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EVALUATION OF A MONOCLONAL ANTIBODY AFFINITY PURIFIED ANTIGEN FOR ZYMODEME SPECIFIC SEROLOGICAL DIAGNOSIS OF *TRYPANOSOMA CRUZI* INFECTION

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Theoretically, serological assays with affinity purified marker antigens can allow strain-specific diagnosis even when parasites cannot be retrieved from an infected host. A Trypanosoma cruzi *antigen was purified by affinity chromatography using a zymodeme (Z) 2 specific monoclonal antibody (2E2C11). An indirect enzyme-linked immunosorbent assay (ELISA) based on the purified antigen could discriminate between sera from rabbits immunized with* T. cruzi *zymodeme clones but could not discriminate between sera from mice infected with different zymodemes.*

Key words: *Trypanosoma cruzi* – zymodemes – diagnosis – monoclonal antibodies

Trypanosoma cruzi, the aetiological agent of Chagas' disease, infects over 20 million people in South and Central America and up to 30% of these may develop heart defects and/or megaviscera. Geographical variations in the prevalence and severity of the clinical forms of the advanced stages of Chagas' disease have been reported by various authors (Koberle, 1974; Coura, 1976; Prata, 1976).

It has been suggested that differences in the genetic make-up of human populations and/or *T. cruzi* strain variations may account for these regional discrepancies (Rezende, 1976), but no association has been reported between clinical form and HLA or isozyme typing of patients (Ferreira et al., 1979; Conti & Kreiger, 1984). Alternatively, clinical manifestations of *T. cruzi* infection may be dependent on the infecting strain of parasite, which can be identified by genotypic (e.g. schizodeme) or phenotypic (zymodeme) characteristics (Gibson & Miles, 1985). Zymodeme 2 (Z2) infections have been tenuously linked with the occurrence of chagasic mega-syndromes (Luquetti et al., 1986). In this paper we investigate whether an antigen affinity purified with a monoclonal antibody (2E2C11), which reacts predominantly with Z2 (Flint et al., 1984), provides the basis for a Z-specific serological assay.

MATERIAL AND METHODS

Parasites – Stocks used in this study were Silvio X10 clone 9 (Z1), Esmeraldo clone 3 (Z2), CAN III clone 1 (Z3), M6241 (Z3) and VIN B5 (Z2). Epimastigotes were grown in a medium containing RPMI 1640, HEPES (2.38g/500ml), Trypticase (1.45g/500ml), haemin (10mg in 0.04% NaOH/500ml) and heat inactivated foetal calf serun (FCS) (50ml). Metacyclic trypomastigotes were obtained by growing parasites in modified insect tissue culture medium (Miles, personal communication). Epimastigotes were harvested by centrifugation (500G, 10 min) and washed (x3) in PBS. Antigens were prepared by freeze-thawing (x3) and sonication (30 sec, 12μ, on ice) or the pellet was re-suspended at a concentration of 10^{-9} organisms/ml in 2% (v/v) Renex 30 (Honeywell Atlas Ltd), 1mM phenyl methylsulfonyl fluoride, 0.1mM N-α-p-Tosyl-L-Lysine chloromethyl ketone (Sigma), 5mM iodoacetamide, 5mM ethylene diaminetetra acetic acid, 5mM Ethyleneglycol-bis-β-aminoethyl Ether) N, N, N', N' – Tetraacetic acid (Sigma), then centrifuged at 10,000 G for 30 min and the supernatant used for enzyme linked immunosorbent assays (ELISA) or affinity chromatography.

Antibodies – Monoclonal antibody 2E2C11 was generated utilizing spleen cells from a mouse immunized with Esmeraldo clone 3 antigen: the antibody had previously been shown to be of IgG1 subclass, to react to the flagellum of epimastigotes and not to bind to *Leishmania* spp., *Trypanosoma rangeli* or *Trypanosoma brucei* in indirect immunofluorescence tests (IFAT) and ELISA (Flint et al.,1984). New Zealand white rabbits were immunized by inoculating 0.5mg of sonicated antigens (X10/9, Esmeraldo clone 3 or CAN III clone 1) emulsified in complete Freund's adjuvant into footpads and intramuscularly. Two further booster doses were similarly administered at two week intervals. Mouse sera were obtained from BALB/c mice infected subcutaneously with 250 metacyclic trypomas-

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tigotes of zymodemes 1, 3 and 2 (X10/9 M6 241 and VINB5, respectively).

ELISA – The indirect ELISA test was performed as described by Voller et al. (1980). Plates were coated overnight at 4°C with antigen diluted in carbonate buffer pH 9.6 and washed (x3) between the coating, blocking, first antibody and conjugate stages, using PBS containing 0.05% Tween (PSB-T). Plates were blocked with 1% BSA in PBS-T for 1 hr at 37°C. First antibody (ascites, supernatant or sera) was diluted in PBS-T containing 1% BSA and left on plates for 2 hrs at room temperature. Conjugate (goat anti-mouse Ig, Dynatech or Goat anti-rabbit Ig, Miles) diluted in PBS-T containing 1% BSA was applied for 2 hrs at room temperature. Substrate (ortho-phenylenediamine, Sigma) (Voller et al., 1980) was added for 30 min, at room temperature, in the dark, the reaction stopped with 2.5N H_2SO_4 and plates read in a Multiscan ELISA reader (Flow Laboratories).

Affinity chromatography – The IgG fraction from ascitic fluid was precipitated by adding saturated ammonium sulphate to a final concentration of 45%, re-suspended in PBS and extensively dialysed against PBS. It was then linked to CNBr-activated Sepharose 4B at a concentration of 20-30 mg of protein/ml of settled gel. The detergent solubilized (Renex 30) antigen was loaded to the column, which was extensively washed with PBS. Bound antigen was eluted utilizing 0.02M glycine/HCl, pH 2.2 and eluted fractions were immediately equilibrated with 1M Tris/HC1, pH 7.2. An aliquot of each eluted fraction was tested in indirect ELISAs and fractions found to be reactive to the monoclonal antibody were pooled, dialysed against PBS and finally concentrated in a vacuum dialysis apparatus. SDS-PAGE was performed as described (Laemmli, 1970) and gels were stained by the silver staining method (Biorad).

RESULTS

In the indirect ELISA, monoclonal antibody 2E2C11 (culture supernatant) bound only to Z2 when titrated against sonicated antigens derived from clones representative of the known principal *T. cruzi* zymodemes (Fig. 1). Similarly, 2E2C11 did not react to Z1 derived soluble antigens irrespective of whether antigens (Fig. 2) or antibody were titrated (data not shown). After passing detergent solubilised antigens through the 2E2C11 affinity column, indirect ELISAs demonstrated that most of the antigens recognised by the monoclonal antibody had been removed (data not shown). The specific antigen was then eluted and monitored in each of the fifty fractions, also by the indirect ELISA. Fractions 16-28 were pooled, dialysed and concentrated. SDS-PAGE demonstrated that the Mr of the purified antigen was approximately 70,000 (not shown).

Rabbit antisera raised against clones representative of Z1 and Z2 were tested for their reactivity, in the indirect ELISA, to Z1, Z2 and Z3 sonicated antigens. Titration curves obtained demonstrated that the antisera tested were equally reactive to all three antigens, although titres were marginally higher against homologous antigens. The same antisera were tested against affinity purified antigen. At high concentrations the anti-Z1 antiserum bound to the purified antigen but overall reactivity was much diminished when compared to the anti-Z2 antiserum. When higher dilutions were considered (e.g. 1/1600) optical densities with anti-Z1 antiserum were 3 times that of the negative control, whereas optical densities with the anti-Z2 antiserum were 14 times that of the negative control (Fig. 3).

Pooled sera from groups of 5 mice that had been infected with metacyclic trypomastigotes representative of zymodemes 1, 3 and 2 were tested against the affinity purified antigen. As the various sera had different anti-*T. cruzi* antibody titres, results at each dilution were ex-

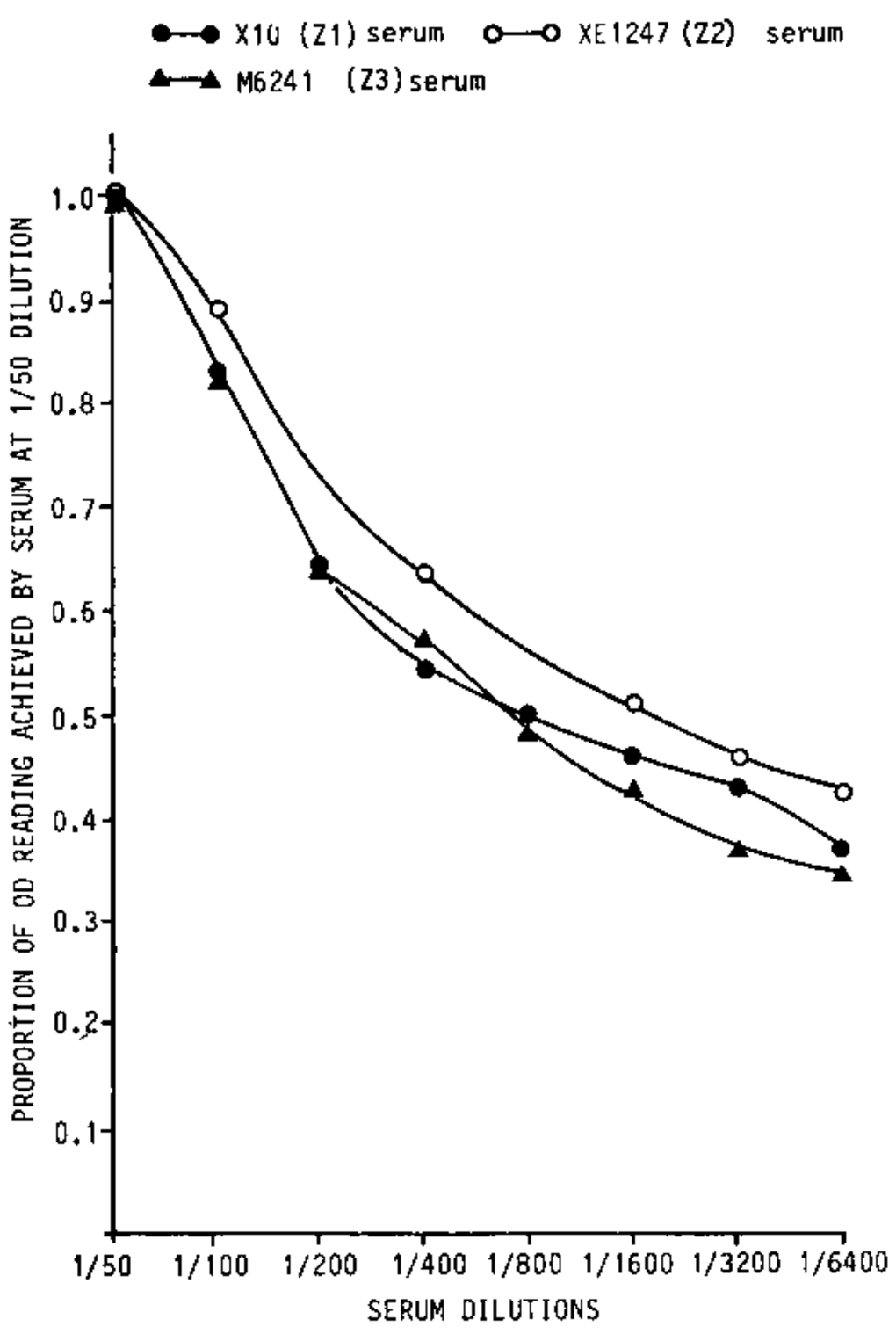

Fig. 1 – Titration of monoclonal antibody 2E2C11 (culture supernatant) to *T. cruzi* sonicated antigens in indirect ELISA test.

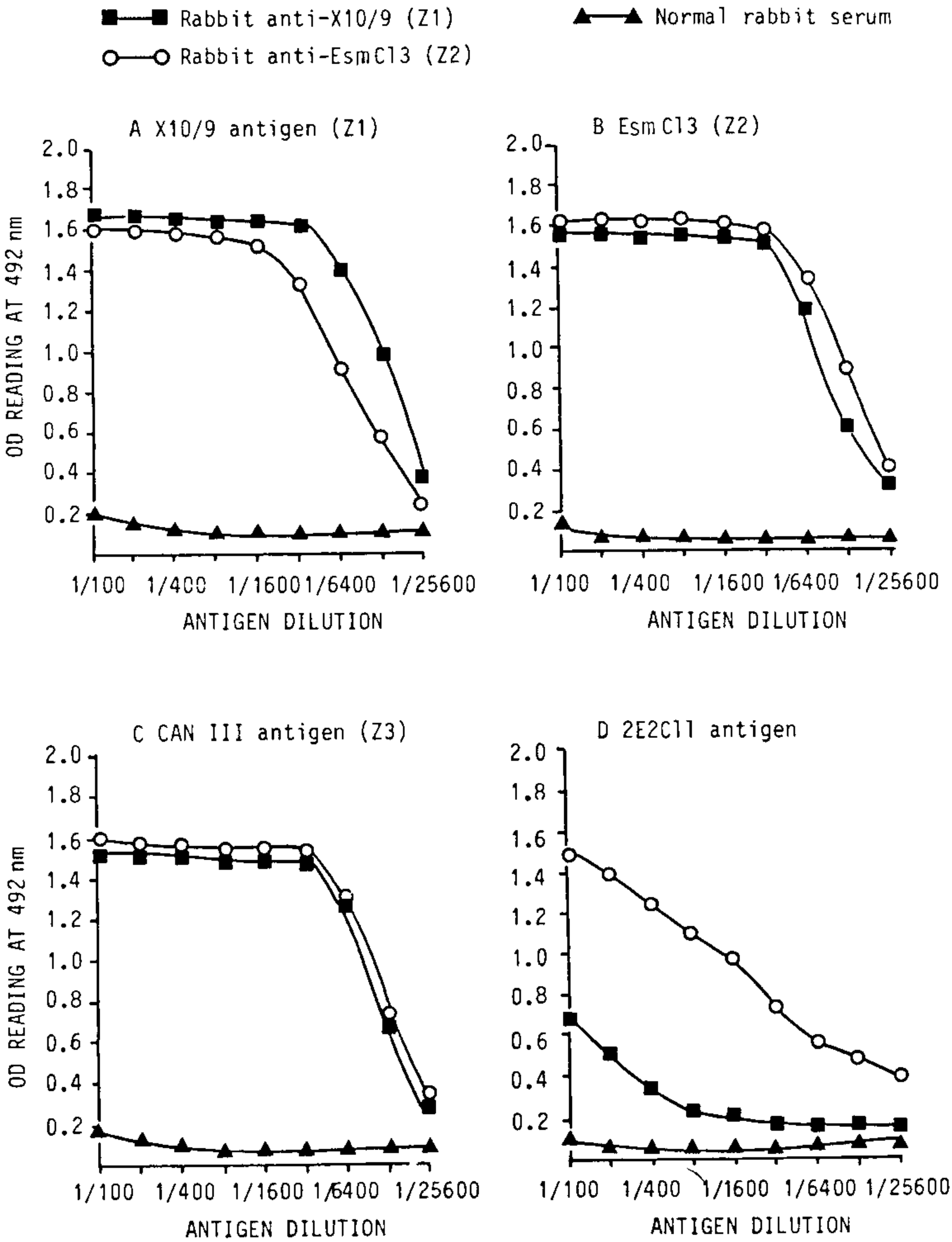

Fig. 3 – Titration of rabbit antisera to various antigens in indirect ELISA tests.

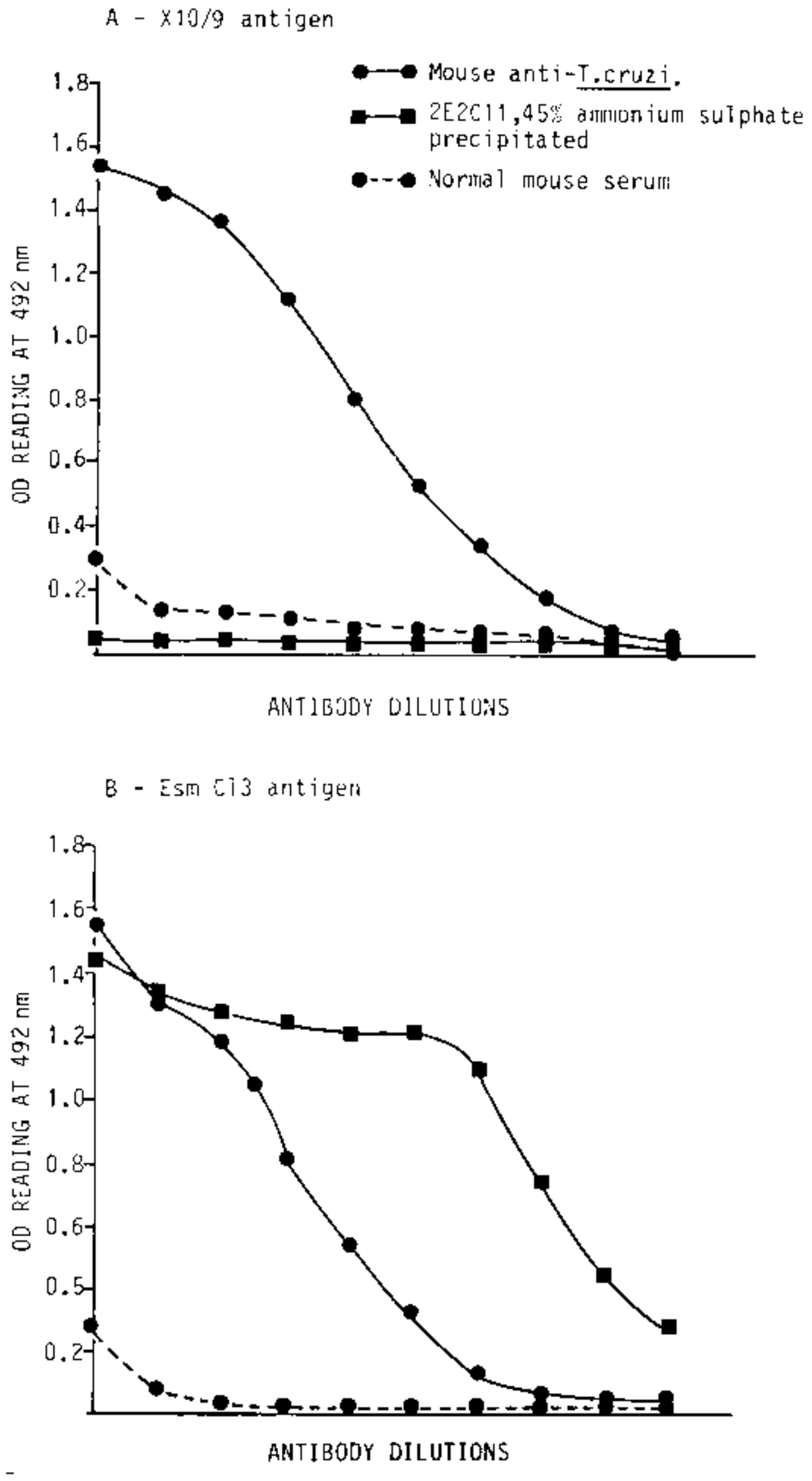

Fig. 2 – **Titration of mouse antisera and monoclonal antibody 2E2C11 to solubilised antigens. Mouse anti-*T. cruzi* and normal mouse serum start at 1/100, whereas 2E2C11 start at 1/2000.**

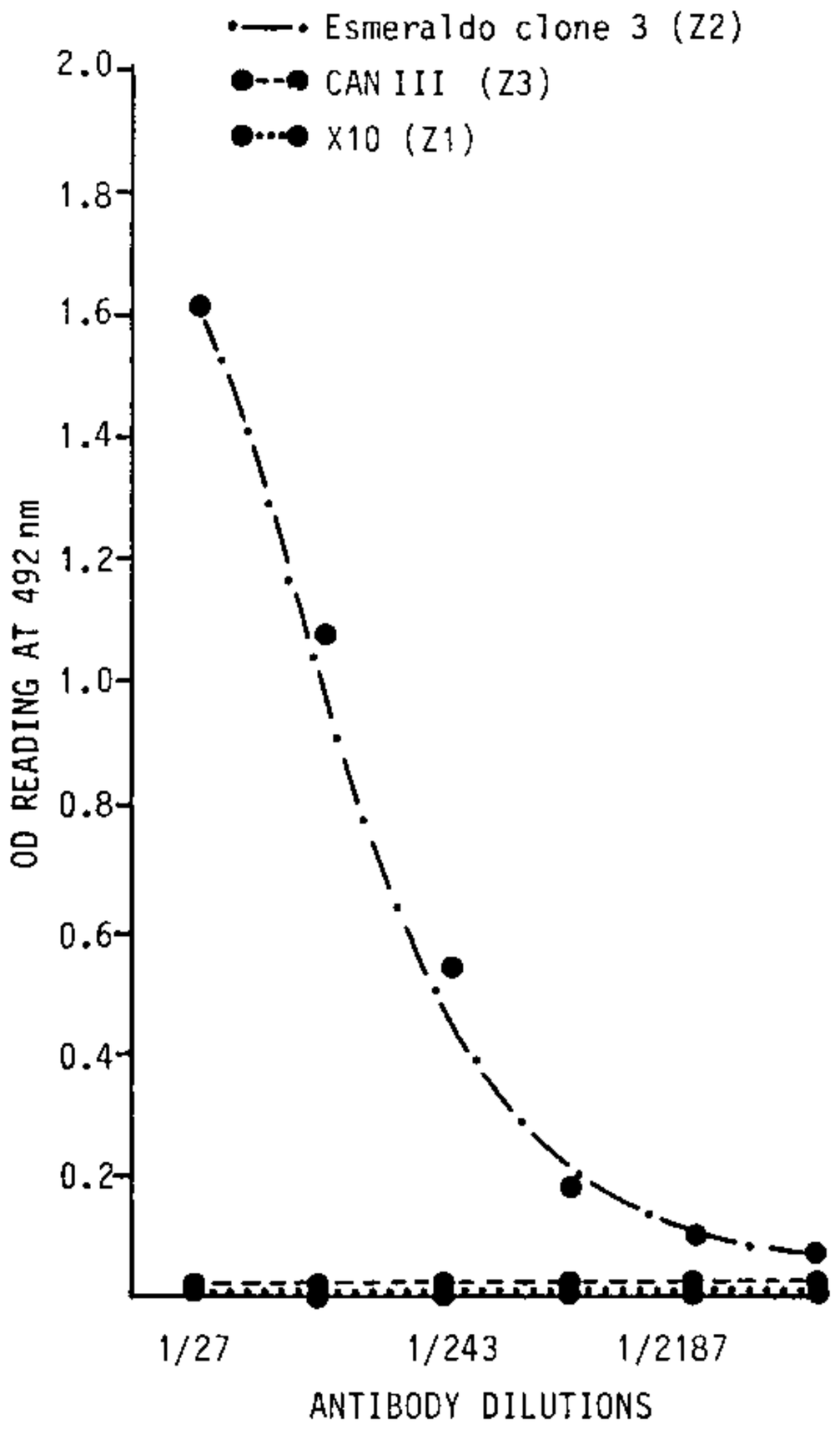

Fig. 4 – Titration of mouse sera to 2E2C11 purified antigen in an indirect ELISA test.

pressed as a proportion of the optical density reading at the highest concentration (1/50) of each serum. The titration curve for the sera from Z2 infected mice was only marginally elevated over the Z1 and Z3 curves (Fig. 4).

DISCUSSION

Differences in the prevalence of megasyndromes (Rezende, 1976; Mota et al., 1984) and chagasic cardiopathy (Coura, 1976) have been observed between countries in the Americas and within Brazil. Different *T. cruzi* strains may, inpart, be responsible for the different clinical manifestations of *T. cruzi* infection (Rezende, 1976; Miles et al., 1981; Brener, 1982; Mota et al., 1984). Both schizodeme and zymodeme analysis provide a means of *T. cruzi* strain identification and *T. cruzi* Z2 has been circumstantially linked to the occurrence of megasyndromes (Miles et al., 1981; Luquetti et al., 1986). Nevertheless, both schizodeme and zymodeme characterization require amplification of *T. cruzi* populations from original hosts and consequent selection of subpopulations (Deane et al., 1984). In view of the need for amplification the full extent of schizodeme and zymodeme heterogeneity in individual host infections is not clear. Theoretically serological assays with purified marker antigens should enable infecting strains to be identified even when parasites cannot be retrieved.

The production of Z-specific monoclonal antibodies has been described previously (Flint et al., 1984) and 2E2C11 was selected as Z2-specific on the basis of IFAT and ELISA with sonicated antigens. Here it was found that the specificity of the monoclonal antibody was not altered by the use of detergent solubilized antigens. The corresponding antigen was purified to see if it would provide a basis for the serological detection of exposure to Z2-specific antigens in patients.

As sera were not available from known

chronic Z1 cases (Luquetti et al., 1986), the discriminative potential of the indirect ELISA with affinity purified antigen was assessed using animal sera.

When antisera from immunized rabbits were analysed, the assay proved to be capable of differentiating between anti-Z1 and anti-Z2 antisera. However, with sera from infected mice antibodies present in animals infected with non-Z2 strains bound to the purified antigen preparation. Although the titration curve for Z2 infected mice was marginally higher than the curve obtained for non-Z2 infected mice, the difference was not of sufficient magnitude as to allow discrimination between the infections.

There are at least three possible explanations for the failure of this assay to discriminate between zymodeme infections in mice: firstly, the antigen may be present in all *T. cruzi* strains but may have enhanced immunogenicity in Z2, under the conditions used for rabbit immunization, and be better able in Z2 to survive sonication, detergent extraction or acetone fixation. Secondly the Z2 defined epitope may be restricted to Z2 but the carrier molecule may not, a situation similar to that known for the *T. cruzi* 72,000 Mr surface glycoprotein (Chapman et al., 1984; Schechter et al., in preparation). Thirdly, it is conceivable that the purification was incomplete and that non-zymodeme specific contaminants were present at undetectable levels.

The results with sera from immunized rabbits indicate that strain-specific diagnostic assays may be an attainable goal. The validity of this approach depends on further understanding of the phenomenon of the heterogeneity of *T. cruzi* and the availability of appropriate purified strain-specific antigens.

RESUMO

Teoricamente, testes sorológicos que utilizem antígenos purificados por cromatografia de afinidade podem permitir diagnóstico cepa-específico mesmo quando parasitas não são isoláveis de hospedeiro infectado. Um antígeno de *Trypanosoma cruzi* foi purificado por cromatografia de afinidade utilizando anticorpo monoclonal (2E2C11) zimodema (Z) específico. Um teste ELISA baseado em tal antígeno mostrou ser capaz de discriminar soros de coelhos imunizados com clones representativos de diferentes zimodemas. No entanto, quando soros de camundongos infectados foram analisados, tal teste mostrou-se incapaz de discriminação.

Palavras-chave: *Trypanosoma cruzi* - zimodemas - diagnóstico - anticorpos monoclonais.

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