Do Archaea and bacteria co-infection have a role in the pathogenesis of chronic chagasic cardiopathy?

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Chronic cardiopathy (CC) in Chagas disease is a fibrotic myocarditis with C5b-9 complement deposition. My- coplasma *and* Chlamydia *may interfere with the complement response. Proteolytic enzymes and* archaeal *genes that have been described in* Trypanosoma cruzi *may increase its virulence. Here we tested the hypothesis that different ratios of* Mycoplasma, Chlamydia *and archaeal organisms, which are frequent symbionts, may be associated with chagasic clinical forms. Materials and methods: eight indeterminate form (IF) and 20 CC chagasic endomyocardial biopsies were submitted to in situ hybridization, electron and immunoelectron microscopy and PCR techniques for detection of* Mycoplasma pneumoniae *(*MP*)*, Chlamydia pneumoniae *(*CP*), C5b-9 and archaeal-like bodies. Results:* MP *and* CP-DNA *were always present at lower levels in CC than in IF (p < 0.001) and were correlated with each other only in CC. Electron microscopy revealed* Mycoplasma, Chlamydia *and two types of archaeal-like bodies. One had electron dense lipid content (EDL) and was mainly present in IF. The other had electron lucent content (ELC) and was mainly present in CC. In this group, ELC correlated negatively with the other microbes and EDL and positively with C5b-9. The CC group was positive for Archaea and* T. cruzi DNA. *In conclusion, different amounts of* Mycoplasma, Chlamydia *and archaeal organisms may be implicated in complement activation and may have a role in Chagas disease outcome.*

Key words: *Chlamydia pneumoniae* - *Mycoplasma pneumoniae* - Archaea - Chagas disease - complement C5b-9

Chagas heart disease is described as a cardiomyopathy secondary to *Trypanosoma cruzi* infection. Antigens (Higuchi et al. 1993a, Bellotti et al. 1996, 1998) and DNA (Jones et al. 1993) from *T. cruzi* are found within the inflammatory infiltrate of the myocardium. The scarce amount of parasite product present is associated with an intense inflammatory infiltrate suggesting alternative mechanisms of pathogenesis (Higuchi et al. 2003a, Marin-Neto et al. 2007). One proposed theory is that the parasite releases polyclonal activators, which would favour the development of autoimmune injury (Gao et al. 2003). Alternatively, the presence of the parasite may alter myosin in such away that it would be recognized as non-self myosin by lymphocytes (Cunha-Neto et al. 1995). One theory not yet explored is the possible concomitance of additional infectious agents with *T. cruzi*.

Interactions between agents and co-infections have been reported in experimental studies with *T. cruzi* and in clinical reports of myocarditis (Andreoli et al. 2006, Cortez et al. 2006, Chimenti et al. 2007, Walder et al. 2007) and non-myocardial diseases. *Mycoplasma* and

Chlamydia are frequent symbionts (Horn & Wagner 2004) and *Mycoplasma* in the cytoplasm of *Trichomonas* causes chronic resistant genital infection (Dessi et al. 2005). Biofilms may be involved in development of plaque vulnerability (Katz & Shannon 2006). We have frequently found *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* antigens in the coronary arteries and valves of normal hearts and in increased amounts in hearts affected by chronic inflammatory disorders involving stenotic aortic valves (Higuchi et al. 2002) and ruptured atherosclerotic plaques (Higuchi et al. 2000, 2003b). We observed that the intimal association between *Mycoplasma* and *Chlamydia* occurred in the presence of archaeal-like forms associated with myxoid matrix in vulnerable plaques (Higuchi et al. 2006b).

Archaea, one of the three domains of life, is a highly diverse and abundant group of prokaryotes and includes a number of "extremophiles" that thrive in such environments as hot springs, salt lakes and submarine volcanic habitats (Madigan et al. 2000). Recent molecular studies have also revealed that archaea, like bacteria, are commonly mesophilic (DeLong 1992).

Multiple archaeal genomes have been sequenced and a large number of Archaeal-like genes have been described in persistent pathogens. It has been suggested that archaeal genes may confer metabolic capabilities as adaptive strategies for survival, even in hostile host niches, by allowing them to breach host barriers that exclude other organisms (Srinivasan & Morowitz 2006). This effort has contributed to a better understanding

of archaeal cellular processes. Some archaeal characteristics, such as M32 metallo carboxypeptidase (MCP) and two types of proteasome similar to those found in a common archaeal-eubacterial ancestor, were described in *T. cruzi* (Niemirowicz et al. 2007, Gille et al. 2003). A microbial consortium between a bacterium and a phylogenetically, distantly related archaeon has been suggested to promote the growth of both microbes in freshwater and marine sediments (Boetius et al. 2000, Raghoebarsing et al. 2006).

However, over half of the archaeal genes encode unique proteins of unknown function and no definitive virulence genes or factors have been described in archaea to date. Archaea share access to their host and are capable of long-term colonization and coexistence with endogenous flora in the host. The detection of anaerobic archaea among the human colonic (Miller et al. 1982, Miller & Wolin 1983), vaginal (Belay et al. 1990) and oral microbial flora (Belay et al. 1988) demonstrates their ability to colonize the human host. Details regarding their survival in such human niches, including their ability to evade the human immune system and compete with normal human flora, however, are practically unavailable (Eckburg et al. 2003).

Lepp et al. (2004) established correlations between the presence of disease and the presence of archaeal DNA, the severity of periodontal disease and the relative abundance of archaeal DNA in subgingival plaque, and between disease resolution and diminished archaeal DNA abundance. Archaeal genomes contain partial tad loci, which may encode Tad-like proteins involved in fibril formation and surface adherence (Lepp et al. 2004).

The complement system is a major component of innate immunity and provides an effective host defence mechanism against pathogens (Muller-Eberhard 1988). *Mycoplasma* may cause chronic disease in part by evading the immune response through antigenic variation and resistance to complement lysis (Simmons et al. 2004). The terminal complement complex C5b-9 was detected in the myocardium of chronic cardiac (CC) chagasic patients (Aiello et al. 2002). C5b-9 participates in several inflammatory and proliferative processes by releasing pro-inflammatory cytokines and growth factors from target cells. Sublytical doses of C5b-9 may promote cell proliferation (Niculescu et al. 1999, Halperin 1993) and participate in the development of severe fibrosis in chagasic cardiomyopathy.

We hypothesized that a consortium involving bacteria, archaea and *T. cruzi* might be related to different chronic chagasic disease outcomes, including CC and indeterminate asymptomatic form (IF). CC is associated with increased complement activation, fibrosis and lymphocytic myocarditis. IF is associated with the presence of *Mycoplasma* and *Chlamydia*, which leads to decreased complement activation and degradation of myosin, preventing an "auto-immune" reaction.

In the present work we investigated whether endomyocardial biopsies of CC or IF chagasic patients present forms and antigens or DNA from *M. pneumoniae*, *C. pneumoniae* or archaeal organisms, and whether they are associated with each other and with C5b-9.

MATERIALS AND METHODS

This work was approved by the Ethical and Scientific Committee of Heart Institute, University of São Paulo, Shool of Medicine, São Paulo, Brazil. We reviewed endomyocardial biopsies from chagasic patients with indeterminate or chronic cardiac forms, performed between 1980-2001 at the Heart Institute of São Paulo. We compared the amount of *M. pneumoniae* DNA (MP-DNA), *M. pneumoniae* DNA (CP-DNA) and archaeal-like bodies in myocardial fragments of chagasic patients with the IF versus CC form using in situ hybridization (ISH), immunoelectron microscopy, electron microscopy and PCR. As the fragments were too small, we could not use the same case for multiple techniques. Therefore, correlations between the results obtained with different techniques were not determined in this study. Archaea-like bodies were quantified according to their morphology by electron microscopy since we do not have specific antibodies or probes. DNA from CC fragments was amplified with specific primers for the Archaea domain.

ISH analysis - in situ myocardial detection of MP-DNA and CP-DNA by light microscopy - Serial sections of paraffin embedded endomyocardial biopsy fragments from 24 patients with Chagas disease were submitted to ISH for identification of MP-DNA and CP-DNA. Of the 24 fragments, seven were of indeterminate form (men age 34 ± 11 years, 3 males), and 17 were associated with heart failure (37 ± 12 years, 11 males).

Cell permeabilization was achieved using a 0.01 M citrate buffered solution, pH 6.0 ± 0.1, in the microwave oven following the protocol for heat-induced target retrieval described for IHC. Endogenous peroxidase was blocked with H_2O_2 at 3% for 20 min at 37º C. Tissue proteins were blocked with serum free protein block (Dako, Carpinteria, CA, USA) for 15 min at 37ºC. Next, 20 μL of hybridization mixture containing the probe, deionized formamide, 50% dextran sulfate, 20X SSC, Denhardt solution, salmon sperm DNA, yeast tRNA, poly A and poly C, and diethyl pyrocarbonate pure water, was applied to the sections and overlaid with a coverslip. The *M. pneumoniae* probe was prepared from a highly specific *M. pneumoniae* clone (Enzo Diagnosis, Farmingdale, NY, USA). The biotin-labelled *M. pneumoniae* oligonucleotide probe was synthesized by GIBCO/BRL (Rockville, MD, USA). The target DNA double-strands were denatured at 95 ± 5ºC for 6 min. DNA hybridization was performed for 18 h in a humidity chamber at 37ºC. After hybridization, the coverslip was removed with 0.0 5M Tris/HCl and 0.003 mM NaCl, pH 7.6, containing 0.1% Tween 20. Non-specific hybrids were excluded with post hybridization washes in 0.2x SSC for 10 min at 50ºC and 2x SSC for 10 min at 37ºC. The signal was amplified using the Catalyzed Signal Amplification system (Dako, Carpinteria, CA, USA). The reaction was visualized with chromogen 3,3-diaminobenzidine (Dako, Carpinteria, CA, USA) for 5 min at RT. Sections were counterstained with Harris' haematoxylin and mounted with Entellan resin (Merck, Darmstadt, Germany). Controls: negative controls were carried out by omitting the probe or the primary antibody. As a positive control for

all reactions, we used human tissue sections known to be positive for *M. pneumoniae* and *C. pneumoniae*. In addition, as a positive control for ISH, we used a repetitive Alu sequence probe (alu1/alu2) by Genetic Research (Al, USA) and for negative control we used a plasmid DNA labelled with biotin (Dako, Carpinteria, CA, USA).

The positive brown dots that represent DNA of *M. pneumoniae* and *C. pneumoniae* were detected by an image analysis system (Quantimet-500 Leica) and the mean percentage area for each DNA in all EMB was obtained.

Electron microscopy quantification of archaea by ultrastructural morphology and M. pneumoniae and C. pneumoniae by immune staining - Araldite embedded endomyocardial fragments collected between 1980-1986 from five patients with the IF and five with the CC form were submitted to semi-thin and thin sectioning and were examined by electron microscopy.

Morphological analysis - The material was photographed at a magnification of 2600X, allowing clear visualization of the myocardial fibre alterations and counting of the archaeal-like bodies.

Immunoelectron microscopy - Immunogold labelling was performed for detection of *M. pneumoniae*, *C. pneumoniae* and C5b-9 from nine biopsy fragments. Of these, five were from patients with indeterminate form (2 males and 3 females, mean age 33 ± 13 years) and four were from patients with chronic Chagas disease with heart failure (3 males and 1 female, mean age 38 ± 22 years).

Ultrathin sections were submitted to immunogold electron microscopy after etching with 12.5% ultrafiltered sodium metaperiodate. The slices were incubated with primary antibodies for 20 h. The primary antibodies use were monoclonal *M. pneumoniae* antibody (clone M2110182, diluted 1:300) from Fitzgerald Industries International Inc, Concord MA, USA, *C. pneumoniae* antibody (clone RR-402, anti-protein from the outer membrane; non diluted) from Dako, Carpinteria, CA, USA, and anti-Human C5b-9-Terminal Complement Complex (rabbit polyclonal antibody; diluted 1:75) from Calbiochem-Novabiochem Corporation, San Diego, CA, USA). The detection was performed with anti-rabbit IgG (whole molecule) gold conjugate, 10 nm (1:20) from Sigma Immunochemicals, Saint Louis, MO, USA.

Morphometric analysis - Five photos at 5.500X original magnification were used for morphometry. We counted the mean numbers of dots (colloidal gold particles of 10nm)/ photo, which correspond to an area of 23 μm^2. We also counted two types of rounded structures enclosed by double membranes. These were not clearly stained, suggestive of archaeal bodies. One had mild electron dense lipidic (EDL) content and the other had an apparently clear empty content. We named these structures respectively: EDL and electron lucent content (ELC) bodies.

The sections were observed by electron transmission microscope Philips EM-301 (Eindhoven, Holland).

PCR technique for detection of Archaea and T. cruzi DNA - Five paraffin myocardial fragment blocks from patients with CC were submitted to the PCR technique for detection of archaeal and *T. cruzi* DNA. DNA from the fragments was obtained using the PureLink Viral RNA/DNA Mini Kit (Invitrogen) following the manufacturer's instructions. Five paraffin myocardial fragment blocks from patients with CC and three normal adult myocardium samples was submitted to the PCR technique for detection of archaeal and *T. cruzi* DNA. For the isolation and purification of genomic DNA we used the PureLink Viral RNA/DNA Mini Kit (Invitrogen) following the manufacturer's instructions. Archaeal DNA amplification was carried out in 25 μL of reaction mixture under the following conditions: 0.2 mM deoxynucleoside triphosphates, 2.5 mM MgCl2, 2.5 U of Platinum Taq DNA Polymerase (Invitrogen), 50 pmol of each primer, 1x PCR buffer and 100 ng of DNA, using universal archaeal primers 1100F (5' -AGTCAGGTAACGAGCGAG-3') and 1400R (5' -GTGCAAGGAGCAGGGAC-3') (Kudo et al. 1997). PCR was performed in a BioRad DNA Engine thermocycler using the following program: denaturation of DNA at 94°C for 3 min, followed by 35 cycles at 94°C for 1 min, 54°C for 1.5 min and 72°C for 1 min, with 10 min at 72°C for extension at the end. Genomic DNA of *Halobacterium salinarum* INCQS A2 (DZMZ 668) (50 ng) was used as a positive control. It was kindly provided by Prof. Maysa Mandetta Clementino from the Bacteria and Archaea Reference Laboratory of the National Institute of Quality Control in Health, Fiocruz, Rio de Janeiro, Brazil. A negative control without DNA was included and the PCR reactions were repeated three times to confirm the reproducibility of the amplification.

T. cruzi DNA amplifications were carried out with 25 μL of reaction mixture under the following conditions: 100 ng of DNA, 50 pmol each primer S34F (5' -TATATTACACCAACCCCAATCGAACC-3') and S67R (5′ - TGGTTTTGGGAGGGGSSKTCAAMTTT-3') (Olivares-Villagómez et al. 1998) 0.2 mM of each deoxynucleoside triphosphate, 1x PCR buffer (pH 9.0), 2,5mM MgCl2 and 2 U of Platinum Taq DNA Polymerase (Invitrogen). Reactions were run in a BioRad DNA Engine thermocycler using the following program: denaturation of DNA at 94°C for 6 min, followed by 45 cycles at 94°C for 40 sec, 57°C for 1 min and 72°C for 45 sec, with 10 min at 72°C for extension. As a positive control, we used genomic DNA from *T. cruzi*. The mixture was run on a 1% electrophoresis agarose gel in 1x TAE buffer (40 mM Tris base, 20 mM sodium acetate, 1.0 mM EDTA, pH 8.0) with a 100-bp DNA ladder (Invitrogen Carlsbad, CA, USA). The gels were stained with ethidium bromide (0.5 mg/mL) to visualize the amplified PCR products under UV illumination.

Statistical analysis - The comparison of DNA quantities and archaeal-like bodies between the two clinical groups was performed using the Student *t*-test. The Pearson correlation test was used to determine the relationships between different microbe DNAs or antigens and complement.

RESULTS

All endomyocardial biopsies were positive for MP-DNA and CP-DNA by light microscopy examination. The mean percent areas detected by ISH in the two

groups of patients are shown in Table I. MP-DNA and CP-DNA were significantly higher in the IF group than in the CC group (p < 0.001). There was a significant positive correlation between MP-DNA and CP-DNA values in the CC group of patients (r = 0.64; p = 0.005) but not in the IF group of patients (r = -0.28; p = 0.547).

Qualitative and quantitative analysis - Endomyocardial biopsies from the IF group revealed myocardial fibres with well preserved morphology, large amounts of glycogen-like granules in the sarcoplasm and moderate dilatation of T tubules, sarcoplasmic reticulum and intercalated discs, which frequently contained membranous elements in the lumen. Fibrosis in the interstitium usually exhibited mycoplasmal-like forms characterized by only one outer membrane with a rounded or cylindrical shape. Chlamydial bodies characterized by a double membrane and dark homogeneous chromatin were rarely seen in myocardial fibres, macrophages or the extracellular matrix (Fig. 1A, B). Many EDL balls were present in the myocytes either at the subsarcolemal region or more internally. These were similar to lysosomes but enclosed by a barely visibly double external membrane with a darker aspect at the periphery. EDLs were always located near mitochondria and glycogen-like granules (Fig. 2A, B).

A second type of round organelle enclosed by a double membrane was also present. These had ELC with irregular thin flat tubules, an irregular folded external membrane and sometimes contained a large, clear periplasmic space. These EDL and ELC bodies are compatible with archaeal morphology and were sometimes fused to each other (Fig. 2A). EDL and ELC bodies presented variable diameters ranging from around 0.10-1.5 μm.

In CC cases, the myocardial fibres presented more intense alterations represented by mitochondrial crystolysis, a clear matrix and dilated reticulum sarcoplasmic organelles frequently presenting irregular membranes in their lumens. There were many foci of myocytolysis usually associated with ELC bodies and a lack of glycogen granules (Fig. 3A, B). There was a severe fibrosis in the interstitium, with mycoplasmal forms among the collagen fibres. There were many forms of ELC among the mononuclear inflammatory cells and in the myocardial fibres. These were associated with mycoplasmal forms and round dark structures that may have been derived from *C. pneumoniae* elements (Fig. 3C, D). ELC structures were sometimes very large (Fig. 4A, B). Glycogen-like granules were usually absent.

Immunogold electron microscopy counting - Immunoelectron microscopy demonstrated that the monoclonal antibodies anti *M. pneumoniae* and *C. pneumoniae* stained surface antigens and free nanolipidic particles suggestive of lipoprotein or lipopolysaccharide from these bacteria, as had been shown in a previous study (Higuchi et al. 2006c). All chagasic EMBs presented high amounts of *M. pneumoniae* and *C. pneumoniae* as extracellular antigens, as well as in the extracellular matrix and myocardial fibres. *M. pneumoniae* and *C. pneumoniae* were apparently associated with lipid nanovesicles.

Table II shows the mean numbers and standard deviation of gold particles/100 μm² detected by immunoelectron microscopy and the number of EDL and ELC bodies detected by transmission electron microscopy in both the IF group and the CC group. The unicaudal Student *t* test showed lower levels of *C. pneumoniae* and higher levels of C5b-9, as well as non-significantly lower levels of *M. pneumoniae* and EDL values, in the CC group than in the IF group. ELC values were higher in the CC group, without statistical significance. The lack of statistical significance may be linked to the small number of samples analyzed.

Table III shows the correlations between the variables in Table II. The CC group had higher R correlation values than the IF group, with significance only between C5b-9 and *C. pneumoniae* antigens (r = -0.96, p = 0.04) and an almost significant negative correlation between EDL and ELC values (r = -0.97; p = 0.06).

PCR technique for detection of archaeal and T. cruzi DNA - All five of the CC paraffin myocardial fragment blocks yielded a 300-bp fragment, including the *H. salinarum* reference strain, after PCR with the universal archaeal primers (Fig. 5). The same samples described above yielded a 122-bp fragment using *T. cruzi* specific primers (Olivares-Villagómez et al. 1998).

DISCUSSION

The exact mechanism for the development of chronic dilated cardiopathy in some individuals infected with *T. cruzi* is a still matter of debate. Our results provide evidence that association of mycoplasmal, chlamydial and/or archaeal elements might be related to this phenomenon.

Optical, electron and immunoelectron microscopy showed that high levels of DNA from *C. pneumoniae* and *M. pneumoniae* and the presence of archaeal-like bodies of the EDL type were associated with IF. Since these values did not correlate with each other and because the morphology was of isolated forms, we conclude that these microbes are not closely associated with IF. Alternatively, fusion of ELC archaeal-like bodies with the microbes described in the IF group was present in the CC group. An intimal interaction is suggested by the following: (i) the high correlation between CP-DNA and MP-DNA, which correlated negatively with ELC values; (ii) the negative correlation between the numbers of ELC with the numbers of EDL and (iii) the positive correlation between ELC and C5b-9 values.

The five samples submitted to PCR analysis yielded fragments compatible with the specific primers. Since we did not have enough material to perform PCR in the IF cases, we could not compare this item. Further studies are needed, including a better characterization of EDL and ELC organelles to determine if they present different archaeal genome sequences.

Mycoplasma can either grow symbiotically with eukaryotic cells without producing profound cytotoxicity or it may provoke a marked host response, acting as a super antigen by triggering apoptosis of the CD4+ T cells (Lo 1992). *Mycoplasma* has the ability to evade the immune system by antigenic variation and resistance to complement lysis (Simmons et al. 2004). However, *My-*

TABLE I

Percentage of area positive for *Mycoplasma pneumoniae* DNA (MP-DNA) and *Chlamydia pneumoniae* DNA (CP-DNA) in endomyocardial biopsies from chagasic patients in indeterminate (IF) or chronic cardiac (CC) forms by *in situ* hybridization technique in light microscopy and Pearson correlation test

Group	% MP-DNA mean (± SD)	% CP-DNA mean (± SD)	Correlation coefficient MP vs. CP
IF (n = 7)	8.75 (4.00)	9.66 (4.22)	- 0.28 (p = 0.547)
CC (n = 17)	2.65 (1.64)	3.43 (2.65)	0.64 (p = 0.005)
p (*t* test)	0.001	< 0.001	

Fig. 2: ultrastructural aspects of an indeterminate asymptomatic form endomyocardial biopsy. A: a panoramic view of a myocardial fiber contain mainly many **electron dense lipidic (EDL) bodies (thin arrows)** and few electron lucent content bodies, one fused with one EDL body (empty arrow); B: higher magnification of an EDL body divided in two lobes (arrow), exhibiting doubled external membrane, electron dense lipidic internal material darker at the periphery.
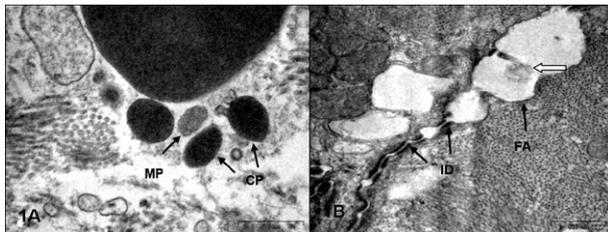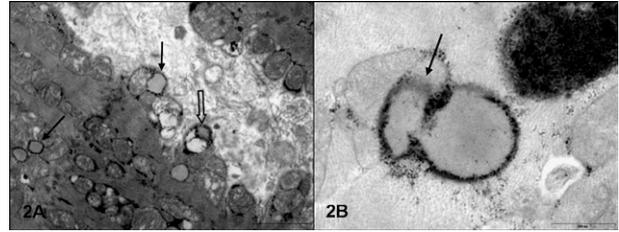

Fig. 1: ultrastructural aspects of an indeterminate asymptomatic form endomyocardial biopsy. A: a group of microbes, suggestive of mycoplasmal and chlamydial bodies, well individualized each other, amidst the fibrosis; B: intercalated disc (ID) with well preserved desmosomes (thin arrows) and dilated fascia adherens containing a membranous element in the lumen (white arrow). CP: *Chlamydia pneumoniae;* FA: *Fascia Adherens* MP: *Mycoplasma pneumoniae.*
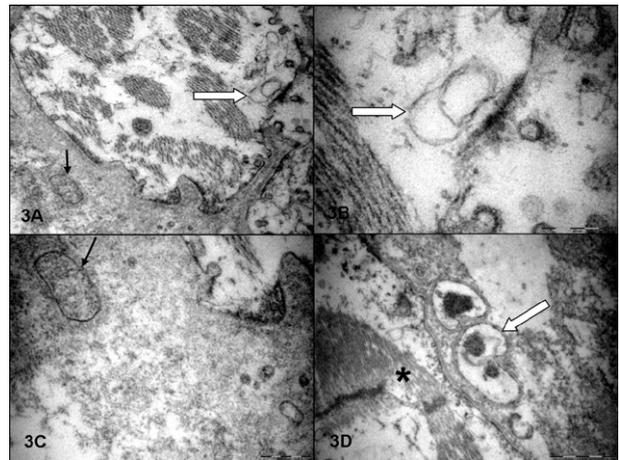

Fig. 3: ultrastructural aspects of a heart failure group endomyocardial biopsy. A: severe myocytolysis of the myocardial fiber. The white arrow indicates an electron lucent content (ELC) structure formed by two compartments, which is in close view in B. The black arrow indicates a mycoplasmal body; C: close view; D: a myocardial fiber with myocytolysis (asterisk), with some apparently symbiotic forms of mycoplasma, ELC and *Chlamydia pneumoniae.*

coplasma may increase the virulence of other microbes (Dessi et al. 2005) and may induce pro-inflammatory cytokines such as TNFα, IL-6 and TGFβ (Blanchard & Bebear 2002, Yang et al. 2004), which are related to heart failure (Sharma et al. 2001, Sugamori et al. 2002). *Chlamydia* is also a frequent symbiont of microbes and has the ability to prevent apoptosis of the host cell (Fan et al. 1998, Sharma & Rudel 2009). Archaea have antioxidative enzymes, such as superoxidismutase, that promote microbe survival (Cannio et al. 2000) and peptidases and proteasomes that contribute to degradation of aggregation-prone proteins and reduce cellular toxicity in mammalian cells (Yamada et al. 2006).

The presence of M32 family MCP, which is known to be specific to the Archaea and bacteria kingdoms, was described in *T. cruzi* (Niemirowicz et al. 2007). The authors suggest that the protozoan has acquired these genes by horizontal transfer of an ancestral archaea gene. Consistent with this hypothesis, the *T. cruzi* MCP has high biochemical similarity with *Archaeon P. furiosus* (*Pfu*). *Pfu* M32 MCP seems to be involved in the utilization of peptides and proteins for the metabolism of the organism (Schul et al. 2003).

Sequences of both the 20S proteasomes and *hsIV* were found in *T. cruzi*, suggesting the presence of endosymbionts, for example, an α-proteobacterial progenitor of mitochondria. Alternatively, horizontal gene transfer by temporal association with bacteria within the intestines of insects could account for the presence of these sequences (Gille et al. 2003). *T. cruzi* undergoes profound morphological changes using proteins from the host cell during its life cycle in the myocardial fibres. An essential role for proteasomes in the degradation of cytoplasmic proteins has been demonstrated (Gonzalez et al. 1996, De Diego et al. 2001).

T. cruzi presents lysosome-related organelles or reservosomes, which contain many enzymes, including carboxypeptidases (Sant'Anna et al. 2008). The lysosome-related organelle or reservosome's morphology is similar to the EDL bodies described in the present study, mainly in IF EMBs. These bodies have a barely visible double membrane, and some types are apparently divided by the presence of two lobes. These findings led us to propose that these EDL bodies could be archaea.

TABLE II

Mean numbers and standard deviation of gold particles/100μm²
Mycoplasma pneumoniae (MP), *Chlamydia pneumoniae* (CP)
and membrane attack complex (MAC) antigens in immuno-
eletron microscopy and archaeal-like bodies/100μm² in elec-
tron microscopy, in indeterminate (IF) and chronic cardiac
(CC) forms

Groups	MP	CP	MAC	EDL	ELC
IF (n = 5)	48 ± 22	48 ± 17	56 ± 13	0.13 ± 0.04	0.04 ± 0.04
CC (n = 4)	39 ± 17	17 ± 8.7	100 ± 43	0.09 ± 0.09	0.06 ± 0.04
p (*t* test)	0.30	0.005	0.04	0.19	0.31

EDL: electron dense lipidic bodies; ELC: electron lucent con-
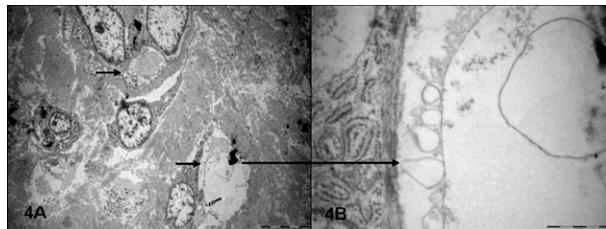tent bodies.

Fig. 4: details of a foci of inflammatory infiltrate in a chronic cardiac
case. A: among the mononuclear inflammatory cells, there are some
suggestive of Archaeal large bodies characterized by rounded struc-
tures surrounded by two membranes, large periplasmic space contain-
ing amorphous and granules content and an internal vacuole (small ar-
rows); B: a close view of the periplasmic space with many clear double
membrane round structures suggestive of nanoarchaea (large arrow).

TABLE III

Correlation coefficient and p value between mean numbers
of *Mycoplasma pneumoniae* (MP*), Chlamydia pneumoniae*
(CP) and membrane attack complex (MAC) antigens at the
immunoelectron microscopy and electron dense lipidic (EDL)
bodies and electron lucent content (ELC) bodies at the elec-
tron microscopy

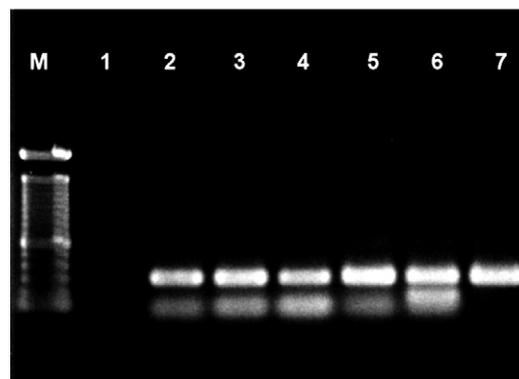|		CP	MAC	EDL	ELC
MP	IF	- 0.51 (0.38)	0.33 (0.59)	- 0.21 (0.73)	- 0.04 (0.95)
	CC	- 0.71 (0.29)	- 0.81 (0.19)	0.77 (0.23)	- 0.85 (0.14)
CP	IF		0.007 (0.99)	- 0.31 (0.61)	- 0.34 (0.57)
	CC		- 0.96 (0.04)	0.34 (0.66)	- 0.64 (0.36)
MAC	IF			0.37 (0.53)	- 0.60 (0.28)
	CC			- 0.33 (0.67)	0.60 (0.40)
EDL	IF				- 0.53 (0.34)
	CC				- 0.97 (06)

Fig. 5: PCR amplifications of Archaea (primers 1100/1400) in tissues
samples of chagasic patients. Lane M: molecular size marker (100-bp
DNA ladder); Lane 1: negative control with no added template DNA;
Lanes 2-6: tissue samples of chagasic patients; Lane 7: positive control
(*Halobacterium salinarum*).

In CC EMBs we observed ELC archaeal-like bodies.
Their morphology is similar to the morphology of those
present in the myxoid matrix of vulnerable atheroma
plaques that are associated with vessel dilatation and
adventitial inflammation in correlation with *C. pneumo-
niae* and *M. pneumoniae* elements (Higuchi et al. 2003b,
2006b). The ELCs were in the interstitium among the
chronic inflammatory infiltrate, sometimes with a huge
periplasmic space containing nanoarchaeal-like bodies.
This morphology is similar to that of *Ignicoccus hospi-
talis* (Junglas et al. 2008).

The unique ether glycerolipids of Archaea can form
vesicles (archaeosomes or liposomes from Archaea) with
strong adjuvant activity for MHC class II presentation.
The archaeosomes are superior adjuvants that induce a
long-term CD8+ cytotoxic T cell response to entrapped
soluble protein in the absence of help from CD4+ T cells
(Krishnan et al. 2000). It has been shown that chronic
chagasic active myocarditis in CC patients presents in-
flammatory infiltrate composed mainly of CD8+ T cells
with increased numbers in the presence of *T. cruzi* an-

tigens, and a lack of CD4+ T cells (Higuchi et al. 1993b,
1997, Reis et al. 1997). We argue that *T. cruzi* antigens
in archaeosomes may not be the cause of this increased
inflammatory infiltrate.

Based on the literature, we may speculate that symbi-
otic forms involving *Mycoplasma*, *Chlamydia* and ELC
organelles in the human chronic infection by *T. cruzi*
causes increased immune activation by complement de-
position and lymphocyte activation leading to the devel-
opment of myocarditis. However, the presence of non-at-
tached mycoplasmal, chlamydial and EDL archaeal-like
bodies leads to reduced myocardial inflammation.

The current view of microbiologists is that prokary-
otes' predominant state of being is inside microbial com-
munities. Direct interaction between archaea has been
proved to mediate exchange of chromosomal DNA, me-
tabolites or substrates from the cytoplasm of one cell
to another (Junglas et al. 2008). The association of dif-
ferent species of microorganisms leads to the develop-
ment of more resistant and productive new associated
forms (Hansen et al. 2007). Also, the presence of one
pathogen may inhibit the growth of others. *Mycoplasma*
inhibited the growth of *Chlamydia* in in vitro studies

(Van Nerom et al. 2000). *Escherichia coli* inhibited the growth of *T. cruzi* (Cortez et al. 2006). High ratios of *M. pneumoniae*/*C. pneumoniae* were related to an increased amount of growth factors and fibrosis in atheroma plaques (Higuchi et al. 2006a).

Limitations and clinical implications - The present work is a retrospective study with a small number of cases that cannot result in definite conclusions. However, our results may suggest a new frontier of investigation in Chagas cardiopathy pathogenesis, specifically the symbiotic interaction among prokaryotes and a trypanosome.

In conclusion, different amounts of *Mycoplasma*, *Chlamydia* and archaeal elements may be implicated in complement activation and may have a role in Chagas disease outcome. IF was associated with isolated forms of these microbes and with the EDL type of archaeal-like bodies. CC was associated with fusion of these microbes and ELC archaeal-like bodies in association with increased myocardial complement deposition. These findings open a new direction for pathogenetic studies and the possibility of new prognostic biomarkers in Chagas disease.

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