Characterization of T Cell Clones from Chagasic Patients: Predominance of CD8 Surface Phenotype in Clones from Patients with Pathology

Washington R Cuna, Celeste Rodriguez Cuna

Departamento de Parasitología, Instituto Boliviano de Biología de Altura, Facultad de Medicina, Casilla 641, La Paz, Bolivia

Human Chagas' disease, caused by the protozoan Trypanosoma cruzi, *is associated with pathological processes whose mechanisms are not known. To address this question, T cell lines were developed from chronic chagasic patients peripheral blood mononuclear cells (PBMC) and cloned. These T cell clones (TCC) were analyzed phenotypically with monoclonal antibodies by the use of a fluorescence microscope. The surface phenotype of the TCC from the asymptomatic patient were predominantly CD4 positive (86%). On the contrary, the surface phenotype CD8 was predominant in the TCC from the patients suffering from cardiomegaly with right bundle branch block (83%), bradycardia with megacolon (75%) and bradycardia (75%). Future studies will be developed in order to identify the antigens eliciting these T cell subpopulations.*

Key words: *Trypanosoma cruzi* - T cell clones - asymptomatic - pathology

Chagas' disease, whose causative agent is the protozoan *Trypanosoma cruzi*, is characterized by an acute often asymptomatic phase which proceeds through a latent period of variable length. A percentage of those infected pass into the more pathological, chronic stage of the disease characterized by damage to the cardiovascular or the digestive system as well as nervous tissue (Amorim et al. 1979, De Rezende 1979, Teixeira 1987, Tanowitz et al. 1992). Cell mediated immune mechanisms have been implicated in the immunopathology of experimental Chagas' disease (Said et al. 1985, Mortatti et al. 1990, Spinella et al. 1990, Ribeiro dos Santos et al. 1992). In contrast, the event mechanisms leading to immunopathology in human *T. cruzi* infections are not fully understood. The approach made in the present work was the development and characterization of TCC derived from T cell lines of four chronic chagasic patients; one asymptomatic and three with pathology. Only one previous study (Britten & Hudson 1985) described the isolation of a *T. cruzi* specific T cell line with the T4 surface phenotype from a chagasic patient. In this work we set out to analyze if the pattern of reactivity described above is characteristic of human *T. cruzi* infections or if there is an association between the clinical manifestations of Chagas' disease and a particular T cell subset. The results of this study show that different patterns of TCC were obtained from these patients in terms of the symptomatology of Chagas' disease.

MATERIALS AND METHODS

Parasites - The Tulahuén strain of *T. cruzi* was used in this work. Tissue culture trypomastigotes were obtained from the supernatants of Vero cells cultured in RPMI 1640 (GIBCO, Grand Island, NY, USA) containing 2% heat-inactivated (56°C, 30 min) FCS initially infected with trypomastigotes and maintained at 37°C, CO_2 for five days.

Antigen - Culture supernatants were centrifuged at 1800 x g for 10 min and incubated at 37°C for 2 hr to yield a supernatant containing highly motile trypomastigotes without debris. Parasites were resuspended at 1 to $2x10^7$ trypomastigotes per ml in RPMI medium containing 10% FCS, 100 IU of penicillin and 100 µg of streptomycin per ml referred to in the text as complete medium. Soluble antigens were prepared through four cycles of freezing (-20°C) and thawing the latter suspension. This material was assayed for protein content, aliquoted and stored at -20°C until used.

Cells - Blood was drawn from four chronic chagasic patients with positive serology for *T. cruzi* (indirect immunofluorescence and ELISA tests) and one patient with positive xenodiagnos. The asymptomatic patient, had normal ECG and chest X-ray and followed a clinical examination. Three

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patients presented clinical symptoms associated with cardiac disease and gastrointestinal lesions. PBMC were purified by centrifugation (400 x g, 20°C, 45 min) over a mixture of Ficoll Hypaque of density 1.077. After two washings with serum free medium, the cells were resuspended at the desired concentration in complete medium. The cell viability of PBMC suspensions was consistently >99% as determined by trypan blue exclusion.

Culture and cloning procedure - Fresh PBMC from the chagasic patients were cultured at 2 x 10^6 cells per ml in complete medium (5% CO_2 , 24 well plate) at 37°C in the presence of antigen. Throughout the study the antigen was used at a protein content of 20 µg/ml final concentration. After eight days of culture, viable cells were separated on Ficoll Hypaque gradient and cultured with antigen in the presence of mitomycin C treated (50 µg/ml) autologous PBMC (maPBMC) at a ratio of 4 x 10^5 viable cells to 1.6 x 10^6 maPBMC. Three cycles of restimulation with antigen were repeated before cloning. Cells were cloned by limiting dilution in 96 well microculture trays by plating 1, 2 or 3 cells/well onto 5 x 10^4 maPBMC in 200 µl of complete medium and antigen. Positive wells were scored by viewing in an inverted microscope after 12-14 days in culture. The T cell clones were subcloned once and then received alternative restimulations in the presence of maPBMC/antigen, in medium supplemented with 10 IU of purified recombinant human interleukin-2 (Genzyme, Cambridge, MA, USA) or 5 x 10^4 fresh allogeneic PBMC treated with mytomicin C as feeder cells and 1 µg/ml phytohaemagglutinin.

Surface phenotyping - Lymphocytes (1.5 x 10^6 per 5 ml tube) were washed twice in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) and incubated in different eppendorf tubes during 30 min at 4°C with monoclonal antibodies defining human T cell (OKT11), human CD4 T cell (OKT4) or human CD8 T cell (OKT8) epitopes (Ortho Diagnostics System, Raritan, NJ, USA). After washing two times with PBS/BSA the cells were incubated (30 min, 4°C) with 100 µl of Ortho fluorescein-isothiocyanate-conjugated goat anti-mouse IgG antibody, diluted 1:20 in cold PBS. After two washings with PBS/BSA the cells were recovered in 100 µl of 90% glycerol in PBS containing 0.1% sodium azide and examined on a fluorescence microscope. Nonspecific mouse immunoglobulin was used as control for nonspecific staining.

RESULTS

The TCC were obtained from three chagasic patients with clinical symptoms and one asymptomatic patient (Table) by stimulation with a soluble extract of trypomastigotes of the Tulahuén strain of *T. cruzi*.

As reported in a previous study (Britten & Hudson 1985) the best cloning efficiencies of the TCC by limiting dilution were obtained from wells plated at 3 cells/culture and were as follows: 28%, 30%, 20% and 18% for patients C, A, F and G respectively. These percentages of efficiency are reflected in the total number of clones obtained from each patient (Table).

The phenotype of the TCC which were maintained in continuous culture for seven months were characterized using a panel of anti-T cell antibodies (Table). Given the limited supply of APC, clones were chosen at random from the original total number which were maintained in culture and further analyzed for its surface phenotype. The results of this characterization are shown in the Table. The TCC derived from the asymptomatic patient C were predominantly CD4 positive (86%). On the contrary TCC obtained from the symptomatic patients were in its majority CD8 positive.

TABLE

Cell surface phenotype of T-cell clones grown from chagasic patients

Patient	Clinical form	No. of TCC	% Positive T cell clones		
			OKT4	OKT8	OKT11
C	Asymptomatic	89(37)	86	14	100
A	Cardiac	72(30)	17	83	100
F	Cardiac-digestive	19(14)	25	75	100
G	Cardiac	26(18)	25	75	100

Number of analyzed TCC shown in parentheses
A: cardiomegaly with right bundle branch block
F: bradycardia with megacolon
G: bradycardia

DISCUSSION

The immediate aim of the present investigation was to produce TCC from chronic chagasic patients with different symptoms and characterize their surface phenotype. Accordingly, PBMC obtained from these patients followed four cycles of *T. cruzi* trypomastigotes stimulation so that the resulting T cell blasts would be comprised exclusively of antigen-specific cells and hence the TCC resulting from these blasts would be of predefined specificity. The *in vitro* model system we used in this work to generate TCC from chagasic patients has allowed us to observe a correlation between the clinical status of the *T. cruzi* infection and the CD4 or CD8 surface phenotype.

The strain and the stage of *T. cruzi* used in this study were chosen because this strain is highly virulent in the mouse model and the trypomastigote is the extracellular infective stage and hence the most likely to be exposed to the immune response of the host. Furthermore, the trypomastigote stage has been shown to be highly potent in terms of stimulating T cells (Nickell et al. 1987).

This work supports previous reports where the inflammatory heart lesions of six chagasic patients were dominated by CD8 positive lymphocytes (D'Avila Reis et al. 1993) and a more recent study by Tostes et al. (1994) in which CD8+ cells were more numerous than CD4+ lymphocytes in myocardial exsudate of necropsied chagasic patients. Equally, this study becomes more relevant in view of the role of CD4 positive T cells in the pathogenesis of experimental Chagas' disease (Ben Younes-Chennoufi et al. 1988, Russo et al. 1988, Ribeiro dos Santos et al. 1990) and mainly the importance of CD8+ T cells in control and immune protection in *T. cruzi* infection in mice (Tarleton et al. 1992, Nickell et al. 1993, Sun & Tarleton 1993).

These results open the possibility of investigating the mechanisms of the pathology of Chagas' disease by defining a model of study which should help to identify the antigens inducing T-lymphocyte immune responses. Future efforts will be directed towards the identification of the relevant parasite molecules.

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