COMPARATIVE MORPHOMETRY OF *TRYPANOSOMA CRUZI* FORMS IN THE INTESTINE OF *RHODNIUS PROLIXUS* AND IN THE NNN MEDIUM

C. GAMALLO*, P. PÉNIN**, R. MAYER**, J. DEL REY***, J. A. DE DIEGO**

SUMMARY

Several significant morphological differences were found between the evolutive forms of *Trypanosoma cruzi* grown in distinct media (culture in NNN or insect's intestine) using a semi-automatic image analysis system. The metacyclic forms cultured in NNN presented greater nucleus and kinetoplast sizes than those from vectors' intestine. The culture forms presented a more anterior position of the nucleus.

This is the first comparative morphometrical study of sixteen parameters of *T. cruzi* forms from NNN medium compared to those from insect's intestine. However, further discriminant studies should be carried out to have a good method to distinguish between distinct forms of *T. cruzi* life cycle.

RÉSUMÉ : Morphométrie comparative des formes de *Trypanosoma cruzi* dans l'intestin de *Rhodnius prolixus* et dans le milieu NNN.

Des différences significatives entre les formes évolutives cultivées sur des milieux différents (milieu NNN et intestin du vecteur) ont été mises en évidence en moyen d'un système d'analyse d'images. Chez les formes métacycliques, les noyaux et le kinétoplaste sont plus grandes chez l'insecte qu'en culture. Les formes des cultures présentaient un noyau à position plus antérieure.

A notre connaissance, ce travail est la première étude morphométrique de seize paramètres de *T. cruzi* appartenant à différents milieux : NNN culture et intestin du vecteur. D'autres études de discrimination seront nécessaires pour avoir une bonne méthode de différenciation entre les formes du cycle de *T. cruzi*.

INTRODUCTION

The original description of *Trypanosoma cruzi*, the causative agent of Chagas' disease, describes basic findings on its development in the vector, reduviid bugs (Chagas, 1909). Since then, further details of the life cycle have been recognized (Brack, 1968; Brener, 1972; Brener and Alvaranga, 1976; Schaub and Lösch, 1988). However, there are a lot of open questions concerning the development of *T. cruzi* in the bug and in the culture media, which can only be solved by more detailed studies.

It is well known that *T. cruzi* metacyclic forms grown in NNN medium and those grown in the intestine of the insect vector have different infective capacity (Neal and Miles, 1977 and Villalta and Kierszenbaum, 1987).

Biochemical differences between these two forms have

been also reported (Giovanni *et al.*, 1987). Moreover, there is a high intra-strain genetic heterogeneity between the different forms of the biological cycle of *T. cruzi* (Engman *et al.*, 1987).

The comparison of the development *T. cruzi* forms in bug and in NNN culture medium, has not been investigated yet. We wanted to establish if there are morphological differences that could make possible a better characterization of stocks and give more information about the differences between the evolution forms of a given stock. The comparative study was made on *T. cruzi* forms (Bolivia stock) with the NNN medium and the *R. prolixus*' intestine.

MATERIAL AND METHODS

I — *T. cruzi*

Bolivia stock. It was isolated from a specimen of *Triatoma viticeps* captured in Vitichi (Bolivia) in 1971 and was kindly given by the Faculty of Pharmacy of the University Complutense of Madrid (Spain) in December, 1986. Thereafter, it has been maintained deep-frozen in our Laboratory.

II — VECTOR INSECT

A stock of *Rhodnius prolixus* from Venezuela was captured in 1970 and has been maintained since that date in our animala-

Unit of Parasitology, Department of Preventive Medicine and Public Health, Faculty of Medicine, Autonom University of Madrid, c/ Arzobispo Morcillo, 4, 28029 Madrid, Spain.

* Dept. de Anatomía Patológica, Hospital « La Paz », 28029 Madrid, España.

** Unidad de Parasitología. Dept. de Medicina Preventiva y Salud Pública. Facultad de Medicina (UAM). 28029 Madrid. España. (Correspondence).

*** Jefe del Dept. de Medicina Preventiva y Salud Pública. Facultad de Medicina (UAM). 28029 Madrid España.

Accepté le : 14 mars 1991.

rium in the following conditions: at $25 \pm 1^\circ \text{C}$ and at $65 \pm 10\%$ relative humidity (Zeledon, 1981 and Abreu Salgado, 1976).

III — MORPHOMETRIC STUDY

a — PREPARATION OF THE MATERIAL

To prepare the material for microscopical examination, we made smears of samples taken from NNN cultures and insect's intestine, which were stained with Giemsa and inspected for the different parasite forms.

We obtained thirty slides of each of the parasite forms observed: short-epimastigotes, long-epimastigotes, sphaeromastigotes and metacyclic trypomastigotes grown in the NNN, culture medium and *R. prolixus*' intestine.

The slides were examined with an image amplifier Leitz Wetzlar type Prado Universal 1: 2.5/90 mm and the drawings were made from the projections of the slides at the above mentioned scale.

b — MORPHOMETRY THROUGH THE IBAS-1 (Image Basic Analytic System)

We took sets of measures from the mentioned images using the IBAS-1, a semiautomatic analytic system. No significant dif-

ferences were recorded in their measures of two observers up to a precision of 90 %.

The measured parameters may be observed in *Table I*.

The average values and the standard deviation for each of these measures were calculated.

c — STATISTICAL STUDY

Thirty subjects belonging to each above mentioned forms were measured. From each one we obtained the values of 16 studied parameters. These data were processed using the statistical package BMDP (V. 7-D: ANOVA-1 analysis).

RESULTS

Tables II and III shows the compared values of the morphometric parameters and the error probabilities corresponding to the statistical study.

The total length, posteronuclear distance and cellular perimeter of short epimastigotes were higher in insect's intestine than in NNN medium. A significant difference in the nuclear indice was recorded: the posteronuclear distance is greater in the intestinal forms of *R. prolixus* and the nucleus of the short epimastigotes from the NNN culture was situated in a much more anterior position.

In large epimastigotes total length, cytoplasmatic length, posteronuclear distance and the nuclear index, were greater in the vector insect than in NNN culture. Cellular and nuclear perimeters were greater in the insect's forms. The kinetoplast's area and perimeter, cellular width and flagellum/cytoplasm ratio were significantly greater in the NNN culture forms.

Sphaeromastigotes showed a nucleus/cytoplasm relation, nuclear index and the area and perimeter of the kinetoplast being greater in the intestinal forms.

TABLE I. — *Legend of the morphometric parameters used.*

L	: Total body length (including free flagellum).
F	: Length of the free flagellum.
NP	: Distance between the nucleus and the posterior extremity.
NA	: Distance between the nucleus and the anterior extremity.
AN	: Nuclear surface.
PN	: Nuclear perimeter.
AC	: Cytoplasmic surface.
PC	: Cytoplasmic perimeter.
AK	: Kinetoplast surface.
PK	: Kinetoplast perimeter.
W	: Cellular width.
NK	: Distance between the nucleus and the kinetoplast.
N/C	: Nucleus/cytoplasm ratio.
IN	: Nuclear index.
LC	: Cytoplasmic length.
F/C	: Flagellum/cytoplasm ratio.

TABLE II. — *Comparative measures of T. cruzi forms from the intestine of R. prolixus versus NNN medium grown (Short and large epimastigotes).*

Parameters	Short Epimastigotes					Large Epimastigotes				
	NNN		Intestine		P	NNN		Intestine		P
	$\bar{X}$	SD	$\bar{X}$	SD		$\bar{X}$	SD	$\bar{X}$	SD	
L	21,90	5,07	27,45	7,52	0,00	36,90	3,56	39,57	3,42	0,00
F	10,70	4,21	14,17	4,97	0,00	16,10	3,37	14,59	5,75	0,21
NP	3,93	1,42	6,05	1,60	0,00	9,36	1,36	12,56	1,43	0,00
NA	6,97	2,26	7,21	2,74	0,64	11,36	2,97	12,32	5,10	0,37
AN	3,45	0,83	3,68	2,15	0,58	5,32	2,34	5,77	1,13	0,34
PN	7,29	0,99	7,08	2,05	0,61	8,79	1,89	10,04	1,27	0,00
AC	15,95	5,12	18,42	7,59	0,04	47,69	13,63	48,64	12,45	0,81
PC	21,84	4,65	26,45	6,24	0,00	41,72	7,57	49,42	11,49	0,00
AK	1,18	0,31	1,28	0,63	0,26	1,52	0,74	1,15	0,21	0,01
PK	4,27	0,55	4,32	1,23	0,86	5,11	1,25	4,18	0,38	0,00
W	2,21	0,48	2,00	0,66	0,16	3,06	0,73	2,59	0,51	0,00
NK	2,06	0,44	1,77	0,42	0,01	2,24	0,64	2,90	0,68	0,00
N/C	0,22	0,06	0,19	0,07	0,69	0,11	0,04	0,13	0,05	0,22
IN	0,66	0,40	0,94	0,41	0,01	0,86	0,22	1,20	0,62	0,02
LC	11,20	2,55	13,28	3,28	0,02	20,79	3,76	24,97	6,22	0,00
F/C	0,99	0,43	1,07	0,27	0,39	0,81	0,27	0,65	0,34	0,05

TABLE III. — Comparative measures of *T. cruzi* forms from the intestine of *R. prolixus* versus NNN medium grown (*Sphaeromastigotes* and *metacyclics*).

Parameters	<i>Sphaeromastigotes</i>					<i>Metacyclic forms</i>				
	NNN		Intestine		P	NNN		Intestine		P
	$\bar{X}$	SD	$\bar{X}$	SD		$\bar{X}$	SD	$\bar{X}$	SD	
L	29,06	3,99	14,78	5,89	0,00	23,72	4,24	26,80	3,53	0,00
F	15,60	5,03	5,50	2,55	0,00	8,47	2,71	6,28	1,48	0,00
NP	4,41	1,23	4,84	3,09	0,48	8,10	1,81	9,30	2,00	0,01
NA	8,97	4,56	4,78	2,68	0,00	6,98	2,14	11,20	2,95	0,00
AN	3,49	0,69	3,15	0,59	0,04	3,04	0,78	4,08	1,05	0,00
PN	7,01	0,67	6,70	0,81	0,11	7,00	0,95	10,09	1,50	0,00
AC	35,13	14,37	25,23	11,09	0,00	17,44	4,34	21,33	5,50	0,00
PC	26,20	7,60	19,21	5,28	0,00	31,00	5,83	39,32	5,91	0,00
AK	1,31	0,51	2,27	0,71	0,00	1,30	0,39	1,91	0,73	0,00
PK	4,98	1,23	5,92	1,02	0,62	4,16	0,59	5,20	1,42	0,00
W	5,22	1,77	4,86	0,71	0,31	1,47	0,28	1,68	0,42	0,02
NK	1,84	0,58	3,59	1,03	0,00	4,72	2,15	6,69	2,24	0,00
N/C	0,11	0,04	0,14	0,05	0,01	0,18	0,07	0,17	0,04	0,25
IN	0,65	0,49	1,49	1,28	0,00	1,24	0,47	0,90	0,35	0,00
LC	17,46	3,95	9,28	3,78	0,00	15,24	3,09	20,52	3,63	0,00
F/C	1,29	0,56	0,51	0,34	0,00	0,57	0,21	0,32	0,10	0,00

The forms found in the insect's intestine had the nucleus and the kinetoplast proportionally greater than those of the NNN medium.

Significant results were found between the NNN and insect's intestine metacyclic forms. Except the nucleus/cytoplasm ratio, all the morphometric parameters showed significant differences, the values of the vector's forms being greater except the length of the flagellum, the ratio flagellum/cytoplasm and the nuclear index which were greater in culture forms. In these last ones, the nucleus was anterior while in those of *R. prolixus* the nucleus was slightly posterior.

DISCUSSION

Villalta and Kierszenbaum (1987) found significant biological differences between the insect metacyclic forms and those of the culture medium (NNN), but for them, they were indistinguishable from the morphological point of view.

Significant differences were shown between forms grown in NNN culture of the intestine of vector insect. The short epimastigote forms presented less significant differences in relation to made of culture.

The comparison of the metacyclic forms of the two media may present important practical implications. The total length, the cytoplasm, nuclear and kinetoplast's areas being greater in the forms of the insect's intestine.

The NNN culture forms are posteronuclear, being the forms of the insect slightly anteronuclear.

The morphometric data of the existent differences between the two above mentioned types of metacyclic forms

may be connected with important physiological differences existing between these forms.

It is well known the major pathogenic action of the insect's forms than those of the NNN medium (Neal and Miles, 1977 and Villalta and Kierszenbaum, 1987), these forms which are less posteronuclear in our study.

The significant differences found in our study between the NNN culture forms and those from the insect's intestine serve us to know something more about the different forms of the *T. cruzi*'s cycle. In our opinion, these differences are interesting in the biological interpretation of *T. cruzi* forms, particularly the metacyclic trypomastigotes fundamental in the host mammal infection.

The use of morphometrical methods to differentiate the distinct forms of the *T. cruzi* life cycle may be useful, in addition to the proposed medical, biological and chemical factors (Dvorak, 1984; Brenière *et al.*, 1985; Tibayrenc *et al.*, 1983, Tibayrenc *et al.*, 1986; Tibayrenc and Ayala, 1988).

After considering the results obtained, we believe that the reported differences in behavior of distinct isolated forms of *T. cruzi* could be related to the morphometric measures. Therefore, we consider that the use of these results obtained by planimetric techniques may be useful in the enhancement of microscopical examination techniques for the diagnosis and prognosis of Chagas's disease.

However, in spite of the great number of significant differences found, this results should not be generalized, since this is the first morphometrical study of sixteen different parameters comparing the developmental forms found in the insect's intestine and in NNN culture medium. Further investigations of other *T. cruzi*/vector and *T. cruzi*/culture systems on the basis of discriminant analysis are needed.

REFERENCES

- Abreu Salgado A de : Xenodiagnosis in the selection of patients and evaluation of treatment. In: American Trypanosomiasis Research, OMS Publ. Cient. n° 318, Washington, 1976, 223-226.
- Brack C. : Elektronenmikroskopische Untersuchungen zum Lebenszyklus von *Trypanosoma cruzi*. *Acta Tropica*, 1968, 25, 289-356.
- Brener Z. : A new aspect of *Trypanosoma cruzi* life-cycle in the invertebrate host. *J. Protozool.*, 1972, 19, 23-27.
- Brener Z., Alvarenga N. : Life-cycle of *Trypanosoma cruzi* in the vector. In: American Trypanosomiasis Research. OMS Publ. Cient. n° 318. Washington, 1976, 83-88.
- Brénière S. F., Llanos B., Tibayrenc M., Désjeux P. : Isoenzymes studies and epidemiological data of *Trypanosoma cruzi* from Arequipa (Perú), pacific side. *Ann. Soc. Belge Med. Trop.*, 1985, 65, 63-66.
- Chagas C. : Nova tripanozomiaze humana. Über eine neue Trypanosomiasis des Menschen. *Mem. Inst. Oswaldo Cruz*, 1909, 1, 159-218.
- Dvorak J. A. : The Natural Heterogeneity of *Trypanosoma cruzi*: Biological and Medical Implications. *J. Cell. Biochem.*, 1984, 24, 357-371.
- Engman D. M., Reddy L. V., Donelson J. E., Kirchhoff J. : *Trypanosoma cruzi* exhibits inter- and intra-strain heterogeneity in molecular karyotype and chromosomal gene location. *Mol. Biochem. Parasitol.*, 1987, 22, 115-123.
- Giovanni S., Pontes L. C., Galvão-Castro B. : Antigenic differences between insect and culture derived *Trypanosoma cruzi* metacyclic trypomastigotes extracts. *Am. J. Trop. Med. Hyg.*, 1987, 37, 61-65.
- Neal R. A., Miles R. A. : The sensitivity of culture methods to detect experimental infections of *Trypanosoma cruzi* and comparison with xenodiagnosis. *Rev. Inst. Med. Trop. São Paulo*, 1977, 19, 170-176.
- Schaub G. A., Lösch P. : *Trypanosoma cruzi*: Origin of metacyclic trypomastigotes in the urine of the vector *Triatoma infestans*. *Exp. Parasitol.*, 1988, 65, 174-186.
- Tibayrenc M., Echalar M., Brénière F., Lemesre J. L., Barnabé C. H., Désjeux P. : Sur le statut taxonomique et médical des souches isoenzymatiques de *Trypanosoma cruzi*. Considérations sur la valeur systématique et immunogénique des différentes isoenzymes. *C. R. Acad. Sci. Paris*, 1983, 296, 721-726.
- Tibayrenc M., Ward A., Moya A., Ayala F. J. : Natural populations of *Trypanosoma cruzi*, the agent of Chagas' disease, have a complex multiclonal structure. *Proc. Nat. Acad. Sci. USA*, 1986, 83, 115.
- Tibayrenc M., Ayala F. J. : Isozyme variability in *Trypanosoma cruzi*, the agent of Chagas' disease: genetical, taxonomical and epidemiological significance. *Evolution*, 1988, 42, 277.
- Villalta F., Kierszenbaum F. : Insect-borne and culture-derived metacyclic *Trypanosoma cruzi*: differences in infectivity and virulence. *Am. J. Trop. Med. Hyg.*, 1987, 36, 529-532.
- Zeledón R. : El *triatoma dimidiata* y su relación con la enfermedad de Chagas. San José de Costa Rica. Ed. *Universidad estatal a distancia.*, 1981, 146.

Des microfiches et des microfilms de cette publication peuvent être obtenus auprès de :

This publication is available in microform from:

MASSON-SPPIF (réf. MIMC), 120, boulevard Saint-Germain, F-75280 Paris Cedex 06.

© Masson, Paris, 1991.

Le Directeur de la Publication : D^r J. TALAMON

Tous droits de traduction, d'adaptation et de reproduction par tous procédés réservés pour tous pays.

Toute reproduction ou représentation intégrale ou partielle, par quelque procédé que ce soit, des pages publiées dans le présent ouvrage, faite sans l'autorisation de l'éditeur est illicite et constitue une contre-façon. Seules sont autorisées, d'une part, les reproductions strictement réservées à l'usage du copiste et non destinées à une utilisation collective, et d'autre part, les courtes citations justifiées par le caractère scientifique ou d'information de l'œuvre dans laquelle elles sont incorporées (loi du 11 mars 1957, art. 20 et 41 et Code pénal, art. 425).

Des photocopies payantes peuvent être réalisées avec l'accord de l'éditeur. S'adresser au : Centre Français du Copyright, 6 bis, rue Gabriel-Laumain, 75010 Paris. Tél. 48-24-98-30.

Masson, éditeur, Paris. — Dépôt légal : 1991.
Imprimé par Imprimerie Barnéoud, à Laval. — 9829.
Printed in France.

N° d'ordre : 6230.

Février 1992

Commission paritaire : n° 54169

© Masson, Paris 1991