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DEVELOPMENT OF CELL MEDIATED IMMUNITY TO FLAGELLAR ANTIGENS AND ACQUIRED RESISTANCE TO INFECTION BY *TRYPANOSOMA CRUZI* IN MICE

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Modulation by BCG and/or cyclophosphamide of sensitization of mice with flagellar fraction (a tubulin-enriched fraction) prevented death of mice challenged with T. cruzi *CL strain trypomastigotes recovered from Vero cells. A methodology was developed to assay specific antigens and to determine optimal doses for sensitization and elicitation of DTH in mice. CL strain is predominantly myotropic strain which does not produce important parasitism of mononuclear phagocyte cells; these cells appear to control infection when activated* in vivo. *Maximum protection was seen in this study when BCG and cyclophosphamide were associated, but protection was observed also when cyclophosphamide, that prevents supressor T cells, was applied 2 days before flagellar fraction sensitization in normal mice. These experiments suggested that the macrophage may have an important role in the early phases of infection particularly when nonspecific stimulation is associated with specific sensitization. A correlation between delayed hypersensitivity to parasite antigens and protection was observed.*

Man and animals infected with *T. cruzi* have signs of cell-mediated immunity (CMI). The involvement of hypersensitivity in chagasic cardiopathy was early pointed out by Magarinos Torres (1929) and Chagas (1934) and was characterized by lymphocytic infiltrates sometimes not correlated to the presence of parasites. The importance of mononuclear phagocytic system in the resistance against *T. cruzi* was demonstrated by Dias (1934), Taliaferro & Pizzi (1954), Kierszenbaum et al (1974) and others. Taliaferro & Pizzi (1954) demonstrated that macrophages have an important role in résistance against *T. cruzi* infection since they observed *in vivo* that macrophages from immune mice could lyse phagocytized trypanosomes whereas those from normal animals were unable to destroy the parasite.

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In *in vitro* experiments, specifically and nonspecifically activated macrophages can control *T. cruzi* multiplication as was demonstrated by Hoff (1975) who used BCG and *Listeria monocytogenes*, but fail to protect *in vivo*. In contrast normal macrophages serve as host cells for *T. cruzi* proliferation.

Positive skin tests elicited by *T. cruzi* antigens in patients with Chagas' disease are very difficult to observe: Muniz & Penna Azevedo (1947) and Pellegrino (1946) found negative response but positive results were reported by Mayer & Pifano (1941); Pessoa & Cardoso (1942); Mazza et al (1943) and Zeledon & Ponce (1974) employing different antigens.

Cell mediated immunity, demonstrated by *in vitro* correlated tests for delayed type hypersensitivity, was shown to be important in acute experimental Chagas' disease, and it seems that there is a relationship between detectable CMI and the degree of tissue invasion by *T. cruzi*. Recently Patruco et al (1978) observed that flagellar antigens give the best response in chronic patients when the leucocyte migration-inhibition test (LIF) was used. Thus evidence exists that CMI plays a fundamental role in control of *T. cruzi* infection, while other studies indicate that CMI mechanisms may participate in tissue damage.

In the present paper we adopted a system to analyse the capacity of *T. cruzi* antigens to elicit T-cell mediated immunological responses in experimental models. In animals artificially immunized with specific antigens we have investigated the relationship between DTH and resistance using flagellar antigens under the influence of immunoregulatory agents. Further experiments are being carried on with other antigenic fractions of *T. cruzi.*

MATERIAL AND METHODS

ANIMALS – Specific-pathogen-free outbred female OF_1 mice were purchased from IFFA-CREDO (Domaine des Oncins, St. Germain Sur L'Arbresle, France). They were 4-6 weeks old. For adoptive sensitization BALB/C mice 3-4 weeks old were used.

PARASITE – They Y and CL strains of *T. cruzi*, maintained at the "Departament de Parasitologie Experimentale – Institut Pasteur" by serial passage in Vero cell culture, were used to infect mice. Trypomastigotes were harvested from infected (5-6 days) culture flasks and purified by centrifugation using 1.000x g for 15 min at 4° C and incubated at 37° C for 15 min. Parasites were washed twice with Hank's balanced salt solution (HBSS), counted and resuspended in PBS. These forms were used for challenge in all experiments. Epimastigotes of Y strain were cultivated in a complex liquid medium for preparation of the flagellar fraction.

HARVESTING OF ORGANISMS – Epimastigotes were grown in 250 ml flasks cointaining a complex liquid medium (LIBIT) which composition is a solution of 68 mM NaC1; 5 mM KC1; 56mM Na_2HPO_4; and a dry mixed composed of glucose 0,4%; tryptose 0,2% and brain heart infusion 0,2%. The basic medium was completed with 50 ml of liver infusion and 50 ml of fetal calf serum per liter and the pH ajusted to 7.4 Hemoglobin (5%) from sheep red blood cell lysate was added before filtration in a Millipore system.

Organisms from 4 day cultures were collected by centrifugation at 4° C 7500x g for 10 min and washed twice under the same conditions with a buffered solution pH 7.0 containing 100 mM NaCl, 20 mM K_2 HPO_4 and 0,5 mM $MgCl_2$. The final pellet was used immediately for cell fractionation.

ISOLATION AND PURIFICATION OS FLAGELLAR FRACTION – Flagellar fraction (FF) from *T. cruzi* epimastigotes was prepared as previously described (Pereira et

al, 1978) and was used here with a slight modification. The washed cell were resuspended in a hypotonic medium containing 50 mM sucrose in 10 mM Tris HCl pH 7.5 (10 ml/g of cells) and Phenyl Methyl Sulfonyl Fluoride Enzyme Inactivator (PMSF) to a final concentration of 0,1 mM. After this the procedure was followed without modifications. Purification of FF was done as described elsewhere (Pereira et al, 1977).

BCG – The Pasteur strain of *Mycobacterium bovis* (BCG) was obtained from Mrs. M. Georghiu, BCG Production Unit, Institut Pasteur. The organisms were grown in dispersed culture as described in previous report (Lagrange, Hutrel & Ravisse, 1978). Dosages were based on viable counts performed by plating into Middlebrook's 7H10 medium (Difco Laboratories, Detroit).

CYCLOPHOSPHAMIDE (Cy) – Cy was kindly supplied by Lucien Laboratories (Colombes, France). The drug was dissolved in sterile phosphate-buffered saline (PBS) membrane filtered (Millipore 0,22 μ) and was injected intravenously as a single dose of 200 mg/kg as was described early (Lagrange et al, 1975).

IMMUNIZATION AND CHALLENGE – Normal mice were immunized with a single subcutaneous (SC) injection of FF. Some groups of mice were pretreated with a single SC footpad injection of 10^6 BCG organisms three weeks prior to the sensitizing dose of FF in the same footpad (left hind footpad-LHF). Some groups of mice of the above schedules were treated with Cy two days before the sensitizing dose of FF. Challenge dose of 5×10^4 trypomastigotes from Vero culture cell was injected six days after sensitizing dose. Purified trypomastigotes were resuspended in 0,04 ml of 0.1 M PBS pH 7.2 and injected in the RHFP. Protection was evaluated by parasitemia and mortality rate. Parasitemia was determined according to the method of Pizzi (1957).

DELAYED-TYPE HYPERSENSITIVITY (DTH) – DTH reaction was measured as described elsewhere (Lagrange et al, 1974). Briefly, variations of the footpad thickness were evaluated 4, 24, 48 and 78 h after an injection of 0,04 ml of saline containing the eliciting FF antigen or the challenge dose of virulent trypomastigotes into the RHFP. Reactions were expressed as the difference in thickness between feet that received injection of eliciting dose and the thickness before injections. Means of 5 or 10 animals were made and standard error of the mean presented.

MEASUREMENT OF CELLULAR RESPONSES IN POPLITEAL LYMPH NODE – The extent of cell proliferation in response to injection of FF antigens into one footpad was measured by ^{125}IUdR incorporation into DNA of cells in the draining popliteal lymph node. At intervals after immunization, five mice from each group received 0,5 ml of 10^{-3} M 5-fluorodeoxyuridine intraperitoneally and thirty minutes later an intravenous pulse of 0,5 ml containing 1 uCi of ^{125}I deoxyuridine (UdR) was given in the tail vein. Two hours later the popliteal nodes were excised, placed in individual plastic tubes and counted individually in a gamma spectometer for 10 min with uptake expressed as counts per minute. Mean background value of 5 tubes was subtracted from each individual count. Results are expressed as the ratio of radioactivity between left and right lymph nodes.

ADOPTIVE IMMUNIZATION – Dissociated lymph node cells from all groups of inbred mice were prepared as described elsewhere (Mackaness, 1969). Two groups of recipient mice were prepared for each of these 4 donor groups: BCG-FF treated, only FF or BCG and saline treated mice. Donor FF-treated mice received optimal sensitization dose and DTH was measured after injection of the FF eliciting dose in part of the group to see the level of DTH in this strain of mice. One recipient group received cells and the other serum. A total of 8×10^7 lymph node cells from immune donors were mixed with FF (10 μg) in 0.04 ml and injected into the LHFP of normal recipient mice. Footpad swelling was measured thereafter.

SERUM TRANSFER – Serum samples obtained from BCG-treated mice or BCG-treated and FF-antigen immunized or FF-antigen immunized as well as saline control mice were membrane filtered (Millipore, 0,22 μ membrane) and injected intravenously in recipient groups of mice in a volume of 0,5 ml. Two hours later these animals received the best eliciting dose of FF antigens in the LHFP and swelling was recorded 4 and 24 h later.

ANALYTICAL PROCEDURES – Proteins were determined by the method of Lowry et al (1951). Precipitation of the trypanosomal proteins with 5% (v/v) trichloroacetic acid (TCA) and resuspension of the pellet in a 0,4 N NaOH was carried out prior to treatment with the Lowry reagent. Bovine serum albumin (Fraction V, Sigma) was used as a protein standard.

RESULTS

BEST DOSES OF SENSITIZATION – As a preliminary, the lymphoproliferative response to FF antigens was investigated, by varying the dose of antigen, since it has been demonstrated that the induction and the magnitude of the cellular response to an antigens is related to its immunogenicity (Kruger & Gershon, 1972). Groups of 15 normal mice were inoculated in the LHFP with varying dilutions of FF-antigens from 1 μg to 100 μg/mouse and one group was inoculated with diluent only (PBS pH 7.2); cell proliferation was measured on days 3, 5 and 7. Fig. 1 shows the relative rate of ^{125}IUdR incorporation into DNA by popliteal draining lymph node cells on day (–3) when doses of 1,10 and 100 μg were applied to different groups of mice.

Since all the doses employed were able to stimulate lymph node cell proliferation, groups of 5 BCG-pretreated mice received varying doses of FF from 1 μg to 100 μg of flagellar protein as sensitizing dose. An eliciting dose was fixed at 10 μg and 6 days after sensitization was applied SC to the immune mice in the nonprimed RHFP; DTH was measured by footpad swelling at 4, 24, 48 and 72 h. The best dose for sensitization as measured at 24 hours was 10 μg; and footpad swelling persisted at 48 hours with this dose (Fig. 2). In preliminary experiments immunization with FF only gave a weak response. Since BCG pretreatment resulted in intensified responses to FF, BCG pretreated mice were used to evaluate dose dependence of DTH to FF.

ELICITING DOSE RESPONSE IN IMMUNE MICE – Several groups of mice under the modulating effect of BCG and immunized with the best dose of sensitization were tested with varying doses of FF-protein content. Doses of 0,1; 1; 5 and 10 μg of FF protein had given increasing amplification of footpad swelling in 24 hours (Fig. 3).

MODULATORY EFFECT OF CY – Groups of BCG-pretreated, and normal mice were treated on day –2 with Cy, injected intravenously. On day zero, all groups received the optimal eliciting dose in the LHFP and footpad swelling was measured 4, 24, 48 and 72 hours later. In these groups we can observe at 24 hours an important potentiating effect of Cy as compared with DTH of sensitized groups not treated (Fig. 4).

TIME COURSE OF DTH REACTION WITH OPTIMAL DOSES – Kinetics of DTH reaction to FF in different immunization schedules using normal, BCG-pretreated and BCG-Cy treated mice were observed with optimal doses of immunization and elicitation. DTH was measured every 2 days in the first week and at 12, 24 and 4 days after sensitization.

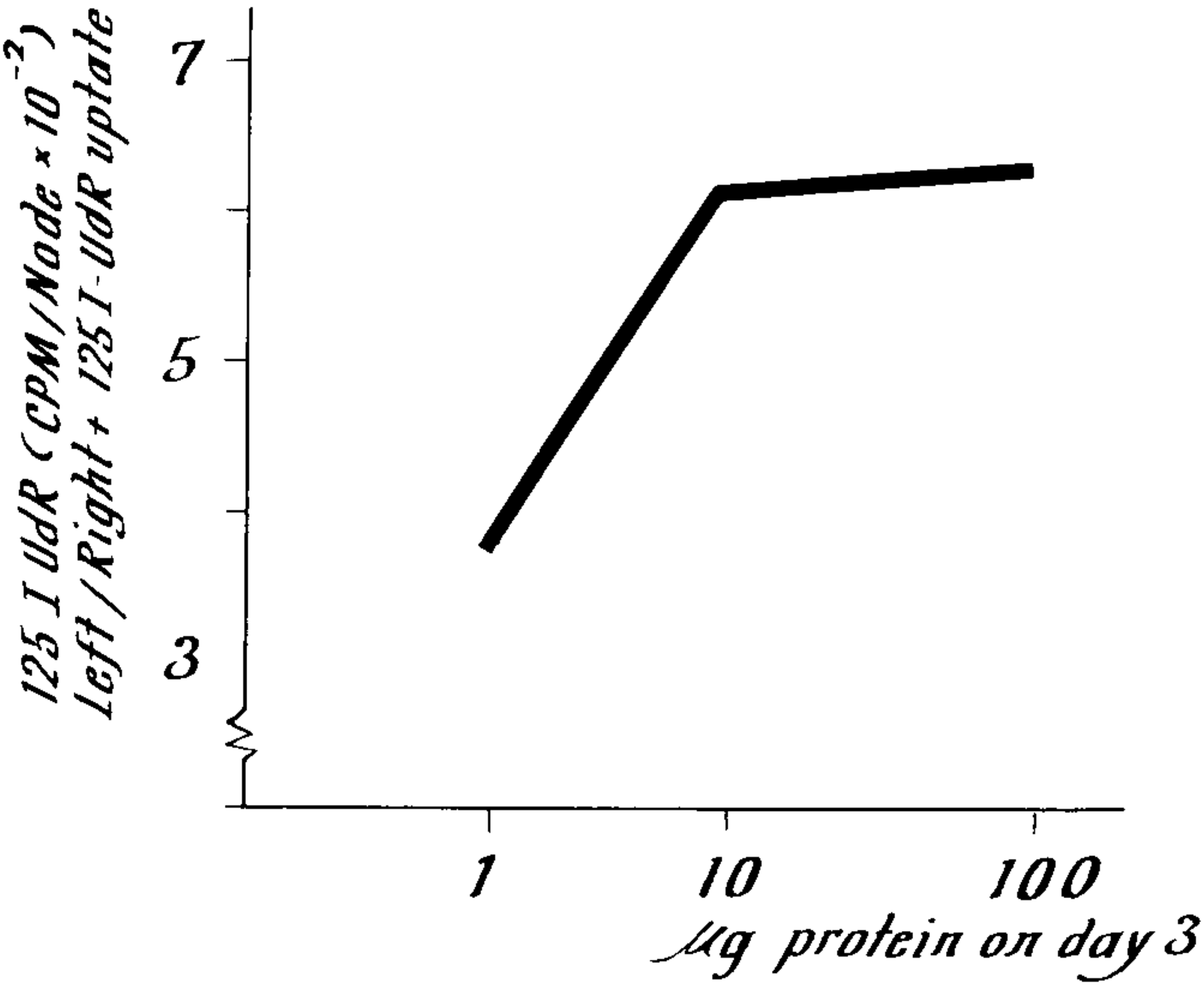

Fig. 1 – Relative rate of ^{125}IUdR incorporation into DNA by draining popliteal lymph node cells 3 days after injection of varying doses of flagellar proteins from *Trypanosoma cruzi* epismastigotes. Left to rigth: ratios (^{125}I) UdR uptake are computed and plotted with the dose of-FF antigen indicated.

As we can see in Fig. 5, BCG-immune-Cy-pretreated mice developed a stronger and prolonged DTH reaction, showing that sensitization persisted for more than one month.

ADOPTIVE CMI – Normal and BCG-pretreated Balb mice sensitized with FF were killed 5 days after and lymph nodes were harvested for cell transfer. The same was done with BCG-pretreated and saline control mice.

When cells were transferred locally to normal recipient mice which were tested 18 hours afterwards significant footpad swelling was observed only for BCG immune, FF-Ag sensitized mice (Fig. 6). No difference was observed at 4 or 24 hours in foot thickness between groups of recipient mice that received immune or control serum.

It is interesting to observe that groups of donor Balb/c mice sensitized with FF-Ag that received the eliciting dose of FF-antigen showed a DTH significantly smaller than that observed in OF_1 outbred mice.

CORRELATION BETWEEN DTH AND PROTECTION – Other goups of FF-immunized mice under modulatory effect of BCG, Cy or by association of both, were challenged with a virulent *T. cruzi* CL strain on day 6. DTH to the challenge dose was measured at 24 hours and the highest level was observed in BCG-Cy pretreated mice (Fig. 7).

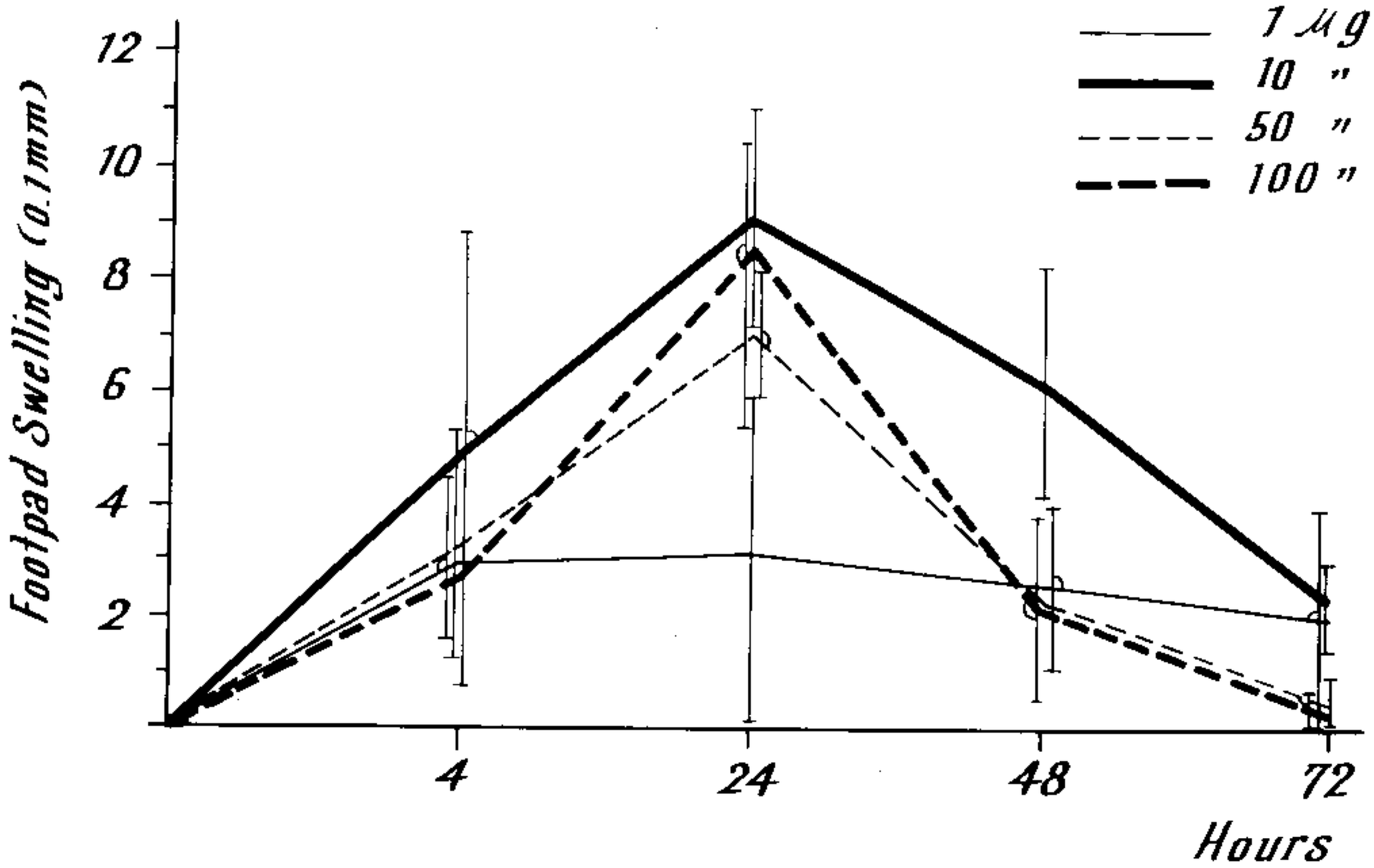

Fig. 2 – Levels of DTH measured in BCG-immune-mice sensitized with different protein content doses of flagellar fraction from *Trypanosoma cruzi* epimastigotes. All mice received an eliciting dose of 10 µg of antigen (FF). Six days after sensitization footpad swelling was measured 4, 24, 48 and 72 hr later. Means of five mice ± standard deviation.

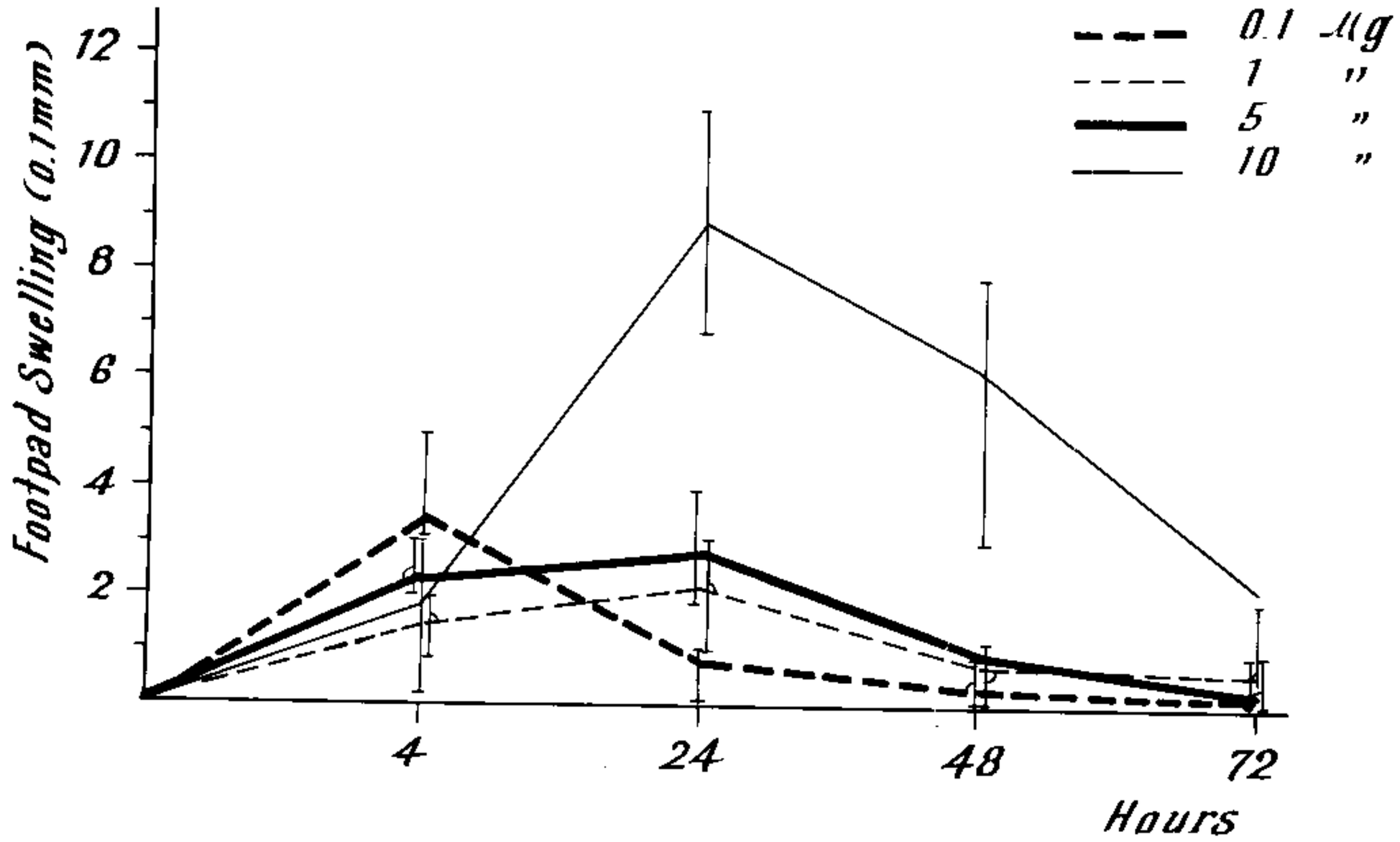

Fig. 3 – Levels of DTH measured in BCG-immune-mice sensitized with 10 µg of flagellar fraction and elicitated with different protein content doses of flagellar fraction from *Trypanosoma cruzi* epimastigotes as expressed in the figure. Mean of five mice ± standard deviation.

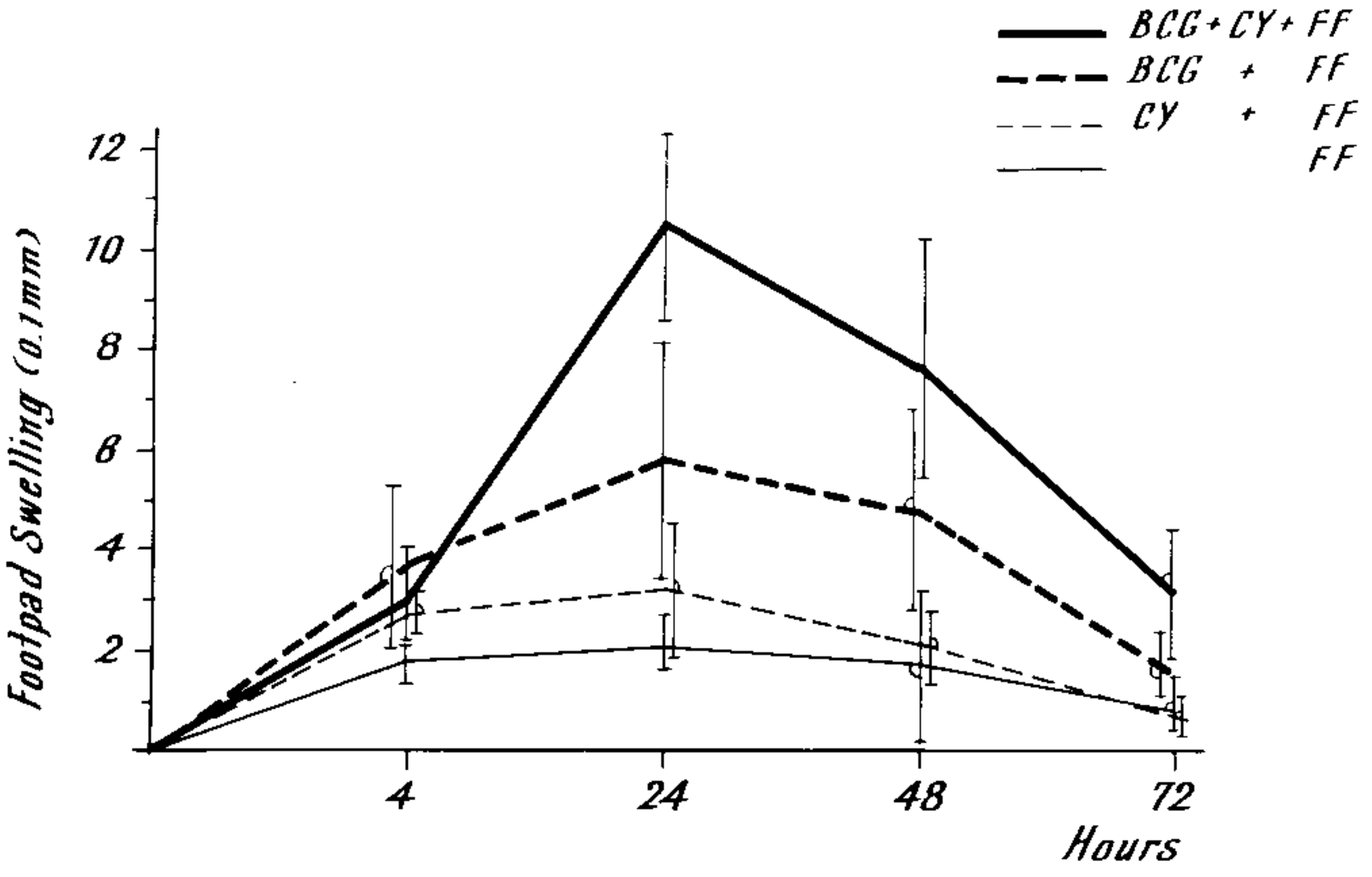

Fig. 4 – Influence of Cy on T–cell responses of mice in which flagellar fraction was used as sensitizing *Trypanosoma cruzi* antigen. This effect was evaluated under modulation of BCG or not, optimal sensitizing and eliciting doses were used. Groups of BCG-pretreated or Cy-treated mice were used as control groups.

Protection was assayed by parasitemia levels as shown in Fig. 8 and mortality expressed in Fig. 9 as percentage of survival. As we can see in Figs. 7 and 8 we have a clear correlation between DTH to challenge dose trypomastigotes and protection.

With the best immune modulated schedule, an experiment was prepared for challenge with the reticulotropic *T. cruzi* Y strain. The modulatory effect of BCG–Cy produced low levels of parasitemia with this strains too (Fig. 10).

DISCUSSION

Recently it was demonstrated that subcellular fractions of *T. cruzi* epimastigotes, such as Flagellar Fraction (Segura, Paulone & Gonzales-Cappa, 1976) may induce an immunity state that can protect mice against virulent trypomastigotes. On the other hand different results were obtained by using different preparations of flagella and schedules of immunization (Leon et al, 1979).

In the present paper the effect of our preparation of flagella on CMI was tested and it was shown that Flagellar Fraction is able to stimulate lymphoproliferative responses *in vivo* but the maximum magnitude of DTH induction was only reached in BCG-immune mice. The difficulty in demonstrating DTH during an artificial immunizations is well known unless the antigen has been chemically modified (Parish, 1972; Dennert & Tucker, 1972) or is given in a dose too small that does not provoke an antibody response (Lagrange, Mackaness & Miller, 1974; Uhr, Salvin & Pappenheimer, 1975). Administration of antigen associated with mycobacterial adjuvant also permits the development of a stable form of DTH in contrast to short-lived response obtained without mycobacterial

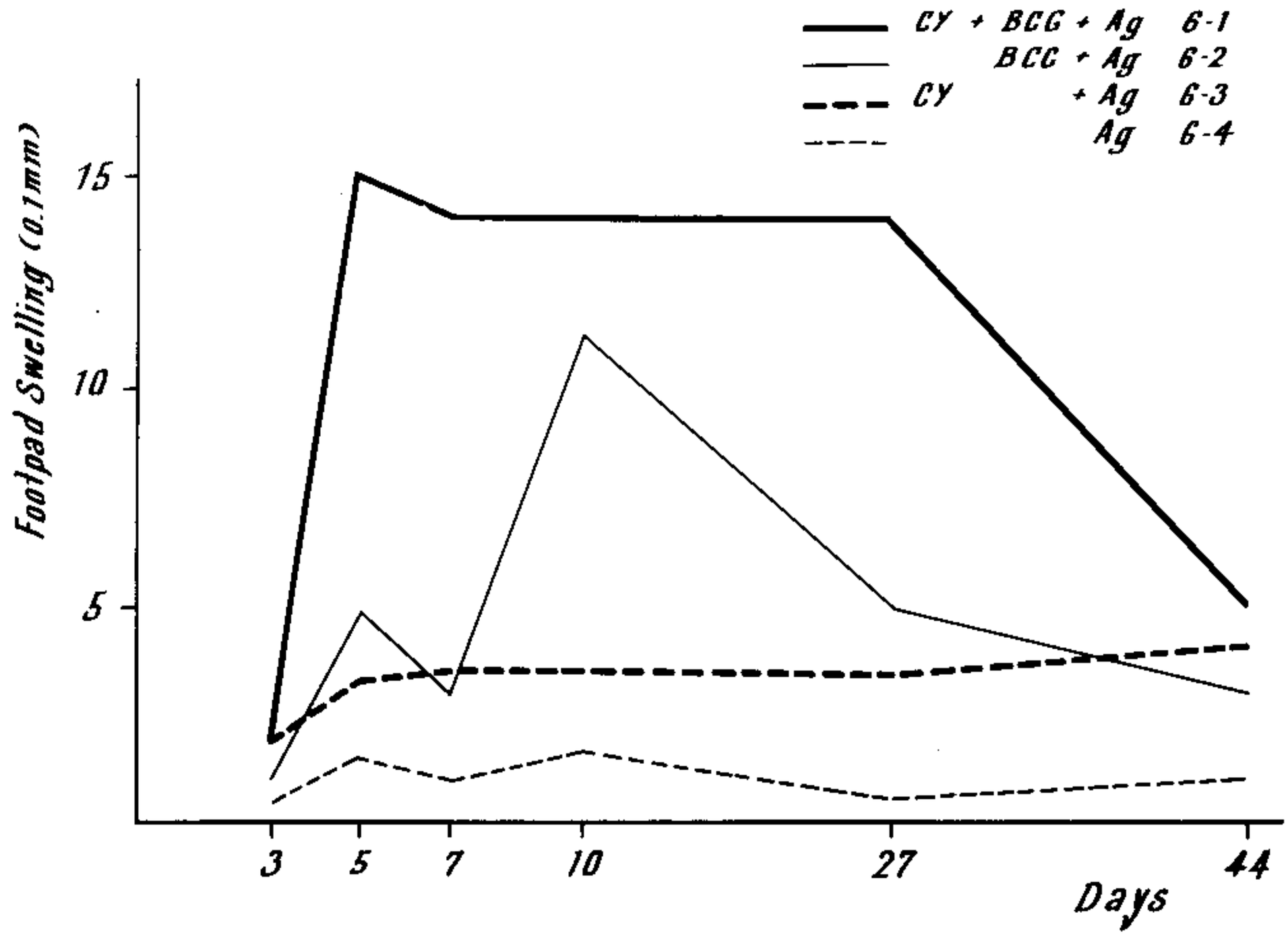

Fig. 5 – Kinetics of 24 hr footpad swelling elicited with *Trypanosoma cruzi* antigen at intervals in different groups of mice. Optimal doses of elicitation and sensitization were used. BCG (Pasteur Strain) immunized mice treated with Cy on day – 2 gave the best DTH level. Tests for DTH were performed on day indicated.

adjuvants. In other models using non-replicating antigen, DTH can be developed without adjuvant (Lagrange, Mackaness & Miller, 1974), and the sensitization levels can be amplified under modulation by BCG Cyclophosphamide or both immunomodulators (Lagrange & Mackaness, 1975; Miller, Mackaness & Lagrange, 1973). In the case of FF-Ag we have a correlation between DTH and resistance, and this fact is interesting to analyse since the best results were obtained with BCG-Cy-pretreated mice while when we analysed this system of immune modulation using an heterologous antigen sheep red blood cells (SRBC) this did not occur (Costa, Hurtrel & Lagrange, 1980). Under modulatory effect of both BCG and Cy, SRBC sensitized mice that received a challenge dose of 10^4 trypomastigote associated with an eliciting dose of SRBC showed a high level of DTH to SRBC and this local inflammatory response gave a low resistance to *T. cruzi* infection. Either in BCG-SRBC immunized mice or in SRBC-sensitized mice the same eliciting dose of SRBC associated with the challenge dose of trypomastigotes confer a high nonspecific level of resistance against *T. cruzi* infection. Thus it seems clear that under modulation by Cy, specific antigens are necessary to enhance the resistance against *T. cruzi* in BCG-pretreated mice.

The enhancement of DTH by Cy injected on day −2 can be attributed to the elimination of suppressor T-cells, while effector T cells do not seem to be affected (Sy, Miller & Claman, 1977). Since it has been reported that the regulation of DTH by suppressor T cells is an age-dependent phenomenon, being prominent in young mouse thymus cells (Mitsuoka et al, 1979) we decide to use 6-8 week old mice in our experiments instead of the 3 week old animals generally used in experimental Chagas' disease.

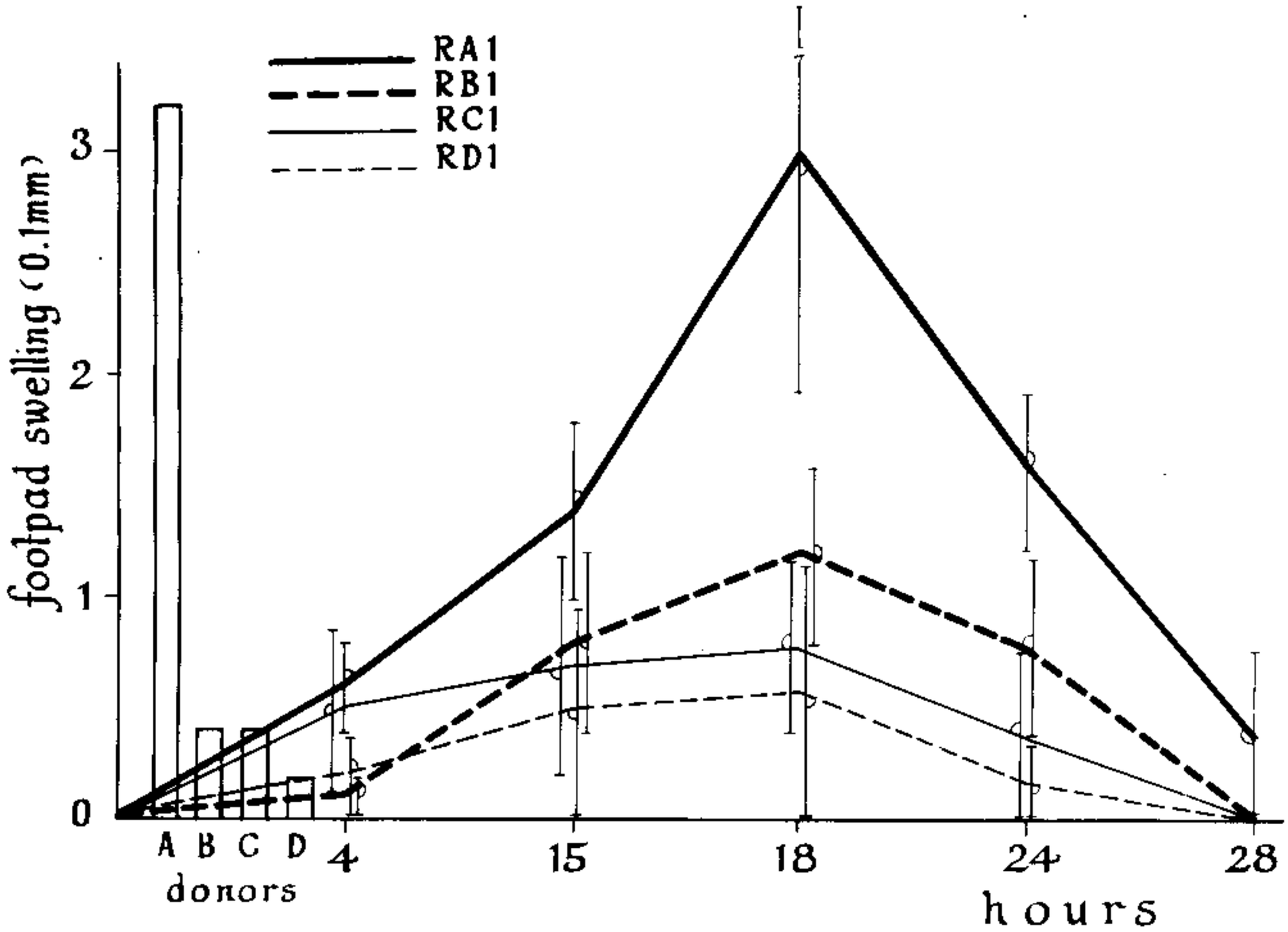

Fig. 6 – Footpad reaction in BALB/C mice tested for DTH after adoptive immunization. On day 5, representative donor mice were tested with eliciting *Trypanosoma cruzi* flagellar fraction antigen for DTH–Group A: BCG–pretreated –FF sensitized mice; B: FF– sensitized mice; C: BCG–pretreated mice; D: saline–control mice. Normal recipient mice (RA1, RB1, RC1 and RD1 groups) were injected with lymph node cells from immune donnors mixed with 10 μg of eliciting antigens and DTH was measured at 4, 15, 18 and 24 hr after injection. Mean of five mice ± standard deviation.

Further investigations must be done, however to better understand this effect of Cy when was associated with specific antigen; perhaps it can act as in the SRBC model by preventing the blockage of DTH cells by antibody or immunecomplex, or may act by interfering with supressor T cells (Mitsuoka et al, 1979). Marchal et al (1978) proposed two different mechanism to explain the effect of Cy upon DTH: permit free circulation of DTH-cells and increase the infiltration by mononuclear cells in test sites. Increased infiltration of mononuclear cells in test sites of BCG-pretreated mice or FF-sensitized mice may furnish additional conditions to explain the enhanced resistance to *T. cruzi* in this model, since in BCG-pretreated mice a great number of mononuclear cells will be activated, and it has been demonstrated *in vitro* that activated macrophages can control *T. cruzi* infection (Hoff, 1976).

We observed a correlation between DTH and protection expressed by parasitemia when a specific Flagellar Fraction antigen was used (compare Figs. 7 and 8). This is interesting to analyse because a better understanding of these phenomena is very important for the progress in this area, since in some cases we observe a correlation between DTH and resistance and sometimes not. This is important to analyse in the Chagas' disease model since hypersensitivity is involved in immunopathological events in patients.

As was discussed by Kierszenbaum (1979) it is very difficult to compare results in immunization assays since experimental conditions are generally not uniform. It is well known that dose route of BCG injection have an important effect upon immuno-

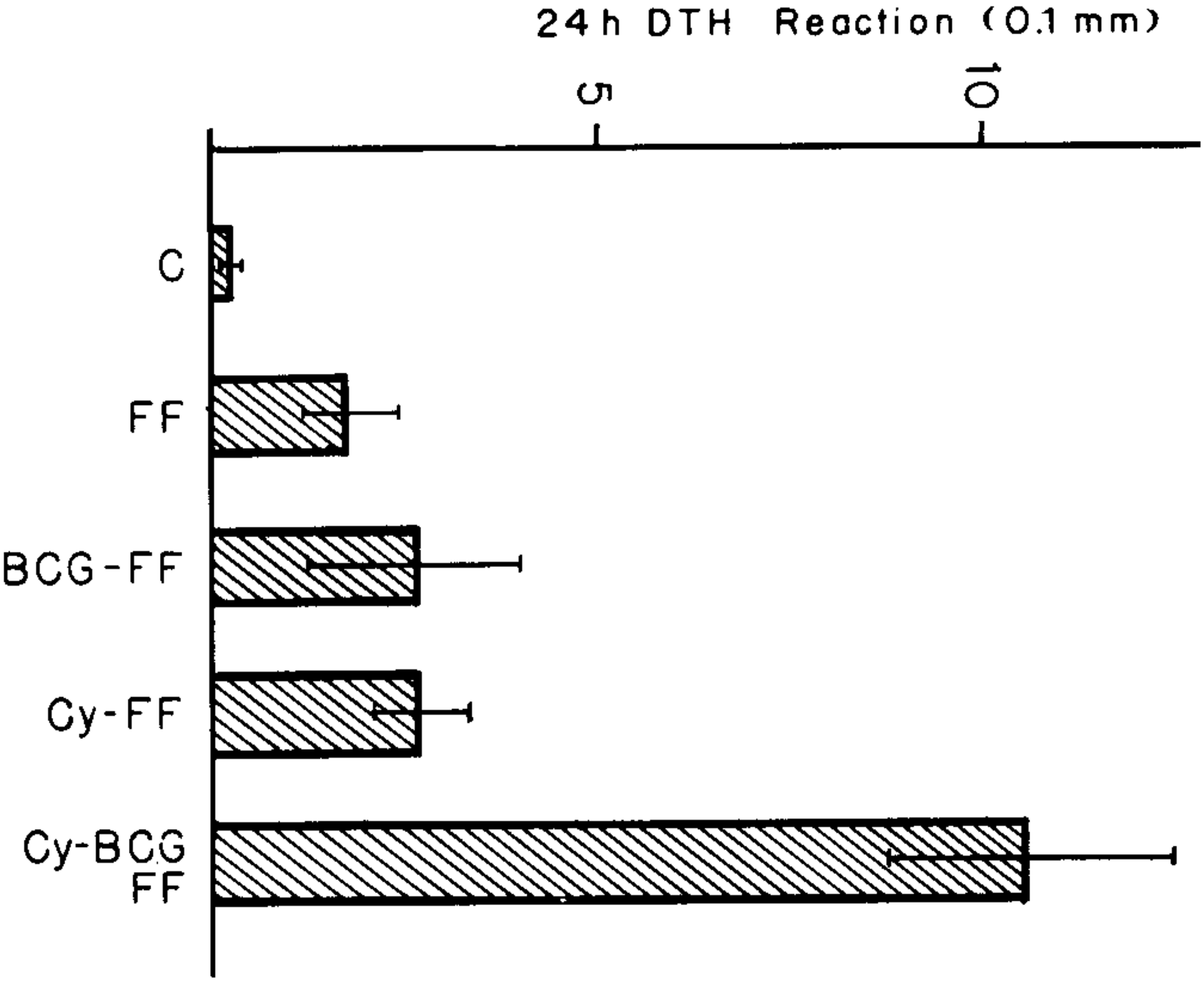

Fig. 7 – Levels of DTH to challenge dose in mice sensitized in one hind footpad following the schedules indicated in the figure and described in material and methods as well and challenged in the other footpad with 10^4 trypomastigotes of CL *Trypanosoma cruzi* strain.

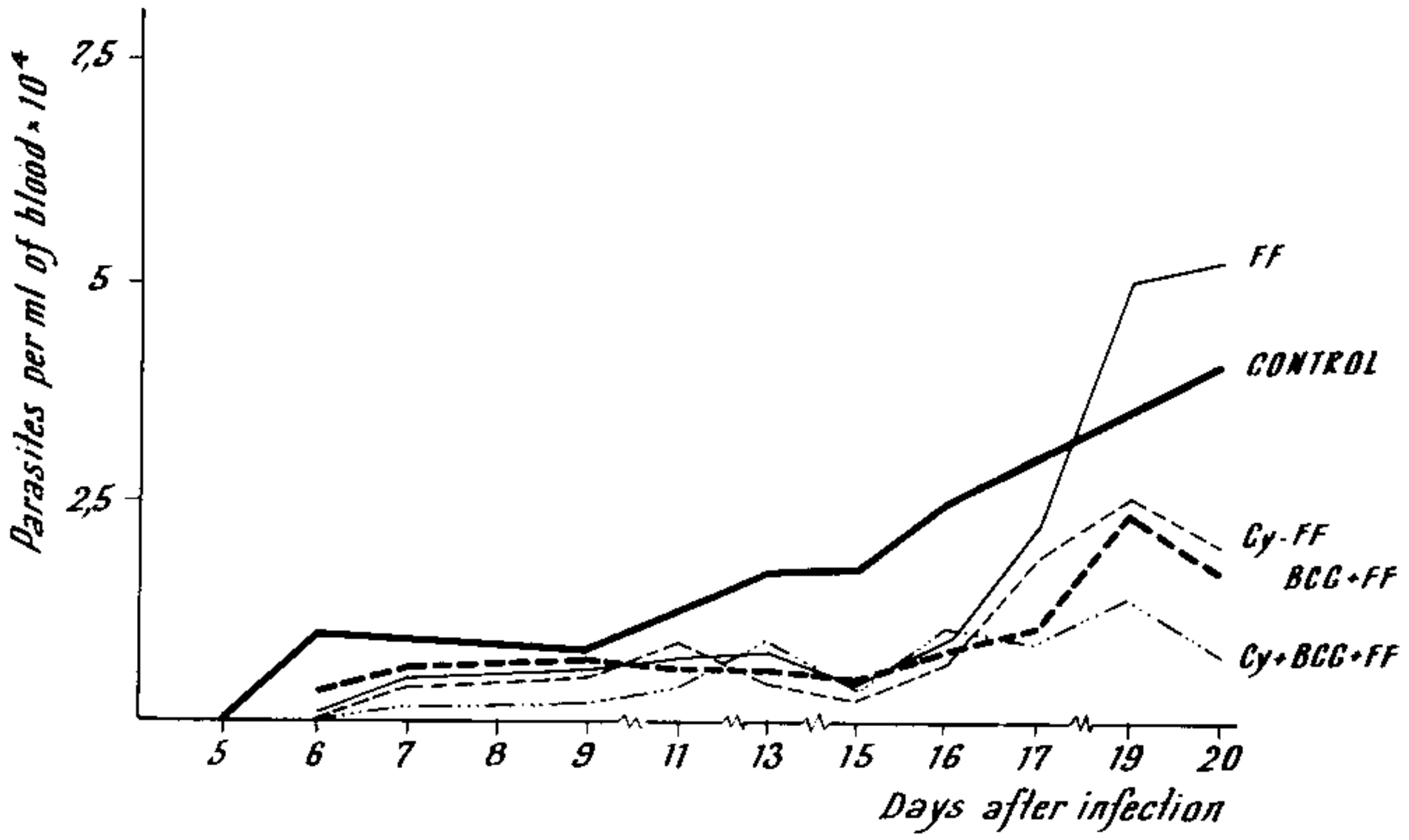

Fig. 8 – Parasitemia of different groups of mice immunized with 10 μg flagellar fraction using the indicated schedules, then challenged with 10^4 trypomastigotes of the CL *Trypanosoma cruzi* strain from Vero–cell culture.

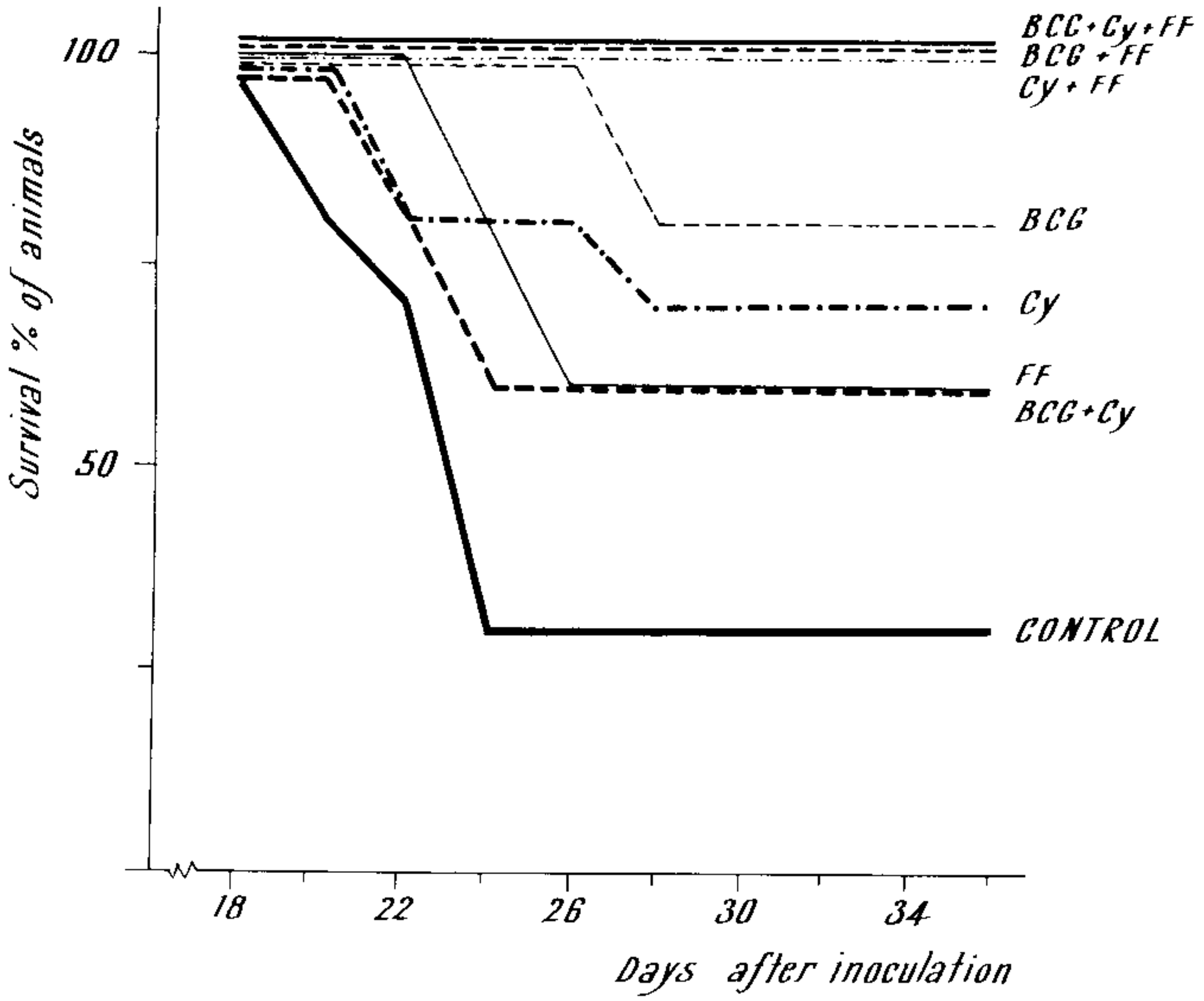

Fig. 9 – Survival rate of different groups of mice immunized with the schedules of Fig. 8 and challenged with 10^4 trypomastigotes of the CL *Trypanosoma cruzi* strains.

logical response (Salvin, 1958; Uhr, Salvin & Pappenheimer, 1957) and this is perhaps the first point to question when we find contradictory results in the literature. In our experiments immunomodulation of FF-sensitized mice by BCG or Cy or both gave 100% of protection in terms of survival rates. This contrasts with the results of Leon et al, 1979 who used FF associated to FCA without success. Several conditions must be established in attempts to standardization. For example, the use of several injections to sensitize mice may lead to desensitization or immunodeviation phenomena. The intraperitoneal route for challenge frequently used in immunization assays in experimental Chagas' disease is artificial as compared with natural exposure; resident peritoneal macrophages are not comparable with peripheral ones.

In our experiment we used Vero cell-cultured trypomastigotes since according to the results of Alcantara, Krettli & Brenner (1979), opsonized blood stream trypomastigotes evade phagocytosis. We believe that this must be pointed out in attempts to vaccination test standardization, since to inject antibody-coated trypomastigotes are also artificial.

Thus working with a Flagellar Fraction preparation we found also a significant level of protection with a small dose of 10 μg and these results agree with those obtained by Segura et al, 1974 e Segura, Paulone & Gonzales-Cappa, 1976. The present preparation (Pereira el al, 1978) is free from other subcellular components and we can say to be a

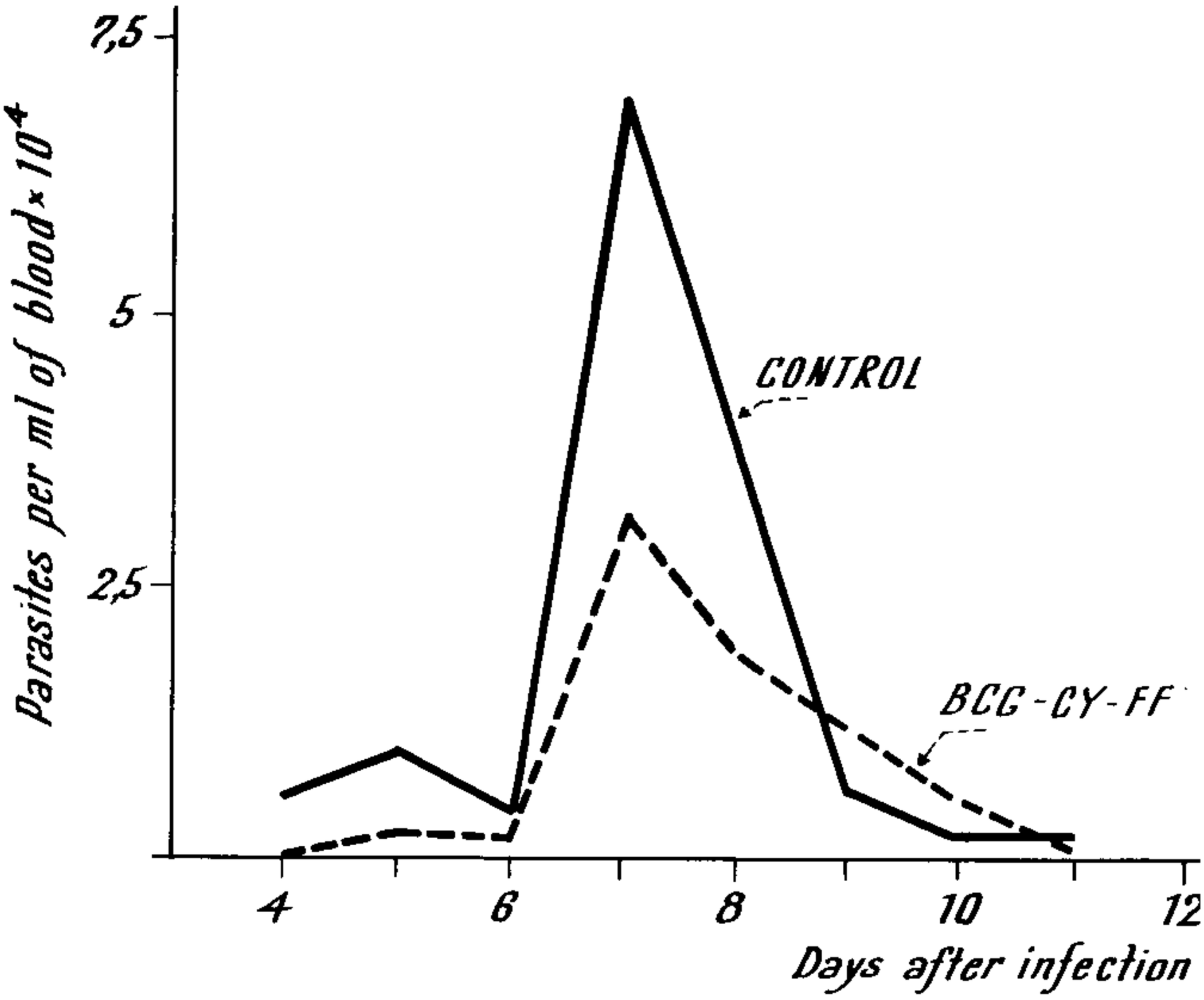

Fig. 10 – The best schedule of experiments presented in Fig. 8 was developed with Y strain of *Trypanosoma cruzi*. As we can see by parasitemia we have also protection when a reticulotropic strain was used for challenge.

tubulin-enriched fraction since most flagellar membrane was lost probably due to the non-ionic detergent treatment. Further studies must be done using pure tubulin, since flagella from *C. fasciculata* can protect mice against *T. cruzi* infection (Pereira et al, 1977) and we must understand the participation of this macromolecular component in CMI in this experimental model. We demostrated *in vivo* the induction of DTH by flagellar fraction and this was correlated with *in vitro* tests developed by Patruco et al 1978 using LIF test in Chagas'patients. This fraction was found by Patruco et al (1978) to be the antigen of choice by its great specifity to the LIF test. We think it is important to point out that FF-sensitized mice modulate by BCG and Cy present a high level of DTH to the challenge dose of live trypomastigote. Thus, we try in this paper to develop a methodology for studies on immunogenicity of parasites antigens.

RESUMO

Camundongos sensibilizados com a Fração Flagelar de formas epimastigotas, desenvolvem um estado de hipersensibilidade retardada medida pelo teste do "Footpad" que pode ser elicitado seis dias após quando se empregam doses ótimas de sensibilização e elicitação. Esta hipersensibilidade retardada pode ser ampliada quando se empregam camundongos pré-tratados por formas vivas de *Mycobacterium bovis* e a ciclofosfamida ou ambos.

O melhor resultado obtido foi registrado quando o BCG e a ciclofosfamida foram empregados em associação, sugerindo que efeitos independentes foram somados. Quando a dose de elicitação da Fração Flagelar foi substituída por uma dose de 10^4 trypomastigotas vivas, esta elicitou a hipersensibilidade retardada de intensidade correlata àquela observada quando a Fração Fragelar foi empregada. Nos diferentes grupos sensibilizados com Fração Flagelar apenas ou modulados pelo BCG ou ciclofosfamida ou ambos, constatou-se um estado de resistência cujo nível avaliado pela parasitemia e mortalidade estava relacionado com o nível de hipersensibilidade retardada medida 24 horas após no local da dose infecção.

A transferência adotiva da hipersensibilidade retardada foi obtida quando células do linfo-nodo de doadores imunes foram injetadas com a Fração Flagelar em camundongos normais. A correlação entre o nível de hipersensibilidade retardada e o grau de resistência à infecção experimental pelo *T. cruzi* poderá ampliar os fenômenos imunológicos envolvidos nos mecanismos de imunoproteção à tripanosomiase americana.

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