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INFECTIVITY OF *TRYPANOSOMA CRUZI* METACYCLIC TRYPOMASTIGOTES FROM CULTURES KEPT IN LABORATORY FOR DIFFERENT PERIODS OF TIME

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SUMMARY

The infectivity of metacyclic trypomastigotes from *T. cruzi* of 5 different cultures of "Y" strain and 4 of "MR" strain kept in laboratory for 1,5 to 18 years without animal passage were studied. As concerns "MR" cultures forms, no correlation could be found between the cultivation period and the infectivity. Data regarding culture forms of "Y" strain, however, apparently suggest such a correlationship. Cultures comprising equal numbers of metacyclic forms may display different infective potentiality. The infectivity of these trypomastigotes is apparently not related to the number of cultivation days since 6 and 11 days old cultures showed similar infectivity. Animals inoculated and presenting patent parasitemia could survive at least 5 months showing a low mortality rate. Metacyclic trypomastigotes of "PF" culture ("Y" strain) induced infection in over 50% of animals inoculated. The infection was detected by hemoculture in "LIT" medium.

INTRODUCTION

T. cruzi strains may display, during cultivation different rates of infectivity to animals. The infectivity of metacyclic trypomastigotes from *T. cruzi* cultures seems to be influenced by the number of passages undergone after isolation from the vertebrate host. The works of PIZZI & PRAGER [12] and PIZZI [13] showed *T. cruzi* strains from cultures with 200 or more passages to be less infective to vertebrate hosts than cultures cultivated for shorter periods. MENEZES [7, 8, 9] also reported loss of virulence in a culture kept for long periods in the laboratory. Nonetheless, PACKCHANIAN & SWEETS [10] had previously demonstrated that a *T. cruzi* strain, kept in culture for 13 years, could infect 100% of animals.

This paper studies the infectivity to mice of *T. cruzi* strains kept in culture ("Blood-agar", "LIT", "HIL" and "Warren" media) for different periods of time (1,5 to 18 years) with no passage through vertebrates.

MATERIAL AND METHODS

T. cruzi strains — *Y* strain, isolated from an acute case of Chagas' disease (SILVA & NUSSENZWEIG [16]) and *MR* strain, isolated from naturally infected *Triatoma infestans* (BRENER & CHIARI [2]). The parasites were kept in mice by repeated i.p. blood passages. At different periods the blood of heavily infected mice was inoculated into "LIT" medium and then transferred to fresh "LIT" medium every 15 days.

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Cultures used in the experiments — *Y* strain — "Y_{34}", having undergone, after isolation from infected mice, 34 passages in "LIT" medium within 1.5 years; "Y_{77}", with 77 passages in "LIT" within 3 years; "Y_{159}", with 159 passages in "LIT" within 7 years; "TCY", received from the Instituto de Medicina Tropical de São Paulo, with unknown cultivation period and then likewise kept in "LIT" for 1.5 years; "PF", cultivated for about 16 years in blood-agar (MENEZES [7]), and then 1.5 years in "LIT" medium in our laboratory.

MR strain — "MR_{44}", with 44 passages in "LIT" within 2 years; "MR_{58}", with 58 passages in "LIT" within 2.5 years; "MR_{86}", with 86 passages in "LIT" within 3.5 years; "MR_{147}", with 147 passages in "LIT" within 6.5 years.

Culture media — "Blood-agar medium", used by PACKCHANIAN & SWEETS [10]; "LIT" ("liver-infusion tryptose") described by CAMARGO [3]; "HIL" ("dog-heart infusion"), described by CASTELLANI et al. [4], "Warren's medium" ("brain-heart infusion") described by WARREN [17]. All cultures were kept at 28°C.

Inoculation of metacyclics trypomastigotes — The number of flagellates in the culture was determined by using a "Fisher Autocytometer", a semi-automatic electric apparatus devised for blood cell counting, with the threshold adjusted to 72.5. The culture was diluted in Paul's solution (PAUL [11]), after being homogenized in a mechanical spinning shaker. The percentage of metacyclic trypomastigotes was determined by microscopical examination of 500 to 1,000 non-selected flagellates in Giemsa-stained preparations. The inocula were intraperitoneally injected in male white mice weighing 14-16 g. When necessary, the cultures were accordingly diluted in 0.9% sodium chloride. All inoculations were performed with cultures either in the 6th or 11th days of growth.

Blood examination and hemoculture — Seven days after inoculation the blood of the animals were examined daily for parasites, under 400 x magnification. Thirty days after inoculation the mice were killed and their blood inoculated into "LIT" or "WARREN" media in order to detect sub-patent infections. The hemocultures were kept at 28°C and examined 15, 30 and 45 days later.

RESULTS

Table I shows results of inoculation with cultures "Y_{34}", "Y_{77}", "Y_{159}", "TCY" and "PF". Apparently, differences could be observed between the infectivity of the different cultures. With culture "Y_{34}", 3 of the 5 mice got infected; with "Y_{77}", all animals developed infection, demonstrated either by blood examination or by hemoculture in "LIT" medium. As regards "Y_{159}", 2 out of 5 mice inoculated displayed infection; with culture "TCY", no infection could be detected and, finally, with culture "PF", only 3 out of the 14 animals were infected. In animals inoculated with "PF" culture blood examination was always negative, infection being the demonstrated only by hemoculture in "LIT" medium. Cultures "MR_{44}", "MR_{58}", "MR_{86}" and "MR_{147}" were also inoculated according to the same techniques. Table II shows that 100% of the animals acquired infection, demonstrated by blood examinations and hemoculture in "LIT" medium. Table III presents data from inoculation of cultures "Y_{77}", "Y_{159}", "TCY", "PF" and "MR_{44}"; the behaviour of those 6-days cultures from "LIT" medium was seen to be pratically identical to that of 11-days cultures. Table IV shows that only by inoculation of 10,000,000 metacyclic trypomastigotes from culture "TCY" infection was achieved and demonstrated by hemoculture. Table V shows data from several inoculations of different inocula of culture "PF", wich infected about 50% of the inoculated animals. Blood examinations always provided negative results infection having been demonstrated only by hemoculture in "LIT" and "Warren" media. Table VI shows the results of hemocultures carried out 1 to 5 months after inoculation of "PF" culture in mice: 15 out of 20 animals were demonstrated to be infected and persistence of infection could be detected even after 5 months of inoculation. Table VII presents overall data on infectivity of the different cultures. The inocula ranged

TABLE I

Showing the infectivity of Y_{34}, Y_{77}, Y_{159}, TCY and PF cultures. Results of inoculation in mice, per intraperitoneal route, of metacyclic trypomastigotes from cultures on the 11th day ("Warren" and "LIT" media)

Culture	Culture media	no. of trypomastigotes inoculated	Positive blood examination/ examined animals	Hemoculture	Animals positive/ inoculated
"Y_{34}"	"LIT"	2,000,000	0/5	3/5	3/5
"Y_{77}"	"Warren"	5,000	1/5	4/5	5/5
"Y_{77}"	"LIT"	5,000	3/5	2/5	5/5
"Y_{77}"	"LIT"	2,000,000	2/5	3/5	5/5
"Y_{159}"	"LIT"	2,000,000	0/5	2/5	2/5
"TCY"	"Warren"	5,000	0/5	0/5	0/5
"TCY"	"LIT"	5,000	0/5	0/5	0/5
"TCY"	"LIT"	2,000,000	0/5	0/5	0/5
"PF"	"Warren"	5,000	0/5	0/5	0/5
"PF"	"LIT"	5,000	0/4	2/4	2/4
"PF"	"LIT"	2,000,000	0/5	1/5	1/5

TABLE II

Showing the infectivity of MR_{44}, MR_{58}, MR_{86}, MR_{147} cultures. Results from inoculation in mice, per intraperitoneal route, of metacyclic trypomastigotes from cultures on the 11th day ("Warren" and "LIT" media)

Culture	Culture media	no. of trypomastigotes inoculated	Positive blood examination/ examined animals	Hemoculture	Animals positive/ inoculated
"MR_{44}"	"Warren"	5,000	5/5	...	5/5
"MR_{44}"	"LIT"	5,000	5/5	...	5/5
"MR_{44}"	"LIT"	2,000,000	5/5	...	5/5
"MR_{58}"	"LIT"	2,000,000	5/5	...	5/5
"MR_{86}"	"LIT"	2,000,000	0/5	5/5	5/5
"MR_{147}"	"LIT"	2,000,000	0/5	5/5	5/5

TABLE III

Showing the infectivity of Y_{77}, Y_{159}, PF, TCY, MR_{44} cultures. Results from inoculation in mice, per intraperitoneal route, of 2×10^5 metacyclic trypomastigotes from cultures on the 6th day ("LIT" medium)

Culture	Positive blood examination/examined animals	Hemoculture	Animals positive/inoculated
"Y_{77}"	0/5	5/5	5/5
"Y_{159}"	0/5	2/5	2/5
"TCY"	0/5	0/5	0/5
"PF"	0/5	3/5	3/5
"MR_{44}"	0/5	5/5	5/5

TABLE IV

Showing the infectivity of "TCY" culture. Results from different inoculations in mice, per intraperitoneal route, of metacyclic trypomastigotes from cultures on the 11th day ("LIT" and "HIL" media)

Culture media	no. of trypomastigotes inoculated	Positive blood examination/ examined animals	Hemoculture	Animals positive/inoculated
"LIT"	2,000,000	0/3	0/3	0/3
"LIT"	4,000,000	0/3	0/3	0/3
"HIL"	4,000,000	0/3	0/3	0/3
"LIT"	6,000,000	0/2	0/2	0/2
"LIT"	8,000,000	0/2	0/2	0/2
"HIL"	8,000,000	0/3	0/3	0/3
"LIT"	10,000,000	0/3	1/3	1/3

TABLE V

Showing the infectivity of "PF" culture. Results of inoculation in mice, per intraperitoneal route, of metacyclic trypomastigotes from cultures on the 11th day ("LIT", "HIL" and "Warren" media)

Culture	Culture media	no. of trypomastigotes inoculated	Positive blood examination/ examined animals	Hemoculture		Animals positive/ inoculated
				"LIT"	"Warren"	
"PF"	"Warren"	100,000	0/9	3/4	2/5	5/9
"PF"	"LIT"	500,000	0/6	2/6	...	2/6
"PF"	"LIT"	1,000,000	0/3	2/3	...	2/3
"PF"	"LIT"	2,000,000	0/6	2/3	...	2/6
"PF"	"HIL"	2,000,000	0/6	2/6	...	2/6
"PF"	"HIL"	4,000,000	0/3	2/3	...	2/3

TABLE VI

Showing infectivity of "PF" culture. Results of inoculation in mice, per intraperitoneal route, of 5×10^6 metacyclic trypomastigotes of cultures on the 11th day ("LIT" medium)

no. of days after	Positive blood examination/examined animals	Hemoculture	Animals positive/inoculated
30	0/5	4/5	4/5
60	...	4/5	4/5
90	...	4/5	4/5
150	...	3/5	3/5

TABLE VII

Overall infectivity results obtained with the inoculation of culture forms of the different cultures in mice. Number of metacyclic trypomastigote forms ranging from 5×10^3 to 24×10^6, inoculation by intraperitoneal route

Culture	Time of maintenance in culture (years)	no. of animals inoculated	no. of animals showing infection
"Y_{34}"	1.5	5	3
"Y_{77}"	3	41	41
"Y_{159}"	7	10	4
"PF"	17	66	35
"TCY"	(unknown)	39	1
"MR_{44}"	2	20	20
"MR_{58}"	2.5	5	5
"MR_{86}"	3.5	5	5
"MR_{147}"	6.5	5	5

from 5,000 to 24,000,000 metacyclic trypomastigotes obtained from "LIT", "HIL" and "WARREN" media. The data obtained show that culture "Y_{77}" as well as all cultures of strain "MR" could infect 100% of the mice. Culture "PF" infected over 50% of the animals and culture "TCY" presented the lowest degree of infectivity, infection being achieved in just one of the 33 inoculated animals.

DISCUSSION

Tables I and II show that, at least as concerns "MR" culture forms, no correlation could be found between the cultivation period and its degree of infectivity, whereas data regarding culture forms of "Y" strain apparently suggest some degree of correlationship. The fact of 6-days cultures presenting similar infectivity to that of 11-days cultures (Table III), shows that the infective potential of the flagellates is not influenced by the number of cultivation days, provided that the metacyclics are apparently normal. Besides variations observed in the degree of infectivity of "Y" and "MR" cultures forms, great differences could also be detected between the infectivity of cultures of the same strain maintained in laboratory for different periods of time.

RUBIO [15] reported that old cultures decrease their virulence, their speed of amastigote multiplication in the tissues and capacity to give origin to blood stream trypomastigotes. ROSEMBERG et al. [14] conducted experiments with *T. cruzi* culture forms maintained, for 5 years, in "WARREN" medium and reported that "CFI" mice could be infected even when inoculated with a small number of trypomastigotes. An inoculum of 5,000 metacyclic trypomastigote induced patent parasitemia in 100% of the animals as well as high mortality rate. A large variation in the proportion of animals presenting patent parasitemia was observed when a standardized 5,000 — trypomastigote inoculum of different cultures was used (Tables I and II). Our data on mortality are not consistent with those from ROSEMBERG et al. [14]. All animals displaying patent parasitemia, in our present work, could survive longer than 5 months after inoculation. Besides the apparently high virulence of the *T. cruzi* strain employed by ROSEMBERG et al. [14] the "CFI" mice were apparently more susceptible to *T. cruzi* culture forms.

MENEZES [7, 8, 9] has repeatedly reported culture "PF" to be non virulent in vertebrates and it induces "transitory infection" and not "an infection disease" in mice. Tables I, III, V, VI, VII show that, as to infectivity, our findings do not support MENEZES [7, 8, 9] findings. In our experience "PF" culture forms actually induced infection in over 50% of the inoculated animals. Bearing in mind that in MENEZES [7, 8, 9] works, as well as in

that by MENEZES & ALBUQUERQUE [9], a large number of experiments on infectivity were carried out with "PF" culture forms from Warren medium, we decided to use this medium, prepared according to ALBUQUERQUE's [9] technique, in order to get inoculum for one of our experiments (Table V). As can be seen, "PF" kept its infectivity after cultivation in "Warren" medium, mice infected with those cultures presenting positive hemocultures in "LIT" and "WARREN" media. Such discrepancy may, perhaps, be accounted for by the fact of hemoculture in "LIT" medium providing better results than in "WARREN".

The data on Table VI show that, by using an inoculum of 50,000 metacyclic trypomastigotes from culture "PF" infection could be demonstrated, through hemocultures in "LIT", 1, 2, 3 and 5 months after inoculation, although blood examination provided negative results. The infection induced by "PF" therefore, is by no means transitory as stated by MENEZES [8].

The overall results recorded on Table VII show that metacyclic trypomastigotes keep their ability to infect vertebrate hosts even after rather large periods of maintenance in artificial media. There is, however, some evidence that the cultivation period has some influence on the infectivity of the cultures, investigators such as PACKCHANIAN & SWEETS [10] and GOBLE [5] did not mention any decrease in the virulence of *T. cruzi* culture forms inoculated in mice after cultivation periods of 13 and 3 years, respectively.

BICE & ZELEDON [1] demonstrated that using five *T. cruzi* strains from Costa Rica, some correlation was observed between the length of time the strains had been maintained in culture and their virulence evaluated both by the parasitemia level and the number of tissue forms. This seems to be due rather to the trypomastigotes characteristics than to a decrease in their number. Working with standard inocula, whose number of metacyclic forms was previously determined, we reached similar conclusion, i.e., we found out that cultures comprising equal numbers of metacyclic forms may display different infective potentiality. BICE & ZELEDON [1] confirmed LAMBRECHT's [16] point of view by stating that loss of virulence could be the result of a natural selection of the forms more fitted to develop in culture media than to infect animals. The present paper also shows that the cultures kept longest in laboratory cultures — "PF" and, probably, "TCY" — were the ones that induced the lowest percentages of infection.

Hemoculture in "LIT" medium has apparently been demonstrated as a dependable technique for detecting infection' in animals inoculated with culture forms.

RESUMO

Infectividade de tripomastigotos metacíclicos do Trypanosoma cruzi *de culturas mantidas em laboratório por diferentes períodos de tempo*

Foi estudada a infectividade de tripomastigotos metacíclicos de *T. cruzi*, sendo 5 diferentes culturas da amostra "Y", e 4 da amostra "MR" mantidas em laboratório (1.5 a 18 anos) sem passagem por vertebrados.

Os resultados obtidos aparentemente sugerem que os metacíclicos das culturas da amostra "Y" retém sua capacidade de infectar vertebrados mesmo após longos períodos de manutenção em meio artificial. Esta capacidade de infectar foi também observada nas culturas da amostra "MR".

Inóculos com o mesmo número de tripomastigotos metacíclicos das culturas "Y" e "MR" apresentaram diferente potencial infectivo. A infectividade destes tripomastigotos não está relacionado com o número de dias de cultivo, desde que os flagelados estejam aparentemente normais, uma vez que culturas de 6 a 11 dias apresentaram infectividade semelhante.

Todos os animais inoculados com culturas que apresentaram parasitemia patente, sobreviveram no mínimo 5 meses após a inoculação e a taxa de mortalidade foi extremamente baixa. Os tripomastigotos metacíclicos da cultura "PF" (amostra "Y") infectaram mais de 50% dos animais inoculados.

A utilização da hemocultura em "LIT" mostrou ser método eficiente para comprovação de infecções sub-patentes do *T. cruzi*.

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