A fraction (FAd) from *Trypanosoma cruzi* epimastigotes depresses the immune response in mice

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Summary. The primary immune response to SRBC in BALB/c mice was depressed when they were injected with a fraction (FAd) obtained from *Trypanosoma cruzi* epimastigotes grown in LIT medium. Plaque-forming cell (PFC) number was 50% less than controls when FAd was injected i.v. 15 min before antigen in doses ranging from 70 µg up to 400 µg of protein. Similar depression was observed when 100 µg FAd was injected up to 6 h before antigen. There was no shift in the peak response to SRBC, neither was depression detected, when a total of 100 µg FAd protein was given in 20 µg amounts twice a day before immunization. Mice injected with FAd fraction only showed no increase in background PFC. Both secondary IgM and secondary IgG PFC were depressed when FAd was given before the boosting injection. However, only IgG PFC were depressed when FAd was injected before the priming dose. The delayed-type hypersensitivity reaction to DNFB was depressed when animals were injected either during the 3 days after sensitization or with a single dose of 100 µg of protein of FAd on day of challenge. Bone marrow colony-forming units in spleens of mice injected with FAd were depressed and nodules in the treated animals were smaller than in controls. We conclude that FAd affects humoral and cell-mediated immune re-sponses by interfering with cell division at some stage of the cell cycle.

INTRODUCTION

Chagas' disease (Chagas, 1909) is one of the most important health problems in Latin America because of its wide geographical distribution and high morbidity.

Although little is known about the antigenic constituents of *Trypanosoma cruzi* and their influence in different host–parasite relationships in man, natural reservoirs or experimental animals (WHO, 1977), intra-specific variations have been described that could be related to differences in the clinical manifestation of the disease (Brener, 1975). These facts have been stressed by WHO experts who emphasized the lack of knowledge concerning the antigenic constituents of parasites in their different forms and the alterations of the immune system of the host during trypanosome infections.

It is known for instance, that salivarian trypanosomes, e.g. *T. brucei*, have a profound effect on the immune system of an infected host. Immunodepression to unrelated antigens can be detected either *in vivo* in different species of laboratory animals (reviewed by Mansfield, 1978) or in *in vitro* experiments (Jayawardena, Waksman & Eardley, 1978).

The mechanism underlying immunodepression has been thought to be either a primary suppressor cell

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elicited by the infection (Jayawardena *et al.*, 1978) or exhaustion of B- and T-cell potential (Corsini, Clayton, Askonas & Ogilvie, 1977; Askonas, Corsini, Clayton & Ogilvie, 1979).

Similar immunodepression has been shown in mice infected with *T. cruzi* (Clinton, Ortiz-Ortiz, Garcia, Martinez & Capin, 1975; Ramos, Lamoyi, Feoli, Rodriguez, Perez & Ortiz-Ortiz, 1978) and similar mechanisms have been put forward (Ramos *et al.*, 1978).

The antigenic structure of trypanosomes studied by analysis of macromolecules extracted from different forms of parasites may help the understanding of mechanisms of differentiation as well as the identification of biologically active substances that could be involved in mechanisms of protection or suppression in the infected host.

Previous studies performed with a crude extract obtained from *T. cruzi* epimastigotes showed a depression in the number of rosette forming cells in the spleen of mice immunized with SRBC. Mice injected with the extract showed a slower clearance of colloidal carbon which could be related to a defect of antigen handling by macrophages (Corsini, unpublished results) as was shown in malaria-infected mice (Loose, Cook & Di Luzio, 1972).

In this paper we report the effects of a fraction (FAd) from epimastigotes of *T. cruzi* on the immune response of mice to SRBC and to DNFB. Both humoral and cell-mediated immune responses were depressed in mice injected with FAd. Possible mechanisms underlying depression are discussed.

MATERIAL AND METHODS

Animals
BALB/c and CBA female and male mice bred and kept under clean conditions in our animal facilities were used when 6–8 weeks of age.

Trypanosome
Trypanosoma (Schizotripanum) cruzi strain Y (Pereira da Silva & Nussenweig, 1953) obtained from Dr Zigman Brener were weekly passaged in Swiss mice according to Brener (1968). An aliquot of infected blood obtained at peak parasitaemia (7th day after inoculation) was dispensed in 250 ml of medium to begin our *in vitro* culture of epimastigotes. Cultures were performed in Roux bottles using Yaeger culture medium (LIT) following the method described by Fer-

nandes & Castellani (1968). Cultures were kept at 28° and seeded every 7 days. Under these conditions 95% of the parasites were epimastigotes.

Trypanosome extract
Parasites from 100 ml culture (approximately 3×10^9 epimastigotes) were washed three times in ice-cold NaCl 0·15 M by centrifugation at 1400 g for 15 min at 4°. The pellet was resuspended in 10 ml distilled water, immediately frozen and lyophilized.

The lyophilized material was delipidated in portions of 250 mg following washes in organic solvents previously distilled and free of peroxides. The preparation was washed in acetone (2 ×), acetone–ethyl ether (1 : 1) (2 ×) and finally in ethyl ether (2 ×). Sedimentable material was deposited by centrifugation at 3020 g for 15 min at 4°. The final pellet was dried in an air vacuum pump and kept in a desiccator at 4°.

Two hundred milligrams of the delipidated material were resuspended in 25 ml ice-cold NaCl 0·15 M and kept in an ice bath with occasional shaking for 10 min. The material was then centrifuged at 12,000 g for 30 min at 4°.

The pellet was resuspended in 10 ml PBS pH 7·22 and incubated in a water bath at 37° for 1 h. It was centrifuged at 12,000 g for 30 min at 4°. The supernatant, called FAd, was kept at −20° in 1 ml vials. Protein content was measured as described by Weichselbaun (1946).

Immunization with SRBC
SRBC harvested in Alsever solution were washed three times in PBS and 4×10^8 cells in 0·2 ml administered i.v. (tail vein) to experimental animals. Where indicated a booster was given 26 days later using the same number of SRBC.

PFC assay
A Jerne PFC assay was performed on slides as described by Dresser (1978). Both primary and secondary responses were assayed on the 4th day after SRBC. Preliminary experiments had shown that the peak response occurred on the 4th day. Indirect plaques were revealed using a rabbit anti-mouse globulin serum.

Sensitization to DNFB
Mice were sensitized to DNFB according to Vadas, Miller, Gamble & Whitelaw (1975). Animals were shaved over the abdomen and painted with 50 μl of a DNFB solution containing 10 mg/ml in acetone–olive

oil (1:1). The mice were challenged 5 days later by painting their left ear with 5 μl of same solution.

Measurement of delayed-type hypersensitivity (DTH) to DNFB

Twenty four hours after ear painting, mice were killed by cervical dislocation and both ears weighed. DTH reactions were assessed by the weight ratio between the ears challenged and unchallenged as described by Corsini, Bellucci & Costa (1979).

Colony-forming units (CFU) in spleen

The technique used was that previously described by Till & McCullogh (1961). Briefly, mice were irradiated (750 rad) from a ^{60}Co source and injected 2 h later with 3×10^4 viable syngeneic bone marrow cells. Viability was assessed by trypan blue exclusion. The number of CFU in the spleen was determined using a magnifying glass (8 ×) 8 days after cell transfer.

Histological examination

Ears were fixed in formaldehyde (4%) and processed as usual for histological haematoxylin–eosin examination.

Statistical analysis

The Student's *t* test was used to analyse the data.

RESULTS

FAd depresses the primary immune response to SRBC

In order to verify the effect of FAd on the immune response to SRBC, BALB/c mice were injected i.v. 15

Table 1. Effect of FAd on the primary immune response to SRBC

FAd dose (μg protein)	PFC/spleen ($\times 10^{-3}$)	Inhibition (%)
0	48·0 ± 6·1	—
10	50·0 ± 4·1	0
35	24·1 ± 10·4	49·8
70	20·3 ± 4·9	57·7
140	21·3 ± 5·7	55·6
280	18·1 ± 8·2	62·3
400	21·6 ± 5·7	55·0

Mice were immunized to SRBC and direct PFC counted 4 days later.

Arithmetic mean ± SD for groups of five animals.

min before antigen with 35, 70, 140, 280 and 400 μg of FAd protein in 0·2 ml of PBS. The weight of FAd used refers always to the protein content in μg. The number of PFC was assessed 4 days after immunization.

Lower PFC numbers were found in mice injected with FAd in a dose range from 70 μg up to 400 μg protein with no significant difference between them. Thirty-five micrograms of FAd inhibited to the same extent only 50% of animals and smaller doses had no effect. The results showed in Table 1 express the arithmetic mean ± SD of groups with five animals/group.

Time interval between FAd injection and SRBC

Preliminary experiments had shown that the time interval between injection of FAd and SRBC was critical to the depression of PFC number. This was studied further in animals injected with 100 μg of FAd protein from 15 min up to 24 h before SRBC or alternatively after antigen administration. Mice immunized with SRBC were injected once with 100 μg FAd protein 8 h after antigen or three times with 100 μg on days 0, 1 and 2 after immunization. Results in Tables 2 and 3 express the arithmetic mean ± SD of groups with five animals/group.

An inhibition of response was shown in mice injected with FAd from 15 min up to 6 h before antigen. FAd injected 24 h before SRBC had no effect in PFC number. The time limit for injection FAd was found to be 12 h before antigen, since at that time the response of only 50% of animals was inhibited (Table 2). One hundred micrograms of FAd protein given in five doses of 20 μg every 12 h before SRBC had no effect on PFC number.

Table 2. Effect of timing of the FAd injection with respect to antigen injections on the primary immune response to SRBC

FAd before SRBC (100 μg of protein)	PFC/spleen ($\times 10^{-3}$)	Inhibition (%)
15 min	21·1 ± 7·4	71·8
3 h	24·8 ± 7·7	66·9
6 h	36·0 ± 5·1	52·0
12 h	49·0 ± 31·5	34·6
24 h	79·0 ± 8·1	0
5 doses 20 μg every 12 h	74·0 ± 6·2	0
Control	75·0 ± 5·1	—

Direct PFC were counted 4 days after immunization.
Arithmetic mean ± SD for groups of five animals.

Table 3. Effect of FAd injection after immunization on the primary immune response to SRBC

FAd after SRBC	PFC/spleen ($\times 10^{-3}$)	Inhibition (%)
8 h single dose (100 μg)	$75 \cdot 0 \pm 14 \cdot 2$	0
3 doses, 100 μg every 24 h	$24 \cdot 7 \pm 9 \cdot 1$	66·8
Control	$75 \cdot 7 \pm 15 \cdot 0$	—

FAd: 100 μg of protein.
Direct PFC were counted 4 days after immunization.
Arithmetic mean ± SD for groups of five animals.

No inhibition was found in mice injected with FAd in a single dose after SRBC but 70% inhibition was detected when animals received 100 μg FAd protein during the 3 days after immunization (Table 3).

FAd depresses secondary immune response to SRBC

In order to study the effect of FAd fraction on the secondary response to SRBC, mice were immunized with SRBC and 26 days later boosted with the same dose of antigen. Experimental animals received 100 μg FAd protein i.v. 3 h before the second dose of antigen. PFC were assayed on the 4th day after the boost. Table 4 presents the results and percentage inhibition for groups of five animals. FAd inhibited both direct (IgM) and indirect (IgG) PFC.

We also investigated whether FAd injected before primary immunization would have any effect on the secondary response to SRBC. Mice were injected with 100 μg FAd protein 3 h before primary immunization and 26 days later received as usual a second antigen dose. PFC assayed on the 4th day showed an inhibition of IgG PFC but no inhibition of IgM PFC (Table 4).

Table 5. DTH reactions to DNFB in mice injected with FAd

Groups	Weight ratio L/R	Statistical significance
Control	$2 \cdot 0505 \pm 0 \cdot 22$	—
FAd, 3 days	$1 \cdot 6713 \pm 0 \cdot 20$	$P < 0 \cdot 01$
FAd on day of challenge	$1 \cdot 3566 \pm 0 \cdot 20$	$P < 0 \cdot 01$

Mice were sensitized to DNFB and received FAd (100 μg protein) either for 3 days after sensitization or on day of challenge. DTH reactions are expressed as the weight ratio between left (challenged) and right (unchallenged) ears. Arithmetic mean ± SD for groups of five animals.
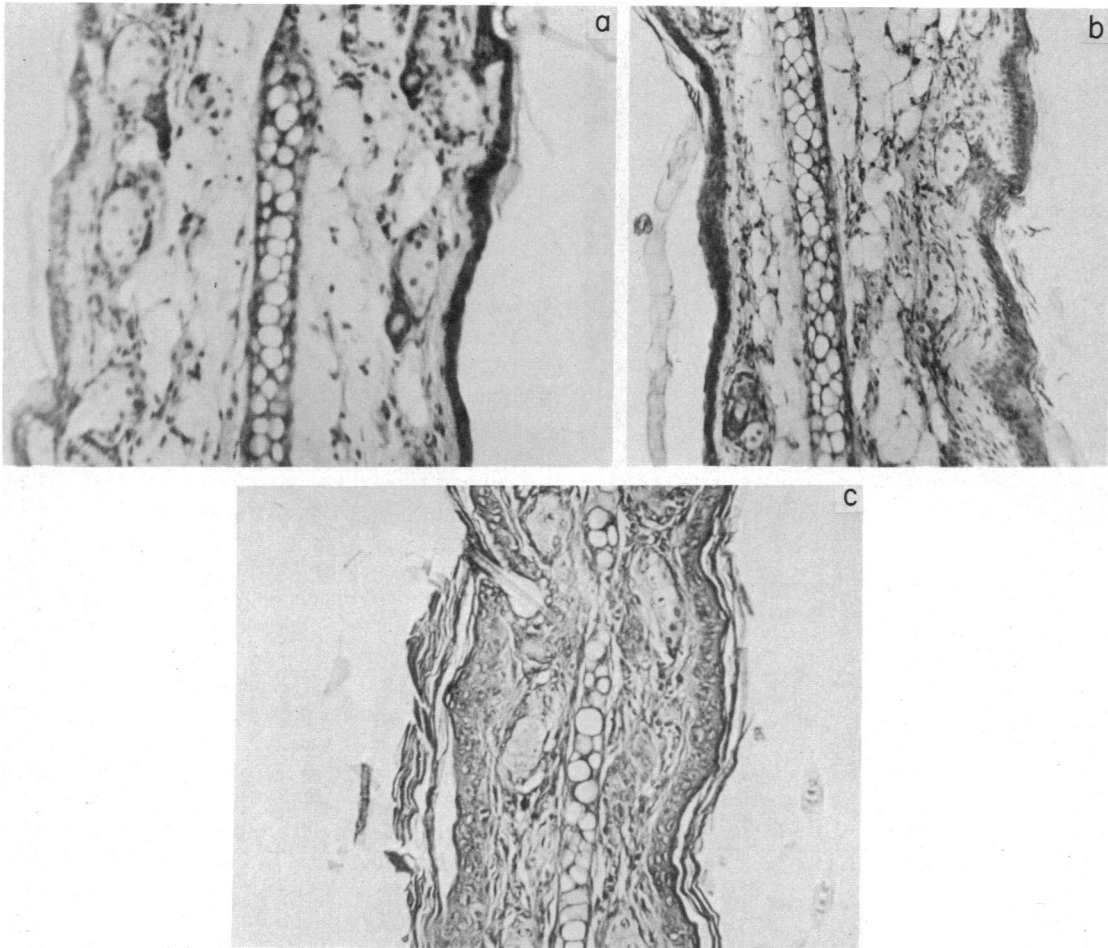
The possibility that a shift in the peak response to SRBC had occurred was investigated but not found. Also plaques were smaller in FAd injected mice than in controls. We conclude that a real depression in primary and secondary response to SRBC occurred in animals injected with FAd fraction.

FAd depresses the immune response to DNFB

The effect of FAd fraction on DTH was studied in mice sensitized to DNFB and challenged 5 days later in the left ear. Experimental animals received 100 μg FAd protein daily during the 3 days after sensitization or one single dose of 100 μg on day of challenge.

A depression of L/R ear ratio ($P < 0 \cdot 01$) was found in animals injected with FAd using either schedule. The results shown in Table 5 express the weight ratio between left and right ears for groups of five animals/ group (arithmetic mean ± SD).

Histological examination of ears showed a smaller mononuclear cell infiltrate and oedema in animals that

Table 4. Secondary response to SRBC in mice injected with FAd

FAd 100 μg of protein	PFC ($\times 10^{-3}$)			
	IgM	Inhibition (%)	IgG	Inhibition (%)
Control	$32 \cdot 71 \pm 1 \cdot 0$	—	$686 \cdot 50 \pm 12 \cdot 9$	—
3 h before priming	$32 \cdot 86 \pm 3 \cdot 3$	0	$468 \cdot 60 \pm 8 \cdot 7$	32
3 h before boost	$7 \cdot 92 \pm 0 \cdot 1$	76	$272 \cdot 30 \pm 5 \cdot 5$	61

Direct and indirect PFC were assayed 4 days after immunization.
Arithmetic mean ± SD for groups of five animals.

Figure 1. Ears of mice sensitized to DNFB. Notice the greater swelling in (a) control sensitized compared to (b) mice injected with FAd. (c) control unsensitized but challenged with DNFB. All the photographs have the same magnification (× 70).

received FAd (Fig. 1). These results confirm that a depression of the DTH reaction occurred in mice injected with FAd.

**FAd interferes with colony-forming units in spleen**

The possibility that FAd interferes with cell proliferation was studied by determining the effect of FAd on CFU in the spleens of irradiated (750 rad) CBA mice injected with 3×10^4 syngeneic bone marrow cells 2 h after irradiation. Experimental animals received a single dose of 100 μg FAd protein or three doses of 100 μg each on days 0, 1 and 2 after cell transfer.

A decrease in CFU number was found in animals injected either with a single dose or three doses of FAd. The latter group of mice besides having the smallest CFU number had smaller nodules (Fig. 2). Table 6 shows the results expressed as the arithmetic mean $\pm$ SD of CFU in spleen of mice (five animals/group).

DISCUSSION

Our results showed a depression of humoral and cell-mediated immune responses in mice injected with FAd fraction. Suppressed responses were found before primary stimulation with SRBC over a large dose range of FAd (measured as protein content). Responses were
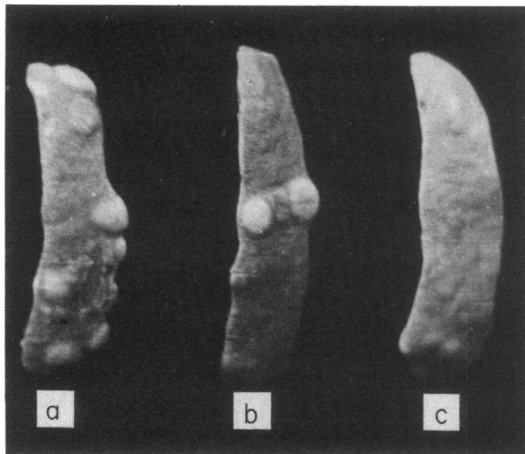
Figure 2. Bone marrow CFU in mice injected with Fad. (a) Normal. (b) FAd on day of transfer, (c) FAd over 3 days (× 8).

Table 6. Bone marrow CFU in mice injected with FAd

Groups	CFU/spleen	Statistical significance
Control	15·0 ± 4·5	–
FAd on day of bone marrow transfer	8·6 ± 3·9	$P < 0.01$
FAd, during 3 days	4·8 ± 2·4	$P < 0.01$

FAd, 100 μg protein.
CFU were assayed as described by Till & McCullogh (1961).
Arithmetic mean ± SD for groups of five animals.

more suppressed when 100 μg FAd protein was injected during 3 days after antigen priming.

A similar depression of the secondary immune response was detected. Again, FAd was more effective when injected during three days after antigen stimulation.

Because FAd injection in a single dose on the 3rd day after antigen priming or boost had no effects on PFC number, it is likely that FAd acts on the early phases of primary and secondary immune responses. This may explain why FAd before priming inhibits both direct (IgM) and indirect (IgG) PFC in the secondary response whereas it affects only IgG PFC when injected just before the booster dose of antigen.

It has been shown by Zauderer & Askonas (1976), Askonas & North (1976), Kemshead, North &

Askonas (1977) that Igs-secreting cells generate *in vitro* only after extensive proliferation of precursor cells. Similar results were previously obtained by Dutton (1975) who showed that generation of *in vitro* PFC needs an extensive B-cell proliferation before differentiation takes place under T command. This antigen driven proliferation of antibody forming precursors is sensitive to irradiation (Dutton, 1975; Kenshead *et al.*, 1977).

Our results suggest that FAd interferes with this proliferative phase of the immune response at some stage of cell cycle thus blocking further cell division, and affecting PFC number. On the other hand, injection of 100 μg FAd protein in doses of 20 μg every 12 h before SRBC had no effect on PFC number. This would suggest that mice can overcome the effect of FAd and explain as well why several doses of FAd after antigen have a greater effect on PFC and CFU numbers.

This block of cell proliferation would explain both humoral and cell-mediated immune depression. Even DTH reactions were depressed particularly when FAd was injected after challenge.

The mechanism has not been defined but we favour a T-cell blockade resulting in a lack of helper cells for antibody-secreting B cells. T cells undergo a proliferative phase after antigen recognition particularly SRBC (reviewed by Katz, 1977).

A direct assay for cell proliferation is available using the technique described by Till & McCullogh (1961). Mice injected with FAd showed smaller nodules and a depression in CFU. This implies that FAd interfered with proliferation in the spleen of mice even when a single dose of 100 μg was given on day of cell transfer (Fig. 2). FAd does not decrease cell viability *in vitro* so the observed decrease in CFU cannot be due to bone marrow cell killing before cell transfer.

Although the CFU experiment suggests a direct cell proliferation blocking effect of FAd we cannot exclude the participation of other factors secreted by a third cell involved in the immune response, e.g. the macrophage, since these cells secrete a large number of immunologically active substances (reviewed by Unanue, 1978). Phagocytosis of SRBC was not impaired when assessed by the ingestion of erythrocytes coated with antibody (EA) in mice injected i.p. 1 h previously with 100 μg of FAd protein. On the other hand, aminopeptidase levels were impaired in peritoneal macrophages harvested from mice injected daily with 100 μg of FAd protein over 3 days (Corsini & Guimarães, unpublished results).

There was no increase in number of background PFC in mice injected with FAd over a large dose range. This suggests indirectly that no mitogenic activity exists in this fraction. FAd may be part of the membrane of epimastigotes since it blocks agglutination of parasites by antiserum raised in guinea-pigs (Rangel *et al.*, unpublished results). We still do not know if this fraction exists in trypomastigotes. Experiments are being carried out in an attempt to demonstrate similar effects with an extract obtained from parasites isolated from infected blood. If FAd does exist in trypomastigotes it may well participate in the immunodepression produced by this parasite infection.

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