 and 47% of these VH genes were found to be hypermutated, suggesting *T. cruzi* driven selection. All results presented herein, strongly suggest that plasma cells found in heart inflammatory lesions migrated into the heart from secondary lymphoid organs. The amplification, cloning, and sequencing of the H chain repertoire is the first step in the construction of complete recombinant Abs from heart B cells. This approach is currently being applied to investigate the specificity of plasma cells from heart sections of patients with Chagas′ heart disease and will be used to study heart B cell specificities in IDC and related conditions. Analysis of kinetic and functional properties of these Abs may advance our understanding of mechanisms underlying pathogenesis of idiopathic as well as infectious cardiomyopathies.

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Disclosures

The authors have no financial conflict of interest.

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