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International Centre for Genetic Engineering and Biotechnology
United Nations Industrial Development Organization



Collaborative Research Programme

TERMINAL EVALUATION REPORT

UNIDO contract # 92/264

ICGEB ref. #: CRP/ ARG92-05

Project initiation: 1992

Project termination: 1995



Collaborative Research Programme

TERMINAL EVALUATION REPORT

Part 1

Title of Project	
"Characterization of an intestinal polypeptide from <i>Triatoma infestans</i> that activates <i>Trypanosoma cruzi</i> adenylyl cyclase and determines the differentiation of epimastigote to trypomastigote forms".	
Keywords: <i>Trypanosoma cruzi</i> , adenylyl cyclase, <i>Triatoma infestans</i> , metacyclogenesis, tripomastigote, epimastigote.	
UNIDO contract # 92/264	ICGEB ref. #: CRP/ ARG92-05
Project initiation: 1992	Project termination: 1995
Principal Investigator's name: Mirtha María Flawiá	
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Abstract:	
<p>A peptide from hindguts of the <i>Triatoma</i> hematophagous Chagas' insect vector activates adenylyl cyclase activity in <i>Trypanosoma cruzi</i> epimastigote membranes and stimulates the <i>in vitro</i> differentiation of epimastigotes to metacyclic trypomastigotes.</p> <p>The following sequence for 20 residues of the amino terminus of this peptide was determined: H₂N-Met-Leu-Thr-Ala-Glu-Asp-Lys-Lys-Leu-Ile-Gln-Gln-Ala-Trp-Glu-Lys-Ala-Ala-Ser-His. This sequence is identical to the amino terminus of chicken a^D-globin.</p> <p>A synthetic peptide carrying the sequence of the 40 amino acids corresponding to the a^D-globin amino terminus (peptide₁₋₄₀), also stimulated adenylyl cyclase activity and promoted differentiation. The ¹²⁵I-labeled synthetic peptide₁₋₁₀ bound specifically to <i>T. cruzi</i> epimastigote cells.</p> <p>The effects of hemoglobin or synthetic peptides carrying a^D-globin fragments on both the growth and transformation of <i>T. cruzi</i> epimastigotes into metacyclic trypomastigotes were studied in the insect vector. Metacyclogenesis did not occur when the insects were fed with plasma. However the differentiation in the insect's gut is expressed when hemoglobin or synthetic peptides corresponding to residues 30-49 and 37-73 of the a^D-globin were added to the plasma diet.</p> <p>A nitric oxide synthase was partially purified from soluble extracts of <i>Trypanosoma cruzi</i> epimastigote forms. The conversion of L-arginine to citrulline by this enzyme activity required NADPH and was blocked by EGTA. The reaction was activated by Ca²⁺, calmodulin, tetrahydrobiopterin and FAD, and inhibited by N^m-methyl-L-arginine. L-glutamate and N-methyl-D-aspartate (NMDA) stimulated <i>in vivo</i> conversion of L-arginine to citrulline by epimastigote cells. These stimulations could be blocked by EGTA, MK-801 and ketamine and enhanced by glycine. A sodium nitroprusside-activated guanylyl cyclase activity was detected in cell-free, soluble preparations of <i>Trypanosoma cruzi</i> epimastigotes. L-glutamate, NMDA and sodium nitroprusside increased epimastigote cyclic GMP levels. MK-801 bound specifically to <i>T. cruzi</i> epimastigote cells. This binding was competed by ketamine and enhanced by glycine or L-serine. Evidence thus indicates that in <i>Trypanosoma cruzi</i> epimastigotes, L-glutamate controls cyclic GMP levels through a pathway mediated by nitric oxide.</p>	

OBJECTIVES/METHODOLOGY

(proposed at the time of the submission of the research proposal)

OBJECTIVES/METHODOLOGY

This project proposes studies on a polypeptide recently found in this laboratory that was purified from hindguts of the insect vector *Triatoma infestans*. The polypeptide, stimulates adenylyl cyclase activity in the epimastigote forms of the Chagas' disease pathogenic agent *Trypanosoma cruzi*, and determines, in vitro, the differentiation of the epimastigote forms (proliferative and non-infectious) to the corresponding trypomastigote forms (non-proliferative and infectious).

The polypeptide will be extracted from several hundreds hindguts from insects fed two days before with chicken hemoglobin and it will be purified to homogeneity by HPLC on C18 and C4 columns.

The peptide will be subjected to amino acid micro-sequencing and the sequence will be analyzed in a data bank to determine putative homologies with known proteins.

Peptides having the sequence of the hindgut factor or those corresponding to homologous known proteins, if any, will be synthesized. The capacity of these peptides to stimulate epimastigote adenylyl cyclase and differentiation will be analyzed. Having a positive effect the corresponding dose-response curves will be determined.

Synthetic peptides will be labeled with ¹²⁵I in the presence of chloramine-T and the corresponding epimastigote receptor will be characterized in cells and membrane preparations. Receptor purification will be performed and, if it is possible, molecular cloning of this receptor will be intended.

Polyclonal antibodies against these peptides or the protein showing homology with the hindgut factor will be prepared. antibodies will be prepared. All these antibodies will be used to study putative effects at the level of infectivity in both, in vivo mouse and in vitro macrophage models.

1. Purification procedure

Triatoma infestans intestines will be excised from adult insects fed with chicken blood, homogenized in PBS and centrifuged. The supernatant fluid will be filtered through a Sephadex G-25 column and the fractions eluting between the exclusion and inclusion peaks will be passed through Sep Pak-C18. After concentration, the material eluting with 15 to 50% acetonitrile will be further purified by HPLC in a C18 column which will be eluted with an acetonitrile gradient (12 to 56%). After concentration of the active peak, eluting at about 43% acetonitrile, the material will be further subjected to HPLC on a C4 column eluted with a acetonitrile gradient (0 to 40%). The homogeneous material eluting at about 32% acetonitrile will be used for further studies.

2. Criteria of purity

Purity of the polypeptide will be asserted by three procedures:

1. Peak symmetry in DO230 topograms in two successive HPLCs;
2. Presence of only one band in 20% PAGE gels stained with silver; and
3. Detection of only one amino-terminal in the sequencing procedure.

3. Aminoacid composition

It will be performed by the procedure involving the generation of PTC-derivatives, from at least 50 pmoles of peptide, in a automated equipment for gas phase hydrolysis and derivatization (Applied Biosystems Model 420A Derivatizer/Hydrolyzer), followed by HPLC separation of amino acid derivatives in a C18 column using a Model 130A equipment from Applied Biosystems provided with a Model 920A data reduction system. All the procedures will be done following the instructions of the manufacturer.

4. Aminoacid sequencing

It will be performed, from at least 200 pmoles of peptide, in a Model 477 Applied Biosystem Sequencer, using the Edman degradation chemistry that generates amino acid-PTC derivatives. Derivatives will be resolved by HPLC by a procedure similar to that described above.

5. Oligonucleotide synthesis

It will be performed with the phosphoramidite chemistry in an Applied Biosystem Model 381-A. After cleavage with ammonia, oligonucleotides will be desalted in Sep-Pak cartridges.

6. PCR

RNA from total insects or from gut will be extracted with guanidinium isothiocyanate and purified by centrifugation on cesium chloride as described by Sambrook et al. (Molecular Cloning, 2nd ed., CSH Laboratory, 1989, p.7.3).

General procedures for PCR and mixed oligonucleotides primed amplification of cDNA will be those described by Lee and Caskey (in PCR Protocols, Academic Press 1990, p.46.).

7. Cloning procedures

Methods for Preparation of libraries and cloning will be those described by Sambrook et al (Molecular Cloning, 2nd ed., CSH Laboratory, 1989, p.7.3.).

8. Peptide synthesis

Peptides will be synthesized by procedures described by Van Regenmortel et al. (Synthetic Polypeptides as Antigens, Elsevier, 1988). Deprotection and coupling will be performed in a Model 431-A peptide synthesizer (Applied Biosystems), with F-MOC chemistry.

9. Peptide iodination

Since preliminary data indicates the existence of at least one tyrosine, triatomine will be labeled with ¹²⁵I with "iodo-beads" (Pierce) according to the procedure of Markwell (Anal. Biochem. 125, 427-432).

RESULTS

(compare against the set objectives)

Peptide purification and characterization of its biological effects.

A peptide from hindguts of the *Triatoma* hematophagous Chagas' insect vector activates adenylyl cyclase activity in *Trypanosoma cruzi* epimastigote membranes and stimulates the *in vitro* differentiation of epimastigotes to metacyclic trypomastigotes. Hindguts were obtained from insects fed two days before with chicken blood. Purification was performed by gel filtration and high performance liquid chromatography on C18 and C4 columns. SDS-PAGE of purified peptide showed a single band of about 10 kDa.

Peptide sequencing.

The following sequence for 20 residues of the amino terminus of this peptide was determined: H₂N-Met-Leu-Thr-Ala-Glu-Asp-Lys-Lys-Leu-Ile-Gln-Gln-Ala-Trp-Glu-Lys-Ala-Ala-Ser-His. This sequence is identical to the amino terminus of chicken α^D-globin. After western blotting, the peptide immunoreacted with a polyclonal antibody against chicken globin D.

Synthesis of peptides and characterization of their biological effects.

A synthetic peptide carrying the sequence of the 40 amino acids corresponding to the α^D-globin amino terminus (peptide₁₋₄₀), also stimulated adenylyl cyclase activity and promoted differentiation. These stimulatory effects were observed at peptide concentrations higher than 10⁻¹⁰ M. Dose-response curves for both effects were coincident.

In order to determine with more precision the primary structure of the active region, some other synthetic peptides were tested for their effect on epimastigote adenylyl cyclase. Peptide 30-49 was a little less active than peptide₁₋₄₀. Peptide 35-73 resulted much less active and peptide 41-73 was inactive. However, the latter peptide enhanced the effect of peptide₁₋₄₀ on adenylyl cyclase.

Labeling of peptides and receptor characterization.

The ¹²⁵I-labeled synthetic peptide₁₋₄₀ bound specifically to *T. cruzi* epimastigote cells. Displacements of the labeled ligand by the unlabeled peptide₁₋₄₀ or peptide 30-49 were identical, giving an estimated dissociation constant of 2 x 10⁻⁹ M and about 2000 receptors per cell. Peptide 35-73 and peptide 41-73 were less efficient in the displacement of the labeled ligand.

Preparation of antibodies.

Further support for the authenticity of the globin-derived peptide was obtained by characterization with a rabbit anti-chicken globin D antibody. After western blotting, the hindgut peptide immunoreacted with this antibody.

In vivo and in vitro characterization.

The effects of hemoglobin or synthetic peptides carrying α^D-globin fragments on both the growth and transformation of *T. cruzi* epimastigotes into metacyclic trypomastigotes were studied in the insect vector. Metacyclogenesis did not occur when the insects were fed with plasma. However the differentiation in the insect's gut is expressed when hemoglobin or synthetic

**Work plan and time schedule
(originally envisaged)**

Working plan.

It has been described in previous sections.

Time schedule.

1st. period: Peptide purification and characterization of its biological effects.
Peptide sequencing.

2nd period: Synthesis of peptides and characterization of their biological effects.

Labeling of peptides and receptor characterization.

3th period: Preparation of antibodies. In vivo and in vitro characterization.
Receptor cloning.

peptides corresponding to residues 30-49 and 37-73 of the α D-globin were added to the plasma diet.

Receptor cloning.

Since the receptor for globin-derived peptides activates adenylyl cyclase activity through a Gs protein its structure, like a β -adrenergic receptor should be analogous to those having seven intramembranous-spanning hydrophobic domains.

In order to initiate work on the cloning of sequences coding for the *T. cruzi* receptor, oligonucleotides having sequences corresponding to the 3th intracytoplasmic loop of the human β -adrenergic receptor were prepared. Using such oligonucleotides in an RT PCR four bands were obtained which are in the process of to be sequenced.

Other work done not related with this project.

A nitric oxide synthase was partially purified from soluble extracts of *Trypanosoma cruzi* epimastigote forms. The conversion of L-arginine to citrulline by this enzyme activity required NADPH and was blocked by EGTA. The reaction was activated by Ca^{2+} , calmodulin, tetrahydrobiopterin and FAD, and inhibited by N^{ω} -methyl-L-arginine. L-glutamate and N-methyl-D-aspartate (NMDA) stimulated *in vivo* conversion of L-arginine to citrulline by epimastigote cells. These stimulations could be blocked by EGTA, MK-801 and ketamine and enhanced by glycine. A sodium nitroprusside-activated guanylyl cyclase activity was detected in cell-free, soluble preparations of *Trypanosoma cruzi* epimastigotes. L-glutamate, NMDA and sodium nitroprusside increased epimastigote cyclic GMP levels. MK-801 bound specifically to *T. cruzi* epimastigote cells. This binding was competed by ketamine and enhanced by glycine or L-serine. Evidence thus indicates that in *Trypanosoma cruzi* epimastigotes, L-glutamate controls cyclic GMP levels through a pathway mediated by nitric oxide.

Work plan and time schedule**(actual)**

Project landmarks, duration of individual tasks (use bar charts); evaluation criteria (publications, patents, services, training)

The plan and the schedule followed details and characteristics described in previous sections.

The most important results in these study is the discovery of the mechanisms used by *Trypanosoma cruzi* for the differentiation of epimastigote to trypomastigote forms. This contribution is absolutely new in the field.

The duration of the individual tasks are those indicated in the time schedule.

Three papers in international journals were published and one more is in preparation. Another paper was published related to metabolic regulation in *Trypanosoma cruzi*, but the characteristics of the studies described in this paper were not included in the project proposal.

One graduate student (now Dr in Chemistry) presented its Ph.D. Thesis in the field covered by this project. Three more are working in their Doctoral Thesis.

NETWORKING

- International Centre for Genetic Engineering and Biotechnology (Trieste-Italy).
- Fundacion Oswaldo Cruz (Rio de Janeiro-Brazil).
- Department of Biological Chemistry, University of Buenos Aires School of Pharmacy (Buenos Aires-Argentina).
- Department of Microbiology and Immunology, University of Buenos Aires School of Medicine (Buenos Aires-Argentina).

PUBLICATIONS

- "An α^D -globin fragment from *Triatoma* hindgut stimulates *Trypanosoma cruzi* adenylyl cyclase and promotes metacyclogenesis " Fraidenraich, D., Peña, C., Isola, E., Lammel, E., Coso, O., Diaz Añel, A., Baralle, F., Torres, H.N., and Flawiá, M.M. . *Biol. Res.*, 26, 279-283, 1993.
- "Stimulation of *Trypanosoma cruzi* adenylyl cyclase by and α^D -globin fragment from *Triatoma* hindgut. Effect on differentiation of epimastigote to trypomastigote forms "Fraidenraich, D., Peña, C., Isola, E., Lammel, E., Coso, O., Díaz Añel, A., Pongor, S., Torres, H.N. and Flawiá, M.M. *Proc. Natl. Acad. Sci. U.S.A.*, 90, 10140-10144, 1993.
- "The nitric oxide transduction pathway in *Trypanosoma cruzi*" Paveto, C., Pereira, C., Espinosa, J., Montagna, A.E., Farber, M., Esteva, M., Flawiá, M.M. and Torres, H.N. (1995) *J. Biol. Chem.* in press.
- "Induction of *Trypanosoma cruzi* metacyclogenesis in the gut of the hematophagous insect vector *Rhodnius prolixus* by hemoglobin and peptides carryin α^D -globin sequences" (1995) Garcia, E.S., Gonzalez, M.S., Azambuja, P., Baralle, E.E., Fraidenraich, S., Torres, H.N. and Flawiá, M.M. (1995) *Exptl. Parasitol.* in press.
- "Globin-derived peptides and hemoglobin act as a novel agonist-antagonist system which modulates *Trypanosoma cruzi* adenylyl cyclase" Fraidenraich, B., Baralle, F., Torres, H.N. and Flawiá, M.M. in preparation.
- "Purificación, secuenciación y caracterización de un péptido presente en el intestino de *Triatoma infestans* que estimula la adenilil ciclasa de *Trypanosoma cruzi* y promueve la metaciclógénesis" (1994) Fraidenraich und Waisman, D., *Doctoral Thesis in Chemistry, University of Buenos Aires, School of Sciences.*

STATEMENT OF EXPENDITURES

To be filled by ICGEB		To be filled by the Affiliated Centre	
Budgets as per original proposal		Summary of expenditures *	
1) Capital equipment	US\$ 6.032.-	1) Capital equipment	US\$
2) consumables	US\$ 40.737,68.	2) consumables	US\$
3) training	US\$--	3) training	US\$
4) literature	US\$ 1.297,85.	4) literature	US\$
5) miscellaneous	US\$ 6.071,95.	5) miscellaneous	US\$
TOTAL GRANT	US\$..... 54.139,48.	TOTAL	US\$.....

Please itemize the following budget categories (if applicable)


Capital equipment

E. Machines, Model 1324-XL Futura SX, 24-Bit accelerated nubus video card; Color monitor complete with software and cable; 4 MB SIMMs FOR MAC IICI (16MB total); 2 MB SIMMs FOR MAC IISI (8MB total); 4 MB SIMMs FOR MAC LCII (8MB total); Daystar universal Power Cache Card, 50 MHZ, with 68882 Math coprocessor; C550D II Si Nubus adapter with math coprocessor nº 68882; SCSI Terminators; R0709 SCSI Peripheral cable, 8 FT; R0707 SCSI Peripheral cable, 3 FT.
 Fax Panasonic MF-F700; transformador D y D W100.
 Horno Microondas BGH, Modelo 13300.

Training (provide names, duration of training, host laboratory)

Literature

Reprints: PNAS, Cell, Current Biology.


 Dra. MIRTHA M. FLAWIA
 INVESTIGADORA PRINCIPAL
 INGENI CONICET

* Please do not send invoices, receipts etc.; these should be kept by the Affiliated Centre for future reference and sent to ICGEB upon request.

** except for invoices that are required in connexion with paragraph 5. of the Contract.

An α^D -globin fragment from *Triatoma infestans* hindgut stimulates *Trypanosoma cruzi* adenylyl cyclase and promotes metacyclogenesis

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A peptide from hindguts of the Triatoma infestans, the hematophagous Chagas' insect vector, activates adenylyl cyclase activity in Trypanosoma cruzi epimastigote membranes and stimulates the in vitro differentiation of epimastigotes (proliferative and non-infectious forms) to metacyclic trypomastigotes (non-proliferative and infectious forms). The peptide was purified from hindguts of insects fed two days before with chicken blood. After purification, the peptide showed upon SDS-PAGE a single band of about 10 kDa. The sequence for 20 residues of the amino terminus of this peptide was: H₂N-Met-Leu-Thr-Ala-Glu-Asp-Lys-Lys-Leu-Ile-Gln-Gln-Ala-Trp-Glu-Lys-Ala-Ala-Ser-His. This sequence corresponds to the amino terminus of chicken α^D -globin. A synthetic peptide with the sequence of the 40 amino acids corresponding to the amino terminus of α^D -globin, also stimulated T. cruzi adenylyl cyclase activity and promoted metacyclogenesis.

INTRODUCTION

Trypanosoma cruzi, the etiological agent of the Chagas' disease, undergoes complex morphological changes throughout its life cycle in both the insect vector and the vertebrate host. Metacyclogenesis is the differentiation process that occurs within the hindgut of the hematophagous *Triatoma infestans* insect vector. This process converts *T. cruzi* epimastigotes, which are proliferative but not infectious, to metacyclic trypomastigotes, the infectious and non-proliferative parasite forms (Brenner, 1973; De Souza, 1984).

Metacyclogenesis can be induced in liquid cultures under a variety of conditions. They include the presence of mammalian serum components (Dusanik, 1980), the use of medium mimicking insect urine (Contreras *et al.*, 1985), and metabolic stress (Castellani *et al.*, 1967) or by growing epimastigotes with *Triatoma infestans* hindgut extracts (Isola *et al.*, 1981, 1986).

Studies on the mechanism that causes *T. cruzi* differentiation revealed that metacyclic trypomastigotes have higher intracellular cyclic AMP levels than epimastigotes (Rangel-Aldao *et al.*, 1987). Other findings showed that

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addition of exogenous cyclic AMP or its analogs to epimastigote cultures causes metacyclogenesis (González-Perdomo *et al.*, 1988). Moreover, our evidences showed that *T. cruzi* epimastigote membranes possess adenylyl cyclase associated to G α_5 subunits (Eisenschlos *et al.*, 1986) and that G α_4 and G β polypeptides are also located in these membranes (Coso *et al.*, 1992).

A basic question arising from these findings is whether a molecule present in *Triatoma infestans* hindgut might be capable to activate epimastigote adenylyl cyclase and thereby to cause metacyclogenesis. Here it is reported that a globin-derived peptide purified from hindgut extracts is responsible of *T. cruzi* epimastigote adenylyl cyclase activation and metacyclogenesis.

METHODS

Parasite and Insect Strains. *Trypanosoma cruzi* epimastigotes of Tulahuén and RA strains were maintained by weekly transfers in bi-phasic medium and harvested during the exponential growth period (Isola *et al.*, 1981, 1986). Cell cultures had less than 1% of metacyclic forms.

Non-infected *Triatoma infestans* in the fifth or adult stages were reared at 28°C and 60% relative humidity and weekly blood fed on chickens. Hindguts were processed 2 days after blood feeding.

Membrane Preparation. *T. cruzi* epimastigote membranes were prepared according to Torruella *et al.* (1986).

Factor Purification from Hindgut Homogenates. Hindguts were homogenized in phosphate buffer/saline (5 ml per g) and centrifuged for 15 min at 10,000 g. Aliquots (10 ml) of the supernatant ("crude extract") were passed through a Sephadex G-25 (medium) column (3.5 x 50 cm), equilibrated with 50 mM Tris-HCl buffer, pH 7.5. Fractions that activate adenylyl cyclase in epimastigote membranes, designated "Sephadex preparation", were pooled and passed through Sep-Pak C₁₈ cartridges (Waters, Milford, MA, U.S.A.; 10 ml per cartridge) equilibrated with water. Each cartridge was washed with 5 ml of water followed by 5 ml of 15% acetonitrile (v:v) in water and subsequently eluted with 10 ml of 50% acetonitrile (v:v) in water. "Sep-

Pak C₁₈ eluates" were pooled and vacuum-concentrated to dryness. The material corresponding to 400 hindguts was resuspended in 0.5 ml of water and further purified by reverse phase C₁₈ and C₄ HPLC as described elsewhere (Fraidenaich, Peña, Isola, Lammel, Coso, Díaz-Afel, Baralle, Pongor, Torres, Flawiá, manuscript in preparation).

Peptide Sequencing. Amino terminal amino acid sequence was determined, from about 250 pmol peptide, by automated Edman degradation using a protein microsequencer Model 477A (Applied Biosystems International).

Peptide Synthesis. Solid-phase peptide synthesis was performed on an Applied Biosystems International automated synthesizer Model 431A, version 1.12, using the Fmoc chemistry (Carpino and Han, 1972) and a two-step deprotection protocol (Tam *et al.*, 1983). Before use, peptide solutions were purified by passage through Sep-Pak C₁₈ cartridges.

Differentiation Assay. Metacyclogenesis was evaluated as described by Isola *et al.* (1981, 1986) in cultures made in modified Grace medium (Junker *et al.*, 1967) supplemented with fractions purified from hindgut homogenates.

Adenylyl Cyclase Assay. Incubations mixtures contained 50 mM Tris-HCl buffer, pH 7.4, 0.2 mM 3-isobutyl-1-methylxanthine, 1 mM cyclic AMP, 1.5 mM MgCl₂, 0.8 mM $\alpha^{32}\text{P}$ -ATP (specific activity, 200 dpm/pmol), 20 μM GTP, 2 mM phosphocreatine, 0.2 mg of creatine kinase, and about 200 mg protein of *T. cruzi* epimastigote membranes plus the indicated additions. The final volume was 0.1 ml. Incubations were performed at 37°C for 10 min on triplicate samples, which were processed as described elsewhere (Torruella *et al.*, 1986).

Protein Sequences. Amino acid sequences of hemoglobin chains were obtained from *Entrez Document Retrieval Software* (Pre-release 6, 1992), National Center for Biotechnology Information, National Institutes of Health, Bethesda, MA, USA.

Other Analytical Methods. Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE) of the gut peptide

was performed according to Laemmli (1970) in 22% acrylamide gels (w:v). Gels were stained with ammoniacal silver nitrate with the procedure of Morrissey (1980) as described by Ausbel *et al.* (1989).

RESULTS

Peptide Characterization and Purification. As it is shown in Table I, crude preparations from hindguts of *Triatoma infestans*, fed two days before with chicken blood, activated adenylyl cyclase in *Trypanosoma cruzi* epimastigote membranes. These preparations also stimulated the differentiation of epimastigotes to metacyclic trypomastigotes. Adenylyl cyclase activation and differentiation were not observed in "Sep-Pak C₁₈ eluates" from a "Sephadex preparation" which have been subjected to a protease treatment. On the other hand, activation and differentiation were maximal after two days of blood feeding and decreased in subsequent days (result not shown). Table I also shows that a preparation from hindguts of insect vectors fed with mouse blood also activated adenylyl cyclase and promoted differentiation.

Purification of the putative peptide factor was further performed by reverse phase chromatography on Sep-Pak C₁₈ cartridges and high performance liquid chromatography on C₁₈ and C₄ columns. A compound with spectral characteristics of a peptide was isolated which activated adenylyl cyclase and stimulated differentiation of the parasite (Table I). Upon SDS-PAGE the peptide showed a single band of about 10 kDa.

The following sequence for 20 residues of the amino terminus of the peptide was determined: NH₂-Met-Leu-Thr-Ala-Glu-Asp-Lys-Lys-Leu-Ile-Gln-Gln-Ala-Trp-Glu-Lys-Ala-Ala-Ser-His, which is identical to the amino terminus of chicken α^D -globin (Dodgson *et al.*, 1981), the minor component of adult chicken α -globin chains (Fig. 1).

Further support for the authenticity of the globin-derived peptide was obtained by assaying a synthetic peptide carrying residues 1 to 40 of chicken α^D -globin (peptide₁₋₄₀). This peptide stimulated adenylyl cyclase activity in *T. cruzi* epimastigote membranes and promoted *in vitro* parasite differentiation. These stimulatory effects were observed at peptide concentrations higher than 10⁻¹⁰ M.

TABLE I

Effects of *Triatoma infestans* hindgut preparations on *T. cruzi* adenylyl cyclase and differentiation. Except for the experiments indicated in the last two rows, all the results correspond to preparations from insects fed with chicken blood

Additions to the Assay	Adenylyl Cyclase pmol/min per mg protein	Differentiation %
None	22 ± 2	4 ± 2
"Crude extract" ¹	40 ± 3	75 ± 10
"Sephadex preparation" ²	74 ± 4	71 ± 9
"Sep-Pak C ₁₈ eluate" ³	78 ± 5	75 ± 2
"Sep-Pak C ₁₈ eluate" ⁴	26 ± 2	4 ± 2
"C ₁₈ HPLC fraction" ⁵	50 ± 3	55 ± 7
"C ₄ HPLC fraction" ⁶	53 ± 3	60 ± 8
10 ⁻⁹ M peptide ₁₋₄₀	36 ± 2	65 ± 7
"Crude extract" from mouse blood-fed insects ¹	55 ± 3	70 ± 8

¹5.0 A₂₈₀; ²2.0 A₂₈₀; ³0.1 A₂₈₀; ⁴0.1 A₂₈₀ from a "Crude preparation" treated with 0.15 mg ml⁻¹ proteinase K for 30 min at 30° C; ⁵0.02 A₂₈₀; and ⁶0.01 A₂₈₀.

Aliquots of 10 μ l of each fraction were used in all assays

	10	20	30	40	50
C α ^D	MLTAEDKKLI	QQAWKAASH	QEEFGAEALT	RMFTTYPQTK	TYFPHFDSLH
C α ^A	MV-SAA--NNV	KGIPT-IAG-	AE-Y---T-E	---TTY-P--	-----L-P
M α	MV-SGE--SNI	KAANG-IGG-	GA-Y---A-E	---ASF-T--	-----V-H
	60	70	80	90	100
C α ^D	GSDQVRGHGK	KVLGALGNAV	KNVDNLSQAM	AELSNLHAYN	LRVDPVNFKL
C α ^A	--A-IK-----	--VA--IE-A	NHI-DIAGTL	SK--D---HK	-----
M α	--A-VK-----	--AD--AS-A	GHL-DLPGAL	SA--D---HK	-----
	110	120	130	140	
C α ^D	LSQCIQVVLA	VHM GKDYTPE	VHA AFDKFLS	AVSAVLAEKY	R
C α ^A	-GQ-FL-VVA	I-HPAAL--E	---SL----C	A-GT--TA--	-
M α	-SH-LL-TLA	S-HPADF--A	---SL----A	S-ST--TS--	-

C α ^D: Chicken α ^D globin chain (141 aa)

C α ^A: Chicken α ^A globin chain (142 aa)

M α : Mouse α globin chain (142 aa)

Fig. 1: Amino acid sequences of chicken and mouse α -globin chains.

DISCUSSION

Results here reported indicate that a peptide which naturally exists in *Triatoma infestans* Chagas' vector hindgut, fed with chicken blood, causes activation of *Trypanosoma cruzi* epimastigote adenyl cyclase and stimulation of metacyclogenesis. The peptide is a breakdown product of α ^D-globin, a minor component of chicken α -globin chains.

A model for *T. cruzi* differentiation can be postulated, taking in consideration these results. Upon binding to a specific receptor present in epimastigote membranes, the peptide ligand causes adenyl cyclase activation through a transduction process that may involve a G_s protein. Thereafter, the rise of intracellular cyclic AMP levels activates protein kinase A which might determine the phosphorylation of specific protein targets involved in the differentiation of epimastigotes to trypomastigotes. Evidences for such a mechanism are supported by the finding in this laboratory of protein kinase A in *T. cruzi* epimastigotes which is similar to type II bovine heart cyclic AMP-dependent protein kinase (Ulloa *et al.*, 1988).

The model here presented is based on the use of insect vectors fed with chicken blood. However, hindgut extracts from insects fed with mouse blood also stimulated epimastigote adenyl cyclase and promoted differentiation.

This could indicate that other fragments from a variety of globin species might have a similar effect.

As it is shown in Fig. 1 globin α chains from chicken and mouse have extensive amino acid homologies (Dodgson *et al.*, 1981; Goodman *et al.*, 1983; Popp *et al.*, 1982). Considering the results obtained with the synthetic peptide (Table I) and accepting that *T. cruzi* has only one receptor entity, specific for a common domain shared by several globin chain species, it could be postulated that this receptor should be specific for peptides having sequences of a globin α chain domain.

These facts are relevant in the pathogenesis of the parasite infection. In an animal host, the risk for a dissemination of infective forms through the whole organism, might be parallel with the capacity to generate globin proteolytic fragments having such domain. In mammals, this function is displayed by macrophages, which constitute the first cellular step for both, hemoglobin degradation and *T. cruzi* infection.

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Stimulation of *Trypanosoma cruzi* adenylyl cyclase by an α^D -globin fragment from *Triatoma* hindgut: Effect on differentiation of epimastigote to trypomastigote forms

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ABSTRACT A peptide from hindguts of the *Triatoma* hematophagous Chagas insect vector activates adenylyl cyclase activity in *Trypanosoma cruzi* epimastigote membranes and stimulates the *in vitro* differentiation of epimastigotes to metacyclic trypomastigotes. Hindguts were obtained from insects fed 2 days earlier with chicken blood. Purification was performed by gel filtration and HPLC on C₁₈ and C₄ columns. SDS/PAGE of the purified peptide showed a single band of about 10 kDa. The following sequence was determined for the 20 amino-terminal residues of this peptide: H₂N-Met-Leu-Thr-Ala-Glu-Asp-Lys-Lys-Leu-Ile-Gln-Gln-Ala-Trp-Glu-Lys-Ala-Ala-Ser-His. This sequence is identical to the amino terminus of chicken α^D -globin. On a Western blot, the peptide immunoreacted with a polyclonal antibody against chicken globin D. A synthetic peptide corresponding to residues 1-40 of the α^D -globin amino terminus also stimulated adenylyl cyclase activity and promoted differentiation. This ¹²⁵I-labeled synthetic peptide bound specifically to *T. cruzi* epimastigote cells. Activation of epimastigote adenylyl cyclase by the hemoglobin-derived peptide may play an important role in *T. cruzi* differentiation and consequently in the transmission of Chagas disease.

A major endemic in Latin America is Chagas disease. Its etiological agent is *Trypanosoma cruzi*, a flagellate protozoan that undergoes complex morphological changes throughout its life cycle in both the insect vector and the vertebrate host (1, 2). The hematophagous *Triatoma* vector ingests circulating trypomastigote forms while feeding on the blood of an infected vertebrate host. In the insect digestive tract, ingested trypomastigotes initially differentiate to epimastigotes; in a second stage, which occurs within the vector hindgut, epimastigotes that are proliferative but not infectious convert to metacyclic trypomastigotes. The latter are infectious and nonproliferative parasite forms (3-5). This differentiation process, known as metacyclogenesis, can be induced in epimastigote liquid cultures by mammalian serum components (6), the use of medium mimicking insect urine (7), metabolic stress (8, 9), *Triatoma infestans* hindgut extracts (10, 11), or cyclic AMP (12).

In addition, metacyclic trypomastigotes exhibit higher intracellular cyclic AMP levels than epimastigotes (13), and evidence from this laboratory has shown that *T. cruzi* membranes possess an adenylyl cyclase associated with the α subunit of the stimulatory guanine nucleotide-binding regulatory protein (G protein) (14). G α_1 and G β polypeptides were also found in these membranes (15).

A basic question arising from these findings is whether a molecule present in *Triatoma* hindgut might be capable of activating epimastigote adenylyl cyclase and thereby causing parasite differentiation.

Here we present evidence showing that a hemoglobin-derived peptide purified from hindgut extracts is responsible for *T. cruzi* epimastigote adenylyl cyclase activation and metacyclogenesis.

MATERIALS AND METHODS

Parasite and Insect Strains. *T. cruzi* epimastigotes of the Tulahuén or RA strain were maintained and harvested as indicated (10, 11).

Noninfected *T. infestans* in the fifth or adult stage were reared at 28°C and 60% relative humidity and were given chickens or mice weekly from which they sucked blood. Hindguts were processed 2 days after feeding.

Membrane Preparation. *T. cruzi* epimastigote membranes were prepared according to Torruella *et al.* (16).

Factor Purification from Hindgut Homogenates. Hindguts were removed and homogenized in phosphate-buffered saline (5 ml/g of tissue). After centrifugation for 15 min at 10,000 \times g, 10-ml aliquots of the supernate ("crude extract") were passed through a Sephadex G-25 (medium) column (3.5 \times 50 cm) and equilibrated with 50 mM Tris-HCl (pH 7.5). Fractions that activated adenylyl cyclase in epimastigote membranes, designated "Sephadex preparation," were pooled and passed through Sep-Pak C₁₈ cartridges (Waters; 10 ml per cartridge) equilibrated with water. Each cartridge was washed with 5 ml of water followed by 5 ml of 15% (vol/vol) acetonitrile in water; subsequently, the factor was eluted with 10 ml of 50% acetonitrile in water. "Sep-Pak C₁₈ eluates" were pooled, and solvent was removed under reduced pressure. The material corresponding to 400 hindguts was resuspended in 0.5 ml of water and further purified by reverse-phase C₁₈ HPLC (Vydac column; 10.0 \times 250 mm; 300 Å pore size). Elution was performed with an acetonitrile gradient from 12% to 56% in water at a flow rate of 2.0 ml/min (see Fig. 1A). The peak fraction eluting at 47% acetonitrile was lyophilized, resuspended in 1 ml of water ("C₁₈ HPLC fraction"), and further purified by reverse-phase C₄ HPLC (Vydac column; 4.6 \times 250 mm; 300 Å pore size). Elution was performed with a gradient of 0% to 40% acetonitrile in water

Abbreviation: G protein, guanine nucleotide-binding regulatory protein.

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at a flow rate of 1.0 ml/min (see Fig. 1B). The peak fraction eluting at about 28% acetonitrile was lyophilized, resuspended in 1 ml of water ("C₄ HPLC fraction"), and subjected to further studies. The amount of active material in the different fractions was expressed as its absorbance at 280 nm (A₂₈₀).

Peptide Sequencing. The hindgut peptide (250 pmol) was subjected to amino-terminal amino acid sequencing by automated Edman degradation using a model 477A protein microsequencer (Applied Biosystems) according to Hewick *et al.* (17).

Peptide Synthesis. Solid-phase peptide synthesis was performed on an Applied Biosystems automated synthesizer (model 431A, version 1.12) using Fmoc chemistry (18). Before use, peptide solutions were purified by passage through Sep-Pak C₁₈ cartridges.

Differentiation Assay. Metacyclogenesis was evaluated as described by Isola *et al.* (10, 11). Briefly, cultures in modified Grace's medium (19) supplemented with fractions purified from hindgut homogenates and inoculated with 4 × 10⁶ epimastigotes per ml were incubated at 28°C, and the percentage of flagellates that differentiated to the metacyclic stage was determined on at least 200 forms in wet preparations and in slides stained with May-Grünwald/Giemsa. Cultures were examined every day, and observations were performed in quintuplicate.

Adenylyl Cyclase Assay. The standard incubation mixture contained 50 mM Tris-HCl (pH 7.4), 0.2 mM 3-isobutyl-1-methylxanthine, 1 mM cyclic AMP, 1.5 mM MgCl₂, 0.8 mM [α -³²P]ATP (specific activity, 200 dpm/pmol), 20 μ M GTP, 2 mM phosphocreatine, 0.2 mg of creatine kinase, and about 200 μ g of protein from *T. cruzi* epimastigote membranes plus the indicated additions. The final volume was 0.1 ml. Incubations were performed at 37°C for 10 min on triplicate samples. Reactions were stopped, and cyclic AMP was purified and counted for radioactivity as described by Solomon *et al.* (20). Under these conditions, the amount of product formed was proportional to the amount of *T. cruzi* membranes and the incubation time.

¹²⁵I Labeling of the Peptide and Binding to Epimastigote Cells. The 40-mer synthetic peptide was labeled with chloramine T following the procedure described by Greenwood (21) with the modifications indicated by Molinolo *et al.* (22).

The incubation mixture for the binding assay contained 50 mM phosphate buffer (pH 7.4), 0.2% albumin, 0.4 pmol of labeled peptide (specific activity 200 μ Ci/ μ g; 1 Ci = 37 GBq), various concentrations of unlabeled peptide, and about 2 × 10⁶ epimastigote cells. The total volume was 0.3 ml. Incubations were performed for 90 min at room temperature. The bound peptide was separated by filtration through glass fiber filters (Whatman GF-C). Nonspecific binding was determined in the presence of 0.5 μ g of unlabeled peptide. The binding constant was calculated according to Cuatrecasas and Hollenberg (23).

Preparation of Globin D and Antibody. Globin D was prepared from chicken adult red cell lysate by chromatography on CM-Sephadex and precipitation with acetone/hydrochloric acid as described by Brown and Ingram (24). Rabbit antiserum was also prepared according to ref. 24.

Other Analytical Methods. The gut peptide was electrophoresed in 22% polyacrylamide gels in the presence of SDS and stained with silver nitrate (25). Western blots were blocked with nonfat milk and incubated with a 1:500 dilution of rabbit antiserum. The immune complexes were detected with the Vectastain ABC-AP kit (Vector Laboratories).

RESULTS

Peptide Characterization and Purification. Preparations from hindguts of *T. infestans*, fed 2 days before with chicken

blood, activated adenylyl cyclase in *T. cruzi* epimastigote membranes. The effect was blocked by guanosine 5'-[β -thio]diphosphate, in either the presence or absence of GTP or 5'-guanylyl imidodiphosphate. These "crude preparations" also stimulated the differentiation of epimastigotes to metacyclic trypomastigotes. Adenylyl cyclase activation and differentiation were not observed in Sep-Pak C₁₈ eluates from a Sephadex preparation that had been subjected to protease treatment (Table 1). On the other hand, activation and differentiation were maximal after 2 days of blood feeding and decreased in subsequent days (results not shown). Table 1 also shows that a preparation from hindguts of insect vectors fed with mouse blood also activated adenylyl cyclase and promoted differentiation.

Purification of the putative peptidic factor was further performed by reverse-phase chromatography on Sep-Pak C₁₈ cartridges and HPLC on C₁₈ (Fig. 1A) and C₄ columns (Fig. 1B). After purification, the compound that activated adenylyl cyclase and stimulated differentiation of the parasite ran as a single band of about 10 kDa on SDS/PAGE (Fig. 2, lane B).

The following sequence for 20 residues of the amino terminus of the peptide was determined: NH₂-Met-Leu-Thr-Ala-Glu-Asp-Lys-Lys-Leu-Ile-Gln-Gln-Ala-Trp-Glu-Lys-Ala-Ala-Ser-His. Computer analysis of this sequence unequivocally showed that it was identical to the amino terminus of chicken α^D -globin (26), the minor component of adult chicken α -globin chains (Fig. 3).

Further support for the authenticity of the globin-derived peptide was obtained by characterization with a rabbit anti-chicken globin D antibody. On a Western blot, the hindgut peptide immunoreacted with this antibody (Fig. 2, lane C).

For practical purposes the peptide was designated GDF (for globin-derived factor).

Table 1. Effects of *Triatoma* hindgut preparations on *T. cruzi* adenylyl cyclase and differentiation

Addition(s)	A ₂₈₀ *	Adenylyl cyclase, pmol per min per mg of protein	% differentiation
None		26 ± 2	4 ± 2
20 μ M GTP		24 ± 2	ND
20 μ M Gpp[NH]p		24 ± 2	ND
Crude extract	5.0	40 ± 3	75 ± 10
Sephadex prep	2.0	74 ± 4	71 ± 9
Sephadex prep + 20 μ M GTP	2.0	78 ± 4	ND
Sephadex prep + 20 μ M Gpp[NH]p	2.0	75 ± 4	ND
Sephadex prep + 20 μ M GTP + 100 μ M GDP[β -S]	2.0	26 ± 4	ND
Sep-Pak C ₁₈ eluate	0.1	78 ± 5	75 ± 2
Sep-Pak C ₁₈ eluate†	0.1	26 ± 2	4 ± 2
C ₁₈ HPLC fraction	0.02	50 ± 3	55 ± 7
C ₄ HPLC fraction	0.01	53 ± 3	60 ± 8
10 ⁻⁸ M chicken hemoglobin		28 ± 3	5 ± 3
Crude extract from mouse blood-fed insects	5.0	55 ± 3	70 ± 8

Except for the experiment in the last row, all the results correspond to preparations from insects fed chicken blood. ND, not done. Gpp[NH]p, 5'-guanylyl imidodiphosphate; GDP[β -S], guanosine 5'-[β -thio]diphosphate. Ten-microliter aliquots of each fraction were used in all assays.

*Of the fraction added.

†The crude preparation was treated with proteinase K (0.15 mg/ml) for 30 min at 30°C.

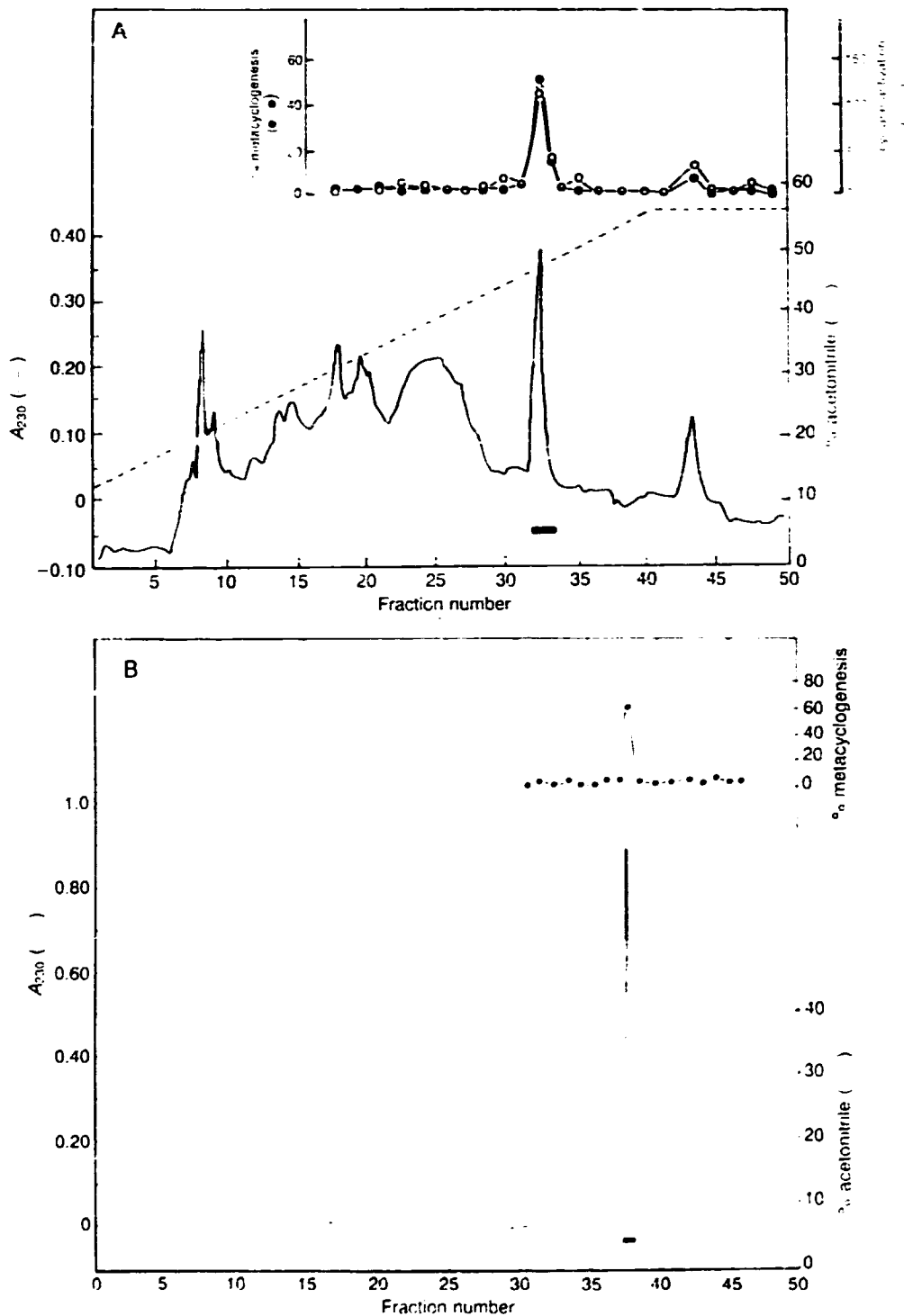


Fig. 1. Purification and characterization of the hindgut peptide. Elution profiles from C_{18} (A) and C_4 (B) HPLC columns are shown.

Biological Activity of Synthetic Peptides. A peptide corresponding to residues 1–40 of chicken α^D -globin was synthesized [peptide-(1–40)]. This peptide stimulated adenylyl cyclase activity in *T. cruzi* epimastigote membranes and promoted *in vitro* parasite differentiation. These stimulatory effects were observed at peptide concentrations higher than 10^{-10} M. Dose-response curves for both effects were coincident (Fig. 4).

To determine with more precision the primary structure of the active region, other synthetic peptides were tested for their effect on epimastigote adenylyl cyclase. As shown in

Fig. 5, peptide-(30–49) was a little less active than peptide-(1–40). Peptide-(35–73) resulted in much less activity, and peptide-(41–73) was inactive. However, the latter peptide enhanced the effect of peptide-(1–40) on adenylyl cyclase.

It is important to point out that chicken hemoglobin was unable to activate epimastigote adenylyl cyclase or to promote differentiation (Table 1).

The 125 I-labeled synthetic peptide-(1–40) bound specifically to *T. cruzi* epimastigote cells. Displacements of the labeled ligand by unlabeled peptide-(1–40) or peptide-(30–49) were identical, giving an estimated dissociation constant of

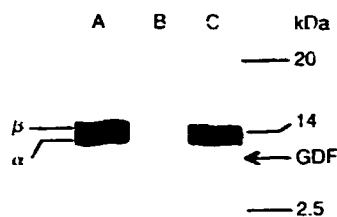


FIG. 2. SDS/PAGE of hindgut fractions. Silver-stained gels of the crude extract (lane A) and C₄ HPLC fraction (lane B) are shown. Lane C shows a Western blot of the crude extract incubated with the anti-globin D antibody. α and β globin chains.

2×10^{-9} M and about 2000 receptors per cell. Peptide-(35-73) and peptide-(41-73) were less efficient in the displacement of the labeled ligand (Fig. 6).

DISCUSSION

Results reported here indicate that a peptide, designated GDF, which naturally exists in hindguts of *Triatoma* fed with chicken blood, causes activation of *T. cruzi* epimastigote adenylyl cyclase and stimulation of metacyclogenesis. The peptide is a breakdown product of α^D -globin, a minor component of chicken α -globin chains.

A possible model for *T. cruzi* differentiation can be postulated. The globin fragment, upon binding to a specific receptor present in epimastigote membranes, causes adenylyl cyclase activation. A G_s protein is probably involved in the signal transduction pathway responsible for the rise of intracellular cyclic AMP levels. Thereafter, the cyclic nucleotide causes protein kinase A activation, which might determine the phosphorylation of specific protein targets involved in the differentiation of epimastigotes to trypomastigotes. Evidence for such a mechanism is supported by the finding in this laboratory of protein kinase A in *T. cruzi* epimastigotes, which is similar to type II bovine heart cyclic AMP-dependent protein kinase (27).

In addition to this, *T. cruzi* transduction pathways might also involve a protein kinase C whose existence in epimastigotes was also demonstrated in this laboratory (28). These facts raise the possibility that Trypanosomatidae differenti-

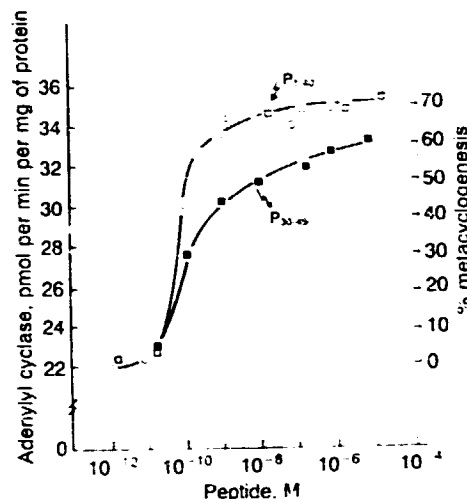


FIG. 4. Stimulation of epimastigote adenylyl cyclase and metacyclogenesis by synthetic peptides (P). \square and \blacksquare . Adenylyl cyclase activity as a function of peptide-(1-40) and peptide-(30-49), respectively; \dots metacyclogenesis as a function of peptide-(1-40) concentration.

ation and proliferation can be controlled by transduction mechanisms resembling those occurring in mammalian cells.

The model presented here is based on the use of insect vectors fed with chicken blood. However, hindgut extracts from insects fed with mouse blood also stimulated epimastigote adenylyl cyclase and promoted differentiation. This could indicate that other fragments from a variety of globin species might have a similar effect. Thus, *T. cruzi* could have several receptors with different specificity for globin-derived peptides, or alternatively, only one receptor specific for a common domain shared by several α -globin chain species.

As shown in Fig. 3, α -globin chains from at least three vertebrate species, chicken, mouse, and human, have extensive amino acid homologies (26, 29, 30). Considering the results obtained with synthetic peptides (Figs. 5 and 6) and accepting that *T. cruzi* has only one receptor entity, it could be postulated that this receptor is specific for peptides having sequences of the α -globin chain domain between positions 30/31 and 62.

These facts are relevant in the pathogenesis of the parasite infection. In an animal host, the risk for a dissemination of infective forms through the whole organism might be parallel to the capacity to generate globin proteolytic fragments having such a domain. In mammals, this function is displayed

	10	20	10	40	50
C _r D	MLTAEDKLI	QQAWEKAASH	QEEFGAEALT	RMFTTYPQTK	TYPPHPDLSH
C _r A	MV-SAA--NNV	KGIFT-IAG-	AE-Y---T-E	---TTY-P--	-----L-P
H _t	MV-SPA--TNV	KAANG-VGA-	AG-Y---A-E	---LSF-T-	-----L-H
M _t	MV-SGE--SNI	KAANG-IGG-	GA-Y---A-E	---ASF-T-	-----V-H
	60	70	80	90	100
C _r D	GSDQVRGHGK	KVLCALGNAV	KNVDNLSQAM	AELSNLHAYN	LRVDPVNFKL
C _r A	--A-IK----	--VA--IE-A	NHI-DIAGTL	SK--D---HK	-----
H _t	--A-VK----	--AD--TN-V	AHV-DMPNAL	SA--D---HK	-----
M _t	--A-VK----	--AD--AS-A	GHL-DLPGAL	SA--D---HK	-----
	110	120	130	140	
C _r D	LSQC1QVVLA	VHMGKDYTPE	VHAAPDKFLS	AVSAVLAEKY	R
C _r A	-GQ-FL-VVA	I-HPAAL--E	---SL----C	A-GT--TA--	-
H _t	-SH-LL-TLA	A-LPAEF--A	---SL----A	S-ST--TS--	-
M _t	-SH-LL-TLA	S-HPADF--A	---SL----A	S-ST--TS--	-

FIG. 3. Amino acid sequences of chicken α^D (C_rD; 141 aa), chicken α^A (C_rA; 142 aa), mouse α (M_t; 142 aa), and human α (H_t; 142 aa) globin chains.

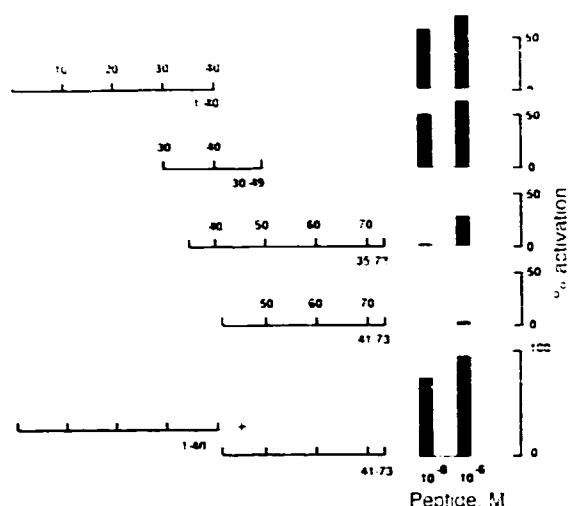


FIG. 5. Effect of synthetic peptides on epimastigote adenyl cyclase activity. Basal adenyl cyclase activity was 22 pmol per min per mg of protein.

by macrophages, which constitute the first cellular step for both hemoglobin degradation and *T. cruzi* infection.

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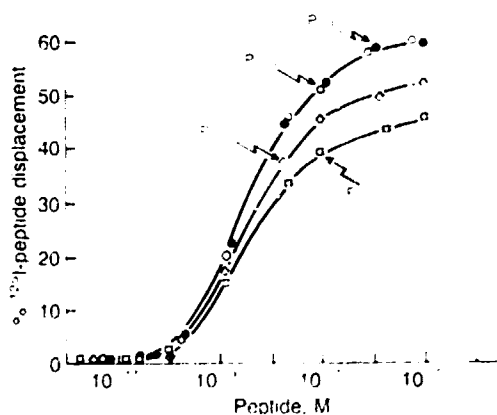


FIG. 6. Competition of ^{125}I -labeled peptide-(1-40) binding to epimastigote cells by unlabeled peptides (P).

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**The nitric oxide transduction pathway in
*Trypanosoma cruzi***

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FOOTNOTES

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RUNNING TITLE

Trypanosoma cruzi NO synthase.

ABSTRACT

A nitric oxide synthase was partially purified from soluble extracts of *Trypanosoma cruzi* epimastigote forms. The conversion of L-arginine to citrulline by this enzyme activity required NADPH and was blocked by EGTA. The reaction was activated by Ca^{2+} , calmodulin, tetrahydrobiopterin and FAD, and inhibited by N^ω-methyl-L-arginine. L-glutamate and N-methyl-D-aspartate (NMDA) stimulated *in vivo* conversion of L-arginine to citrulline by epimastigote cells. These stimulations could be blocked by EGTA, MK-801 and ketamine and enhanced by glycine. A sodium nitroprusside-activated guanylyl cyclase activity was detected in cell-free, soluble preparations of *Trypanosoma cruzi* epimastigotes. L-glutamate, NMDA and sodium nitroprusside increased epimastigote cyclic GMP levels. MK-801 bound specifically to *T. cruzi* epimastigote cells. This binding was competed by ketamine and enhanced by glycine or L-serine. Evidence thus indicates that in *Trypanosoma cruzi* epimastigotes, L-glutamate controls cyclic GMP levels through a pathway mediated by nitric oxide.

In mammalian cells L-arginine is metabolized to yield nitric oxide (NO), also known as endothelium-derived relaxing factor (1, 2). Within the neural or endothelial cells where it originates, or in neighboring cells, NO activates heme-containing soluble guanylyl cyclase (3, 4), thereby acting either as an intracellular or an intercellular signaling molecule. Consequently, NO formation is associated with an increase in cyclic GMP levels (5).

NO synthases are the enzymes responsible for the conversion of L-arginine to NO and citrulline. These enzymes require NADPH and possess binding sites for heme, tetrahydrobiopterin, flavin adenine dinucleotide and flavin adenine mononucleotide. Two groups of isoforms are usually defined for these synthases: constitutive and inducible. NO synthases of the first group, found in endothelium and neurons, are regulated by agonist-induced elevation of intracellular Ca^{2+} (6-9). NO synthases of the second group are induced at the transcriptional level by bacterial toxins and some cytokines and are found in macrophages, vascular smooth muscle cells, fibroblasts and hepatocytes (10-12).

In neural cells, constitutive NO synthase is modulated by the activity of a L-glutamate receptor subtype specific for N-methyl-D-aspartate (NMDA). Receptors of this subtype control the voltage-dependent uptake of Ca^{2+} (13, 14).

From an evolutionary viewpoint, evidence indicates that the NO transduction signaling pathway is operative only in higher eukaryotic organisms. The present studies provide the first demonstration that this pathway is also present in the lower eukaryotic organism *Trypanosoma cruzi*, the ethiological agent of the Chagas' disease. The existence of Ca^{2+} -stimulated NO synthase, a nitroprusside-activated guanylyl cyclase, as well as NMDA receptors in

epimastigote forms of the parasite is demonstrated. In addition, evidence indicating that L-arginine and NMDA increase NO production and cyclic GMP levels in epimastigote cells is also presented.

MATERIALS AND METHODS

Materials

L-[2,3-³H]arginine (53 Ci/mmol), [³H](+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine hydrogen maleate (MK-801; 20 mCi/nmol), [³H](±)-3-(2-carboxypiperazin-4-yl)-propyl-*i*-phosphonic acid (CPP; 40 mCi/nmol), [α-³²P]GTP, [³H]cyclic GMP and a cyclic GMP [¹²⁵I]RIA kit were obtained from NEN/DuPont (Boston, MA, USA). Amino acid analogs were purchased from Research Biochemicals Incorporated (Natick, MA, USA). Components of the *T. cruzi* growth medium were obtained from Difco Laboratories (Detroit, MI, USA) and AG50WX-8 resin from Bio-Rad (Hercules, CA, USA). Other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture and homogenates

Trypanosoma cruzi epimastigote forms (Tulahuen 2 strain) were cultured 7 days at 28°C in a medium containing per liter Bacto Liver, 35 g, tryptose, 10 g, yeast extract, 3 g, glucose, 5 g, Na₂HPO₄, 8 g, NaCl, 4 g, KCl, 0.4 g and hemine 20 mg (15). The pH was adjusted to 7.8. All the components of this medium were autoclaved 15 min at 118°C. Standing cultures were carried out 7 days at 28° up to the late-exponential phase in 1-liter Erlenmeyer flasks containing 100 ml of medium.

Cells were collected by centrifugation at 1000 x g, washed three times with 0.25 M sucrose containing 5 mM KCl, and homogenized in the same solution (10 ml

g⁻¹ of wet cells) with a Sorvall Ribi press operated at 34.5 MPa (5000 lb/ in⁻¹) under a N₂ atmosphere.

Membrane preparation

After cell homogenization, the extract was centrifuged 15 min. at 1000 x g. The membrane pellet was resuspended in 0.25 M sucrose containing 5 mM KCl and layered onto a discontinuous gradient containing 1.58, 1.90 and 2.20 M sucrose. After centrifugation in a Beckman SW-40 rotor for 60 min. at 90,000 x g, membranes were recovered from the interface of 1.58 and 1.90 M sucrose and stored at -70°C.

NO synthase purification

After homogenization, cell debris was discarded by centrifugation at 1000 x g for 10 min. The supernatant fluid, adjusted to 0.5 mM phenylmethylsulfonyl fluoride, 25 units ml⁻¹ aprotinin, 0.01% leupeptin (w:v) and 0.2 mg ml⁻¹ soybean trypsin inhibitor, was further centrifuged 60 min at 105,000 x g. The supernatant fluid, referred to as "soluble crude extract" was immediately processed to avoid proteolytic degradation.

The "soluble crude extract" (25 ml) was loaded onto a DEAE-cellulose column (1 x 10 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride and 25 units ml⁻¹ aprotinin (buffer A). The column was then washed with 50 ml of buffer A and eluted with 100 ml of a linear gradient of 0 to 400 mM NaCl in buffer A. Fractions of 2 ml were collected. The NO synthase activity peak (6 ml) was mixed with 1 ml of 2'5' ADP-agarose slurry equilibrated with 10 mM Tris-HCl buffer containing 1 mM EDTA and 5 mM mercaptoethanol (buffer B). The mixture was shaken 12 h s

at 4°C. The slurry was then poured into a column assembled in a Pasteur pipet, and washed with 25 ml of buffer B containing 0.5 M NaCl followed by 10 ml of buffer B. The column was then eluted with 3 ml of buffer B supplemented with 10 mM NADPH.

NO synthase assay.

Enzyme activity was measured by following the conversion of L-[³H]arginine to [³H]citrulline according to the procedure described by Bredt and Snyder (16). Incubation mixtures contained 50 mM Tris-HCl buffer, pH 7.5, 1 μM L-[³H]arginine (0.2 μCi per assay), 0.1 mM CaCl₂, 10 μM tetrahydrobiopterin, 1 μM FAD, 1 μM NADPH, 1 mM dithiothreitol and 10 μg/ml bovine brain calmodulin in a total volume of 0.1 ml. Incubations were performed 2 min at 25°C and stopped by the addition of 2 ml of ice-cold 20 mM Hepes buffer, pH 5.5, containing 1 mM EDTA. Samples were immediately applied to 2 ml-columns of AG50WX-8 resin (Na⁺ form) and washed with 2 ml of water. Percolate plus wash from each column (4 ml) were mixed with 12.5 ml of Bray's scintillation cocktail and counted for radioactivity. Enzyme activity was proportional to incubation time for the first two minutes, as well as to the amount of "soluble crude extract" protein up to 1 μg per assay.

The authenticity of the radioactive citrulline formed during the reaction was ascertained by comigration with a citrulline standard on a silica gel 60 plate developed with CHCl₃/MeOH/NH₄OH/H₂O (1:4:2:1; vol:vol) according to Iyengar *et al.* (17). Under these conditions, no other radioactive product was detected.

***In vivo* conversion of L-[³H]arginine to [³H]citrulline and production of NO.**

Trypanosoma cruzi epimastigotes were resuspended in Krebs-Henseleit medium, and aliquots (1 ml; about 10⁷ cells) were incubated 45 min at 28°C in the presence of 1 μM L-[³H]arginine (1 μCi per assay). After the addition of the indicated amino acid derivative, incubation was continued for 15 min. Reactions were stopped by the addition of 0.1 ml of ice-cold 70 % trichloroacetic acid (w:v). After 3 cycles of freeze-thawing, mixtures were centrifuged 10 min at 1000 x g and the supernatant solutions extracted 4 times with 4 ml of diethyl ether to eliminate trichloroacetic acid. Aliquots (0.2 ml) of the aqueous phases were then mixed with 2 ml of 20 mM Hepes-NaOH buffer, pH 6.0, and purified by passage through AG50WX8 columns as described above.

NO generation by epimastigote cultures was monitored by the formation of NO₂ using the Griess reagent, as described by Bredt and Snyder (16). In this case, concentration of L-arginine was 1 mM.

Determination of cyclic GMP levels

Incubations of epimastigote cells were performed as described above for 3 min in the presence of the indicated excitatory amino acid. Reactions were then stopped and processed according to this procedure. After extraction with diethyl ether, samples were subjected to acetylation and assayed for cyclic GMP using a radioimmunoassay kit (New England Nuclear) following the instructions of the manufacturer.

Guanylyl cyclase assay

Enzyme activity was assayed following the conversion of [α - 32 P]GTP to [32 P]cyclic GMP. Incubation mixtures contained 50 mM Tris-HCl buffer, pH 7.5, 0.1 mM 3-isobutyl-1-methylxanthine, 1 mM cyclic GMP, 5 mM MgCl₂, 0.1 mM [α - 32 P]GTP (specific activity, 200 cpm/pmol), 2 mM phosphocreatine, 0.2 mg creatine kinase, and appropriate volumes of the enzyme preparation. Assays were performed in the presence or absence of 0.1 mM sodium nitroprusside in a total volume of 0.1 ml. Incubations were performed 5 min at 30°C on triplicate samples. Reactions were stopped by the addition of 0.1 ml of a solution containing 10 mM [3 H]cyclic GMP (1500 cpm/mmol) followed by boiling for 2 min. Cyclic GMP was purified and counted for radioactivity as described by Birnbaumer *et al.* (18). Under these conditions, reactions were proportional to the amount of enzyme protein and incubation time.

Receptor binding assays

Incubation mixtures for the binding assay contained 10 mM Tris-HCl buffer, pH 7.5, 10 nM to 100 μ M [3 H]-labeled ligand (MK-801 or CPP) and approximately 50 μ g of membrane protein or 10⁵ epimastigote cells, in a final volume of 0.1 ml. Incubations were performed 90 min at room temperature. The bound peptide was separated by filtration through nitrocellulose disks (S&S, BA-85). Non-specific binding was determined in the presence of 0.1 mM unlabeled ligand. Binding constants were calculated according to Cuatrecasas and Hollenberg (19).

RESULTS

NO synthase activity in cell-free preparations.

A soluble NO synthase activity was purified and characterized from cell-free extracts of *Trypanosoma cruzi* epimastigotes. The purification protocol, which included ion exchange chromatography on DEAE-cellulose and affinity chromatography on ADP-agarose, was similar to the one employed for the purification of the rat cerebellum enzyme (20). As a result of these steps, enzyme activity was purified about 2000-fold (Table I). NO synthase specific activity in epimastigote soluble extracts was in the same order of magnitude as that found for cerebral tissue (20).

Addition of protease inhibitors to the crude extracts is absolutely necessary to preserve NO synthase activity. The high proteolytic activity, which is characteristic of these extracts, might explain the 5-fold difference in total activity observed between the two first steps of purification (Table I).

Under the assay conditions described by Bredt and Snyder (16) for neural tissues, conversion of [³H]arginine to [³H]citrulline by the purified *T. cruzi* enzyme required NADPH and was blocked by EGTA. Reaction could be stimulated by calmodulin, tetrahydrobiopterin, flavin adenine dinucleotide and flavin adenine mononucleotide (Table II). This NO synthase activity was also blocked by N^ω-monomethyl-L-arginine; half maximal inhibition was observed at about 40 μM of this amino acid derivative. Most of these properties are very similar to those of the neural synthase (20).

Conversion of L-[³H]arginine to [³H]citrulline and production of NO by epimastigote cells.

Table III shows that in epimastigote cells, conversion of L-[³H]arginine to [³H]citrulline is stimulated by L-glutamate and by NMDA. The stimulation was effectively blocked by EGTA and non-competitive NMDA antagonists such as MK-801 and ketamine (21). AP-5, which has been described as a competitive L-glutamate antagonist (22), slightly decreased the effects of this amino acid and NMDA. On the other hand, glycine, which has been reported to be a potentiator of L-glutamate responses at the level of the NMDA receptor (23), enhanced the L-glutamate effect.

The effect of excitatory amino acids was also studied by monitoring the concentration of NO in the incubation medium as accumulation of NO₂⁻. As shown in Table IV, 1 mM L-arginine slightly stimulated NO production. Under such conditions, L-glutamate and NMDA efficiently increased NO₂⁻ accumulation.

Modulation of guanylyl cyclase activity and cyclic GMP levels.

Guanylyl cyclase activity was detected in cell-free preparations from *Trypanosoma cruzi* epimastigotes. Enzyme specific activity in the "soluble crude extract" was about 1 to 2 pmoles/min per mg protein. Specific activity increased approximately 5-fold in the presence of 0.1 mM sodium nitroprusside, which acts as a NO donor.

The effects of amino acids and sodium nitroprusside on cyclic GMP levels was studied in *Trypanosoma cruzi* epimastigote cells. As shown in Table V, glutamate and NMDA were the most potent agents in increasing such levels.

Sodium nitroprusside had a slightly smaller effect, while L-arginine alone was much less active.

Receptor binding studies.

[³H]MK-801 bound specifically to *T. cruzi* epimastigote cells and membranes. Binding could be displaced 95 percent by 0.1 mM unlabeled MK-801 or ketamide. Displacement studies of the labeled ligand by the unlabeled compound gave an estimated dissociation constant of 7×10^{-9} M and about 10^4 receptors per cell. As shown in Table VI, [³H]MK-801 binding was strongly enhanced by glycine and L-serine and only slightly by L-glutamate.

The binding of [³H]CPP to epimastigote cells or membranes was also determined. The compound bound poorly and nonspecifically, making it impossible to determine any binding parameter.

DISCUSSION

Results reported in this article show that *Trypanosoma cruzi* epimastigotes have a NO synthase activity similar to that previously described for mammalian endothelium and nervous tissue (6-9). Enzymes from these tissues show Ca^{2+} and calmodulin dependence.

Some of the excitatory amino acids, well known to affect the conversion of L-arginine to citrulline and NO in neural tissue, also influence the *T. cruzi* NO synthase *in vivo*. Remarkable stimulatory effects of L-glutamate and NMDA could be observed in epimastigote cells, suggesting that *T. cruzi* epimastigotes have L-glutamate (NMDA) receptors of the type described for nervous tissue (13). As occurs in neural cells, *T. cruzi* NMDA receptors should be major entities controlling cytosolic Ca^{2+} levels.

A well known feature of neural NMDA receptors is also found in *T. cruzi* epimastigote membrane receptors. This is the [3H]MK-801 binding capacity, strongly enhanced by glycine and L-serine. It has been postulated that [3H]MK-801 binds within the ion channel of the NMDA receptor (25).

On the other hand, the failure to detect specific binding at the level of the L-glutamate-NMDA site in the receptor may be attributable to the usually very low affinity of this site for ligands such as L-glutamate, NMDA, CPP or AP5 (24).

The NO pathway, controlled through NMDA receptors in neural cells, possesses a heme-containing soluble guanylyl cyclase as its effector. This enzyme can be activated by sodium nitroprusside through the generation of NO (3, 4). This also seems to be the case of *T. cruzi* epimastigotes, since NMDA and excitatory

amino acids such as L-glutamate, that activate NO synthase *in vivo*, also increase intracellular levels of cyclic GMP in epimastigote cells.

It is known that NO generated by macrophages is cytostatic or cytotoxic for a variety of pathogens, including *Trypanosoma brucei* and *Trypanosoma cruzi*. (25, 26). Moreover *T. cruzi* infection in mice increases the capacity of splenic cells to produce NO (27). Obviously, the relationship between the two NO-generating systems, in the parasite and the mammalian cell, remains unknown.

Finally, it is rather suprising that a neural control mechanism such as the long-term potentiation involved in memory (28) has in *Trypanosomatidae* such an old evolutionary precedent. Both cases involve NMDA receptors, a Ca²⁺-calmodulin-dependent NO synthase and a nitroprusside-stimulated guanylyl cyclase. The effectors of such a pathway should be a cyclic GMP-dependent protein kinase and unknown phosphate protein acceptors. The characteristics of these entities in *Trypanosoma cruzi* and their cellular effects require further studies.

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TABLE I

Purification of NO synthase from Trypanosoma cruzi epimastigotes

NO synthase activity was assayed in triplicate samples by following the conversion of L-[³H]arginine to [³H]citrulline as described under Materials and Methods.

Step	Protein (mg)	Total Activity (nmoles)	Yield (%)	Specific Activity (nmoles/min per mg protein)	Purification (fold)
"Soluble crude extract"	125.0	12.5	100	0.1	1
DEAE-cellulose	20.4	60.0	480	3.0	30
ADP-agarose	<0.05	10.0	80	>200.0	>2,000

TABLE II

Factors affecting Trypanosoma cruzi NO synthase activity

Assay conditions were as described under Materials and Methods. Standard errors of the means are indicated. Student's *t* test was used to compare values corresponding to each group (addition or omission) versus to the control (none). P values were <0.01 versus control.

Addition or Omission	NO Synthase activity (%)
None	100±9.4*
2.0 mM EGTA	0
Minus calmodulin	55±5.1
Minus NADPH	0
Minus tetrahydrobiopterin	21±1.3
Minus FAD	59±3.8
Plus 1 mM N ^ω -monomethyl-L-arginine	2±0.4

*"DEAE Preparation", specific activity 3±0.28 nmoles min per mg protein

TABLE III

Modulation by amino acid derivatives of the conversion of L-[³H]arginine to [³H]citrulline by Trypanosoma cruzi epimastigote cells

Assays were performed in triplicate samples as described under Materials and Methods. P<0.01 for 0.1 or 1.0 mM L-glutamate or 0.1 mM NMDA versus the control; P<0.05 for glutamate plus MK-801 (or ketamine or AP5 or glycine), versus control; P<0.05 for NMDA plus EGTA (or MK-801 or ketamine or AP5), versus control.

Compound and concentration (mM)	³ H Citrulline formation	
	(pmoles/min per 10 ⁷ cells)	Stimulation (%)
None	0.18±0.015	0
0.1 mM L-glutamate	0.31±0.017	72
1.0 mM L-glutamate	0.44±0.020	144
1.0 mM L-glutamate + 1.0 mM MK-801	0.18±0.014	0
1.0 mM L-glutamate + 1.0 mM ketamine	0.26±0.013	44
1.0 mM L-glutamate + 1.0 mM AP5	0.40±0.015	122
1.0 mM L-glutamate +	0.51±0.022	182

1.0 mM glycine		
0.1 mM NMDA	0.29±0.011	61
1.0 mM NMDA	0.43±0.021	142
1.0 mM NMDA + 1.0 mM EGTA	0.23±0.017	31
1.0 mM NMDA + 1.0 mM MK-801	0.22±0.011	22
1.0 mM NMDA + 1.0 mM ketamine	0.25±0.012	38
1.0 mM NMDA + 1.0 mM AP5	0.41±0.019	125

TABLE IV

Effects of L-arginine and excitatory amino acids on NO production by epimastigote cells

Assays were performed as in Table III, except that 1 mM L-arginine was used instead of the labeled amino acid and NO generation was measured by accumulation of NO₂ in the medium. P<0.01 for arginine versus control or for arginine plus glutamate (or NMDA), versus arginine.

Compound (mM)	NO ₂ accumulated (μM)	Stimulation (%)
None	1.1±0.10	0
1 mM L-arginine	1.5±0.15	36
1 mM L-arginine + 1 mM L-glutamate	3.1±0.18	181
1 mM L-arginine + 1 mM NMDA	2.9±0.14	163

TABLE V

*Influence of amino acids and sodium nitroprusside on cyclic GMP levels in
Trypanosoma cruzi epimastigotes*

Assays were performed in triplicates on duplicate samples as indicated under Materials and Methods. P<0.05 for 0.1 mM arginine versus control; P<0.01 for 1.0 mM arginine or nitroprusside or NMDA or glutamate, versus control.

Compound (mM)	Cyclic GMP levels (pmoles per mg protein)	Stimulation (%)
None	1.28±0.12	0
0.1 mM L-arginine	1.53±0.12	19.5
1.0 mM L-arginine	1.75±0.11	36.7
0.1 mM Na nitroprusside	2.00±0.10	56.3
1.0 mM Na nitroprusside	3.13±0.12	144.5
0.1 mM NMDA	2.18±0.19	70.3
1.0 mM NMDA	4.08±0.25	218.8
1 mM L-glutamate	3.40±0.17	165.6

TABLE VI

Modulation of [³H]MK-801 binding in epimastigote membranes by amino acids

Conditions were described under Materials and Methods. P<0.01 for glycine or serine (with or without glutamate), versus control.

Compound and concentration (mM)	[³ H]MK-801 binding (fmoles per mg protein)	Stimulation (%)
None	19.0±0.82	0
0.10 mM L-glutamate	21.5±0.64	8.0
0.01 mM Glycine	30.5±0.57	60.5
0.01 mM L-serine	28.5±1.35	50.0
0.10 mM L-glutamate + 0.01 mM Glycine	34.0±2.40	80.0
0.10 mM L-glutamate + 0.01 mM L-serine	29.5±0.92	55.2

INDUCTION OF *TRYPANOSOMA CRUZI* METACYCLOGENESIS IN THE GUT OF THE HEMATOPHAGOUS INSECT VECTOR, *RHODNIUS PROLIXUS*, BY HEMOGLOBIN AND PEPTIDES CARRYING α^D -GLOBIN SEQUENCES.

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* Department of Biochemistry and Molecular Biology, Oswaldo Cruz Foundation, Av. Brasil 4365, 21045-900, Rio de Janeiro, Brazil; [#] Department of General Biology, Universidade Federal Fluminense, Niterói, 2400, Brazil; ⁺ International Centre for Genetic Engineering and Biotechnology, Patriciano 99, 34012 Trieste, Italy; [♦] Instituto de Investigaciones en Ingeniería Genética y Biología Molecular (INGEBI-CONICET), Obligado 2490, 1428 Buenos Aires, Argentina

Garcia, E. S., Gonzalez, M. S., Azambuja, P., Baralle, F. E., Fraidenraich, D., Torres, H. N. and Flawiá, M. M. (1994). Induction of *Trypanosoma cruzi* metacyclogenesis in the gut of the hematophagous insect vector, *Rhodnius prolixus*, by hemoglobin and peptides carrying α^D -globin sequences. *Experimental Parasitology* 00, 000-000. *Trypanosoma cruzi*, a protozoan responsible for the american trypanosomiasis (Chagas disease), multiplies and differentiates in the gut of triatomine insect vectors. The effects of hemoglobin and synthetic peptides carrying α^D -globin fragments on both the growth and transformation of *T. cruzi* epimastigotes (non-infective) into metacyclic trypomastigotes (infective forms) were studied. This differentiation in the insect's gut is expressed when hemoglobin and synthetic peptides corresponding to residues 30-49 and 35-73 of the α^D -globin were added to the plasma diet. However, synthetic peptide 41-73 does not induce the differentiation of epimastigotes even in presence of the two former synthetic peptides. Thus, these data delineate an unusual molecular mechanism which modulates the dynamics of transformation of epimastigotes into metacyclic trypomastigotes in the triatomine vector's gut.

Index Descriptors: *Trypanosoma cruzi*, *Rhodnius prolixus*, metacyclogenesis, insect vector, hematophagous insect, triatomine, vector-parasite relationship, hemoglobin.

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Introduction

CHAGAS disease, a major endemic in Latin America, caused by the flagellate protozoan *Trypanosoma cruzi*, is transmitted by triatomine hematophagous insect vectors (Chagas, 1909). The parasite displays different morphological and functional forms, alternating between vertebrate and invertebrate hosts. It also alternates between dividing stages (epimastigotes in the vector midgut and amastigotes in mammalian cells) and nonreplicative but infective forms (metacyclic trypomastigotes in the insect vector and bloodstream trypomastigotes in mammals). The invertebrate cycle of the parasite begins with the ingestion of bloodstream trypomastigotes by the vector and continues with differentiation into epimastigotes, which proliferate in the gut and eventually transform into metacyclic trypomastigotes, which are eliminated together with feces and urine and are able to infect vertebrate hosts (Brener, 1973; Zeledon, 1987; Garcia and Azambuja, 1991). A crucial step in the life cycle of *T. cruzi* is the conversion of epimastigotes to metacyclic trypomastigotes. Such a process, designated as metacyclogenesis, occurs in the vector hindgut (Brener, 1973; Zeledon, 1987; Garcia and Azambuja, 1991; Garcia and Dvorak, 1982; Garcia et al., 1989a,b).

Recently, it has been demonstrated that metacyclogenesis is promoted *in vitro* by an α^D -globin-derived peptide corresponding to residues 1-40 amino terminals found in the vector hindgut. Peptides having such a sequence and recognizing a surface receptor in epimastigote cells stimulate *T. cruzi* adenylyl cyclase (Fraidenraich et al., 1993). Very few insects have a feeding behaviour similar to triatomines. Usually, in just one feeding triatomines take a very large bloodmeal, which represents an ingestion of a great amount of hemoglobin. These findings prompted us to investigate the role of hemoglobin and synthetic peptides containing different sequences of α^D -globin amino terminal on the differentiation of *T. cruzi* epimastigotes into infective forms *in vivo* experiments. Here, we show that hemoglobin and synthetic peptides having such sequences are effective *in vivo*, in promoting or blocking the metacyclogenesis in the vector gut.

Materials and Methods

Fifth-instar larvae from a colony of *Rhodnius prolixus* reared and maintained in the laboratory at a r.h. of 60-70% at 28 °C, as previously described by Garcia et al. (1984) were used. *Trypanosoma cruzi* Dm28c clone was obtained and kept in the laboratory as previously described (Garcia et al., 1989a).

In our experiments randomly chosen starved larvae, 45 days following ecdysis, unless otherwise stated, were allowed to artificially feed upon citrate decomplemented human blood or other meals containing 15×10^4 epimastigotes

ml⁻¹ of the *T. cruzi* Dm28c clone grown in liver infusion tryptose medium (Garcia et al., 1989a,b). The plasma diet was prepared by centrifugation of the decompartmented citrated human blood just before feeding the insects, and diluted in 0.01M phosphate-buffered 0.15M NaCl to the original volume of blood from which it was prepared. Hemoglobin was prepared according to Azambuja et al. (1993). Basically, fresh washed red cells were mixed with 40 volumes of cold 50 mM NaCl and centrifuged at 10,000 g for 20 min to remove debris. The supernatant containing human hemoglobin (and other minor erythrocyte components) was concentrated by pressure dialysis using Visking tubing (Scientific Instrument Centre Ltd. London, U.K.), dialysed at the same time against several changes of 0.01M phosphate-buffered 0.15M NaCl pH 7.2, and purified by passage in HPLC. This hemoglobin was added to human plasma at different concentrations. Only fully gorged insects were used; partially fed ones were discarded.

At different intervals after feeding and infection with *T. cruzi* epimastigotes, the entire intestinal tract (crop, midgut and rectum) was removed and then gently homogenized in 1 ml phosphate-buffered saline (PBS; pH 7.2) using a small homogenator. The total number of parasites, and the percentage of metacyclic trypomastigotes, identified by morphology, were counted in a Neubauer hemocytometer. The classification of the distinct morphological forms of *T. cruzi* (epimastigote or metacyclic trypomastigote) was based on the cell shape and motility (trypomastigote has an undulate membrane which makes the parasite appear to have a tender and typical body motility), the point of emergence of the flagellum from the cell body and whether the kinetoplast is anterior or posterior to the nucleus. In the present paper the transitional forms of the parasites in the insect gut (usually <10%) were considered as epimastigotes. Each experiment had at least six insects per day of evaluation.

The peptide synthesis was performed on a solid-phase Applied Biosystems automated synthesizer (model 431A, version 1.12) using Fmoc chemistry according to Fraidenaich et al. (1993). Before use, peptides corresponding to residues 30-49, 35-73, and 41-73 of chicken α^0 globin, were purified by passage through Sep-Pak C18 cartridges.

Data were compared using the two-way analysis of variance method and X² test (Snedecor, 1964).

Results and Discussion

In order to obtain clear and comparable results on the percentage of metacyclogenesis in the gut, fifth-instar larvae of *R. prolixus* with different intervals of starvation (15, 30 and 45 days), were allowed to feed on plasma containing epimastigote forms of *T. cruzi* Dm28c clone. On the last day of evaluation (day 20 after infection) the groups with different times of 15, 30 and 45 days of starvation presented 85%, 65% and 45% of metacyclogenesis,

respectively (Figure 1). The lowest percentage of transformation of epimastigotes into metacyclic trypomastigotes occurred in the group with 45 days of starvation. Thus, we decided to use this parameter in all experiments to analyse the process of metacyclogenesis.

The following experiment describes the importance of hemoglobin on the transformation of epimastigotes into metacyclic trypomastigotes. Groups of fifth-instar larvae of *R. prolixus* experiencing 45 days of starvation, were fed on *T. cruzi* infected whole blood or infected plasma containing 1 g, 7 g, 14 g and 21 g of hemoglobin/100 ml and infected plasma alone (for details of the diet preparation and infection see Materials and Methods). Table 1 shows the results obtained when the infection in the gut was evaluated 15 days after parasite ingestion. The lowest percentage of metacyclogenesis is observed in the group fed on plasma alone and the group fed on plasma containing 1 g of hemoglobin/100 ml ($p > 0.25$, X^2 test). However, as shown in Table 1, insects fed on plasma containing 1 g hemoglobin/100 ml show a significant difference in the number of parasites in the gut when compared with the infection observed in the group fed on plasma alone ($p < 0.01$). Hemoglobin added at concentrations higher than 1 g/100 ml plasma are more effective in increasing the percentage of transformation of epimastigote forms into metacyclics when these groups were compared with group fed with plasma alone ($p < 0.01$). Furthermore, the percentages of metacyclogenesis were related to hemoglobin concentrations in the plasma (Table 1). Thus, it seems that hemoglobin is an important factor to complement plasma nutritional-deficiency necessary for both the growth and to give the maximal efficiency of the *T. cruzi* differentiation in the invertebrate host.

To find out a reproducible method to study the effects of synthetic fragments of α^D -globin on the metacyclogenesis in the gut of the insect vector, we designed experiments that mimicked the natural infection in the triatomine. *T. cruzi* survival and differentiation to metacyclic trypomastigotes were evaluated in insects fed upon whole blood or plasma for 0, 10, 20 or 30 days after infection with about 27,000 epimastigote forms. Results shown in Figure 2, demonstrate that in the blood fed group, the parasite effectively survives and proliferates. In addition blood feeding is much more effective than plasma in supporting *in vivo* metacyclogenesis and development ($P < 0.01$). Thus, we decided to use in the next experiments, decomplexed plasma containing different synthetic peptides and epimastigotes to feed the triatomine vector.

The effect on *T. cruzi* metacyclogenesis of synthetic peptides carrying α^D -globin sequences and supplementing plasma feeding of *R. prolixus* larvae was studied. Immediately after feeding, six insects of each group were killed and the numbers and forms of parasites in the crop determined. There was no statistically significant difference in the number of parasites ingested by these groups ($P > 0.25$). However, as shown in Figure 2, 10 days after infection more than 95% of the parasites had transformed from epimastigotes to metacyclic trypomastigotes in the groups fed upon plasma containing the synthetic peptides 30-49 and 35-73. The high percentage of metacyclogenesis was promoted in

both groups until the end of the experiment, 30 days after feeding. There were no significant differences between these latter groups and blood ingested controls ($P > 0.25$). In contrast, insects fed upon plasma containing the synthetic peptide 41-73 had a very low metacyclogenesis as compared with blood ingested controls or insects which fed on plasma containing the synthetic peptides 30-49 and 35-73 ($P < 0.01$). The percentage of metacyclogenesis in the group fed on plasma containing peptide 41-73 remained below the values observed with plasma alone. Furthermore, the total number of parasites (epimastigotes + metacyclic trypomastigotes) per insect in this group was similar to the insects fed on plasma alone or plasma containing peptide 30-49 or 35-73. These data indicate that the reduction in the transformation of epimastigotes into metacyclic trypomastigotes was not due to the toxicity of peptide 41-73 on either epimastigotes and metacyclics. However, none of the synthetic peptides were able to establish the rate of parasite infection as observed in blood fed controls (Figure 2).

In another experiment, we mixed two synthetic peptides in the plasma meal and showed that the metacyclogenesis blocking effect of synthetic peptide 41-73 was still observed in plasma feedings supplemented with synthetic peptides 30-49 and 35-73 (Figure 3). There were significant differences between these latter groups and the groups fed on plasma containing only the peptide 30-49 or 35-73 (compare Figure 2 and Figure 3, $P < 0.01$). Thus, it seems that the synthetic peptide 41-73 binds, better than the two former synthetic peptides, the membrane receptor present in epimastigote forms and blocks the stimulation of metacyclogenesis as induced by these peptides.

Chagas disease, as transmitted by triatomines, is dependent on a high degree of the interaction between parasite and vector. *In vivo*, *T. cruzi* undergoes two differentiation processes in the insect gut vector. The first is the transformation of ingested trypomastigotes into epimastigotes, the multiplicative forms of the parasite; the second is the differentiation of epimastigotes into metacyclic trypomastigotes, the infective form of the parasite (Zeledon, 1987; Garcia and Azambuja, 1991; Garcia and Dvorak, 1982; Garcia et al., 1989a,b). In the present study, only epimastigote forms were used to infect *R. prolixus* larvae, thus, the analyzed process is just the metacyclogenesis in the vector's hindgut.

No considerable advances have been made for a better understanding of the many factors which influence the development of *T. cruzi* in its insect host since the fundamental discovery of the trypanosomid basis of this human disease (Chagas, 1909). Recently, however, evidence from *in vitro* studies showed that a globin-derived peptide, purified from *Triatoma infestans* hindguts as well as synthetic peptides carrying amino terminal globin sequences and containing a conserved domain spanning amino acid residues 30 to 40, stimulate the epimastigote adenyl cyclase of *T. cruzi* epimastigotes (Fraidenraich et al., 1993).

In the studies reported here, experimental conditions have been selected to study metacyclogenesis *in vivo*. Evidence shows that whole blood feeding or hemoglobin added to plasma at concentration higher than 1 g/100ml are much more efficient than plasma alone in supporting parasite survival and differentiation to metacyclic trypomastigotes. This raises an important question about the role of hemoglobin to the parasite life cycle in the vector gut. Assuming that human blood contains 14 g hemoglobin/100 ml of blood, even a concentration of hemoglobin of 7 g/100 ml, was able to significantly increase the percentage of metacyclogenesis, and an amount of 1g hemoglobin/100 ml induced 10-fold more development of parasites in the gut if these insects are compared with the group fed on plasma alone (Table 1). We suggest, therefore, that the hemoglobin does not have only a nutritional effect but it also seems to be important in inducing the differentiation of epimastigotes to metacyclic trypomastigotes. The fact that in plasma fed insects two synthetic peptides carrying amino terminal sequences of α^D -globin have the capability to support metacyclogenesis at least to the same extent as whole blood, indicates that a globin-derived peptidic fragment(s) is playing a critical role in *T. cruzi* differentiation.

In conclusion, these results provide the first basis for delineating the *in vivo* molecular mechanisms that are involved in promoting the metacyclogenesis of *T. cruzi* in the vector's gut. Based on the present model we postulate that nutritional state and the availability of globin fragments released by proteolysis, modulate, promoting or blocking, the dynamics of *T. cruzi* differentiation in the vector's intestine. This hypothesis assumes that some peptides corresponding to fragments of α^D -globin induce the differentiation of *T. cruzi* epimastigotes to the metacyclic trypomastigote forms in the vector's gut. As previously reported, direct evidence for this conclusion comes from the fact that binding of the specific globin fragment(s) to a receptor entity present in epimastigote membranes causes adenylyl cyclase activation. A Gs protein is likely to be involved in the signal transduction pathway responsible for the rise of intracellular cyclic AMP levels (Eisenschlos et al., 1986; Coso et al., 1992) which in turn induces differentiation (Rangel et al., 1987; Gonzalez-Perdomo et al., 1987) through the activation of type cyclic II cyclic AMP-dependent protein kinase (Ullca et al., 1988).

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LEGENDS TO FIGURES

FIGURE 1. *Trypanosoma cruzi* and metacyclogenesis in fifth-instar larvae of *Rhodnius prolixus* subjected to different days of starvation (in days after the last molting) and fed on plasma. The insects of each group were allowed to feed on infected plasma only on the last day of starvation of each group. Each value represents the average of at least six insects.

FIGURE 2. *Trypanosoma cruzi* and metacyclogenesis in fifth-instar larvae of *Rhodnius prolixus* subjected to different feedings containing or not different synthetic peptides. Each value represents the average (mean \pm SE) of three experiments, with at least 6 insects need per experiment. The experiments were repeated three times. Peptide concentration was 10^{-6} M.

FIGURE 3. *Trypanosoma cruzi* and metacyclogenesis in fifth-instar larvae of *Rhodnius prolixus* subjected to plasma meal containing or not two different synthetic peptides. Each value represents the average (mean \pm SE) of three experiments, with at least 6 insects need per experiment. The experiments were repeated three times. Peptide concentration was 10^{-6} M.

Table 1. *Trypanosoma cruzi* and metacyclogenesis in fifth-instar larvae of *Rhodnius prolixus* subjected to infected plasma feeding containing different hemoglobin concentrations. The gut infection was evaluated 15 days after infected feeding. Each value represents the average (mean±SE, n= 8 insects).

Feeding (g hemoglobin/100ml)	Parasite number per insects (X10 ⁴)	Metacyclic forms (%)
Plasma alone	0.9 ±0.07	34
Plasma + 1 g	9.2±1.1	32
Plasma + 7 g	8.5±0.9	45
Plasma + 14 g	6.0±0.7	64
Plasma + 21 g	4.2±0.6	72

Fig. 1

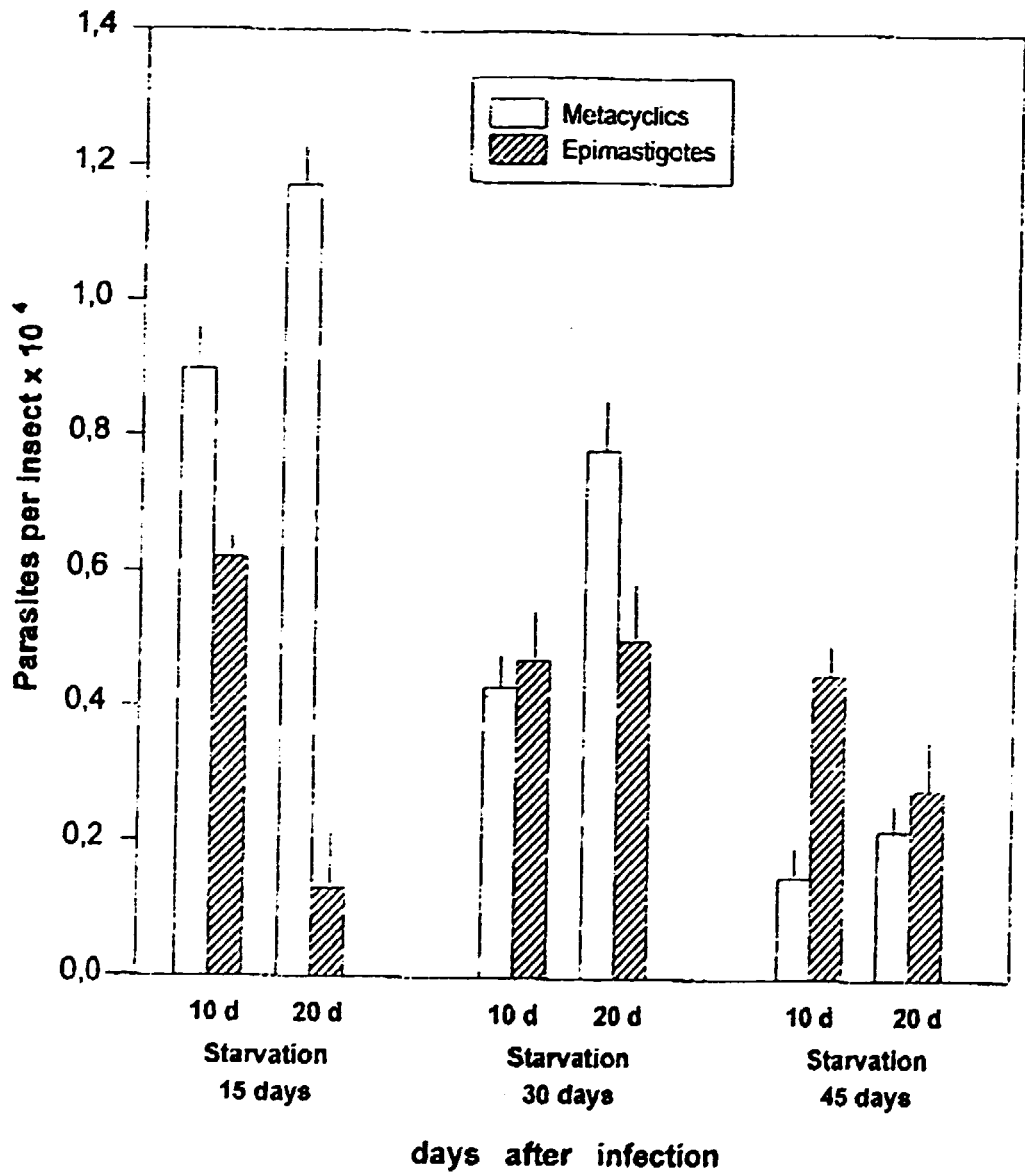


Fig. 2

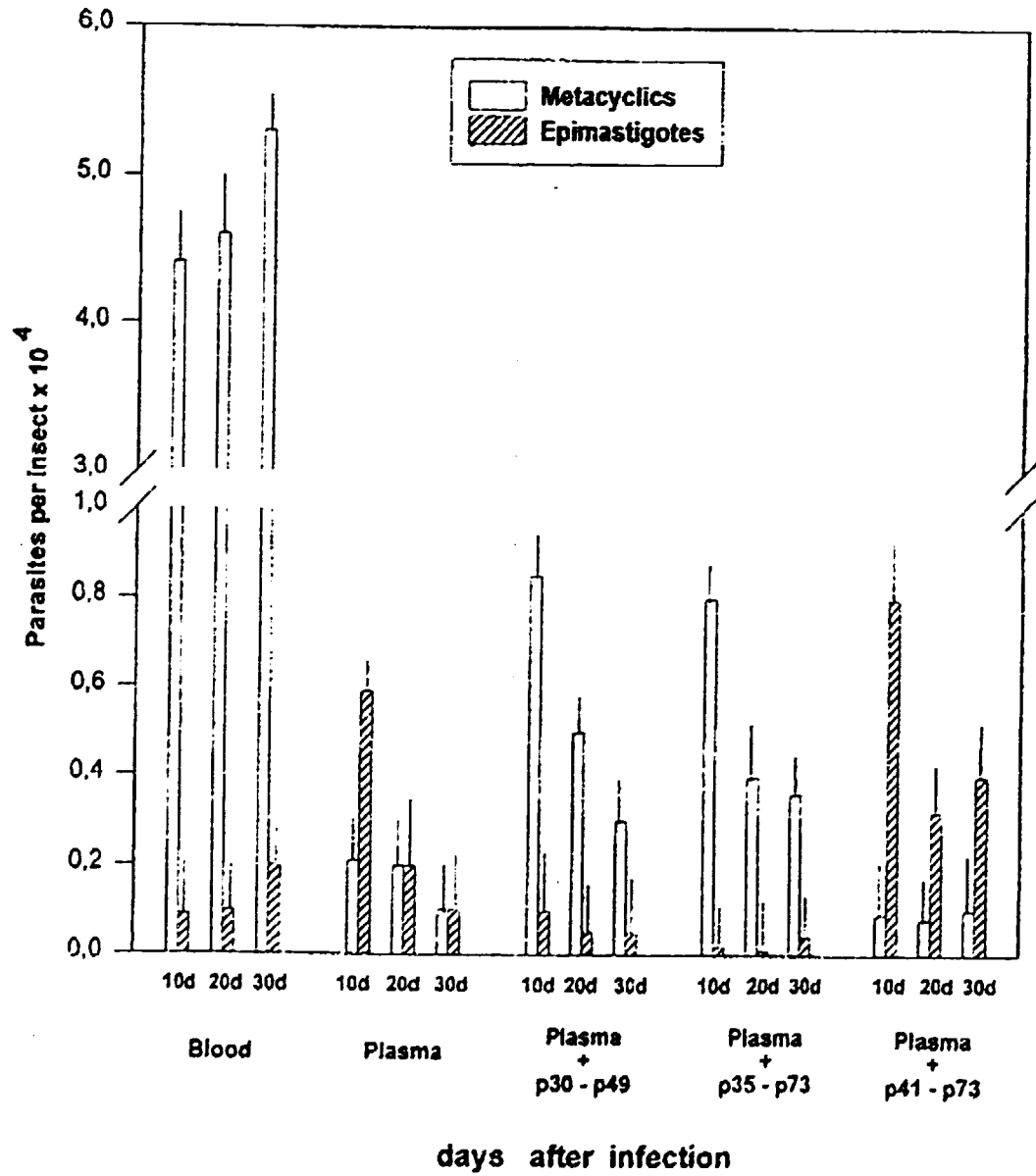


Fig 3

