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The Vaccine Potential of Cell Surface Glycoproteins From Trypanosoma cruzi [and Discussion]

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The vaccine potential of cell surface glycoproteins from *Trypanosoma cruzi*

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An experimental *Trypanosoma cruzi* 90 kDa cell surface glycoprotein (GP90) vaccine, previously shown to be protective in mice is similarly effective in marmosets (*Callithrix jacchus jacchus*). Protection in the mouse is completely dependent on the adjuvant saponin and immunological studies confirm that GP90 is intrisically poorly immunogenic. Both specific antibody and cell mediated immunity are potentiated strongly by saponin and the resulting protective immunity is long lasting (six months). It is effective against the naturally infective, insect-metacyclic, form and a range of heterologous *T. cruzi* strains including a low mouse passage human isolate. Sterile immunity (that is, complete elimination of parasites) was not, however, achieved. Evidence is presented that the levels of tissue damage associated with acute infection, as measured by production of auto anti-tissue immunoglobulins, are significantly reduced in GP90-immunized mice. These and other results are discussed in terms of the desired characteristics for vaccine use of *T. cruzi* antigens.

Introduction

The parasitic, protozoan flagellate *Trypanosoma cruzi* is the cause of American trypanosomiasis (Chagas' disease). Some 35 million people live in endemic areas and 10–12 million are estimated to be infected (World Health Organization 1960). The pattern of infection in humans is an early acute phase with high blood parasitaemias subsiding into a chronic phase with parasites persisting at sub-patent levels throughout life. A similar pattern of infection occurs when experimental animals are infected with human forms of *T. cruzi* and, with such models, immunization against *T. cruzi* has readily been achieved using various avirulent, attenuated, or killed whole organism preparations. However, it is unlikely that any whole organism-based vaccine will ever be used in man since *T. cruzi* contains antigens that cross-react with mammalian tissues and these are implicated in the autoimmune pathogenesis of Chagas' disease (reviewed Scott & Snary 1982).

Cell surface antigens are likely to be important components of sub-unit vaccines since they are the first to be encountered by the host's immune system. The experimental results presented here describe recent immunological studies designed to define further the vaccine potential of a 90 kDa cell surface glycoprotein (GP90) isolated from *T. cruzi* (Snary & Hudson 1979) which has already been shown to be protective in mice and apparently lack identity with antigens known to cross-react with mammalian tissue (Scott & Snary 1979; Scott *et al.* 1982). The present findings, with other results, form the basis of a general discussion of the desired characteristics for vaccine use of *T. cruzi* antigens.

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Materials and methods

Animals

CBA mice and marmosets (*Calithrix jacchus jacchus*) from our own breeding colonies were used.

T. cruzi strains

The Y strain of *T. cruzi*, originally isolated from a human patient in 1950 in Brazil (Andrade 1974), was used to prepare glycoptoetin and for blood trypomastigote challenge in most experiments. Epimastigote forms were cultured at 25 °C in Boné & Parents (1963) medium containing 5% rabbit serum, penicillin (200 units per cubic centimetre) and streptomycin (100 units per cubic centimetre). Blood trypomastigotes were obtained from mice infected by serial transfer of infected heparinized blood. Metacyclic trypomastigotes were obtained by loosely homogenizing in phosphate buffered saline the abdomens of *Rhodnius prolixus* fed one month previously on infected mice.

Other strains of *T. cruzi* were maintained in various forms in our laboratories and adapted to blood passage in mice for the current experiments. By titration the challenge dose of blood trypomastigotes for each strain which approximated in lethality to that the Y strain was determined. Strains Peru and Colombiana were isolated from patients in Peru and Columbia in 1963 and 1964 respectively (Andrade 1974). Strain BG was isolated in 1926 in Brazil (Goodwin *et al.* 1950) and M1 from a patient in Argentina in 1962. Unlike the above strains a more recent clinical isolate, LUMP 754, has not been extensively passaged in mice. It was isolated from a patient in Brazil in 1972 by feeding *T. infestans*. Metacyclics were inoculated into an immunosuppressed mouse and the blood stored frozen until 1982. Before use in the present experiments the blood was passaged once in lethally irradiated CBA mice.

Parasite counting

Parasites in infected mouse tail blood samples were counted microscopically using ammonium chloride to lyse red cells as described (Scott 1981*a*). Whole blood samples (5 µl) from infected marmosets were counted.

Xenodiagnosis

Third or fourth instars of *Triatoma infestans* were fed on mice as described (Scott 1981*a*).

Glycoprotein (GP90)

This fraction was prepared from Y strain epimastigotes by lectin affinity chromatography as described by Snary & Hudson (1979). Dilutions for injection were in saline.

Saponin

Quillaia saponin was from Food Industries Ltd, Wirral, Merseyside, U.K.

Radioimmune assays for antibody

Flexible PVC plates were coated with either *T. cruzi* antigens; freeze-dried Y strain epimastigotes extracted in 0.05 M bicarbonate buffer pH 9.6 (100 µg ml^{-1}), or mouse tissue antigens; hearts (four per 10 ml). brains (two per 10 ml), liver (two per 10 ml), spleen (four

per 10 ml) homogenized in the same buffer and filtered to remove debris. Samples of 50 µl were added to each well and the plates incubated for 90 min at room temperature and then washed by immersion in 0.2 % BSA, 0.05 % Tween 40 for 90 min. Then 50 µl test antisera were added to each well and the plates incubated for 30 min and then washed up 10 min by immersion in buffer; 50 µl of an ^{125}I-labelled F(ab′)$_2$ of an immune purified rabbit antimouse Ig antibody were then added to each well (2×10^5 counts per minute per well). After a final wash plates were drained and bound radioactivity counted. All titrations were performed in duplicate and, where given, titres represent the highest dilution of test serum with counts per minute more than twice those of the corresponding dilution of normal serum.

Delayed hypersensitivity

Appropriately sensitized mice were injected in the footpad with *T. cruzi* antigen and 3 and 24 h footpad swelling measured as described (Scott 1981 *b*).

Results

Optimal conditions for protective immunization against homologous challenge

Table 1 summarizes the results of experiments in mice to optimize doses of GP90 and saponin, and frequency of injection: 100 µg GP90 mixed with 50 µg saponin afforded the best protection and two injections were better than one. Although for economy of space only peak parasitaemia data are shown, the mice were monitored throughout infection and profiles similar to those shown in figure 1 obtained.

Duration of immunity in mice

Full protection following two injections of GP90 and saponin was afforded up to six months after challenge. Figure 1 also shows the failure of either GP90 or saponin alone to protect.

Table 1. Effect on protective immunication in mice of varying the dose of either glycoprotein (GP90) or saponin, and frequency of injection

immunization†	parasitaemia 16 days after infection 10^5 (parasites) cm^{-3}	proportion of mice surviving
100 µg GP90 + 50 µg saponin	3.4 ± 1.4	5 of 5
100 µg GP90 + 10 µg saponin	42.8 ± 13.8	3 of 5
100 µg GP90 + 2 µg saponin	244 ± 24.3	0 of 5
no treatment	118 ± 8.7	0 of 5
50 µg saponin + 100 µg GP90	3.4 ± 1.8	5 of 5
50 µg saponin + 30 µg GP90	32.4 ± 8.9	2 to 5
50 µg saponin + 10 µg GP90	37.8 ± 20.1	2 of 5
no treatment	178.0 ± 28.9	0 of 5
100 µg GP90 + 50 µg saponin	0.8 ± 0.3	5 of 5
100 µg GP90 + 50 µg saponin (× 1)	6.6 ± 1.9	3 of 5
no treatment	58.8 ± 23.3	0 of 5

† Mice received two s.c. injections of the stated dose except where only one dose is shown. Challenge with 10^6 Y strain blood trypomastigotes subcutaneously was 14 days later.

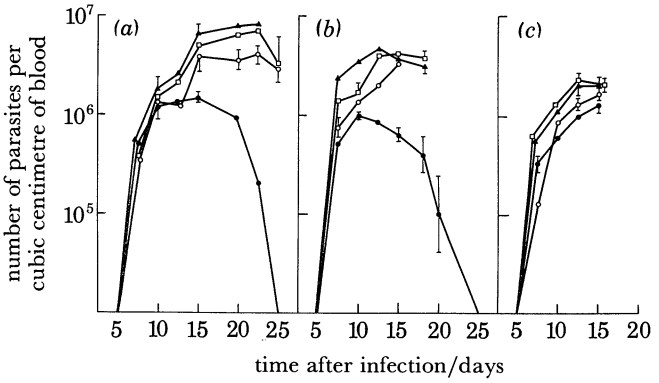

FIGURE 1. Groups of five mice were immunized subcutaneously at days 0 and 14 with 100 μg GP90 plus 50 μg saponin (●–●), GP90 alone (○–○), saponin alone (▲–▲), or were untreated (□–□). Three months (*a*), six months (*b*) or 12 months (*c*) after the second immunization they were challenged subcutaneously with 10^6 live Y strain blood trypomastigotes and parasitemias monitored.

Adjuvant effect of saponin

The control of blood parasitaemia in *T. cruzi* infection was known to depend upon the development of specific immunity, thus the complete failure of GP90 without saponin to protect indicated the former to be a very poor immunogen and the latter a potent immune adjuvant. This was demonstrated in studies of three immune parameters; there was no detectable primary immune response to an injection of 100 μg GP90 as measured by increase in draining lymph node mass, production of specific antibody detected by sensitive radioimmuneassay, or development of cell mediated immunity (delayed hypersensitivity). Saponin strongly potentiated all these parameters (figure 2).

Protection against metacyclic forms and other T. cruzi strains

In the above experiments the Y strain-derived GP90 has been protecting mice against homologous blood form trypomastigote challenge. Figure 3*a* shows that it is equally effective against Y strain insect-derived metacyclic trypomastigotes, that is, the naturally infecting form.

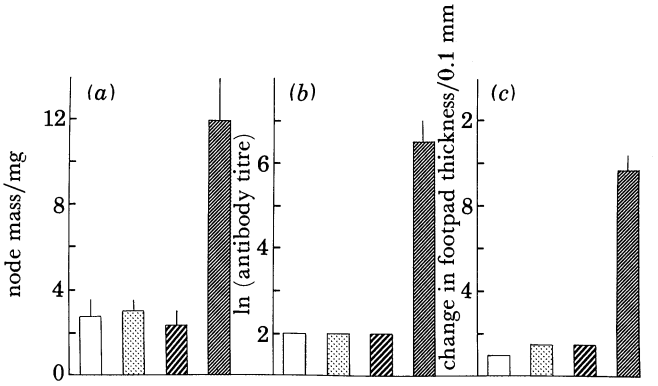

FIGURE 2. Groups of 15 mice were immunized subcutaneously in the right flank with 100 μg GP90 plus 50 μg saponin (thin diagonal), GP90 alone (thick diagonal), saponin alone (dots) or were untreated (blank). Five mice from each group were assayed as follows: (*a*) six days after immunization ipsilateral inguinal lymph nodes were removed and weighed; (*b*) two weeks after immunization serum antibodies against *T. cruzi* were assayed; and (*c*) ten days after immunization mice were challenged with *T. cruzi* antigen in the footpad and delayed hypersensitivity (24 h footpad reaction) measured. Responses at 3 h were insignificant.

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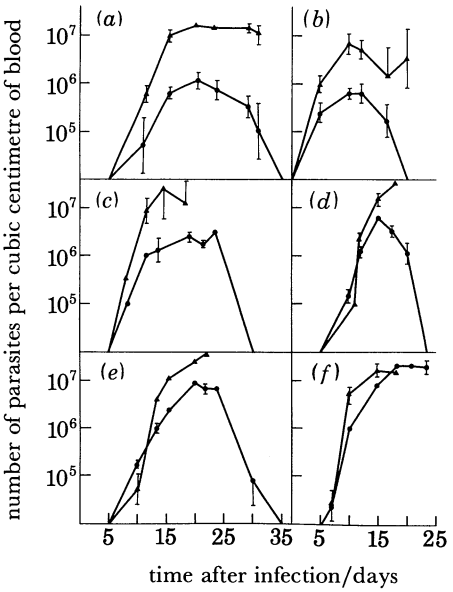

FIGURE 3. Groups of five mice were immunized subcutaneously at days 0 and 14 with 100 μg GP90 plus 50 μg saponin (●–●) or were untreated (▲–▲). At day 28 they were infected with equivalent lethal doses of various live blood trypomastigotes as follows: (*a*) metacyclic Y strain; (*b*) strain LUMP 754; (*c*) strain M1; (*d*) strain BG; (*e*) strain Colombiana; and (*f*) strain Peru. Blood parasitaemias were monitored.

Also shown in figure 3 are the protective effects of Y strain GP90 immunization against several heterologous *T. cruzi* strains. Many of these represent well established laboratory strains that have undergone numerous culture and mouse passages; however, the strain LUMP 754 is a relatively recent human isolate that has undergone minimal manipulation (see Materials and methods) and full protection was observed (figure 3*b*). Of the other strains (figure 3*c–f*) only strain Peru eventually killed immunized mice but a significant increase in mean survival time was observed; 23.0 ± 1.4 and 18.4 ± 1.3 days for immunized and non-immunized mice respectively.

Protection against low dose challenges: failure to achieve sterile immunity

Immunized mice protected against lethal strain blood trypomastigote challenge have all proved xenodiagnosis-positive (parasites detected by feeding the insect vector on them) when tested up to year later. It was considered that this failure to achieve sterile immunity may be due to the unnaturally large challenge doses used. Figure 4 shows the effect of GP90 immunization against a low dose (10^2), non-lethal Y strain challenge; the acute blood phase parasitaemia was significantly reduced by immunization but all mice were xenodiagnosis-positive six months later. In a further experiment using a nominal challenge of 1 trypomastigote per mouse, three of five immunized and three of five non-immunized mice were xenodiagnosis-positive 100 days after infection.

Immunization of marmosets

To obtain primate data a protection study in marmosets were carried out using an immunization regimen based on the obtained mouse data. Immunized animals received two subcutaneous injections of 1 mg GP90 mixed with 0.5 mg saponin four weeks apart and were challenged subcutaneously with a non-lethal dose (10^6) of Y strain blood trypomastigotes.

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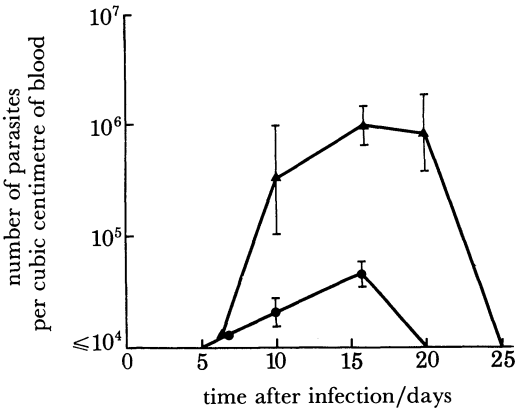

FIGURE 4. Groups of five mice were immunized subcutaneously with 100 μg GP90 plus 50 μg saponin at days 0 and 14 (●–●) or were untreated (▲–▲). At day 28 they were challenged subcutaneously with a non-lethal dose (10^2) live Y strain blood trypomastigotes and blood parasitaemias monitored.

Control animals were unimmunized. There was a marked reduction in acute phase blood parasitaemia (figure 5); however, all marmosets which are being maintained for chronic pathology studies, are xenodiagnosis-positive 60 weeks after infection.

Effects of immunization on T. cruzi-induced anti-tissue reactivity in mice

Normal mice recovered from a low-dose non-lethal Y strain *T. cruzi* infection showed high levels of immunoglobulins against mouse heart tissue preparations. If, however, the mice had been immunized before infection then the subsequent level of anti-heart immunoglobulins was markedly reduced (figure 6). No anti-tissue reactivity resulted from immunization alone. It is apparent from absorption studies (table 2) that the phenomenon is a general autoimmune anti-tissue response in that the heart antigens are represented on a range of tissues. They are also not cross-reactive with *T. cruzi* antigens.

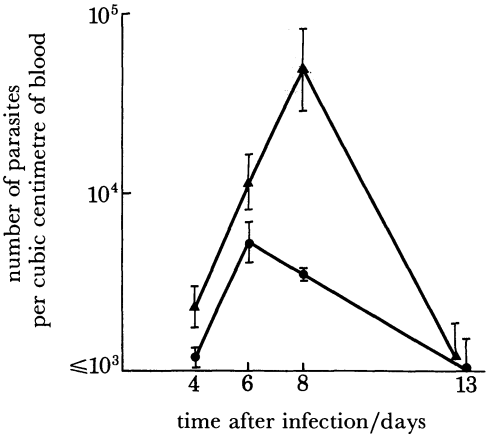

FIGURE 5. Groups of three male marmosets were immunized subcutaneously at days 0 and 28 with 1 mg GP90 and 0.5 mg saponin (●–●) or were untreated (▲–▲). At day 56 they were challenged subcutaneously with 10^6 live Y strain blood trypomastigotes and blood parasitaemias monitored.

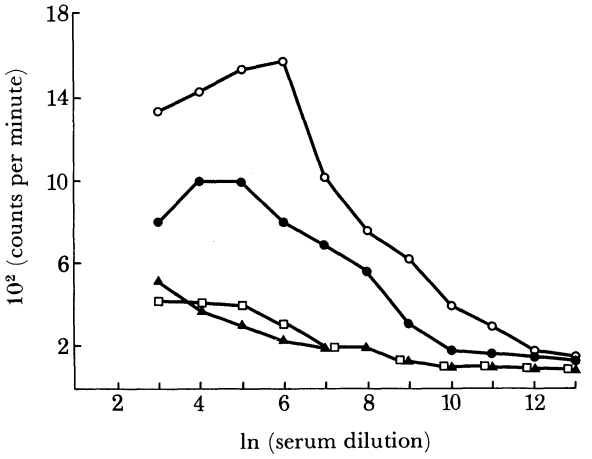

FIGURE 6. Radioimmunoassay of anti-heart antibodies in mice immunized (• •), or unimmunized (○–○) and infected as in figure 4. Control mice were immunized and not infected (□–□) or untreated (▲–▲). Serum samples were taken 12 weeks after infection.

TABLE 2. LACK OF BOTH TISSUE SPECIFICITY AND CROSS-REACTIVITY WITH *T. CRUZI* OF ANTI-HEART ANTIBODIES IN MICE RECOVERED FROM ACUTE INFECTION

anti-heart serum absorbed with...	ln titre heart	brain	liver	against *T. cruzi*
—	8	6	8	10
heart	< 2	< 2	< 2	10
T. cruzi	8	n.d.	n.d.	3

n.d., Not done.

DISCUSSION

Parasite cell surface antigens are logical candidates for subunit vaccines because it is they that first encounter the host's immune system. Recent developments in fractionation procedures, particularly hybridoma technology, have given impetus to studies characterizing *T. cruzi* cell surface antigens, but there are few data so far concerning the evaluation of such antigens for vaccine potential. This had been the theme of our own studies with the *T. cruzi* GP90 antigen and this discussion will take the form of using our GP90 results to exemplify the desired characteristics of *T. cruzi* antigens for vaccine use, relating where possible to other reported cell surface glycoproteins.

GP90, isolated by lectin affinity chromatography from lysed, Y strain, culture epimastigotes, has been shown earlier to be a major cell glycoprotein present throughout the life cycle of the parasite (Snary & Hudson 1979). It does not undergo antigenic variation and is present on geographically distinct strains of *T. cruzi* (Snary 1980). Mice vaccinated with GP90 mixed with either saponin, or Freund's complete adjuvant, have been protected against subsequent lethal *T. cruzi* infection and further aspects of such protective immunity have been reported here. Protection conferred by GP90 is completely adjuvant-dependent, and the poor intrinsic immunogenicity of GP90 is confirmed by immunological studies; no primary response to GP90 alone was detected by either lymph node, antibody or cell mediated immune assay. Saponin strongly promoted all these responses. Given the frequent non-specific lymphoproliferative

activity of adjuvants, it was interesting that saponin did not itself induce increased lymph node mass, this response resulting entirely from its interaction with GP90. The adjuvant requirements of GP90 have been studied and are discussed elsewhere (Scott *et al.* 1984) but they affirm that the choice of a suitable adjuvant may be critical to the successful development of parasite subunit vaccines for human use. The fact that the adjuvant requirements of subunit vaccines seem to be more demanding than their intact counterparts is probably due to their lacking the intrinsic adjuvanticity conferred by associated molecules.

The reported presence of GP90 on different forms and strains of *T. cruzi* has here been translated into the *in vivo* demonstration of effective immunization against several different *T. cruzi* strains including the naturally infective, insect metacyclic form. Cross-strain protection is an important characteristic for a vaccine to have wide applicability, and to find this in a single component vaccine is encouraging. Cross-stage protection is similarly important; for example, another epimastigote-derived *T. cruzi* cell surface glycoprotein (GP72) is present on metacyclic but not blood trypomastigotes and protects only against metacyclic challenge (Snary 1983). Such a vaccine would not be effective against the significant numbers of patients infected with blood trypomastigotes through transfusion of contaminated blood, and it would need to be 100% effective against infecting metacyclics since any escaping to the blood form would be uncontrolled. A cell surface GP25 isolated recently by Scharfstein *et al.* (1983) is similar to GP90 in being present on all parasite forms and different strains. Antibodies against GP25 have protected human muscle cells from *T. cruzi* infection *in vitro*, which suggests that it too may prove to be protective *in vivo*.

GP25, GP72 and GP90 are all recognized strongly by antibodies in sera from Chagas' patients (Scharfstein *et al.* 1983; Snary 1983), indeed such antibodies were used to isolate GP25. However, such a characteristic need not be a prerequisite for a successful vaccine antigen or antigens since the immune response in natural infection can be considered inadequate in allowing the chronic persistence of low levels of parasites. Presentation of a minor or novel antigen may prove effective in this respect.

The accepted trend in parasite vaccine research is towards the production of vaccines comprising only relevant protective antigen or antigens, but in the case of *T. cruzi*, there is a particularly compelling reason for the avoidance of whole organism-based vaccines. This is the existence of parasite antigens that cross-react with mammalian tissue and have been implicated in the pathogenesis of chronic Chagas' disease. Although the extent to which such antigens are so involved remains to be determined, it is important than candidate vaccines do not contain them. The original demonstration of cross-reacting antigens concerned auto antibodies with e.v.i. (endocardium, vascular, interstitial) reactivity in Chagas' patients which could be absorbed with *T. cruzi*. Absorbtion studies have shown that GP90 lacks identity with those responsible for e.v.i. activity (Scott & Snary 1979). More recently, two different *T. cruzi*-tissue cross-reacting monoclonal antibodies CE5 (Wood *et al.* 1982) and 5H7 (Snary *et al.* 1983) have been raised. GP90 is not recognized by CE5 (Scott *et al.* 1982) and the discrepancy in molecular masses between GP90 and the antigens recognized by 5H7 (35 and 58 kDa) suggest these too lack identity, although this needs to be demonstrated formally. An antigenic determinant of *T. cruzi* that cross-reacts with muscle sarcoplasmic reticulum is apparently located in the interior of the parasite (Sadigursky *et al.* 1982).

That immunization with GP90 has failed to produce sterile immunity is disappointing. A vaccine capable of reducing acute infection but not completely eliminating all parasites will

only be acceptable if it reduces the incidence or severity of chronic Chagas' disease. The tacit assumption is that the low level, persisting parasitaemia must contribute to the development of chronic pathology. However, there is evidence that the late manifestations of chronic pathology arise from tissue damage inflicted during the acute phase of infection (Koberle 1974; Machado *et al.* 1979). Although autoimmune pathology is clearly implicated, to what extent such sensitization arises from cross-reacting antigens or from autosensitization following acute-phase tissue damage has not been resolved. A reduction in the level of acute infection may therefore modify the development of chronic pathology. We have not detected characteristic Chagas' pathology in our infected CBA mice but have been able to assess acute-phase tissue damage as evidenced by production of auto anti-tissue antobodies. These were significantly reduced if GP90 immunization preceded infection. GP90 immunization alone did not elicit anti-tissue antibodies supporting the data that it lacks cross-reacting determinants.

These findings are encouraging and accord with a recent report of a significant reduction in the development of chronic histopathological lesions in mice following immunization with an attenuated whole organism vaccine (Basombrio & Besuschio 1982). They cannot, however, substitute for a definitive demonstration of reduced pathology in an appropriate animal model. In our hands chronically infected small laboratory animals have so far neither readily nor consistently shown characteristic chronic pathology but results in mice and rabbits have been reported (Laguens *et al.* 1981; Teixeria *et al.* 1983). Infected primates do seem to develop chronic Chagas' disease (Miles *et al.* 1979; Milei *et al.* 1983) and we await the outcome of our own ongoing pathology studies with the immunized marmosets.

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Discussion

W. E. Ormerod (*London School of Hygiene and Tropical Medicine, London*). I must express doubt about the efficacy of a weak antigen as a vaccination agent for Chagas' disease. Although *Trypanosoma cruzi* infects man in much of South and Central America, Chagas' disease only occurs to any significant extent in a relatively few areas, mainly in Brazil, Argentina and Peru; elsewhere it is sporadic (provided the distinction between infection and disease is made). The essential environmental difference between areas where overt disease occurs and those where infection with minimal disability is seen lies in the former having long-term agricultural degradation with inappropriate techniques, poor soil, erosion and leaching of minerals, where a relatively high rural population is malnourished and carries a heavy load of other parasites and infectious diseases. In such areas the response to 'artificial' vaccination is unlikely to result in increased immunity any more than it is able to provide resistance to the original disease. I have discussed the distribution and pathogenicity of *T. cruzi* in more detail in a review article (Ormerod 1979).

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M. J. Turner (*MRC Biochemical Parasitology Unit, Molteno Institute, Cambridge*). On this question of adjuvants, Tony Allison and his colleagues at Syntex have developed a new adjuvant, claimed to be suitable for use in humans but with many of the desirable properties of Freund's Complete. I understand that it is based on muramyl dipeptide, but the key seems to be in the use of synthetic detergents as emulsifers that form single-phase solutions at room temperature but phase-separate at 37 °C to form very effective oil-in-water emulsions. Have you considered trying this new generation of adjuvants?

M. T. Scott. We have not tried the adjuvant you describe and it would be interesting to do so. However, we have tried, without success, muramyl dipeptide and several of its analogues, both with and without oil, using Tween 80 as emulsifier (Scott *et al.* 1984).

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