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Trypanosoma cruzi strain-specific monoclonal antibodies: identification of Colombian strain flagellates in the insect vector*

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Abstract

Spleen cells from mice immunized with insect-derived *Trypanosoma cruzi* metacyclic trypomastigotes were used to obtain Colombian strain-specific monoclonal antibodies. At least 4 different strain-specific antigens were recognized by the monoclonal antibodies on epimastigotes or metacyclic trypomastigotes. There was no reactivity with other stages of Colombian strain *T. cruzi*, nor with any stage of 15 other *T. cruzi* strains or isolates, nor with 22 other Trypanosomatidae. One of the monoclonal antibodies was used to identify, by indirect immunofluorescence, Colombian strain flagellates in cryostat sections or glass-slide smears of the insect vector's intestine.

Introduction

Trypanosoma cruzi, the protozoan parasite that causes American trypanosomiasis (Chagas disease) has been shown to be heterogeneous according to several criteria. Thus, isolates or strains with distinct biological behaviour, such as different tissue tropism, infectivity and pathogenicity, have been well characterized. Moreover, different strains can show marked variations in their sensitivities to chemotherapy (ANDRADE, 1979; CANÇADO & BRENER, 1979; ANDRADE *et al.*, 1985), making the identification of the *T. cruzi* strains in a given geographical region or patient of great epidemiological and clinical relevance.

T. cruzi strains can be identified by zymodeme and schizodeme analyses. However, these methods have the disadvantage of having to depend on *in vitro* cultivation of the parasite, which may select clones and thus give rise to parasite populations which do not necessarily correspond to the initial isolates (GONÇALVES *et al.*, 1983; BRUN & JENNI, 1985). In this paper the production of *T. cruzi* Colombian strain-specific monoclonal antibodies (MAbs) and their use in identifying *T. cruzi* flagellates *in situ* in the insect vector (i.e., without requiring previous *in vitro* proliferation of parasites), are described.

Material and Methods

T. cruzi *strains*

The Colombian strain (Federici *et al.*, 1964) of *T. cruzi* was used in the preparation of MAbs. This and 15 other strains (Y, Y2, CL, 12SF, F, W, Dm 28c, WA-250/1, Can III/1, Tulahuén, R, T, 259, Paraiba, and 17 Montalvania) were maintained both in LIT (liver infusion tryptose; Difco Laboratories, Detroit USA) and by serial passage in outbred Swiss mice.

Epimastigotes were obtained from exponential growth phase LIT cultures. Culture-derived metacyclic trypomastigotes (obtained from stationary growth phase, <25% trypomastigotes) were separated from epimastigotes by the procedure of AL-ABBASSY *et al.* (1972). Bloodstream trypomastigotes, from acutely infected immunosuppressed mice (SMITH *et al.*, 1982), were purified to >98% trypomastigotes on a DEAE-cellulose column (SOUZA, 1983). Amastigotes and trypomastigotes were obtained from cultures of LLC-MK2 cells (Rhesus monkey kidney epithelium) infected with bloodstream trypomastigotes (SANTIAGO *et al.*, 1981). Colombian strain epimastigotes and insect-derived metacyclic trypomastigotes were also obtained from *Triatoma infestans* or *Dipetalogaster maximus* faeces, 20 to 30 d after feeding on infected mice, as described elsewhere (LUGO, 1973; ZELEDON *et al.*, 1977). Briefly, the parasites were washed by centrifugation (2500*g*, 15 min, 4°C) with 8mM phosphate buffer, pH 6, containing 190mM NaCl. The final sediment was allowed to stand for 30 min at 37°C, and the supernatant, containing viable parasites, was transferred to other tubes. After an additional wash, the parasites were finally suspended in 0·15M phosphate-buffered saline, pH 7·2 (PBS) for the immunofluorescence tests or stored at −70°C for preparation of antigen extracts. This process yielded a preparation of 98% trypomastigotes.

Other Trypanosomatidae

Epimastigotes of *T. rangeli* and *T. conorhini* were obtained from cultures in NNN medium. Epimastigotes of 2 trypanosome species from *Phyllostomus hastatus* were raised in LIT cultures (BAKER *et al.*, 1978).

Promastigotes of *Leishmania mexicana amazonensis* (MHOM/BR/73/M2269), *L. donovani chagasi* (MCAN/BR/80/CRI), *L. brasiliensis panamensis* (MHOM/PA/71/L594), *L. brasiliensis guyanensis* (MHOM/BR/75/M4147), *L. mexicana aristedesi* (MORY/PA/68/GHLB), *L. mexicana pifanoi* (MHOM/VE/57/LL1), *L. donovani donovani* (MHOM/IN/80/DD8), *L. tropica* (MHOM/SU/74/K27), *L. major* (MHOM/IL/67/JERICHO II), *L. hertigi hertigi* (MCOE/PA/65/C8), and *L. gerbilli* (MCAV/BR/45/L88) were obtained from cultures in a biphasic medium consisting of rabbit blood-agar (BHI; Difco Laboratories, Detroit, Michigan, USA).

Crithidia fasciculata (ATCC 11745), *C. deanei* (ATCC 30255), *C. oncopelti* (ATCC 30264) and *Blastocrithidia culicis* (ATCC 14806) were maintained in LIT cultures.

Parasite extracts

These were prepared by sonication (4 pulses of 18 μm amplitude, 30 sec each) of the parasite (2×10^9/ml of PBS) in an ice bath. The protein concentration was assessed by the

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method of LOWRY *et al.* (1951), using bovine serum albumin as standard.

Preparation of antisera and sensitized spleen cells

2-month-old female BALB/c mice, raised in our own animal facilities, were injected intraperitoneally with an extract of Colombian strain insect-derived metacyclic trypomastigotes (>98%) and epimastigotes (<2%) containing 360 μg of protein emulsified in incomplete Freund's adjuvant (IFA). A further 450 μg in IFA was given 15 d later and again, in saline, 30 d later. 60 d after the last injection, 2 mice were injected intravenously with a soluble extract (150 μg of protein) to provide sensitized spleen cells for hybridization.

Indirect immunofluorescence (IIF)

The general technique was carried out according to CAMARGO (1966). Briefly, culture supernatants containing MAb were incubated with (i) parasites fixed to glass slides by drying PBS suspensions, (ii) live parasites suspended in RPMI-1640 with 5% foetal calf serum, (iii) amastigotes in tissue sections from infected mice, (iv) epimastigotes and trypomastigotes in cryostat sections (3 μm) of infected bug (*Triatoma infestans, Dipetalogaster maximus, Rhodnius prolixus* and *Panstrongylus megistus*) intestine and (v) smears prepared from cut intestine of infected bugs (25, 50 and 80 d after infection).

Rabbit anti-mouse immunoglobulin conjugated to fluorescein (final dilution 1:80) was obtained from Cappel Laboratories (Cochranville, PA, USA). Controls with RPMI-1640 or unrelated IgM MAb instead of the specific MAb were used.

Preparation of monoclonal antibodies

4 d after the final intravenous injection of insect-derived trypomastigote antigens, spleen cells from the immunized mice were fused with SP2/0-Ag14 myeloma cells (SHULMAN *et al.*, 1978) as described by KOHLER & MILSTEIN (1975) and cultured in 24-well Coster plates in a CO_2 incubator. 10 000 BALB/c peritoneal exudate cells per well were used as a

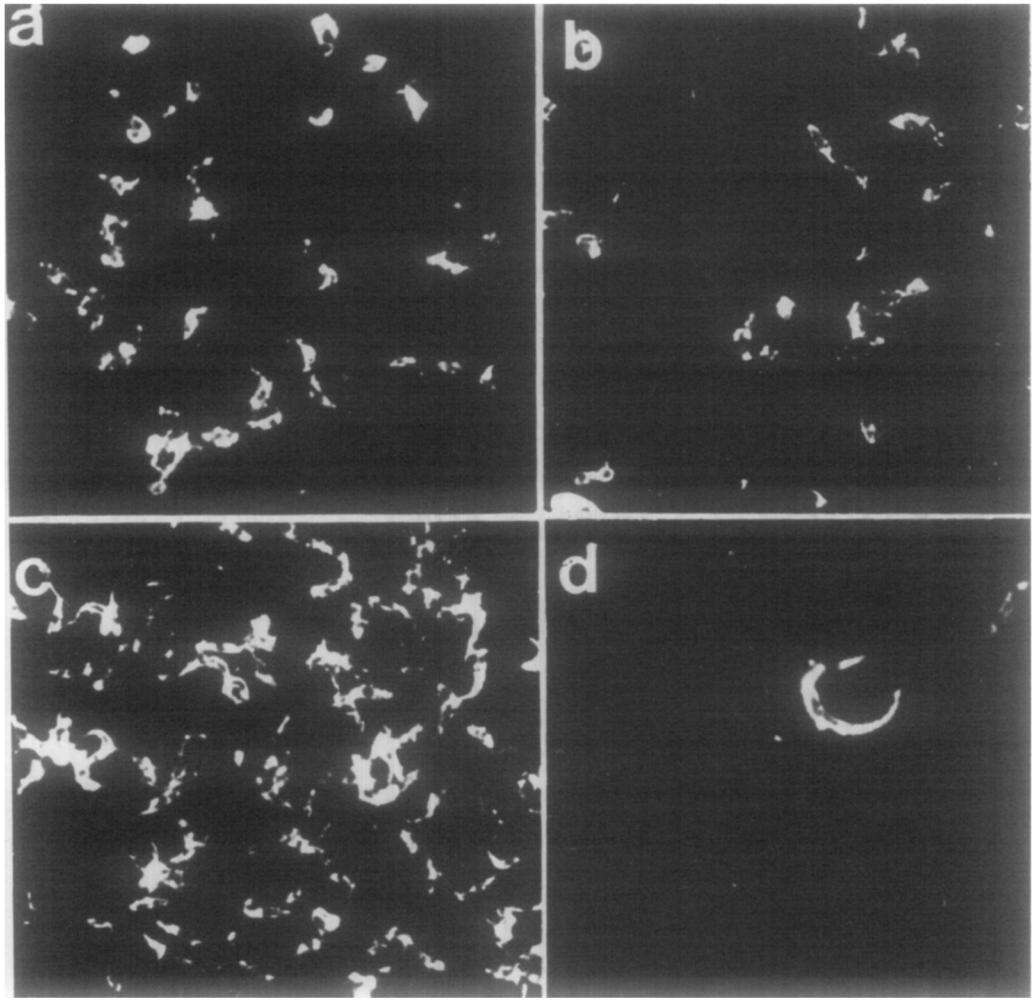

Fig. 1. Indirect immunofluorescence patterns produced by monoclonal antibodies (MAbs) on fixed *T. cruzi* epimastigotes and trypomastigotes. (a) Many individual fluorescent granules in the cytoplasm, × 500 (this MAb did not bind to live parasites). (b) Very bright patches associated with the cell membrane, × 500 (this MAb did not bind to live parasites). (c) Many fluorescent cytoplasmic granules with a sharply stained cell membrane, × 500 (this MAb bound to live parasites). (d) Fluorescent granules exclusively associated with the cell membrane, × 1000 (this MAb bound to live parasites).

feeder layer. Antibody-producing hybrid cells were screened by IIF using insect-derived trypomastigotes adsorbed to glass slides as antigen. Hybrid cells from 4 antibody-positive wells were cloned by limiting dilution, plating 0·5, 1 and 3 cells per well. Limiting diluting conditions were confirmed by Poisson analysis (DE BLAS *et al.*, 1983). Ascitic fluid with 5 to 10 mg of MAb ml^{-1} was obtained from BALB/c mice that had been pre-treated with Pristane (Aldrich Chemical Co., Dorset, UK) and injected intraperitoneally with clones cultured *in vitro*.

Determination of the Ig class of the MAbs

This was done by (i) IIF using IgG and IgM specific anti-immunoglobulin antibodies coupled to fluorescein (Cappel Laboratories), (ii) double immunodiffusion in gel (OUCHTERLONY & NILSON, 1978) with isotype-specific (IgG1, IgG2ab, IgA and IgM) anti-immunoglobulin sera (Cappel Laboratories) and (iii) assessment of the ability to bind to protamine (Sigma Chemical Co., St Louis, USA), an IgM specific reagent (HUDSON & HAY, 1980), coupled to Sepharose-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) by the method of AXEN *et al.* (1967).

Results

Specificity and Ig class of MAbs

10 clones of hybrid cells producing antibodies to *T. cruzi* insect-derived trypomastigotes, detectable by IIF, were isolated. IIF and double immunodiffusion tests performed with immunoglobulin class-specific antisera showed that all the clones produced IgM antibodies. This was further confirmed by the specific binding of the MAbs to protamine-Sepharose.

The MAb specificities were assessed by IIF on a panel of Trypanosomatidae comprising different stages of 15 *T. cruzi* populations isolated from different geographic areas, 6 other *Trypanosoma* species, 12 *Leishmania* species or sub-species, 3 *Crithidia* species and *Blastocrithidia culicis*.

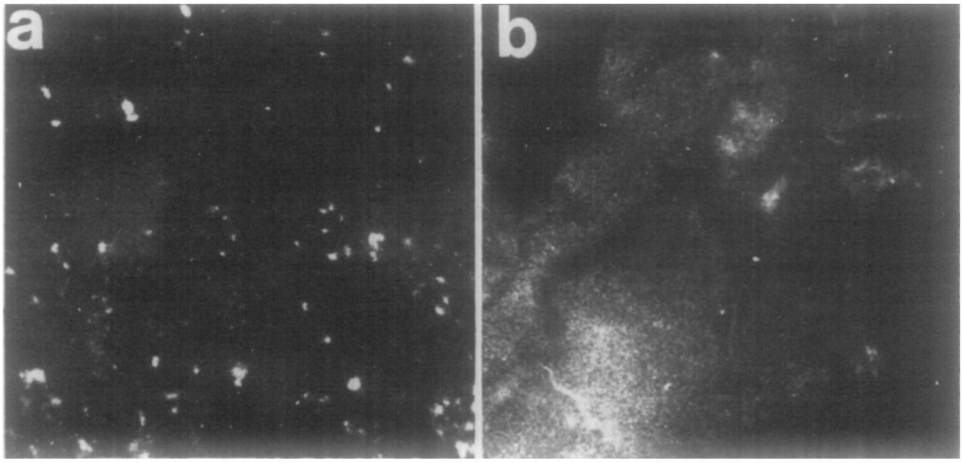

Fig. 2. Identification of the *T. cruzi* strain infecting *Triatoma infestans* by indirect immunofluorescence (IIF) on cryostat sections of the bug intestine. A hybridoma culture supernatant containing MAb D11 was used. The bugs were infected 28 d before the IIF test. (a) Fluorescent flagellates from bug infected with Colombian strain. (b) No fluorescence in bug infected with strain W; × 250.

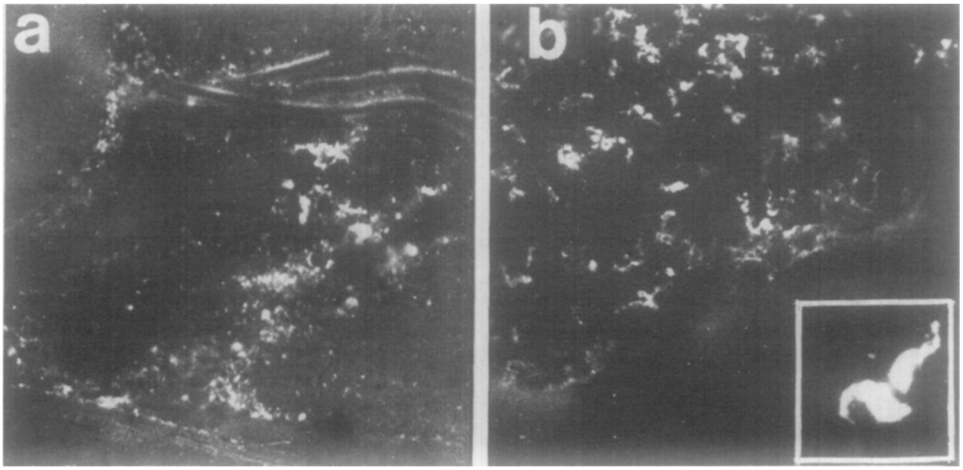

Fig. 3. Indirect immunofluorescence test carried out with undiluted MAb supernatants on glass-slide smears of infected *Dipetalogaster maximus* intestines. The bugs were dissected 25-30 d after feeding on infected mice. No fluorescent staining was observed in control smears, made from uninfected bug intestines (not shown). (a) × 160; (b) × 250; insert, × 1000.

All the MAbs reacted only with Colombian strain *T. cruzi* parasites. Insect vector stage-specificy could also be demonstrated: only epimastigotes or trypomastigotes obtained from the insect vector or from axenic cultures were stained.

At least 4 different IIF patterns on culture-derived parasites were observed. (i) MAbs G6, G12 and H5, which seem to be identical, gave a pattern of many cytoplasmatic granules (Fig. 1a). The IIF on live parasites was negative. (ii) MAbH1 gave rise to very bright patches associated with the cell membrane (Fig. 1b). No fluorescence could be seen on live parasites. These results are compatible with an antigen on the internal side of the cell membrane. (iii) MAbs B10, D11, D12 and G9 stained live parasites and gave a pattern of many fluorescent cytoplasmic granules on fixed parasites with a sharply stained cell membrane (Fig. 1c). (iv) MAbs A5 and B2 also stained live organisms, but there was no intracellular staining on fixed parasites. The terminal portion of the flagellar pocket was brighter than the remainder of the parasite (Fig. 1d). It appeared therefore that at least 4 distinct antigenic specificities were recognized by the 10 MAbs.

Chemical nature of the antigen recognized by the MAbs

In order to investigate whether the MAbs reacted with carbohydrate structures, parasites adsorbed to glass slides were treated with 10mM periodate for 10 min. After 2 washings with PBS, the binding abilities of the MAbs were analysed by IIF. MAbs A5 and B2 stained the treated parasites, whereas the reactivities of MAbs B10, D11, D12, G6, G9, G12, H1 and H5 were abolished (data not shown), indicating that these last MAbs were recognizing carbohydrate determinants.

Identification of parasites in the bug

IIF tests using the surface-reacting MAb D11 permitted the demonstration of Colombian strain *T. cruzi* in intestine sections of 4 different Triatominae species (Fig. 2). No fluorescence was observed in the intestines of uninfected bugs or of bugs infected with another strain of *T. cruzi* (W strain). Furthermore, the same MAb produced sharply defined fluorescence when used in IIF tests on smears of intestines from bugs infected with Colombian strain parasites (Fig. 3) and not on smears of uninfected (control) intestines. Positive IIF results were obtained by this technique in most of the infected bugs up to 80 days after infection (Table).

Table—Frequency of positive IIF results in injected bug intestine smears with the use of Colombian strain-specific monoclonal antibodies

Days after infection	No. bugs with fluorescent parasites /total no. of bugs
25	10/10
50	10/10
80	8/10

Discussion

In this paper we described the preparation of at least 4 different MAbs which were specific for the invertebrate host stages of *T. cruzi* Colombian strain. The specificity of the MAbs was such that they discriminated between two *T. cruzi* populations (Colombian and 17 Montalvania) that have similar biological properties and identical isoenzymic patterns, and have been classified as type III *T. cruzi* strains (ANDRADE *et al.*, 1983; and S. G. Andrade, personal communication. The feasibility of obtaining *T. cruzi* strain-specific MAbs is consistent with the known existence of antigenic differences among *T. cruzi* strains (FLINT *et al.*, 1984). All the MAbs obtained were of the IgM isotype, recognizing carbohydrate antigenic determinants. Whether the use of insect-derived trypomastigotes as immunogens, or some other particularity of the immunization procedure, preferentially induced a carbohydrate-specific IgM response in the immunized mice, should be the subject of future studies.

Marked variation in the sensitivities of *T. cruzi* strains to chemotherapy have been reported (ANDRADE, 1974; CANÇADO & BRENER, 1979; ANDRADE *et al.*, 1985), and different pathological features of Chagas disease have a regional distribution and could be associated with distinct parasite strains (BRENER, 1982). Therefore, the identification of the *T. cruzi* strains in a given geographical region or patient could be of great epidemiological and clinical relevance and the MAbs described in this paper could constitute one of the first reagents in an MAb battery selected for characterizing *T. cruzi* strains in the insect vector. MAbs have been used in immunoradiometric assays to identify the (sub)species of *Leishmania* in epidemiological studies (PRATT *et al.*, 1982). Furthermore, the fact that the present MAbs react with invertebrate host forms of *T. cruzi* makes them of potential use in assays for demonstrating and characterizing Colombian strain *T. cruzi* in insect vectors, as has been described for *Plasmodium* sporozoite-specific MAbs (COLLINS *et al.*, 1984). Indeed, as described above, one of our MAbs was successfully applied to the identification of Colombian strain flagellates in frozen sections and smears of bug intestines by IIF, and the technique could be of use in screening naturally infected bugs. This technique can be particularly suitable for large scale application, and studies are underway to adapt it for use with enzyme-conjugated MAbs in immunoperoxidase microscopy.

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