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Anti-Striated Muscle Antibody Activity Produced by *Trypanosoma cruzi*[1] (41571)

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Abstract. We have previously shown that *Trypanosoma cruzi* shares antigenic determinants with preparations of the calcium-sequestering adenosine triphosphatase of sarcoplasmic reticulum. The cross-reacting antigen (SRA) is also apparently present on the sarcolemma of cardiac myofibers. Using highly specific reference antisera to either the small membranes of *T. cruzi* or to a tryptic fragment of striated muscle SRA, it was shown that SRA is present in the striated muscle of animals representative of the evolutionary scale ranging from nonhuman primate to fish. The small membranes of nine different *T. cruzi* strains isolated from widely divergent areas of the American continents also reacted with the reference antisera. This indicates that SRA is present in these *T. cruzi* strains and may be prevalent among all *T. cruzi* strains. The shared *T. cruzi*-striated muscle antigen, SRA, may be a heteroantigen present in all *T. cruzi* strains and in the striated muscle of all classes of animals. Immunization of rabbits (three of five) or chickens (five pairs of five pairs) with striated muscle membrane preparations of different classes of animals, particularly those of nonhuman primate, chicken, and turtle, gave rise to IgG anti-allogeneic striated muscle antibody activity. Immunization of rabbits (four of nine) and chickens (five pairs of six pairs) with the small membranes of different *T. cruzi* strains also produced IgG anti-allogeneic striated muscle. These data indicate that *T. cruzi* shares cross-immunogenicity with striated muscle SRA. Since SRA is apparently present on the sarcolemma of cardiac myofibers, it may be implicated in the immunopathogenesis of Chagas' disease.

Previous investigation has shown that a clone of *Trypanosoma cruzi* (Maria Cristina strain) originally isolated from a living patient with Chagas' disease, shared antigenic determinants with preparations of mammalian striated muscle sarcoplasmic reticulum (SR) (1). The shared *T. cruzi*-muscle antigen (SRA) is present in purified preparations of SR rich in the magnesium-activated adenosine triphosphate (ATPase) implicated in calcium ion membrane transport during muscle relaxation (2, 3). Unlike that of skeletal muscle fibers, the sarcolemma of myocardial cells has calcium transport properties (4), and a calcium-sequestering ATPase of cardiac sarcolemma has recently been described (5). Antisera to either *T. cruzi* membrane or to skeletal muscle SRA localized antibody on the sarcolemma of cardiac myofibers by immunofluorescence tests (1). Furthermore, by gel immunoprecipitation, antiserum to heart membrane preparation and antiserum to *T. cruzi* membrane formed two lines of identity with purified SRA (1). Other studies have shown that lymphocytes obtained from *T. cruzi*-sensitized rabbits strongly interact with heart cell surface antigens (6, 7). These animals also have antiheart antibodies reacting with the sarcolemma (7). Biopsy of hearts of *T. cruzi*-infected patients has shown autogenous antibody bound to the sarcolemma of cardiac myofibers (8). In 11 of 25 *T. cruzi*-infected patients (44%) who had developed moderate to severe cardiomyopathy, abnormally high levels of IgG anti-muscle SRA activity were measured (9). These cell-mediated and antibody cross-reactions with putative heart myofiber surface antigen may be related to the development of chronic Chagasic cardiomyopathy (6–10). We believe that the precise cross immunogenicity of *T. cruzi* with muscle may play an important role either during the induction phase or during the repair of the myocardial lesion often associated with chronic Chagas' disease.

With these considerations in mind, the study reported here was designed to answer the following questions: (1) Is SRA present in

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all *T. cruzi* strains isolated from widely spaced geographic areas? (2) Is SRA present in the muscle SR of animal species representative of the evolutionary scale? (3) What is the degree of specificity of the immune response to striated muscle SRA and to *T. cruzi* membrane SRA?

MATERIALS AND METHODS. *Trypanosoma cruzi strains.* Nine different strains were used: FH5, FR4, skunk, and raccoon strains isolated from North American sylvatic animals were supplied by Dr. Irwin Kagan, Center for Disease Control, Atlanta, Ga.; the others were originally isolated by xenodiagnosis from humans and include: the Enriqueta strain (Dr. Jorge Tay, Universidad de Mexico); the Colombia strain (Dr. Sonia Andrade, Universidade Federal da Bahia, Brazil); the José Cardoso strain from Northeast Brazil (Dr. Rodney Hoff, Harvard University School of Public Health); the Y strain from Central Brazil (Dr. Antonio Teixeira, Universidade da Brasilia); and the Argentina strain (Dr. Patricio M. Cossio, Centro de Estudios Médicos e Investigaciones Clínicas [CEMIC], Buenos Aires). All were grown in liver infusion broth supplemented with fetal calf serum as outlined previously (1), and all had been passed more than 20 times before use. Actomyosin-extracted small membranes of *T. cruzi* were prepared as previously described (1).

Classes of animal striated muscle and muscle SR microsomes. The striated muscle of the following classes of animals were used for the isolation of SR microsome antigens: *Cyprinus carpio* (goldfish), *Rana clamitans* (green frog), *Chrysemys picta* (painted turtle), *Gallus domesticus* (chicken), *Oryctolagus cuniculus* (rabbit), and *Cercopithecus aethiops* (African green monkey). Muscle from all classes of animals was obtained immediately after killing, except for nonhuman primate striated muscle which was shipped fresh frozen (Pel-Freez Biologicals, Inc., Rogers, Ark.). Actomyosin-extracted SR microsomes were obtained as described by MacLennan (2).

Shared T. cruzi-striated muscle antigen. Rabbit striated muscle SR microsomes were further purified according to MacLennan (2) in order to obtain preparations of SRA. The serologic antigenicity of SRA declines rapidly within 1 month of storage at −20°C.

Immunization schedule of chickens and rabbits. Twenty-two 6- to 8-week-old Leghorn chickens (Rockland Farms, Gilbertsville, Pa.) and fourteen 6- to 8-month-old male white New Zealand rabbits (Triple-R Rabbitry, Manasquan, N.J.) were used for immunization. Animals were checked prior to immunization by serologic testing for natural antibody to muscle SRA. For immunization, 500 μg of each antigen was homogenized in 1 ml of complete Freund's adjuvant (CFA) (Grand Island Biologicals Co., Grand Island, N.Y.) and given in six equal doses 1 week apart by injection into multiple subcutaneous, intramuscular, and intraperitoneal sites. Animals were bled 10–14 days after the last antigen injection. Rabbit preimmunization sera gave ≤1:20 titers when tested against muscle SRA; chicken preimmunization sera, ≤1:80. In testing that a significant antibody response after immunization had occurred, the sera of all animals yielded titers ≥1:640 with the homologous immunogen.

Reference cross-reacting anti-SRA sera. Dr. David H. MacLennan, Montreal, Canada, kindly supplied highly specific chicken anti-M_r = 55,000 tryptic fragment of rabbit skeletal muscle SRA (3). Rabbit anti-*T. cruzi* (Maria Cristina clone) membrane serum, previously characterized by us (1), was also used.

Control antisera. Preimmunization samples, rabbit anti-human cardiac tropomysin (Dr. Carl G. Becker, Department of Pathology, Cornell University Medical College), rabbit anti-mouse kidney extract antigen, anti-ox RBC, and anti-sheep RBC were used as control sera (1). All antisera were obtained after immunization with CFA and antigen in a similar schedule to that described above.

Enzyme-linked immunoassay (ELISA). Goat anti-rabbit IgG and rabbit IgG anti-chicken IgG (Miles Laboratories, Inc., Elkhart, Ind.) were linked to alkaline phosphatase (Bovine Intestine Type VII, Sigma Chemical Co., St. Louis, Mo.) in a ratio of 100 units/mg immunoglobulin, according to published procedures (12). Ten micrograms of purified rabbit skeletal muscle SRA was applied to each well of a polystyrene microtiter plate (Ace Scientific Supply Co., Linden, N.J.). SR microsomes and small membranes of *T. cruzi* were applied in 30 μg quantities. All sera were titrated in twofold dilutions, beginning

TABLE I. CROSS-*T. cruzi* STRAIN SPECIFICITY OF CHICKEN ANTI-MUSCLE SR MICROSOME ANTIGENS[a]

Reference antiserum to:	North American strains				Central American strain	South American strains			
	FH5	FR4	Skunk	Raccoon	Enriqueta	Colombia	Cardoso	Y	Argentina
T. cruzi membrane	1:20,480	1:40,960	1:40,960	1:5,120	1:20,480	1:40,960	1:40,960	1:20,480	1:40,960
Rabbit muscle SRA	1:5,120	1:5,120	1:20,480	1:10,240	1:5,120	1:10,240	1:10,240	1:5,120	1:10,240

[a] Chicken control sera: <1:80 prior to immunization; positive titer ⩾ 1:320.

at 1:20, and in duplicate. Known positive and negative antisera were run on each plate. Titers were reproducible within one twofold dilution and titers were read without knowledge of the source of the serum (9).

Protein determination. The amount of protein was measured by Kjeldahl nitrogen determination as previously described (1).

Data analysis. Titers were considered positive when antisera of each animal were greater than two standard deviations from the mean of preimmunization bleedings.

RESULTS. *Cross specificity of the shared muscle-T. cruzi antigen.* The small membranes of the four strains of *T. cruzi* isolated from North American sylvatic animals and the small membranes of the five strains of *T. cruzi* isolated from infected humans in Central and South America, reacted with chicken anti-*T. cruzi* membrane reference serum (Table I). The reference chicken antiserum to the tryptic fragment M_r = 55,000 of rabbit skeletal muscle SRA also reacted with the small membrane fragments of all nine different strains of *T. cruzi* (Table I).

The actomyosin-extracted SR microsomes of skeletal muscle of five of six types of animal shared antigenic determinants with the small membranes of *T. cruzi* (Table II). Muscle SR microsomes derived from two orders of Mammalia, monkey—a nonhuman primate—and rabbit, and from chicken, frog, and carp, reacted with chicken anti-*T. cruzi* membrane serum, whereas no significant cross-reaction was detected with turtle muscle SR microsomes. The reference chicken antiserum to the tryptic fragment M_r = 55,000 of rabbit skeletal muscle SRA also reacted with five classes of muscle SR microsome preparations with the exception of frog (Table II). When anti-M_r = 55,000 was used, nonhuman primate and chicken muscle SR microsome preparations yielded the highest titers. Anti-allogenic striated muscle SR antibody activity was detected; that is, both reference chicken antisera reacted with chicken actomyosin-extracted skeletal muscle SR microsomes (Table II).

The actomyosin-extracted muscle SR microsomes of all other classes of animals with the exception of nonhuman primates may contain significantly less SRA per unit weight than the small membranes of *T. cruzi*. This is suggested by comparing the titers of chicken

TABLE II. CROSS-CLASS SPECIFICITY OF CHICKEN ANTI-MUSCLE SR MICROSOME ANTIGENS[a]

Reference antiserum to:	Mammals		Aves[b]	Reptile	Amphibian	Fish
	Nonhuman primate (monkey)	Lagomorph (rabbit)	(chicken)	(turtle)	(frog)	Osteichthyes (carp)
T. cruzi membrane	1:320	1:640	1:320	1:160 NS[c]	1:320	1:640
Rabbit muscle SRA	1:2560	1:320	1:1280	1:640	1:160 NS	1:640

[a] Chicken control sera: <1:80 prior to immunization: positive titer ⩾ 1:320.
[b] Allogeneic antigen: actomyosin-extracted chicken muscle SR microsomes (30 μg/well).
[c] Not significant.

TABLE III. PRODUCTION OF CHICKEN IgG ANTI-ALLOGENEIC MUSCLE SR ACTIVITY

Antiserum to:	Immune titer against heterogeneic (rabbit) muscle SRA[a,b]	Immune titer against allogeneic muscle SR[b]
Muscle SR		
Nonhuman primate	1:2560	1:5120
Lagomorph	1:5120	1:5120
Aves	ND	ND
Reptilia	1:640	1:5120
Amphibian	1:1280	1:40960
Osteichthyes	1:640	1:1280 (5/5)
T. cruzi membrane		
FH5	1:80 NS[c]	1:640
FR4	1:160 NS	1:1280
Colombia	1:640	1:5120
Cardoso	1:80 NS	1:160 NS
Y	1:160 NS	1:1280
Argentina	1:320	1:1280 (5/6)

[a] Allogeneic antigen: actomyosin-extracted chicken muscle SR microsomes (30 μg/well).
[b] Chicken control sera: ≤1:80 prior to immunization; positive titer ≥ 1:320.
[c] Not significant.

anti-M_r = 55,000 rabbit skeletal muscle SRA against 30 μg/well of muscle SR microsomes of the classes of animal in Table II with the titers obtained against an equal amount of small membranes of the nine *T. cruzi* strains in Table I. However, comparisons of this kind may not be valid since there is evidence that the shared muscle SR antigen may be unavailable beneath the exposed surface of *T. cruzi* membranes (1).

Production of IgG anti-allogeneic muscle SR. All chickens immunized with actomyosin-extracted striated muscle SR microsomes of different types of animal showed significant serum IgG anti-allogeneic chicken skeletal muscle SR microsome activity (Table III). These same chicken antisera gave rise to high IgG titers when tested against SRA derived from rabbit striated muscle (Table III). When chicken IgG anti-chicken muscle SR microsome activity was measured in sera of chickens immunized with actomyosin-extracted membranes of different strains of *T. cruzi*, five of six pairs of these sera showed anti-allogeneic striated muscle SR antibody activity (Table III). Two of six pairs of these sera also showed significant titers of IgG anti-heterogeneic rabbit striated muscle SRA activity.

Immunization of rabbits with actomyosin-extracted muscle SR microsomes of classes of animals representative of the upper evolutionary scale (nonhuman primate, chicken, and turtle) produced anti-allogeneic striated muscle SRA antibody activity, whereas immunization with frog and carp muscle SR microsomes did not (Table IV). When rabbit IgG anti-rabbit striated muscle SR activity was measured in sera of rabbits immunized with actomyosin-extracted small membranes of different strains of *T. cruzi*, four of nine rabbits showed low-titer anti-allogeneic striated muscle activity (Table IV).

A variety of control antisera (rabbit anti-human cardiac tropomyosin, rabbit anti-mouse kidney extract antigen, anti-ox RBC, and anti-sheep RBC) showed no significant IgG anti-chicken microsome activity nor any significant IgG anti-rabbit muscle SR activity (≤1:40).

DISCUSSION. The data reported here indicate that the antigen shared by *T. cruzi* and striated muscle (SRA) is present in striated

TABLE IV. PRODUCTION OF RABBIT IgG ANTI-ALLOGENEIC MUSCLE SR ACTIVITY

Antiserum to:	Immune titer against allogeneic muscle SRA[a]
Muscle SR	
Nonhuman primate	1:5120
Lagomorph	ND
Aves	1:1280
Reptilia	1:5120
Amphibian	1:20 NS[b]
Osteichthyes	<1:20 NS (3/5)
T. cruzi membrane	
FH5	<1:20 NS
FR4	<1:20 NS
Skunk	1:80
Raccoon	1:80
Enriqueta	<1:20 NS
Colombia	1:160
Cardoso	1:160
Y	<1:20 NS
Argentina	<1:20 NS (4/9)

[a] Allogeneic antigen: SRA preparations 10 μg/well. Rabbit control sera: <1:20 prior to immunization; positive titer ≥ 1:80.
[b] Not significant.

muscle SR of classes of animals representative of the evolutionary scale, ranging from nonhuman primate (monkey) to fish (Osteichthyes). Furthermore, nine different strains of *T. cruzi*, including strains isolated from sylvatic animals of North America, also showed cross-reactivity with highly specific anti-striated muscle SRA reference serum. These data indicate that SRA is present among all *T. cruzi* strains tested and suggested that the cross-reactive muscle determinant may be prevalent among all *T. cruzi* strains of the American continents.

None of a variety of hyperimmune sera to muscle tropomyosin, ox RBC, murine kidney extract, or sheep RBC reacted with the small membranes of nine *T. cruzi* strains nor with the actomyosin-extracted muscle SR of monkey, rabbit, chicken, turtle, frog, or carp. These results indicate that the shared *T. cruzi*-muscle SR antigen is not related to ubiquitous blood group or Forssman determinants, or to components of the muscle contraction/relaxation system.

The data show that muscle SR immunogens of different classes of animal, particularly those of nonhuman primates, chickens, and turtles, give rise to IgG anti-allogeneic muscle SR activity. More importantly, however, animals of both classes, chicken and rabbit, develop IgG anti-muscle microsome activity when immunized with *T. cruzi* strain membranes. Five of six pairs of chickens and four of nine rabbits immunized with different *T. cruzi* strain membranes produced antibody to allogeneic muscle SR.

The shared *T. cruzi*-striated muscle antigen may exist as a heteroantigen present in all *T. cruzi* strains and all classes of animals. The degree of immunologic identity between the heteroantigen present in *T. cruzi* and that present in the host may modulate the nature of the immune response evoked by the infective organism. An exact identity between the two may result in recognition as self and lack immunogenicity. On the other hand, partial identity between the two may elicit an immune response to the "mismatched" heteroantigen resulting in autoreactive immunologic phenomena. Since SRA is evidently present on the sarcolemma of cardiac myofibers (1), these autoimmune reactions may involve the heart. In this regard, heart biopsies of patients with *T. cruzi* infection have shown autogenous antibody bound to the sarcolemma of cardiac myofibers (8). Lymphocytes from *T. cruzi*-infected rabbits or from rabbits immunized with *T. cruzi* membranes interacted with and destroyed allogeneic cardiac cell cultures (6, 7), and sera from these animals reacted with the sarcolemma of heart fibers (7). Rabbits immunized with *T. cruzi* membranes developed a cardiomyopathy (7). In addition, sera from 11 of 25 patients (44%) with chagasic cardiomyopathy had abnormal high anti-SRA antibody activity (9).

An immunopathogenic role cannot be assigned to SRA unless it can be shown that immunization with SRA induces the production of autoimmune reactions and that these SRA-directed reactions cause myocardial damage. Recent studies in our laboratory have shown that immunization of inbred mice with the shared *T. cruzi*-striated muscle SRA elicited the production of cytotoxic T-lymphocytes which destroyed syngeneic neonatal cardiac myofibers *in vitro* (13). Furthermore, immunization of mice with heterologous nonhuman primate SRA in complete Freund's adjuvant produced a severe myocarditis with myocardial fiber destruction attended by intermediate-sized lymphocytes (9). These data, showing the elicitation of autoimmune reactions and the subsequent destruction of cardiac myofibers both *in vitro* and *in vivo*, suggest that SRA is indeed immunopathogenic.

Further characterization of the shared *T. cruzi* striated muscle antigen is needed to fully understand the immunologic responses it elicits and its role in the immunopathogenesis of the heart disease associated with *T. cruzi* infection.

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