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BRIEF COMMUNICATION

SIMULTANEOUS IDENTIFICATION OF *Trypanosoma cruzi* SURFACE AND INTERNAL ANTIGENS REACTIVE TO DIFFERENT IMMUNOGLOBULIN CLASSES (RADIO-IMMUNOBLOTTING).

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SUMMARY

A radioactive Western-blotting technique was developed by which the reactivity of Immunoglobulins (Igs) from different classes to both membrane radiolabelled and internal parasite antigens is simultaneously identified. The method includes radioiodination of parasites, polypeptide fractionation by SDS-PAGE, Western-blot transfer and autoradiography of the immunoblots developed with anti-Igs conjugates labelled with enzymes. The analysis is then performed by the comparison of common bands on the autoradiograms and the respective substrate stained nitrocellulose blots. This technique was used to analyse **T. cruzi** trypomastigote surface labelled antigens reactive to IgM, IgA and IgG specific antibodies. A different pattern of reactivity with acute Chagas' disease patients sera was thus obtained.

KEY WORDS: Trypanosoma cruzi; Chagas' disease; Surface antigens; Western-blot; Immunoblotting; IgG classes

INTRODUCTION

Surface antigens of parasites are considered to be important both for induction of host protective immune response and for activation of immunological effector mechanisms which recognize and either destroy parasites (ANDERS et al, 1982).

Of profound importance as well, is the pattern of Ig isotype (SHAKIB & STANWORTH, 1980) elicited in the antibody response, which may even decide between parasite rejection and acceptance (GRZYCH et al, 1984). Actually, in the **S. mansoni** system, rat monoclonal antibo-

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Abbreviations

M.W. — Molecular Weight; Igs — Immunoglobulins, SDS — Sodium Dodecyl Sulfate; TBS- 50 mM Tris — HCl, pH 8,0, 150 mM NaCl; TCT — Tissue Culture Trypomastigotes.

dies of IgG_{2c} subclass which specifically inhibited the protective response mediated by rat monoclonal IgG_{2a} antibodies, both directed to the same surface antigen, have been described. Besides, the existence of blocking antibodies has been characterized through their isotype constitution also in human parasitic infections (HOFSTETTER et al, 1982; KHALIFE et al, 1986).

Regarding these observations, the identification of class (and subclass) antibodies specific to surface antigens of parasites is of interest.

The technique of choice for studying the host immune response directed to plasma membrane antigens is immunoprecipitation of the surface radiolabelled antigens, using a Protein A immunosorbent (cf KESSLER, 1981).

However, when this technique is used to analyze Igs isotypes (or subclasses) which do not bind to Protein A, the corresponding anti-Igs are usually added in a second incubation step, leading either to loss of detection of the reactive antigens or to non-specific reactivity (ANDERS et al, 1982).

Other approaches have been used to analyze immunoreactivity of parasite membrane antigens. For **Trypanosoma cruzi**, SCHECHTER & NOGUEIRA (1988) performed the excision and elution of surface labelled proteins from SDS-gels and used them for dot-blot tests. LANAR & MANNING (1984) compared the molecular weight and the isoelectric point of surface polypeptides from a two dimensional electrophoresis (2D) of radioiodinated parasites with the immunoreactive data obtained with Western-blot of 2D gels of unlabelled parasites incubated with specific antibodies. However since internal and membrane-located antigens can show similar molecular weight (KURTNER et al, 1988) the use of a simpler technique to define the subcellular origin of parasitic antigens is of great interest. Here we describe such a technique, which permitted the simultaneous identification of IgA, IgM and IgG antibodies (from acute disease patient sera) directed to **T. cruzi** surface internal antigens.

MATERIAL AND METHODS

Tissue culture-derived trypomastigotes (tct) of **Trypanosoma cruzi**, Y strain, were obtained as described (ZINGALES et al, 1982) $3x10^8$ parasites were radioiodinated with $Na^{131}I$ by the Iodo-gen method (ZINGALES et al, 1982). After washing, cells were resuspended in 500 μl of electrophoresis sample buffer (LAEMMLI, 1970), containing 1 mM N — $\propto$ tosyl-L-chloromethyl ketone and 1 mM phenylmethylsulfonyl fluoride and heated for 5 min at 100°C. Samples were immediately submitted to electrophoresis (SDS-PAGE) in 7.5% (w/v) polyacrylamide gels (0.75 mm width), according to LAEMMLI (1970). The sample was applied in a single central slot (11.5 cm lenght) flanked by a narrow well (0.7 cm lenght) where M.W. standards (Pharmacia Fine Chem.) were placed. After electrophoresis, the sample was transferred overnight to nitrocellulose membranes (Trans-blot apparatus; 1.2A; 40 V) according to TOWBIN et al (1979). The membrane was cut into strips which were blocked in Blotto buffer (5% non fat dry milk in TBS) (TBS = 50 mM Tris-HCl, pH 8.0; 150 mM NaCl) and incubated overnight with a pool of three human sera from acute phase of Chagas' disease (1:200 dil. in Blotto buffer). After 5 rinses (5 min each) with TBS, the strips were incubated independently for 2 hs with peroxidase labelled anti-human IgA, IgM or IgG sera (heavy chain specific, Cappel, USA) conjugated with peroxidase. The antisera were pre-titered in the same system. After washing with TBS the strips were developed with H_2O_2 30% (5μl/ml) and DAB (3,3' -diaminobenzidine, Sigma) (0.166 mg/ml). The developed nitrocellulose strips were then submitted to autoradiography (Kodak X-OMAT films). The M. W. standards and a strip containing the transferred **Trypanosoma cruzi** polypeptides were stained with Amido-Schwartz (0.1% w/v) and destained in 2% (v/v) acetic acid in ethanol.

Surface antigens recognized by the Igs subclasses were identified placing side by side, on a transilluminator, the peroxidase-developed strip and the correspondent autoradiography.

RESULTS AND DISCUSSION

The presence of anti-**Trypanosoma cruzi** IgA and IgM antibodies has been often observed in chagasic patients by indirect immunofluorescence and Elisa techniques (PRIMAVERA et al, 1988; SÁ FERREIRA et al, 1983). In other parasitic models (**e. g. Schistosoma mansoni, Trichi-**

nella spiralis, Onchocerca gibsoni) it has been described the differential recognition patterns of human and murine immunoglobulin classes to parasite antigens with a possible relevance for protection, pathology and diagnosis (GRZYCH et al, 1984; BUTTERWORTH & HAGAN, 1987; ALMOND & PARKHOUSE, 1986 and CABRERA et al, 1986).

Aiming to identify the surface antigens of trypomastigotes reactive to IgA and IgM antibodies, we tried to use immunoprecipitation techniques via Staph A using a second incubation step with specific anti-Igs. These experiments, however, were unsuccessful mainly because a complete clearance of IgG — specific antibodies could not be obtained (data not shown). It is clear that Western-blot analysis of parasite whole extracts, incubated with total sera and developed with class-specific anti-Igs conjugate, does not lead to an identification of membrane antigens. Therefore, we devised a technique, which combines radioiodination of parasites and Western-blot analysis. The comparison between the antigens developed with enzyme-conjugated specific second antibodies and the autoradiographic pattern leads to the identification of the surface antigens.

Figure 1 exemplifies the results obtained when we analyzed the reactivity of tct antigens to a pool of sera from patients in the acute phase of Chagas' disease. Lanes 1, 2 and 3 correspond, respectively, to the reactivity of IgA, IgM and IgG antibodies visualized with peroxidase-labelled second antibodies. It can be seen that the patterns are different, e.g. bands A, B and C being recognized by the IgA (lane 1) but not IgG antibodies, whereas bands a, b, c and d are highly reactive to IgG (lane 3). The IgM immunoglobulins (lane 2) show a mixed reactivity pattern with bands in common to the IgA and IgG antibodies. Lanes 1', 2' and 3' show the correspondent autoradiograms of the nitrocellulose strips. Comparison of lanes 1 and 1' indicates that most of the anti-**T. cruzi** specific IgA antibodies present in the acute phase of the disease are directed to surface antigens. On the other hand, it can be concluded that the majority of IgG — reacting antigens are internal components (lanes 3, 3'). In the case of IgM antibodies (lanes 2, 2') an intermediary pattern is also obtained.

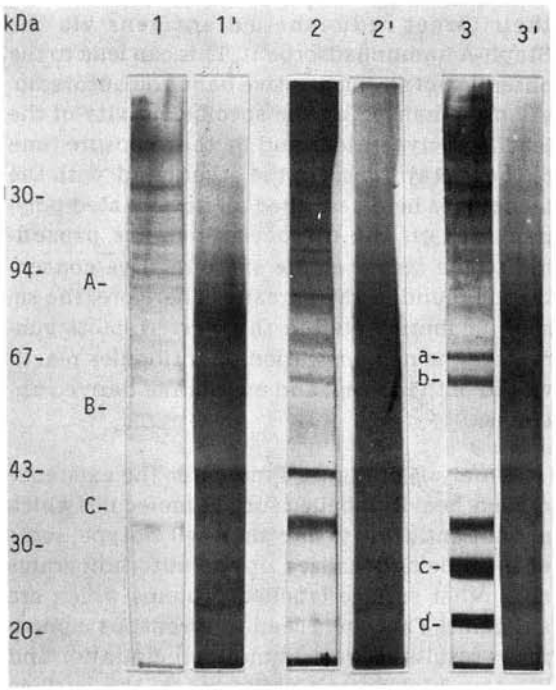

Fig. 1 — "Radio-immunoblotting": Surface radioiodinated tissue culture trypomastigotes from **T. cruzi** were run on SDS-PAGE and tansferred to a nitrocellulose membrane. The strips were incubated with a pool of human sera from the acute phase of Chagas' disease and revealed independently with peroxidase anti-human IgA (lane 1), IgM (lane 2) and IgG (lane 3). Lanes 1', 2' and 3' show the correspondents autoradiograms. Molecular weight markers (kDa) on the left.

The pattern of trypomastigote surface antigens recognized by anti-**T. cruzi** IgG here described, differs from the data of the literature obtained by immunoprecipitation analysis. In this latter case, strong reactivity of high molecular weight polypeptides as well as of antigens of 90 kDa, 80 kDa, 72 kDa and 58 kDa was reported for chronic chagasic or hyperimmune sera (NOGUEIRA et al, 1981; ZINGALES et al, 1982; PIRAS et al, 1983; ANDREWS et al, 1984). The difference observed could in fact be resultant from the different phases of the Chagas' disease from which the sera were obtained (acute versus chronic), opening up new perspectives for the investigation of this disease. On the other hand, the different patterns could arise from characteristics of the employed methodology. In this sense, one should remind that the immunoprecipitation reaction consists of a serum incubation step, where antigen-specific immunoglobulins, even if present in small amount, are concentrated on

their target radiolabelled antigens via the Staph-A immunoadsorbent. This can lead to the obtention of strong positive bands on autoradiograms, enhanced by the specific activity of the labelled polypeptides and by the exposure time to the X-Ray films. On the other hand, with the techniques here described all fractionated polypeptides on the nitrocellulose are presented to the serum at the same relative concentration found in the parasite. Therefore, the serum incubation step in the Western-blots guarantees the demonstration of antibodies reactivity to both internal and membrane-derived antigens.

Analysis of Figure 1 indicates the existence of some heavily labelled surface molecules which are not antigenic to any analyzed isotype, some of them being localized on the autoradiograms near other surface labelled proteins which are antigenic. This could lead to erroneous comparative results between immunoprecipitation and Western-blot data. Furthermore, the surface non-antigenic components of **T. cruzi** here identified, deserve further studies in order to verify their participation in the host immune response in different phases of the Chagas' disease.

In conclusion, the main advantage of this technique is to permit the analysis of both membrane and internal antigens reacting with class-specific antibodies. This information will be useful in the understanding of the humoral immune response to parasitic as well as to other infectious disease.

RESUMO

Identificação simultânea de antígenos internos e de superfície de Trypanosoma cruzi **reativos para diferentes classes de imunoglobulinas (radio-immunoblotting).**

Classes e subclasses de anticorpos apresentam diferentes funções, influenciando a resposta imune humoral de um hospedeiro, frente a um agente infeccioso. Na maioria dos sistemas, o alvo principal é representado pelos antígenos de membrana do parasita. Entretanto, a identificação de antígenos de superfície de parasitas, reativos para classe (e subclasse) de imunoglobulinas que não se ligam a proteína-A implica em imunoprecipitações sucessivas, que levam a perda de antígenos e/ou reações inespecíficas.

Visando esse estudo, foi desenvolvida uma técnica denominada "radio-immunoblotting", através da qual a reatividade de imunoglobulinas de diferentes classes para antígenos de membrana (e/ou internos) foi analisada simultaneamente. O método constitui na marcação prévia da superfície dos parasitas por radioiodação, fracionamento dos polipeptídeos por SDS/PAGE, transferência das frações para nitrocelulose, reação com soros e conjugados anti-Igs — peroxidase e autoradiografia dos mesmos, a análise é feita comparando-se os antígenos comuns evidenciados na autoradiografia e nas tiras de nitrocelulose coradas com o substrato da peroxidase.

Essa técnica foi utilizada para analisar antígenos de superfície de formas tripomastigotas de **T. cruzi** reativas para IgG, IgM e IgA provenientes de soros de pacientes com doença de Chagas na fase aguda. Obtiveram-se distintos padrões de reatividade para as diferentes classes de anticorpos provenientes de um mesmo soro humano.

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