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SPECIFIC AND SENSITIVE IMMUNOLOGICAL DIAGNOSIS OF CHAGAS' DISEASE BY COMPETITIVE ANTIBODY ENZYME IMMUNOASSAY USING A *TRYPANOSOMA CRUZI*-SPECIFIC MONOCLONAL ANTIBODY

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Abstract. Coexistence of Chagas' disease with leishmaniasis and *T. rangeli* infection in endemic areas and cross-reactivity between corresponding etiological agents can confuse the immunodiagnosis of Chagas' disease. A discriminative serological test could therefore represent a major advance in specific immunodiagnosis. A competitive antibody enzyme immunoassay against a component 5-enriched preparation, using a *T. cruzi* species-specific monoclonal antibody has allowed development of a specific serodiagnosis of Chagas' disease with high sensitivity (96.6% in undetermined and chronic phases of infection). This test can differentiate Chagas' disease from other cross-reacting parasitic diseases in areas where concomitant infections are unknown or suspected.

The parasitic protozoan *Trypanosoma cruzi* is the causative agent of Chagas' disease (American trypanosomiasis). At least 24 million people in Central and South America are estimated to be infected with *T. cruzi*.[1]

In the initial acute phase of Chagas' disease, lethal in about 10% of cases, trypanosomes can usually be detected in the circulating blood. In the undeterminate and chronic phases of infection, parasitemia is very low and immunoserological diagnosis is required.

In vast areas of Central and South America, cutaneous, mucocutaneous[2,3] or visceral leishmaniasis[4] and *T. rangeli* infections[5–7] are associated with *T. cruzi* infection. Due to a variable degree of cross-reactivity between the corresponding etiological agents[8–10] the precise diagnosis of Chagas' disease using classical serological tests is not effective in areas where these diseases are coendemic.

Attempts have been made to overcome such cross-reactivity using amastigote or trypomastigote forms of *T. cruzi*,[11,12] "live" *T. cruzi* antigen,[13] human sera absorption,[14] a monospecific serum anti-*T. cruzi* component 5 to identify specific antibodies,[15] and defined *T. cruzi*-specific antigens.[16,17] The World Health Organization has emphasized the need for a discriminative serological test ensuring the specific diagnosis of South American trypanosomiasis.

In previous studies, a *T. cruzi*-specific component with regard to other Trypanosomatidae (*T. rangeli, L. donovani, L. mexicana, L. braziliensis*)[8,15] so called "5," was identified. Moreover, anti-component 5 precipitating antibodies frequently were found in sera from patients chronically infected with *T. cruzi*.[15,18,19] In recent papers,[20–22] we reported the production of murine monoclonal antibodies directed against component 5 of *T. cruzi* and the characterization of target antigens corresponding to the 72 Kd glycoprotein and its maturation products (51 Kd, 43 Kd and 24 Kd).

In the present work, we describe a simple, highly specific and sensitive serological test for Chagas' disease by competitive (antibody) enzyme immunoassay (CEIA). It uses a 24 Kd-enriched fraction as antigen and a species-spe-

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cific anti-*T. cruzi* monoclonal antibody, allows the detection of anti-component 5 antibodies in the sera of chagasic patients, and can differentiate Chagas' disease from leishmaniasis.

MATERIALS AND METHODS

Antigen preparation

Trypanosoma cruzi (Tehuantepec strain) epimastigotes cultured in GLSH medium as previously described[8] were collected by centrifugation at 400 × *g* for 15 min at 4°C and washed four times with Hank's balanced salt solution. Six grams (wet weight) of epimastigotes frozen in 100 ml 0.1% NaCl were disintegrated by four passages through an hydraulic press (X press, Bio-LKB) at 18,000 psi. It was then centrifuged at 26,000 × *g* for 1 hr at 4°C. The supernatant was dialyzed against distilled water for 24 hr at 4°C, lyophilized and used as a total soluble extract of *T. cruzi* (TSE). Twenty mg of TSE were resuspended in 4 ml distilled water and added with an equal volume of chloroform/methanol solution (2:1). The mixture was shaken and centrifuged at 1,000 × *g* for 30 min at 4°C. The aqueous phase was collected and extracted twice in the same way. Organic solvents were evaporated and the remaining aqueous fraction was precipitated by the addition of 3 volumes of ethanol for 4 hr at −20°C. After centrifugation, the precipitate was washed with ethanol, dried, and resuspended in 2 ml distilled water. The solution was centrifuged, dialyzed against distilled water for 24 hr at 4°C and lyophilized. This successive extraction by chloroform/methanol and ethanol precipitation created a component 5-enriched preparation (C_5-EP) as verified by immunoelectrophoresis results of mouse sera immunized with this fraction.[21]

Monoclonal antibody (Mab)

A murine IgG_1 Mab (II-190/30) produced in our laboratory has been demonstrated to recognize the component 5 corresponding to the 72 Kd glycoprotein and its maturation products (51 Kd, 43 Kd and 24 Kd).[21,22] Ascites were produced in BALB/c mice and immunoglobulins were isolated from the ascitic fluid by precipitation with 50% saturated ammonium sulfate. The pellet was dissolved in 0.9% NaCl and dialyzed overnight. The solution was then submitted to ion exchange chromatography on a DEAE-Trisacryl column. The purified Mab was labeled with alkaline phosphatase (grade I from calf intestine, Boehringer) by the one-step glutaraldehyde method previously described.[23]

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Total soluble extract and C_5-EP were dissolved in 40 μl of sodium dodecyl sulfate (SDS)-containing slab gel buffer with 5% β-mercaptoethanol and boiled for 3 min. Insoluble material was removed by centrifugation at 2,000 × *g* for 10 min before samples were applied to the gel. SDS-PAGE was carried out on vertical slab gels according to the method of Laemmli[24] using 10% polyacrylamide gel. After electrophoresis the gel was stained with Coomassie blue, destained and then dried under vacuum.

Competitive antibody enzyme immunoassay (CEIA)

Polypropylene beads (6.5 mm) were incubated overnight at room temperature by gentle agitation in 0.015 M carbonate-0.035 M bicarbonate buffer (pH 9.6) containing the antigen (see Results for concentrations). After washing three times in PBS-Tween (0.01 M phosphate buffer, pH 7.2, 0.1% Tween 20), the beads were incubated with PBS + 0.1% bovine serum albumin for 2 hr and washed twice in PBS-Tween, then once in phosphate buffered saline (PBS).

CEIA was performed in disposable polystyrene tubes. Coated beads were incubated for 3 hr at 37°C in a mixture of 100 μl diluted Mab labeled with alkaline phosphatase, 100 μl diluted human serum sample and 150 μl PBS-Tween. Tubes were emptied by suction and beads were washed three times in PBS-Tween and transferred to another tube; the amount of enzyme fixed to the beads was determined using 300 μl of enzyme substrate (1 mg/ml 4-nitrophenylphosphate in 0.5 M Na_2CO_3, 0.001 M $MgCl_2$ buffer, pH 10.4). After 1 hr incubation at 37°C, further reaction was stopped by addition of 300 μl 2 N NaOH and the resulting yellow color was measured in a spectrophotometer at 405 nm. The CEIA test involved the inhibition of binding of

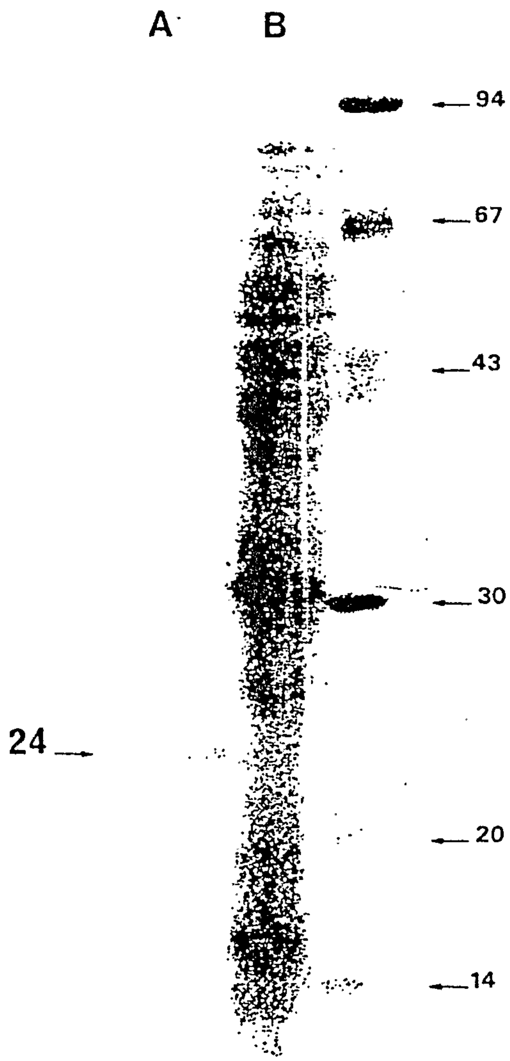

FIGURE 1. Protein patterns after Coomassie blue staining of 500 μg of total soluble extract of *T. cruzi* (B) and 50 μg of the component 5-enriched preparation (A) by SDS-PAGE. The positions of migration of the marker proteins ($M_r \times 10^{-3}$) are indicated.

alkalin phosphatase-labeled Mab by sera from *T. cruzi*-infected patients; low extinction values indicate the presence of anti-component 5 antibodies in human serum. A total of 1,750 sera can be tested using 2.5 mg of C_5-EP and 3.5 mg of purified Mab.

Human sera

The control group was comprised of 50 healthy patients with a negative serology for *T. cruzi*, including 20 Europeans, 20 Bolivians and 10 Argentinians.

The *T. cruzi*-infected group included 15 Argentinian children (1–10 years) with evident acute phase Chagas' disease (Romaña sign, general edema, presence of blood parasites) and 89 Bolivian patients with chronic phase Chagas' disease and a positive serology for *T. cruzi* (immunofluorescence, complement fixation test, immunoelectrophoresis and ELISA). They lived in lowland areas of Bolivia where leishmaniasis has never been found.

The third group was infected by other Trypanosomatidae, including *Leishmania braziliensis* (15 Bolivian patients with evident lesions of mucocutaneous leishmaniasis and a negative serology for *T. cruzi* who lived in areas where Chagas' disease had never been found), *L. tropica* (6 African patients with evident cutaneous leishmaniasis), *L. donovani* (14 mediterranean patients with visceral leishmaniasis), and *T. gambiense* (11 African patients with sleeping sickness).

A fourth group was infected by other protozoa including *Toxoplasma gondii* (14 European patients), *Plasmodium* (10 African patients), and *Entamoeba histolytica* (3 African patients).

The fifth group was comprised of patients living in areas with possible mixed infection: 40 Brazilians clinically and serologically confirmed as being infected with *Schistosoma mansoni* and 47 Bolivian patients with clinical and serological confirmation of *L. braziliensis* infection, living in the Yungas Valley.

RESULTS

Composition of the C_5-EP antigen

The C_5-EP was analyzed by SDS-PAGE followed by Coomassie blue staining. Figure 1 shows a comparison between protein patterns of the *T. cruzi* total soluble extract (TSE) (500 μg, lane B) and the C_5-EP (50 μg, lane A). More than 20 polypeptide chains could be clearly distinguished in TSE pattern and a major band of 24,000 daltons with some minor contaminating proteins were identified in the C_5-EP profile. Approximately 2.5 mg of C_5-EP was obtained from 20 mg of TSE.

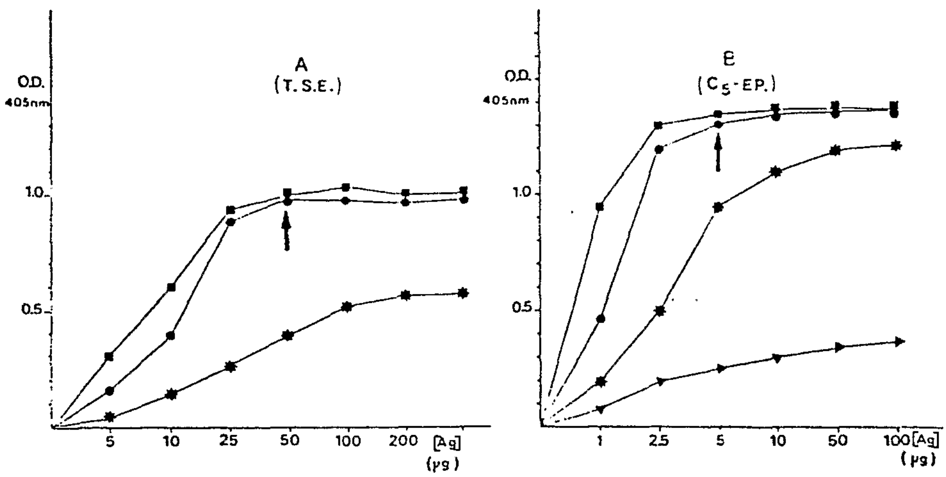

FIGURE 2. Direct binding assays of various dilutions (■ 1/50, ● 1/100, ★ 1/200, ▲ 1/400) of the conjugate (alkaline phosphatase labeled Mab) to polypropylene beads coated with different concentrations of *T. cruzi* total soluble extract (A) or component 5-enriched preparation (B).

TSE and C_5-EP in CEIA

Various dilutions of the conjugate (alkaline phosphatase-labeled Mab) and different concentrations of TSE and C_5-EP were used in preliminary studies. The highest range of optical density (OD) values was obtained with $\frac{1}{100}$ diluted conjugate (Fig. 2A and B). The optimal antigen concentrations were 50 μg/ml of TSE (Fig. 2A) and 5 μg/ml of C_5-EP (Fig. 2B).

In these conditions, CEIA test was performed with sera from *T. cruzi*-infected patients and from controls. In both cases, optimal sensitivity of the test was achieved using the $\frac{1}{5}$ diluted serum (Fig. 3A and B), which obtained a 2.3 higher difference in OD values between positive and negative sera with C_5-EP than with TSE. This increase in the sensitivity of the test was used in all subsequent studies, using the 5 μg ml C_5-EP coating.

Sensitivity and specificity of CEIA

Figure 4 shows the CEIA OD values in the control group (0.92 ± 0.17) significantly higher than those of the acute (0.50 ± 0.30, $P < 0.001$) and those with the chronic phase (0.19 ± 0.14, $P < 0.001$). There was a clear cut-off point be-

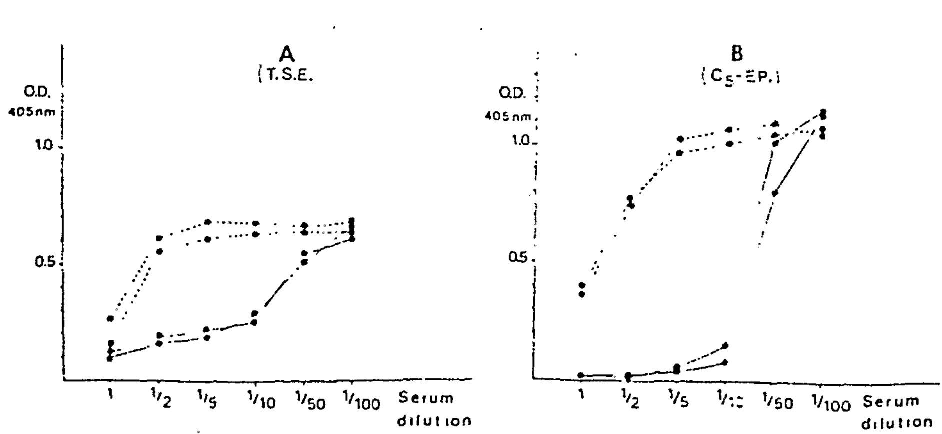

FIGURE 3. Human serum dilution curves (●——● chronic Chagas' disease serum; ●— — —● control serum) in the CEIA test performed with *T. cruzi* total soluble extract (A) and component 5-enriched preparation (B).

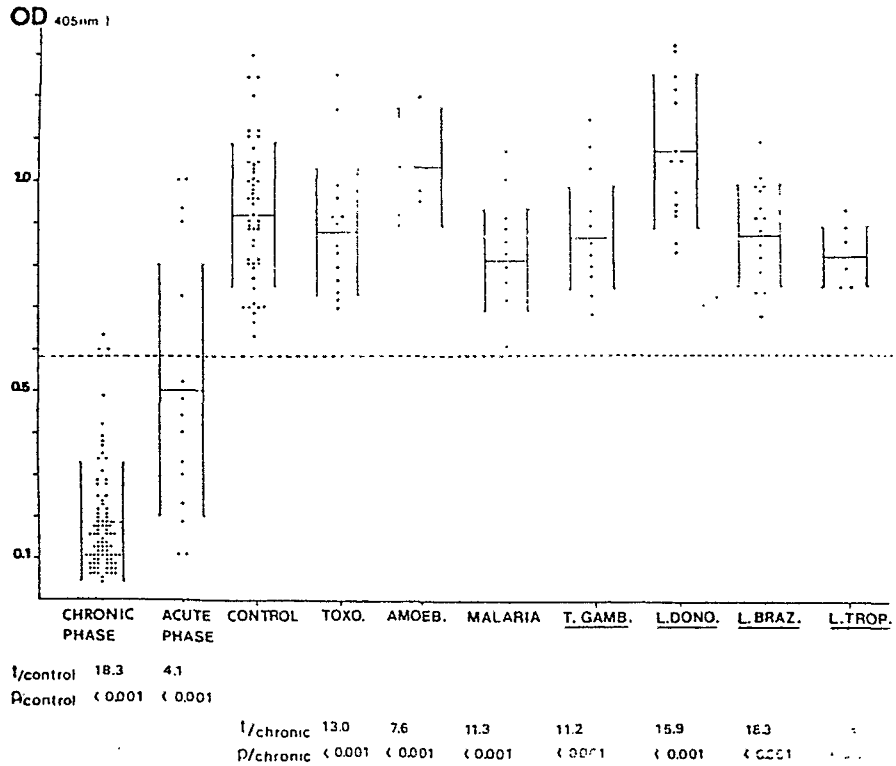

FIGURE 4. Sensitivity and specificity studies of CEIA using component 5-enriched preparat[illegible] anti-component 5 Mab and sera from different patient groups. Chronic phase = patients with chronic [illegible]. Acute phase = patients with acute phase of the Chagas' disease; Control = healthy subjects from [illegible] Bolivia and Argentina; Toxo = Toxoplasmosis; Amoeb. = Amoebiasis; malaria; *T. gamb.* = African try[illegible]; *L. dono.* = visceral leishmaniasis; *L. braz.* = mucocutaneous leishmaniasis; *L. trop.* = cutaneous leishmaniasis. The Student's *t*-test shows a significant difference between Chagas' disease group and other groups. – – – Optical density cut-off point.

tween chagasic and non-chagasic patients using the OD 0.58 [calculated as m (control group)-2 SD; i.e., 0.92 − 2 × (0.17) = 0.58]. The sensitivity of CEIA positive detection in relation to the cut-off value was 66.7% in acute infection and attained 96.6% in the chronic phase of Chagas' disease.

As shown in Figure 4, CEIA OD values in sera from patients with other Trypanosomatidae or protozoal infections were between 0.6 and 1.3 and showed a significant difference from those of chronic Chagas' disease.

Detection of Chagas' disease in areas with possible mixed infections

In the two groups of Brazilian and Bolivian sera with possible mixed infections, CEIA detected anti-*T. cruzi*-specific antibodies in 3 and 15 sera samples, respectively (Fig. 5), giving evidence of Chagas' infection in 7.5% of schistosomiasis cases and in 31.9% of leishmaniasis cases.

DISCUSSION

In areas where mucocutaneous and or visceral leishmaniasis are coendemic with Chagas' disease, the classic serological tests do not allow a differential immunodiagnosis. Consequently, various methods have been proposed to increase the specificity of the serodiagnosis of *T. cruzi* infection.[11–13] But the increase in specificity with these tests is often at the expense of efficiency[14] and sensitivity.[15] In the present investigation, we propose a specific and sensitive enzyme immunoassay using a Mab that provides a potential serodiagnosis for Chagas' disease, particularly in areas with concomitant infections.

Previous studies, using immunoelectrophoresis[18] and a microdouble diffusion test,[15] have demonstrated the high frequency of anti-component 5 antibodies in sera of patients with Chagas' disease. More recently, murine Mabs (II-190/30, III-160/18 and I-35/67) have been produced against component 5[20,21] which recognized a glycoprotein of 72 Kd (GP72) and its maturation products (51, 43 and 24 Kd). These molecules are exclusively expressed in epimastigote and amastigote forms of *T. cruzi*[21] and not on other trypanosomatid parasites of humans (*Leishmania* and *T. rangeli*).[8] Preliminary results[21] using Mab II-190/30 in a competitive radioimmunoassay suggest its potential value for specific serodiagnosis of Chagas' disease. Thus, the use of a species-specific Mab in a competitive enzyme immunoassay allows precise immunodiagnosis.[25] It possesses all the advantages of constant specificity and reproducibility of a monoclonal reagent. Finally, this enzyme immunoassay is sensitive, requires low reagent consumption (microassays) and can be automated for large scale applications.[26,27]

Recently, two sensitive and specific tests using purified antigens of *T. cruzi* (25 Kd and 90 Kd) have been developed.[16,17] However, they are very expensive and require large quantities of purified antigens, precluding a large field application. The present simple chemical extraction procedure from a total hydrosoluble extract of *T. cruzi* can isolate a measurable quantity of 24 Kd-enriched fraction. The 24 Kd antigen, one of the maturation products of the GP72,[21,22] seems to be produced by a proteolytic degradation occurring during extraction.

No false positive reactions were observed with the CEIA test using sera from healthy individuals as well as from patients with other parasitic infections. This was due to the specificity of the component 5 of *T. cruzi* with regard to other Trypanosomatidae as previously demonstrated.[8] However, further evaluations of the usefulness of this test are required in areas where *T. rangeli* infections are associated with Chagas' disease.

More than 95% of the patients with undeterminate and chronic phases of Chagas' disease presented specific high levels of anti-component 5 antibodies. This high sensitivity was not only dependent on the monoclonal reagent but also on the use of a component 5-enriched fraction

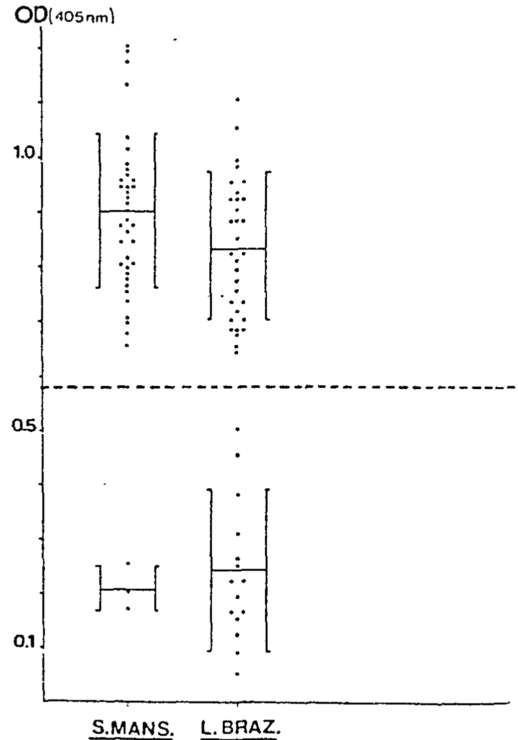

FIGURE 5. Detection of mixed infections in two groups of sera samples from South American patients (*S. mans.* = Brazilian patients with schistosomiasis; *L. braz.* = Bolivian patients with mucocutaneous leishmaniasis). --- Optical density cut-off point.

In fact, when a crude extract of *T. cruzi* was used, the sensitivity of the CEIA test was lower.

On the other hand, only 66.7% of the patients with acute Chagas' disease showed anti-component 5 antibodies. Several hypotheses can account for these results: a) the low level of anti-component 5 antibodies; b) the eventual weak affinity of corresponding IgG, or c) the presence of IgM antibodies and/or immune complexes in sera of acute cases.

The level of associated Chagas' disease detected in coendemic areas (schistosomiasis and leishmaniasis endemic areas, respectively) demonstrates that the CEIA test provides an extremely precise diagnosis of Chagas' disease. Indeed, it is very important to obtain such confirmation of *T. cruzi* infection since the therapeutic management of leishmaniasis and Chagas' disease are very different.

In view of its simplicity, specificity and sensitivity, the CEIA test is recommended for a specific diagnosis and for large screening of undeterminate and chronic phases of Chagas' disease, especially in areas where several parasitic diseases are coendemic.

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