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Stabilization and preservation of the antigenic specificity of *Trypanosoma (Trypanozoon) brucei* variant specific surface antigens by mild fixation techniques

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Summary

Living bloodstream trypansomes fixed by suspension in a 1% formalin solution maintain both their morphology and the immunological specificity of their variant specific surface glycoprotein, so allowing precise identification of the variant types present in a trypanosome population by direct or indirect immunofluorescence combined with phase microscopy. The technique is simple, adaptable to the study of low parasitaemias and should facilitate analysis of the phenomenon of antigenic variation both in the field and the laboratory.

Key words: Trypanosoma (T.) brucei – preservation of surface antigens – typing of antigenic variants.

Introduction

Antigenic variation during the course of infection of the mammalian host is now a well recognized mechanism by which salivarian trypanosomes evade the immune response of the host, and it is a serious obstacle to the development of effective immunization. This phenomenon has been the subject of extensive investigations reviewed most recently by Gray and Luckins (1976) and Doyle (1977).

Despite the increasing amount of information on several aspects of this phenomenon, the nature and extent of the variation process in the field is largely unknown. The range of antigenic diversity of a given species in a defined locality or country and the relevance of its possible limitation by the appearance of basic antigens following cyclical transmission is not adequately known.

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In studying the phenomenon of antigenic variation in the field it is essential to examine the antigenic character of the trypanosomes at the time of the isolation. At present the parasites isolated in the field are either initially preserved in liquid nitrogen and/or maintained in laboratory animals before antisera are raised for the study of the antigenic characteristics of the isolates.

Access to liquid nitrogen in the field in Africa is a serious limitation to cryopreservation. Maintenance of the parasites in laboratory animals, however, does not offer the solution to this problem because the isolates so maintained may undergo antigenic changes.

As regards the identification of the antigenic types of the parasites, the indirect immunofluorescent technique described by van Meirvenne et al. (1975) appears to offer the best solution at present. Using a mono-variant specific antiserum, positively stained trypanosomes can be clearly identified in a mixed variant population of parasites. Obviously negative parasites, however, are difficult to identify because of the background fluorescent staining while the trypanosome morphology is not sufficiently preserved for identification using phase contrast microscopy.

Previous work on mammalian cells (Bubbers and Henney, 1975; Drake et al., 1972; Gold et al., 1958; Kudo et al., 1974; Lin et al., 1969; McLean and Nakane, 1973, 1974; Moskowitz and Carb, 1958), suggested to us that stabilization of the surface antigens on trypanosomes by mild fixation might improve on the immunofluorescent technique to the point where variant specificity could be retained without distortion of the parasite morphology. We further thought that trypanosomes so treated might be stored under simple laboratory conditions without loss or alteration of both the parasite structure and the surface antigens, thus making the technique simple and applicable to studies under field conditions. As the variant specific surface antigens of *T. brucei* have been shown to be glycoprotein in nature (Cross, 1975), two protein fixatives, formaldehyde and glutaraldehyde were tested along with a fixative devised specifically for glycoproteins (Periodate-lysine paraformaldehyde – McLean and Nakane, 1973).

Materials and methods

Trypanosomes: Parasitized whole blood was obtained from lethally irradiated C57BL mice infected with either of two strains of *T. brucei* (LUMP 227 and 427) or clones derived from these strains.

Antisera: Variant specific antisera were raised in rabbits either by infection or immunisation with isolated clone specific surface glycoprotein (Cross, 1975). In the case of the former, rabbits were infected intravenously with 10^6 trypanosomes of a clone, bled 6 days later and the antiserum extensively absorbed against the other clones used in these experiments. In the latter case, the rabbits initially received 1 mg of isolated glycoprotein in complete Freund's adjuvant subcutaneously followed one month later by a booster dose of a similar amount in incomplete Freund's adjuvant. The rabbits were bled 2–4 weeks later and the IgG fraction of the antiserum obtained by salt precipitation and ion-exchange chromatography.

Immunofluorescent conjugates: The IgG fraction of the variant specific antiserum raised to the isolated glycoprotein was conjugated to either FITC or TRITC (BBL. Cockeysville, Maryland 21030, USA) by dialysis and the conjugates subsequently fractionated by ion exchange chromatography as described previously (Doyle et al., 1974). An IgG fraction of a goat antiserum to rabbit immunoglobulins was likewise labelled with either fluorochrome. Fluorescein conjugates used had molar fluorescein: protein ratios of 3.47–5.14 and rhodamine conjugates O.D. ratios of 0.64–0.28.

Fixation reagents

a) *Formalin:* (37% formaldehyde in aqueous solution, Fisher Scientific Company, New Jersey 07410, USA) was diluted to 0.05%, 0.1%, 0,5%, 1% and 10% with 0.01 M phosphate-buffered saline (PBS) pH 7.2.

b) *Glutaraldehyde:* (LADD Research Industries, P.O. Box 901, Burlington, Vermont 05401, USA) 70% (w/v) was diluted to 0.03%, 0.04%, 0.05%, 0.06%, 0.07%, 0.08%, 0.09%, 0.1%, 0.2% and 1% in 0.01 M phosphate buffered saline (PBS) pH 7.2.

c) *Periodate-lysine-paraformaldehyde (PLP):* The mixture was constituted as described by McLean and Nakane (1973). The concentration of paraformaldehyde was reduced to 0.5%, 1%, 2% or 4%.

Fixation techniques

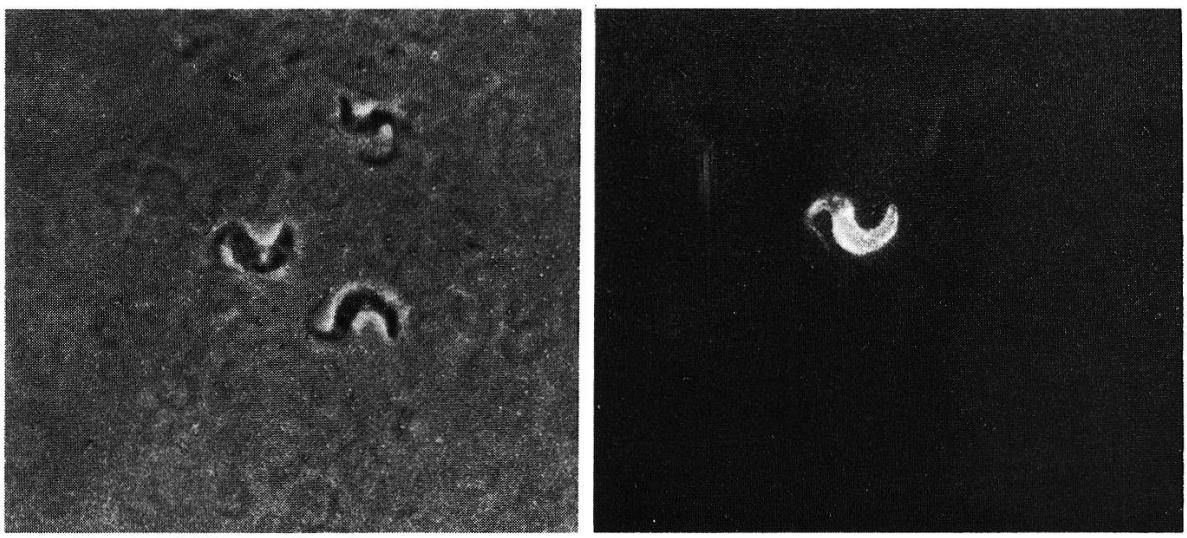
a) *Formalin fixation:* A small quantity (0.1 ml) of freshly collected parasitized mouse blood was added to 0.9 ml of formalin solution in a test tube and left at 4° C with occasional agitation for periods varying form 1 h to 10 days. The tubes were then transferred to the centrifuge and spun at 350 *g* at 4° C for 10 min, and the supernatant discarded. The pellet, containing trypanosomes and red blood cells was resuspended and washed twice in PBS pH 7.⅓. After the final washing the pellet was resuspended in 10 μl of heat inactivated foetal bovine serum and thin smears made for immunofluorescent microscopy. Alternatively, the pellet was resuspended in 10 μl of PBS transferred into haematocrit tubes, sealed, and spun in the haematocrit centrifuge for 10 min. The buffy coats from 2 or 3 haematocrit tubes were recovered and resuspended in 5 μl of heat inactivated foetal bovine serum on a microscope slide, and thin or thick smears made. The thin smear of fixed parasites in whole blood was for the purpose of enumerating the trypanosomes of any particular variant type in a mixed parasite population. The thin or thick smear of the buffy coat was used when screening for any particular variant in very low parasitaemia states (below 10^5 per millilitre of whole blood).

The slides were air dried at room temperature (20° C) for 10–15 min and reaction zones marked out on the slides with nail varnish.

b) *Glutaraldehyde fixation:* Parasitized mouse blood (0.1 ml) was added to 0.4 ml of glutaraldehyde solution in a test tube and allowed to react, with gentle agitation, for a time period varying from 30 sec to 5 min. After the appropriate reaction time 9.5 ml PBS pH 7.2 was added to the tube to give a twenty five-fold dilution of glutaraldehyde and the tube centrifuged at 350 *g* for 10 min at 4° C. The pellet was resuspended and washed three times in PBS. Smears were made as described for formalin fixation.

c) *Periodate-lysine-paraformaldehyde fixation:* 0.1 ml of parasitized mouse blood was added to 0.9 ml of PLP, fixation allowed to proceed for one hour at room temperature, and washed twice with 10% sucrose in PBS. Smears for immunofluorescence were made as described for formalin.

Immunofluorescence: The stability of surface antigens of the trypanosomes was assessed by direct and indirect immunofluorescence. In the direct immunofluorescent test suitable dilutions of conjugated variant-specific antiserum were added to the appropriate reaction zones on the slides and allowed to react for 15 min. Excess antiserum was washed off with PBS using a wash bottle. The slides were then dipped a few times in a bath of PBS pH 7.2, washed for 5 min in another bath, and changed to the final bath for 30 min of further washing. The slides were mounted in 50% glycerol in tris-saline pH 9. Indirect immunofluorescence was performed as described by van Meirvenne et al. (1975) with some modifications. The slides were air dried at room temperature for 15 min. Variant



a b

Fig. 1. a) Phase contrast of trypanosome smear showing total number of trypanosomes/field. b) Immunofluorescence of same field showing only 1 positive parasite. – Total magnification: ×380. Conjugate: TRITC labelled anti-variant specific glycoprotein. O.D. ratio 0.62. Protein concentration 26.1 mg/ml. Dilution 1:80.

specific antiserum dilutions were added and allowed to react for 15 min. Excess antiserum was washed off with PBS and the slides transferred to a bath for 5 min and then to a second bath for 20 min. Fluorescein labelled goat anti-rabbit 1 g (see Table 2) was added and allowed to react for 15 min, the slides washed as before and mounted in 50% glycerol in tris-saline pH 9 for microscopy.

Microscopy was done using the Orthoplan fluorescence microscope (Ernst Leitz GmbH, D-6330 Wetzlar). Readings were made with the ×40 fluorescent phase contrast oil objective and ×6.3 periplan eyepieces, under epi-ploem illumination with a 200 W ultra-high pressure mercury vapour lamp. The filter combinations used were as follows: a (2 mm BG-36 and KP560 and K530) excitation filter, TK580 dichroic beam splitting mirror, and a K580 suppression filter for Rhodamine (TRITC) conjugates; and a (2×KP490 and 1 mm GG 455) excitation filter, a TK510 dichroic beam splitting mirror and a K515 suppression filter for fluorescein (FITC) conjugates.

Results

Effect of fixation on the antigenic stability of surface antigens

All three techniques of fixation were assessed in respect of their effect on parasite morphology and antigenic stability of their surface variant antigens.

a) *Formalin:* It was found that a concentration of 1% formalin consistently gave the best results in terms of preservation of both trypanosome morphology and surface antigens. At the appropriate dilution of variant specific antiserum unreacting trypanosomes were clearly negative and did not show any background fluorescent staining, in contrast to the smooth and uniform positive antigenic variants (Figs. 1 a and b).

At lower concentrations of formalin there was loss of morphology while at higher concentrations there was loss of antigenicity of the variant antigens as evidenced by diminishing specific immunofluorescence.

All observations were made using the same dilution of conjugate. The duration of fixation was not critical. Results obtained after 10 days of fixation were comparable to those obtained after 3 h fixation. The minimum fixation time used was 1 h. The application of 1% formalin solution to air dried blood films did not give satisfactory fixation and stabilisation of trypanosome morphology or variant antigen specificity.

b) *Glutaraldehyde:* Results obtained with glutaraldehyde showed a concentration of 0.05% to be optimal. There was a loss of morphology at lower concentrations, whereas concentrations above 0.05% led to a loss of antigenicity as demonstrated by a diminishing level of specific fluorescence. Furthermore, a heavy background fluorescent staining was present at higher concentrations of glutaraldehyde. The optimal fixation time was found to be 30–60 sec. Above 60 sec of fixation, specific fluorescence diminished markedly, making it difficult to distinguish the positive from the negatively stained parasites.

c) *Periodate-lysine-paraformaldehyde:* The optimum concentration of paraformaldehyde was 2%. At lower concentrations there was a loss of morphology while at a higher concentration antigenicity was not well preserved. Overall, the results of fixation with PLP were inferior to the formalin and glutaraldehyde because the trypanosomes contracted and became smaller than in the controls. The only part that maintained its morphology and antigenicity was the flagellum. This technique was not pursued further.

Effect of storage on the stability of surface antigens

After 18 h of fixation with 1% formalin, several smears were made as already described and some slides stored at +4° C and others at −80° C, with or without a dessicating agent (silica gel).

Smears of 0.05% glutaraldehyde-fixed trypanosomes were made after 30 sec of fixation and stored in a similar manner. Unfixed were included as controls.

The slides were recovered, air dried at room temperature, stained and examined every 14 days to assess the effect of the various conditions of storage on the morphology and retention of surface antigenicity.

The results obtained show that trypanosomes treated with 1% formalin retain their morphology and surface antigens at +4° C in the absence of a dessicating agent for a period of 7 months. Working with a multivariant population of trypanosomes, the proportion of organisms that reacted with a monovariant specific antiserum remained constant over the period of 7 month.

The consistency of the method was examined using both direct and indirect fluorescent techniques. The direct test (Table 1) shows a clear plateau titre of the labelled anti variant Ig at titres of over 1:160. The double titration in the indirect immunofluorescence technique shows the consistency and specificity of the method even while using an antiserum raised by rabbit infection (Table 2).

Table 1. Checkerboard titration of labelled antiserum* to variant 49 in direct IFAT using a mixed trypanosome population

Reciprocal of antiserum dilution	Number and percentage of positive trypanosomes found per 200 trypanosomes	
	No.	%
40	49	25
80	36	18
160	44	22
320	31	16
640	28	14
1280	29	15
2560	26	13

* Conjugate: FITC labelled IgG fraction of antiserum raised to isolated variant specific glycoprotein of clone 49. Protein concentration 29.5 mg/ml. F:P_m 3.13. Plateau titre 1/320–1/640.

Table 2. Double titration of FITC labelled anti-rabbit Ig and specific antiserum to variant 9 raised by rabbit infection. Percentage of positive trypanosomes found in 200 trypanosomes of a mixed population

Reciprocal of antiserum dilution	Reciprocal of conjugate* dilution				
	20	40	80	160	320
160	77	76	75	75	70
320	82	77	71	81	77
640	75	85	76	77	78
1280	80	75	72	80	77
Mean percentage ± one standard deviation	78.5 ± 3.1	78.2 ± 4.5	73.5 ± 2.4	78.2 ± 2.7	75.5 ± 3.7

* Conjugate: FITC labelled IgG fraction of goat antirabbit Ig. F:P_m ratio 5.14. Protein concentration 58.8 mg/ml. – Mean and standard deviation of positive parasites counted per 200 trypanosomes in 20 areas = 154.1 ± 6.9.

In both direct and indirect immunofluorescent techniques the anti variant antisera were titrated till the plateau titre was found (Table 1) and such sera subsequently used at or above this dilution. Anti-rabbit immunoglobulin conjugates were titrated similarly. Controls for specificity routinely included in each test were in the case of direct immunofluorescence an antigenically unrelated trypanosome population and in indirect immunofluorescence, normal rabbit serum and phosphate buffered saline were also used to assess the specificity of the anti-immunoglobulin conjugate.

Discussion

The ideal fixative for surface cell membrane antigens should preserve antigenicity as well as cell morphology. In immunohistochemistry this is not always possible because a fixation system suitable for one antigen may not necessarily be suitable for another. Thus, while glutaraldehyde may preserve cellular ultrastructure, antigenicity is usually lost. Paraformaldehyde which, on the other hand, preserves the antigenicity of some antigens is not as good at preserving ultrastructural details (Leduc et al., 1969).

The conventional fixatives like glutaraldehyde and paraformaldehyde interact strongly with proteins and thus often denature protein antigens. To circumvent this problem, Mc Lean and Nakane (1973, 1974) developed the periodate-lysine-paraformaldehyde (PLP) fixative. This fixative operates on the theoretical supposition that given a carbohydrate-containing antigen, periodate would oxidize the carbohydrate moities to form aldehyde groups. Lysine, a divalent amine, then would cross-link the carbohydrate containing molecules by reacting with the aldehyde groups. Paraformaldehyde is added simply to achieve a stabilization of proteins and lipids. This technique has been used successfully in immunoelectron microscopy (McLean and Nakane, 1973, 1974).

In the case of the fixation techniques described in this paper, the most consistent results were obtained with 1% formalin. Using glutaraldehyde there was loss of antigenicity as well as parasite morphology, despite the good results reported in the preservation of the antigenicity of mastocytoma cells by Bubbers and Henney (1975). Likewise, fixation with periodate-lysine-paraformaldehyde did not preserve the morphology of trypanosomes, although antigenicity was preserved.

The ability of formalin to stabilize surface membrane antigens of mammalian cells has been utilized in the preservation of the antigenicity of tumor cells (Drake et al., 1972; Kudo et al., 1974; Lin et al., 1969), and in the preservation of blood group antigens for blood group serology (Gold et al., 1958; Moskowitz and Carb, 1958). Similarly, the results presented in this communication show that fixation of living bloodstream trypanosomes by suspension in a 1% formalin solution preserves both their morphology and the antigenicity of their variant specific surface glycoprotein as judged by immunofluorescent techniques. This method offers certain important advantages over the technique originally developed by van Meirvenne et al. (1975). Using a combination of phase microscopy and immunofluorescence, unstained parasites can clearly be located among positively stained parasites. This is necessary for example when assessing the homogeneity of a cloned population using monospecific antiserum. Such fixed trypanosomes also retain their morphology and antigenicity for long periods (over 7 months at present) under simple storage conditons, $+4°$ C in a refrigerator, and do not require ultra low temperature storage in the presence of a dessicating agent (van Meirvenne et al., 1975).

This simple, inexpensive technique which is readily adaptable to the study of low parasitaemic states provides a useful specific tool for the investigation of the phenomena of antigenic variation both in the field and in the laboratory.

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