Rev. Inst. Med. trop. São Paulo
36 (5): 423-431, setembro-outubro, 1994.

MURINE VIRUS CONTAMINANT OF *TRYPANOSOMA CRUZI* EXPERIMENTAL INFECTION

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SUMMARY

The possibility that some virus contaminants could be altering host response to *Trypanosoma cruzi* experimental infection was investigated.

Data obtained showed that CBA/J mice infected with stocks of parasite maintained in mice (Y_{IUEC}) presented higher level of parasitemia and shorter survival times than those infected with a stock (Y_{ITC}) which was also maintained in mice but had been previously passaged in cell culture. Mouse antibody production tests, performed with the filtered plasma of mice infected with Y_{IUEC}, indicated the presence of mouse hepatitis virus (MHV) while no virus was detected when testing the plasma of Y_{ITC} infected mice. Filtered plasma of Y_{IEUC} infected mice was shown to contain a factor able to enhance the level of parasitemia and to reduce the mean survival time of mice challenged with 10^5 Y_{ITC}. This factor, that could be serially passaged to naïve mice was shown to be a coronavirus by neutralization tests.

KEYWORDS: Chagas' disease; *Trypanosoma cruzi*; Immune response; Coronavirus

INTRODUCTION

Since Chagas' disease was discovered it was observed that mice were susceptible to infection with *Trypanosoma cruzi*, its causal agent [8]. For this and other reasons - like facility to breed and handle - mouse has been largely used to investigate the immune response to and the pathology of this infection [1, 5, 6, 19].

Results obtained to date indicate that evolution of the disease depends both on some traits of parasite and on host susceptibility. Indeed, when testing different parasite isolates in a given strain of mice, it can be seen that some these isolates are highly pathogenic, inducing high parasitemia and high mortality rates while others, although being able to induce a long-lived infection, are unable to kill host [6, 22].

When studying host susceptibility, it was seen that genetic background, sex, and environmental conditions were important factors for developing disease [9, 23, 25, 26]. However, to our knowledge, no information is available on the influence of microbiological status of host on the infection with *T. cruzi*.

Breeding mice under barrier-sustained conditions was only recently available to some laboratories. Thus earlier isolates of the parasite were obtained by using

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conventionally reared mice which are usually infected by different pathogens [12]. This fact prompted us to investigate if any of these naturally occurring pathogens was being transmitted along with *T. cruzi* experimental infection and if some features of the experimental Chagas' disease was altered by these contaminant pathogens.

MATERIALS AND METHODS

Parasites. The **Y** [20] and **CL** [7] strains of *T. cruzi* were used. Different stocks of the **Y** strain were obtained as described below and maintained, unless otherwise stated, by the intraperitoneal injection, every week, with 10^5 blood parasites, in naïve mice. When aseptic conditions were used animals were injected and maintained within Trexler isolators. Otherwise animals were kept under normal hygienic conditions by using sterilized cages, food and shavings which were changed twice a week.

The stock of **Y** strain originaly received from Prof. Brener in 1972 was labelled as Y_{IUEC} and, since then until 1989, was maintained in our laboratory by infecting conventionally bred Swiss mice. From 1989 to date, this stock was maintained in CBA/J mice obtained from colonies bred under barrier sustained conditions.

$\mathbf{Y}_{ITC}$ stock was obtained by culturing $\mathbf{Y}_{IUEC}$ parasites in monolayers of LCC-MK_2 cells in RPMI 1640 medium supplemented with 10% inactivated fetal calf serum. After 10 passages in cell cultures, parasites were haversted and 10^5 trypomastigotes were injected in CBA/J mice that were maintained under aseptic conditions. Parasites were then maintained by weekly transfer of infection, under aseptic conditions, to naïve CBA/J.

$\mathbf{Y}_{INNN}$ stock was obtained by culturing $\mathbf{Y}_{IUEC}$ parasites at 28°C for 15 days in Nicolle-Novy-McNeal (NNN) medium, to which some drops of liver infusion tryptose (LIT) medium were added. After 10 passages in NNN media, parasites were harvested and 10^5 trypomastigotes were inoculated in CBA/J mice, and then serially passaged, from mouse to mouse under aseptic conditions.

CL strain was kindly supplied by Dr. T. Kipnis from Instituto de Ciências Biomédicas, Universidade de S. Paulo. This stock was labelled CL_1 and was maintained by infecting of CBA/J mice, every 15 days with 10^4 blood parasites given intraperitoneally.

Animals. Eight to twelve week old mice, of both sexes, of the CBA/J, and BALB/c strains were used throughout. Animals were obtained from cesarian-derived colonies reared under barrier-sustained conditions at Cemib-Unicamp. Serologic tests to detect virus infection in these colonies, performed every 3 months since January 1989, showed no positive result for any of the following viruses: mouse hepatitis virus (MHV-3), Theiler's mouse encephalomielytis virus strain GDVII (TMEV-GDVII), pneumonia virus of mice (PVM), Sendai virus, minute virus of mice (MVM), vaccinia, lymphocytic choriomeningitis virus (LCMV), mouse adenovirus, polyoma virus, K-virus, reovirus (Reo-3), mouse cytomegalovirus (MCMV), rotavirus and lactate dehydrogenase-elevating virus (LDHV). Detection of virus infection was carried out by performing hemagglutination inhibition tests [17] for K-virus and polyoma virus, LDH-elevation end point [10] for LDHV and indirect immunofluorescence tests [14] for the other above-mentioned viruses.

Unless otherwise stated, experiments were performed in triplicate, by using groups of five mice.

Viruses: The following viruses were obtained from Prof. Dr. V. Kraft from Zentralinstitut für Versuchstierzucht Hannover: MHV-3, TMEV-GDVII, PVM, Sendai virus, MVM, Vaccinia, LCMV, mouse adenovirus, polyoma virus, K-virus, Reo-3, MCMV. Rotavirus was obrtained from Prof. Silvia Gatti, from Department of Microbiology and Immunology (Unicamp).

Since $\mathbf{Y}_{IUEC}$ stock had been maintained in conventionally bred mice it was assumed that mice infected 7 days earlier with 10^5 blood parasites of this stock also could be infected with a virus, provisionally labelled virus X. The plasma of these mice was pooled, filtered on 0.2 μ Millipore membrane and used as primary source of this virus. No parasite was detected when injecting, ip., 0.2 ml portions of the filtered plasma in groups of 5 CBA mice and looking thereafter for blood parasites at days 7, 14 and 21. Virus X was serially passaged to naïve mice, kept under aseptic conditions, by injecting, ip., 0.2 ml aliquots of the filtered plasma of mice infected 7 days earlier.

Antibodies. Antibodies specific for each of the above indicated viruses were prepared and tested as indicated by KRAFT & MAYER (1986) [14]. Immunization was carried out by using BALB/c mice, housed in Trexler isolators and each batch of immune serum obtained was tested with every virus above indicated to determine its

specificity. Positive reactions were observed only when the homologous antigen was used.

Antibodies for virus X were obtained by immunizing 10-12 week old CBA/J or BALB/c mice with 0.2 ml aliquots of plasma obtained from mice infected 7 days earlier with the 5th passage of virus X. Animals received 2 i.p. injections, fortnightly spaced, and were exsanguinated 7 days after the last dose.

Unless otherwise stated, immune sera were heated at 56°C for 45 min and kept at -20°C until used.

Mouse Antibody Production Test (MAP Test). The material to be tested, e. g., plasma of mice infected with *T. cruzi*, was centrifuged, filtered in 0.2 μ Millipore membrane and inoculated i. p. in CBA/J mice housed in Trexler isolators. After 28 days animals were exsanguinated under the ether anesthesia and sera obtained tested for the presence of antibodies.

Immune electron microscopy. The method of MILNE & LUISONI (1975) [16] was used to perform immune electron microscopy experiments.

Sucrose Gradient Fractionation. Purification of viral particles was performed as follow: samples of spleens or livers from mice infected seven days earlier with $\mathbf{Y}_{IUEC}$ stock of *T. cruzi* or with virus X were homogenized in RPMI medium (10% w/vol) and centrifuged (3000xg for 30 min at 4°C). Supernates were harvested and layered onto a 15% (w/w) sucrose solution in TNE buffer (0.05M Tris hydrochloride, 0.1 M NaCl and 1mM EDTA) on top of a 50% (w/w) sucrose cushion. After centrifugation (3 h, 50.000xg, at 4°C) the light-scattering band at the sucrose interphase was collected and processed for electron microscopy.

Samples obtained from sucrose gradient fraction were dialyzed against phosphate buffered saline and spotted onto copper grids. Particles were allowed to settle for at least 30 min after which they were negatively stained, using 2% phosphotungstic acid, and visualized in a Zeiss EMS9 transmission electron microscope, at a 27,700x grid magnification.

Neutralization Tests. These tests were carried out by mixing equal volumes of immune sera and a virus solution containing 10 LD_{50}. Control mixtures were run out simultaneously, by using sera of normal mice. After incubating mixtures, at room temperature, for 45 min, 0.2 ml aliquots were injected, i. p., in groups of 5 mice, 4 weeks old, and survival rate determined after 10 days.

Pathogenicity Tests. These tests were carried out to see if the plasma of some mice contained some factor that could influence the level of parasitemia and the mean survival time of mice challenged with 10^5 $\mathbf{Y}_{ITC}$. The pooled plasma to be tested were filtered through 0.2 μ Millipore membrane and immediatelly used. Groups of 5 CBA/J mice were injected, i. p.; with 0.1 ml aliquots of the plasma to be tested. Animals were then challenged one hour later, by injecting i.p. 10^5 blood parasites of the $\mathbf{Y}_{ITC}$ stock. Two control groups were carried out: one of these groups received the plasma to be tested but was not challenged and the other group received plasma of normal mice and was challenged. The level of parasitemia, checked 7 days after challenge, and the mean mortality time were determined.

Parasitemia and Mortality. Parasitemia was determined, at the 7th day after infection, by direct light microscopy of 5 μl tail blood, as described by BRENER (1962). Mortality was registered, daily during a period of 30 days. Parasitemia and mortality rate figures presented in Tables take in account the accumulated data four different experiments, when a total of 20 mice per group was used.

Statistical Methods. Student t test was determined as indicated in ZAR (1984) [27].

LD_{50}. Determination of LD_{50} of virus preparations was made according to method of REED & MUENCH (1938) [18] by using groups of 5 mice, four week old, which were injected, i. p., with 0.1 ml aliquots of different concentration of virus. An observation period of 10 days was established since no death was observed after this period.

RESULTS

Data presented on Fig. 1 show that CBA mice infected with the $\mathbf{Y}_{IUEC}$ stock had a higher level of parasitemia and shorter survival time than those infected with $\mathbf{Y}_{ITC}$ stock. MAP tests, performed with the filtered plasma of mice infected with $\mathbf{Y}_{IUEC}$, indicated the presence of anti-MHV antibodies while no antibody was detected when testing the plasma of $\mathbf{Y}_{ITC}$ or $\mathbf{Y}_{INNN}$ infected mice with the different viruses indicated in Material and Methods.

Pathogenicity tests (Fig. 2) indicated that the

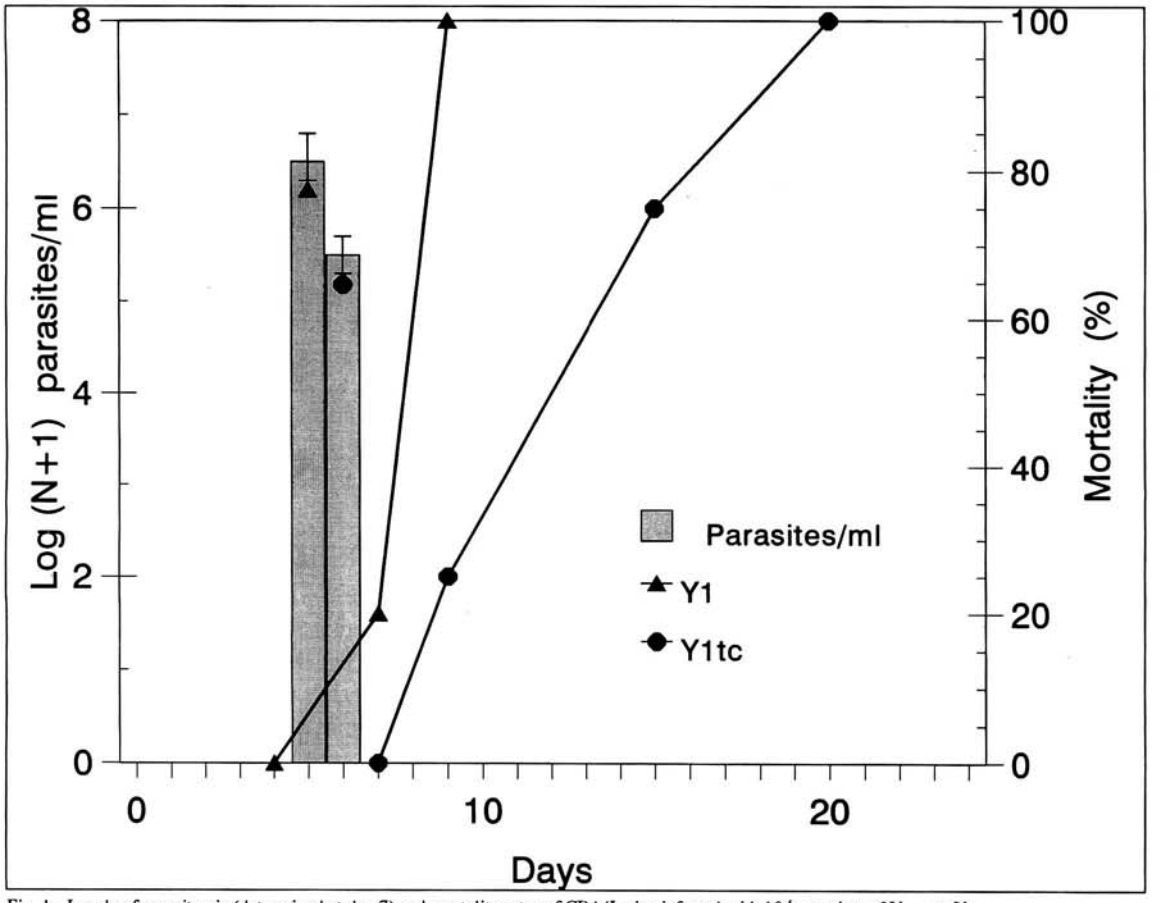

Fig. 1 - Levels of parasitemia (determined at day 7) and mortality rates of CBA/J mice infected with 10^5 parasites of $\mathbf{Y}_{IUEC}$ or $\mathbf{Y}_{ITC}$

plasma of $\mathbf{Y}_{IUEC}$ contained a filterable factor that was able to enhance the level of parasitemia and to decrease the mean survival time of mice injected with 10^5 $\mathbf{Y}_{ITC}$. The plasma of normal or $\mathbf{Y}_{ITC}$ infected mice was unable to induce similar effects.

To verify if the filtered factor could be replicated in a susceptible host, experiments of serial transfer in CBA mice were carried out as indicated in Material and Methods. Pathogenicity tests, made with the plasma obtained after first, second and third passages of the serial transfer, are summarized in Table 1 where can be seen that the filterable factor could be maintained by serial transfer in susceptible hosts.

Parallel experiments (data not shown) showed that this factor, provisionally labelled virus X, enhanced its pathogenicity during serial transfer, since four week-old mice injected only with the third passage of virus X died before the seventh day while those injected with the first passage survived the twenty days observation period.

Virus X lost its ability to kill CBA/J mice and enhance the parasitemia of mice injected with $\mathbf{Y}_{ITC}$ when previously heated at 56°C for 45 min. Animals could survive a challenge of 10 LD_{50} provided that they were primed 15 days before with 0.2 ml of heated (56°C for 45 min) plasma of mice infected with virus X (5^{th} passage). Mice that survived this challenge also survived a second challenge of 50 LD_{50}, given 7 days later. Plasma obtained from those mice that survived the two challenges, when testd by immunofluorescence tests with different viruses, presented positive reactions only when MHV antigen was used.

Neutralization tests showed that mice can be protected from dying after a challenge of 10 LD_{50} of virus X when either anti-MHV or anti-X antibodies were used, while antibodies specific for other viruses were unable to induce any protection (Table 2).

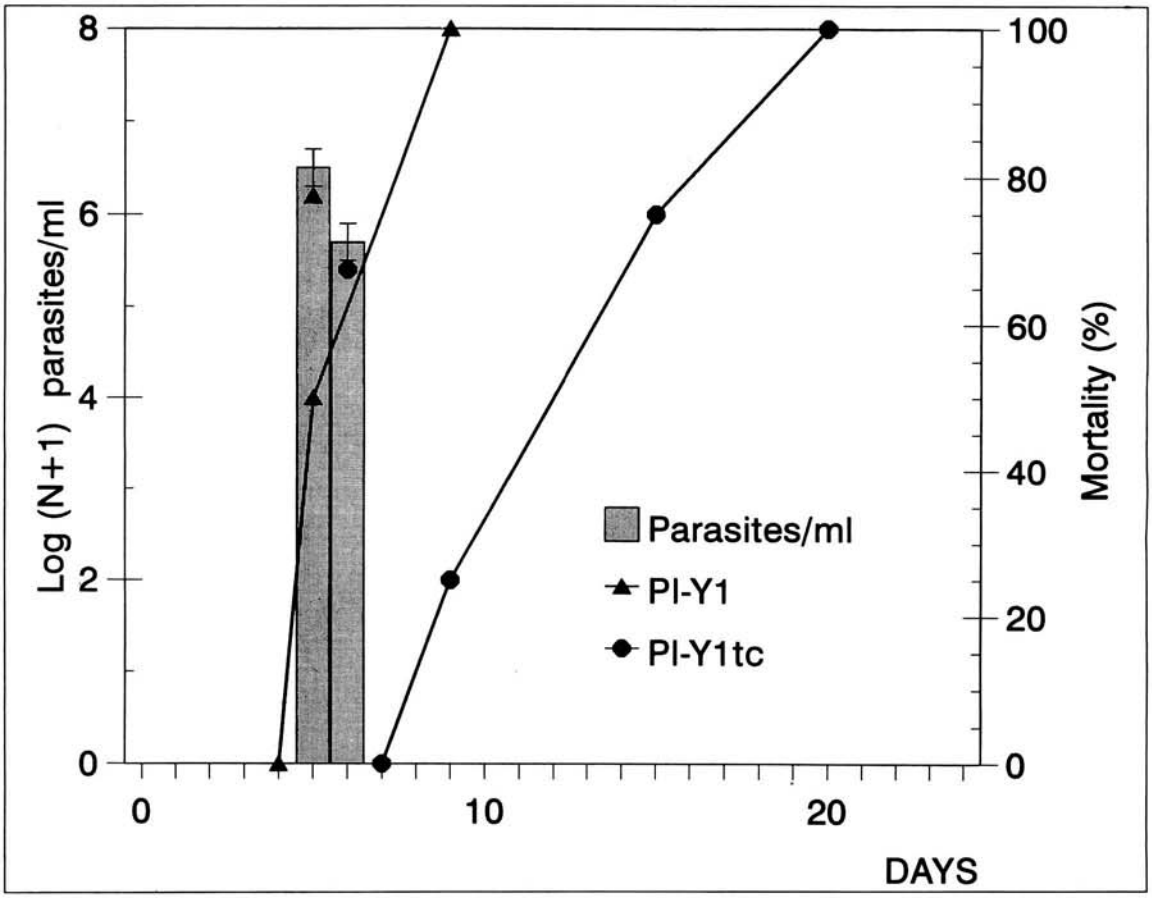

Fig. 2 - Levels of parasitemia (determined at day 7) and mortality rates of CBA/J mice injected with P1-$\mathbf{Y}_{IUEC}$ or P1-$\mathbf{Y}_{ITC}$ and challenged with 10^5 parasites from the $\mathbf{Y}_{ITC}$ stock.

Immune electron microscopy experiments performed by using anti-X or anti-MHV antibodies permitted to detect virus particles in the plasma and in the purified fractions obtained from liver or spleens of mice infected seven days earlier with either virus X (third passage) or $\mathbf{Y}_{IUEC}$ (Fig. 3). No virus particles was detected when using antibodies specific to other viruses.

The action of antibodies specific for different viruses on the evolution of experimental infection with a $\mathbf{Y}_{IUEC}$ was investigated by injecting groups of 5 CBA mice with mixture, previously incubated at 37°C for 45 min, of equal volumes of blood sample obtained from mice infected 7 days earlier with $\mathbf{Y}_{IUEC}$ and specific anti-virus antibody.

As shown in Table 3 the level of parasitemia and the mean survival time of mice infected with blood samples containing 10^5 $\mathbf{Y}_{IUEC}$ that were mixed with equal volume of either anti-X or anti-MHV, were similar to those infected with 10^5 $\mathbf{Y}_{ITC}$ and significantly different from those infected with $\mathbf{Y}_{IUEC}$ mixed with normal sera. These sera were also able to induce lower parasitemia and longer survival time in **CL** infected mice (Table 3).

DISCUSSION

It is well established that laboratory animal genetics, health and environment largely influence results in biomedical research [15]. For this reason, presently these animals are bred under controlled conditions in Laboratory Animal Centers where genetic, health and environment factors are monitored and controlled.

Different strains of *T. cruzi* were isolated and maintained, during a period when mice bred under controlled conditions were not available in our country [7, 8, 20]. Indeed only recently a Laboratory Animal Center (CEMIB/UNICAMP) - able to provide specific pathogen-free mice and rats for research - was inaugurated in Cam-

TABLE 1

Action of filtered plasma, obtained from *T. cruzi* infected mice and serially passaged in naïve mice, on the level of parasitemia and mortality rate of CBA/J mice inoculated with 10^5 parasites of Y_{ITC} stock.

Plasmas injected	Log n° parasites/ml	Mortality rate (%) at days 7	10	15	20
1rst passage plasma	6.0 ± 0.0 (**)	50	100		
Normal plasma	5.4 ± 0.1	20	40	80	100
2nd passage plasma	6.6 ± 0.2 (**)	50	100		
Normal plasma	5.7 ± 0.2	40	80	100	
3rd passage plasma	5.7 ± 0.1(#)	100			
Normal plasma	6.1 ± 0.1	40	80	100	

(**) $P < 0.01$

(#) Only two records were made since almost 100% of mice were dead at that time.

TABLE 2

Neutralization test using different antibodies

Sera tested	Mortality ratio
anti-MHV	1/5
Anti-X	0/5
Anti-LCM	5/5
Anti-Rota	5/5
Normal sera	5/5

pinas University. Thus these strains of *T. cruzi* were isolated and maintained by using convertionally bred mice which are usually found to be affected by natural infections caused by different ectoparasites, endoparasites, fungi, bacteria and viruses [12] it could be expected that when serially passaging blood forms of *T. cruzi*, some other pathogens were also being transmitted.

Present data confirm the expectation that a stock (Y_{IUEC}), originally maintained in conventionally bred mice, is contaminated with a virus - probably a coronavirus, in spite of the fact that this parasite stock has been maintained in SPF mice for 5 years. Work in progress shows that this virus is also present in others stocks of both Y and CL strains. Indeed it was shown that both Y_{IUEC} - a parasite stock which was maintained by serially passaging only in mice - present higher parasitemia and mortality rate than those stocks (Y_{ITC}, Y_{INNN}) which had been submitted to serial passages either in tissue culture (TC) or in axenic media (NNN) before being maintained by serial passage in SPF mice. This finding could not be ascribed to selection of a clone of low pathogenicity due to selective pressures of cell culture or axenic media, since it was shown that the filtered plasma of mice infected with Y_{IUEC} stock contained a factor which was able to restore, in mice infected with either Y_{ITC} or Y_{INNN} stocks, the high level of parasitemia and mortality rates seen among Y_{IUEC} infected mice. This factor, provisionally labelled as virus X, was undoubtly a virus since it could be serially passaged in naïve CBA and BALB/c mice, was inactivated by heating and was neutralized by antibodies which were specific to a coronavirus (anti-MHV-3). The presence of this virus in **CL** can be inferred from the fact that anti-X antibodies induced a significant reduction on the level of parasitemia and increased the median death time of infected animals.

The serial passage of virus X into naïve mice represents indeed a biological purification of this factor. If

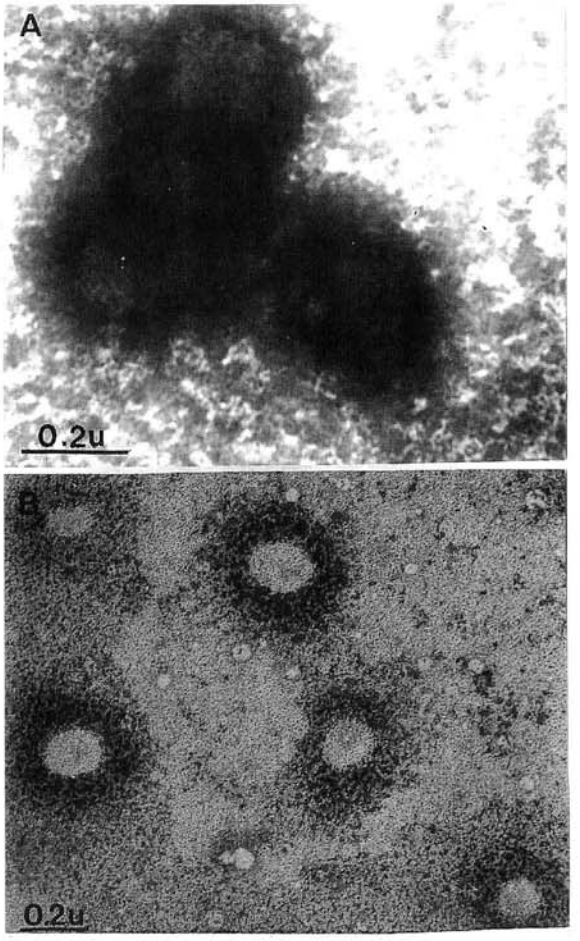

Fig. 3 - A) Electron micrography of virus X particles obtained from plasma of mice infected with Y_{IUEC} stock of *T. cruzi*, on immune electron microscopy tests by using anti-X. B) Electron micrography of sucrose gradient purified particles obtained from livers of mice infected with Y_{IUEC} stock of *T. cruzi*.

TABLE 3

Levels of parasitemia and mortality rates of CBA/J mice inoculated with parasite stocks previously mixed with either anti-X or anti-MHV antibodies.

Parasite stock	Sera admixed	Log n° Parasites/ml	Average day of death ± s.d.
Y_{IUEC}	Anti-X	5.4 ± 0.2(**)	12 ± 2
	Anti-MHV	6.4 ± 0.7	10 ± 1
	Normal sera	6.6 ± 0.4	8 ± 1
CL_1	Anti-X	5.3 ± 0.1	20 ± 4
	Anti-MHV	5.6 ± 0.2	15 ± 2
	Normal sera	5.8 ± 0.3	14 ± 2

(**) $P < 0.01$ when compared with normal sera

we consider that when injecting mice with 0.2 ml volume we are diluting injected antigens at least by a factor of 1/25, at the fifth serial transfer antigens would be diluted to $(1/25)^5$, practically 1 to 10 millions. Then we can practically discard the presence of any *T. cruzi* antigen in the sera of mice infected with the 5th passage of virus X, unless this antigen was replicating during each transfer. Thus the fact that anti-X antibodies was prepared by using the fifth serial transfer of the virus in naïve mice permit to exclude the possibility that these antibodies have some specificity to *T. cruzi* structural antigens.

Data presented evidence that the *T. cruzi* strains test are contamined with a virus that is able either to increase the pathogenicity of some parasites stocks or alter the immune response of the host.

Some parasitic protozoa can be found infected with viruses which alter some of their biological characteristics [24]. However, to our knowledge no virus was detected in *T. cruzi* and the fact that virus X serially passaged in mice in absence of *T. cruzi* infections rule out the hypothesis that this virus would have a direct action on the parasite.

Neutralization experiments strongly suggest that virus X is a coronavirus, since mortality was drastically reduced when either anti-X or anti-MHV was used and both sera were able to neutralize the action of virus X on *T. cruzi* infected mice. However, anti-MHV, apparently, had no effect on the levels of parasitemia of Y_{IUEC} infected animals while anti-X did. This fact can be interpreted by assuming that two different viruses are present or that virus X is a coronavirus with a type specificity different from that MHV-3. Since no other virus was detected in MAP tests it is highly probable that only coronavirus is present and that MHV-3 and virus X have some biological differences. Murine Coronaviruses are, in fact, a large family of related viruses that vary in virulence, genetic and antigenic composition [2, 3].

No death was observed among mice injected with the filtered plasma obtained from mice infected with Y_{IUEC} seven days earlier which were shown to be also infected with virus X. This fact, suggesting that adult mice can cope with the virus infection transmitted under conditions used to maintain the parasite, would permit to assume that mice maintained a low level infection which was carried over the years with *T. cruzi* inoculations. However LD_{50} determinations performed by using 4 weeks old mice showed that virus enhanced its pathogenicity when serially transmitted to naïve mice. No explanation presently exists to this apparent contradiction which suggest that virus infection is somewhat limited in parasite infected mice.

Overall data indicate that some stocks of *T. cruzi* can be contaminated with a virus, which is antigenically related with MHV-3 and, as already shown to occur with others coronaviruses [11, 13, 21], alter the immune response of host. It would be interesting to know if some phenomena ascribed to *T. cruzi* experimental infection are the result of a concomitant infection with the virus or not.

RESUMO

Virus murino como contaminante na infecção experimental pelo *Trypanosoma cruzi*

A possibilidade de que alguma contaminação por virus poderia estar alterando a resposta do hospedeiro à infecção experimental por *Trypanosoma cruzi* foi investigada.

Os dados obtidos mostraram que camundongos CBA/J infectados com estoques de parasitos mantidos em camundongos (Y_{IUEC}) apresentavam maior parasitemia e menor período de sobrevivência do que os infectados com um estoque (Y_{ITC}) que também foi mantido em animal mas tinha sido previamente passado em cultura de células. Testes de produção de anticorpos em camundongos (Map tests) realizados com o plasma fliltrado de camundongos infectados com Y_{IUEC} indicaram a presença do virus da hepatite do camundongo (MHV) enquanto nenhum virus foi detectado no plasma de animais infectados com Y_{ITC}. O plasma filtrado obtido de camundongos infectados com Y_{IUEC} continha um fator capaz de aumentar o nível de parasitemia e reduzir o tempo médio de sobrevivencia dos camundongos desafiados com 10^5 Y_{ITC}. Testes de neutralização mostraram que este fator que podia ser passado seriadamente para camundongos era um coronavirus.

ACKNOWLEDGEMENTS

This work was made possible by virtue of a special program of FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) which implanted the UNICAMP Laboratory Animal Center - CEMIB/ UNICAMP. We thank the collaboration of Prof. V. Kraft and B. Meyer from Zentralinstitut für Versuchtierzucht, Hannover, which permitted to establish a service, in this Center, for the control of virus disease. We are grateful for the technical assistance of C. Colombari, D. V. Cunha Jr. and D. L. Gabriel. We thank the finantial support from FAPESP, FAEP (Fundo de Apoio ao Ensino e a Pesquisa da Unicamp), CNPq (Conselho Nacional para o Desenvolvimento Científico e Tecnológico, Brazil) and KFA (Kernforschungsanlage, Internationale Büro, Jülich, Germany).

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Recebido para publicação em 03/01/1994.
Aceito para publicação em 16/06/1994.