AN IMMUNOCYTOTOXIC ASSAY FOR
TRYPANOSOMA CRUZI

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(Received 14 May 1975)

Abstract—KUHN R. E. and VAUGHN R. T. 1976. An immunocytotoxic assay for Trypanosoma cruzi. International Journal for Parasitology 6: 129–134. Trypanosoma cruzi culture forms grown for 8 days in LIT medium fortified with 14C-labeled amino acids will incorporate the isotope into membranous and soluble components of the parasite. On lysis, labeled cytoplasmic elements released into the surrounding medium can be measured and reflect the degree of destruction of the parasites. Materials released as a result of trypanosome destruction are not re-utilized by mouse spleen cells which supports the use of this assay for studies on lymphocyte mediated cytotoxicity in experimental Chagas’ disease. Experiments on complement dependent antibody-mediated lysis of T. cruzi demonstrate utility of the assay in examining both agglutinating and cytotoxic capacities of immune and normal serums. It is suggested that this assay is applicable to other protozoan parasite systems and may have some utility in diagnosis.

INDEX KEY WORDS: Trypanosoma cruzi; Chagas’ disease; immunity; antibody; immunocytotoxicity.

INTRODUCTION

VARIOUS immunizations and vaccinations have been shown to effect some degree of immunity in experimental animals infected with Trypanosoma cruzi (Kagan & Norman, 1962; Seneca, Peer & Hampar, 1966; Yanovsky, Traversa, Taratuto, Schmunis, Gonzalez Cappa & Parodi, 1969). Both humoral and cell-mediated mechanisms apparently contribute to immunity against the parasite (Culbertson & Kolodny, 1938; Roberson, Hanson & Chapman, 1973; Vilches, Katzin, Golfera & Schmunis, 1973). Although mechanisms by which these trypanosomes are rejected are not understood, it is known that T. cruzi is susceptible to destruction by both macrophages (Dvorak & Schmunis, 1972; Tanowitz, Wittner, Kress & Bloom, 1975) and complement dependent antibody activity (Budzko, Pizzimenti & Kierszenbaum, 1975). The possible role of anti-T. cruzi lymphocyte mediated cytotoxicity in Chagas’ disease, however, has not been shown.

A major difficulty in studying antibody or lymphocyte mediated cytotoxic responses against protozoan parasites is the problem of measuring and quantifying destruction of the parasite in in vitro experiments. While much information has been gained using visual observations of the interactions of parasites and host immune components, these procedures can be somewhat self-limiting in terms of time, sample sizes, and accuracy of measurement. A sensitive, reproducible assay which would allow indirect measurement of parasite destruction could prove useful in elucidation of immune mechanisms responsible for parasite rejection.

We report here a procedure which we believe to be applicable for studies on immune mediated destruction of Trypanosoma cruzi in vitro. The basic rationale for this procedure is as follows: Trypanosomes grown in the presence of 14C-labeled amino acids will incorporate the label into membranous and soluble fractions. At trypanosome lysis, 14C-labeled cytoplasmic components released into the surrounding medium can be measured and the amount of the isotope released will reflect the degree of immune destruction of target parasites.

MATERIALS AND METHODS

Parasites. Maintenance of culture forms of the Brazil strain of Trypanosoma cruzi used in these experiments has been described (Kuhn, Vaughn & Iannuzzi, 1974).

Radiolabeling procedures. Uniformly labeled 14C-amino acids (Chlorella hydrolysates, 57 mCi/mAtom; Amersham/Searle) were added to standard LIT medium at a concentration of 0.5 μCi/ml in initial experiments (Figs. 1 & 2) and 2.0 μCi/ml for all others. Radiolabeling was initiated by the addition of 0.4 x 10^6 culture-form parasites per ml of isotope containing LIT medium (14C-LIT). Incubations were done for specified periods of time at 28-C. At the end of the incubation period 14C-labeled parasites were harvested by centrifugation and washed three times in cold phosphate buffered saline (PBS). These washed, labeled parasites were then used for the experiments described. Unless otherwise specified,
14C-labeled trypanosomes will refer to parasites grown for 8 days in 14C-LIT.

Scintillation cocktails (10 ml) contained Fluorolroyl and 10% Biosolv BBS solubilizer (Beckman Instruments, Inc.) in a toluene base. Soluble fractions were counted as 0.2 ml aliquots. Particulate, cellular and precipitate fractions were hydrolyzed in 0.5 ml of 0.2 N NaOH overnight and then neutralized with 4 drops of 5% glacial acetic acid before being added to the scintillation cocktail.

Leakage determination. Metabolically active parasites labeled in the manner described above will spontaneously release some of the isotope during post-labeling incubations. To determine the amount of this leakage, tubes containing 8 x 10^7 14C-labeled trypanosomes were incubated at 37°C for 8 and 24 h in 1 ml of RPMI-1640 medium (Hepes buffer) fortified with 16% fetal calf serum, 100 μg penicillin G and 50 μg streptomycin sulfate/ml (RPMI-1640f). At the end of the incubation period, parasites were separated from the incubation medium by centrifugation and the amount of radiolabel in the parasites and supernatant determined.

TCA precipitation. In some experiments the amount of isotope in trichloroacetic acid (TCA) precipitable material in soluble fractions was determined. Precipitation was done in an ice bath with a final concentration of 10% TCA. Precipitates were washed 3 times with 1 ml of cold 5% TCA, hydrolyzed, neutralized and counted.

When bovine serum albumin (BSA) was used as a carrier for TCA precipitation, 0.2 ml of a PBS solution containing 1.0 mg of BSA was added per 0.5 ml of medium prior to precipitation.

Re-utilization experiment. It was of interest to determine the capacity of normal mouse spleen cells to re-utilize 14C-labeled T. cruzi components which would be released as a result of parasite disruption. 5 x 10^6 Trypanosomes labeled for 8 days in 14C-LIT (20 μCi/ml) were washed in cold Locke's solution and resuspended in 3.0 ml RPMI-1640f. The parasites were then subjected to sonication (30 s, 10% intensity, 3/8 in probe; Biosonik III) in an ice bath. Membranous and particulate components of the parasites were removed by centrifugation and the resulting supernatant fluid passed through a sterile 0.45 mm Milipore filter. Four ml of fresh RPMI-1640f was added to the 2.4 ml of label-containing medium recovered after filtration; the total volume was mixed thoroughly and 0.9 ml aliquots placed in sterile tubes. To each aliquot was added 0.1 ml of RPMI-1640f containing 2 x 10^6 normal C3H(He) female mouse spleen cells (Kuhn & Durum, 1975). The tubes were capped and incubated for 8 and 24 h at 37°C. At the end of the incubation period the cells were separated from the medium by centrifugation, washed 4 times in cold PBS and the amount of 14C-uptake determined.

Distilled water lysis of labeled trypanosomes. The amount of isotope released as a result of distilled water lysis of labeled trypanosomes was determined. Labeled parasites were removed from 14C-LIT, washed 3 times in cold PBS and resuspended in 1.0 ml of glass distilled water. After standing overnight at 4°C, the particulate and soluble fractions were separated by centrifugation, the particulate fraction was washed 3 times in PBS and the amount of isotope in the washed, particulate fraction and in the soluble fraction determined.

Antiserum. Immune serum was raised in two New Zealand white female rabbits. The animals were bled prior to immunization for normal serum. Immunizing doses contained 5 x 10^7 heat killed (56°C for 1 h) culture-form trypanosomes in 0.25 ml of PBS, were emulsified with 0.25 ml of Freund's complete adjuvant and were injected into two bilateral sites in 0.25 ml aliquots. Each dose was injected at 10 day intervals. The first injections were in the footpads while the second and third injections were administered intramuscularly and subcutaneously, respectively. Ten days following the third challenge, the rabbits were bled from the marginal ear vein and the serum collected. Ten days after the first bleeding, a fourth challenge was given subcutaneously and 12 days later the animals were bled by cardiac puncture for serum.

Appropriate sera were pooled, frozen in 2 ml aliquots and inactivated (56°C for 30 min) prior to use in experiments.

Cytolytic antibody assay. Parasites grown for 8 days in 14C-LIT were harvested, washed 3 times with cold PBS and resuspended in veronal buffer at a concentration of 12.5 x 10^6 trypanosomes per ml. 0.2 ml of this suspension (2.5 x 10^6 labeled parasites) was added to 0.2 ml of veronal buffer containing dilutions of either normal or immune serum. After this combination was incubated for 15 min at 37°C, 0.1 ml of adsorbed, diluted guinea-pig serum was added to each tube and the incubation was continued for an additional 45 min. The tubes were then placed in an ice bath for 15 min, centrifuged at 1500 g for 2 min, and the supernatant removed. The amount of isotope released into the supernatant was determined and the data expressed by the following formula:

\[
\text{c.p.m. in supernatant} \times 100 = \% \text{ release} \\
\text{Total c.p.m.}
\]

Lyophilized guinea-pig serum (GIBCO, Grand Island, NY) was reconstituted and used as the complement source. For use in these experiments it was adsorbed 5 times at 0°C for 15 min with 0.2 ml of packed culture-form parasites per 30 ml of guinea-pig serum. Adsorbed complement was stored at -20°C and was used within one month of adsorption. Complement was used at 1/30 dilution.

All stated dilutions of sera and complement were actual dilutions of the 0.5 ml incubation volume and were done in veronal buffer (pH 7.5). Controls for each experiment included buffer alone and 1/30 dilution of adsorbed complement.

Measurements of agglutinated parasites. In one experiment slides were made of the parasites after the cytolytic antibody assay had been performed. Following removal of 0.2 ml of the supernatant to measure the release of label, each tube was gently swirled to resuspend the pelleted parasites. One drop of this suspension was then placed on a microscope slide, air dried, and stained with Wright-Giemsa stain. The slides were projected onto a screen and the size and number of "clumps" of parasites was determined in three randomly chosen fields of view. The average diameter of "clumps" of parasites was recorded as length plus width divided by two. A 6 cm projected distance was an actual distance of 0.1 mm.

Correlation of % release to actual trypanosome destruction. For this experiment the cytolytic antibody assay was done as described above except that only 1/1280, 1/2560
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and 1/5120 dilutions of immune serum were examined. Two sets of triplicate tubes at each dilution were used. One set was used for the 14C-release assay. The other set of samples was treated the same way except that at the end of the incubation period the tubes were not centrifuged. Instead, sedimented parasites were resuspended by gentle swirling, two 4-μl aliquots of each sample were placed uniformly on a slide under an 18-mm circular coverslip, and the number of motile parasites in 50 fields in each aliquot was determined. The number of parasites per sample was then calculated. A complement (1/30 dilution) control was used and considered to indicate 0% cytolysis of parasites.

Calculations of percent trypanosomes killed by visual determinations were done using the following formula:

\[ \text{percent killed} = \frac{\text{motile parasites in sample}}{\text{motile parasites in control}} \times 100 \]

Calculations of percent trypanosomes killed by the 14C-release assay were done using the following formula:

\[ \text{percent killed} = \frac{\text{c.p.m. in sample}}{\text{max release}} \times 100. \]

The c.p.m. released by a standard aliquot of labeled parasites in 1.1 ml of distilled H2O for 3 h at 37°C was used as maximum release.

RESULTS

The first set of experiments was designed to determine the correlation between parasite population growth and uptake of 14C-amino acids as well as the amount of label which would be released from parasites on distilled water lysis. As seen in Fig. 1, population growth and total 14C-uptake were found to be concomitant throughout the 10 day period. In Fig. 2, it can be seen that the amount of label in the lysate maintained a close parallel to the c.p.m. in the membrane fraction until day 10 but that this relationship was not maintained for day 20 or day 31 of the incubation period. This was probably due to increasing numbers of dead trypanosomes, the membranes of which retained activity but which lacked cytoplasm to release at lysis since at death cytoplasm would have been lost into the surrounding medium.

Additional data were also derived from this initial set of experiments. Day 10 lysates were analyzed for the amount of isotope in the TCA precipitable fractions and it was found that 62.3% of the radiolabel in the lysate was precipitable. All post-incubation 14C-LIT media were examined for TCA precipitable c.p.m. which would reflect trypanosome release or labeled components during culture. No significant activity was found in the TCA precipitable material until day 20 and 31 when a small but detectable amount was measured. The appearance of labeled TCA precipitable material on these days supports the suggestion that dead trypanosomes had released their contents into the medium (see Fig. 2). It may also reflect the active release of labeled cytoplasmic material by these parasites.

Fig. 1. 14C-uptake and population growth of T. cruzi culture-forms in LIT medium containing 0.5 μCi/ml. n = 3; 2.5 ml volume/tube; data presented with ± s.e.

Fig. 2. The amount of isotope in membrane and lysate components of 14C-labeled T. cruzi culture-forms. These data were derived from replicate sets of samples as presented in Fig. 1 (n = 3).
FIG. 3. Complement-dependent antibody-mediated $^{14}$C-release from labeled T. cruzi culture-forms by normal and immune rabbit serum.

The amount of spontaneously released isotope was measured under conditions considered appropriate for some in vitro experiments. It was found that labeled parasites (8 x $10^7$/ml in RPMI-1640F) leaked 15.46% and 19.51% of the isotope at 8 and 24 h, respectively. It was further determined that the material spontaneously released after 24 h incubation contained 29.04% of the activity in the TCA precipitable fraction. This would indicate that the leaked material consisted mainly of waste products and other small metabolites.

In anticipation of using $^{14}$C-labeled trypanosomes as target cells for in vitro lymphocyte mediated cytotoxicity (LMC) studies, an experiment was done to determine the capacity of normal mouse spleen cells to re-utilize soluble materials released from labeled parasites as a result of sonic destruction. If effector lymphocytes could re-utilize labeled materials released from killed trypanosomes in vitro, measurement of LMC would be difficult since released material would not be free in the medium for detection. It was found that 2 x $10^6$ normal spleen cells incubated in 2 ml of RPMI-1640F containing 110,700 c.p.m. in the soluble, filtered parasite material re-utilized 0.83% (n = 6) of the label at 8 h and 1.12% (n = 6) at 24 h. The lack of significant re-utilization of released label supports the use of $^{14}$C-labeled trypanosomes for LMC experiments.

The results of a typical experiment on complement-dependent antibody mediated cytotoxicity against labeled trypanosomes is presented in Fig. 3. Although it was anticipated that normal rabbit serum would have heterophile antibody activity, it was unexpected that immune rabbit serum would mediate less $^{14}$C-release at low dilutions than at higher dilutions or that at low dilutions normal serum would cause greater release than the low dilutions of immune serum. (The prozone-like effect with immune serum was reproducible.) Visual observations of post-reaction parasites suggested that at low antibody dilutions (1/10-1/320) the trypanosomes were in large "clumps" and that clump size decreased with increasing dilutions. Parasites in larger versus smaller clumps would release less $^{14}$C, even in the presence of greater antibody concentration, because of decreased surface area available for release and less release from lysed parasites deep within a large clump.

To corroborate this suspicion a subsequent experiment was performed and the number and size of clumps of parasites was determined at the end of the cytolytic antibody assay. Both stained and fresh, living parasites were observed. The results of this experiment are presented in Fig. 4. The size of the clumps decreased as the number of clumps increased with increasing antibody dilutions. It was noted that maximum $^{14}$C-release was observed at dilutions resulting in minimal agglutination.

Microscopic observation of the contents of each incubation tube at the end of the cytolytic assay revealed that the antiserum was not 100% effective...
in mediating lysis of all the target parasites. Even at the lowest dilution (1/10) an occasional living trypanosome was observed on the periphery of large, amorphous masses of killed parasites. These survivors were, perhaps, trypomastigote forms which may be resistant to lysis by antibody raised against the predominately epimastigote forms of LIT cultures.

The use of any indirect assay to estimate the immune destruction of target cells necessitates the establishment of a correlation between the indirect parameter and a measurable, direct indicator of destruction. To establish this correlation, the cytotoxic assay was performed using rabbit anti-T. cruzi antiserum at dilutions which would result in minimal agglutination (1/1280, 1/2560 and 1/5120) and which would effect a significant release of 14C from target parasites (see Figs. 3 & 4). Triplicate samples were used at each dilution for both 14C-release and visual estimates of trypanosome destruction. The results of this experiment are shown in Fig. 5. A direct relationship between measurement of immune destruction by visual examination and the 14C-release technique was observed. The visual estimate indicated greater destruction than was suggested by the 14C-release data. This was not unexpected since only motile parasites were scored as alive. Living parasites which were inactive at the moment of observation were excluded from the living population. This resulted in a net overestimation of destruction when using the visual determination.

**DISCUSSION**

We believe that the 14C-release assay described herein can be used effectively in studies to elucidate host immunocytotoxic responses to *Trypanosoma cruzi* infection. The technique is sensitive, provides an accurate index of parasite destruction and is relatively simple to perform. By providing a numerical rather than a visual basis for estimation of parasite destruction, comparative experiments using large sample sizes can readily be performed.

For antibody studies, the combined cytotoxic and agglutinating activity of a serum can be determined in a single titration. Region A in Fig. 6 is representative of high antibody concentration. Release of label is low here because the trypanosomes are in large, agglutinated clumps which effectively reduces the surface area for 14C-release. In Region B smaller numbers of parasites are agglutinated in each clump. A consequent increase in isotope release is possible since greater numbers of trypanosomes are exposed to cytolytic activity and can release the isotope into the surrounding medium. Point C represents the end-point agglutination titer. Lytic action is no longer obscured by agglutination of target parasites. This point will shift to the right or left with decreasing or increasing antibody concentration, respectively.

Region D of the curve requires special consideration. Decreasing release of isotope at these titers reflects the actual decreasing concentration of cytolytic antibody. The slope of the curve is suggestive of the effectiveness of the test serum. With high IgM concentration, for instance, the slope would be reduced while antiserum containing high concentrations of non-C1-fixing IgG (e.g. human IgG4) would dictate a greater slope in the curve.

Using this assay, therefore, different sera can be compared as to agglutination titer (Point C), maximum isotope release (Point C), and, possibly, the cytolytic effectiveness of the antiserum (Region D).

We are particularly interested in using this technique to investigate lymphocyte mediated cytotoxicity in experimental Chagas' disease. In other
areas of immunology, particularly tumor and transplantation immunology, *in vitro* cytotoxicity assays have been employed to generate significant information on the nature of mechanisms involved in the rejection of foreign cellular antigens (Berke & Amos, 1973; Cerottini & Brunner, 1974).

It is known that cell-mediated immune mechanisms contribute to immunity against *Trypanosoma cruzi*. Macrophages play a significant role (Taliaferro & Pizzi, 1955; Dvorak & Schmunis, 1972; Tanowitz et al., 1975) and *in vitro* correlates of lymphocyte activity have been demonstrated (Teixeira & Santos-Buch, 1975). The ability of sensitized lymphocytes to mediate direct cytolysis of the parasite, however, has not been shown.

It is also thought that this technique could be adapted for use with other protozoan parasites and that it may have some usefulness as a diagnostic assay.

**Acknowledgements**—This work was supported by a Brown-Hazen Grant from Research Corporation.

**REFERENCES**


