CAPPING OF VARIABLE ANTIGEN ON 
TRYPANOSOMA BRUCEI, AND ITS 
IMMUNOLOGICAL AND BIOLOGICAL 
SIGNIFICANCE

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SUMMARY

Pathogenic trypanosomes undergo antigenic variation, whereby the glycoprotein molecules constituting the cell surface coat are changed, the parasite thus evading the host's immune response. On application of homologous antiserum in indirect immunofluorescence to a given variable antigen type of Trypanosoma brucei, the surface variable antigen moves to the flagellar pocket region, which overlies the Golgi apparatus. This redistribution, or capping, is temperature-dependent, occurring at 37 °C but not at 0-4 °C. Patching does not occur at either temperature. Immediately after capping no homologous or heterologous variable antigen, or host plasma or blood cell antigens, can be detected by immunofluorescence on the cell surface outside the cap; only trypanosome membrane common antigens can be found.

It seems unlikely for two reasons that this antibody-induced redistribution is relevant to antigenic variation. Capping of the coat requires the indirect, rather than the direct, immunofluorescent method; a single layer of antibody, as available in nature, would appear to be ineffective. Also, capping of variable antigen of one type is followed within 3 h by appearance of antigen of the same, and not another, type.

The necessity for 2 antibody layers is usually thought of as meaning that the individual molecules of the cell surface antigen are spaced further apart than the binding sites of an individual antibody molecule, so that the necessary cross-linked lattice cannot be formed, but on T. brucei the surface variable antigen molecules are very closely packed. It is proposed that one layer of antibody is ineffective for steric reasons; the dimensions of the exposed face of each variable antigen molecule may not permit the binding of more than one molecule of immunoglobulin, or perhaps the antigen molecules are so closely packed that most of the antigenic determinants are hidden from antibodies. To test this hypothesis, an attempt was made to cap variable antigen on trypanosomes transforming in vitro from the bloodstream to the procyclic (insect midgut) stage; such forms have a much less densely packed surface coat. Patching was observed, indicative of lattice formation, but these trypanosomes did not survive the in vitro manipulation long enough to permit any possible capping.

T. brucei differs structurally from most other eukaryotic cells. It has no detectable microfilaments under the plasma membrane, except at the desmosomes in the region of flagellar binding, and it also has a pellicular cortex of microtubules. Capping of its surface antigen would appear then to differ from that on mammalian cells, either in the cellular components involved or in that specialized areas of the plasma membrane are involved.

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INTRODUCTION

The protozoan parasite *Trypanosoma brucei* undergoes a series of variations in surface antigens during the course of an infection of a mammal (reviewed by Vickerman, 1978). The variable antigens are located in the 12-15 nm-thick glycoprotein surface coat which completely envelopes the organism (Vickerman, 1969a; Vickerman & Luckins, 1969; Fruit et al. 1977), and the change in antigen specificity would appear to involve a change in the glycoprotein molecules composing the coat (Cross, 1975, 1978; Baltz et al. 1977).

Although our understanding of antigenic variation at the molecular level is increasing, nothing is as yet known of what actually triggers antigenic change. One possible mechanism could be that host antibodies induce redistribution of the surface variable antigen and thus initiate the change in expression. Such redistribution occurs in the 'capping' phenomenon, first observed on lymphocytes by Taylor, Duffus, Raff & de Petris (1971) (reviewed by Nicholson, 1976; Loo, 1977). Antibodies have long been suspected of triggering the change in surface antigens (Ehrlich, 1909) and trypanosomes are naturally exposed to surface-specific antibodies formed by their hosts. Capping has been demonstrated on various protozoa including *Entamoeba histolytica* (Pinto da Silva, Martinez-Palomo & Gonzales-Robles, 1975), *Naegleria gruberi* (Preston, O'Dell & King, 1975) and *Leishmania enriettii* (Doyle, Behin, Mauel & Rowe, 1974; Dwyer, 1976). This last species also displayed reappearance of surface antigen removed to the poles of the organism in the capping process.

In this paper capping of variable antigen on *T. brucei* is described, and its relation to antigenic variation discussed. The surface and cortical structure of this parasite are very different from that of other cells, and the nature of redistribution of its variable antigen poses some questions about the fundamental nature of capping in general. Preliminary accounts of this work have been given previously (Barry, 1975; Barry & Vickerman, 1977).

MATERIALS AND METHODS

*Trypanosomes*

A clone of *Trypanosoma brucei* of variable antigen type (VAT) ETat 1 (Lumsden & Herbert, 1975) was received as a frozen stabilate (LUMP 63; Lumsden & Herbert, 1975), recloned, and the VAT of the new clone verified as ETat 1 by immunofluorescence and trypanolysis (Van Meirvenne, Janssens & Magnus, 1975) using a reference antiserum. This clone was highly infective and virulent for laboratory rodents and showed little sign of pleomorphism (Vickerman, 1971). ETats 2 and 5 (see below) were cloned relapse VATs derived from ETat 1 (McNeilage, Herbert & Lumsden, 1969). A pleomorphic clone of different origin, with VAT GUTat 1 (Barry & Vickerman, 1979), was used to study surface changes taking place during transformation to the culture (procyclic) form of *T. brucei in vitro*.

Bloodstream forms of trypanosomes were obtained by infecting rats (Hooded Lister, 300 g) intraperitoneally with cryopreserved infected blood and separating the parasites from blood cells 3 days later on DEAE cellulose columns (Lanham, 1968) using phosphate saline glucose buffer pH 8.0 (PSG; Lanham, 1968). Upon elution the parasites were pelleted (1600 g for 10 min) and resuspended in PSG containing 10% heat-inactivated foetal calf serum on ice.

Culturing and harvesting of trypanosomes in modified Pittam's Medium were carried out as described in Barry & Vickerman (1975). Capping experiments on trypanosomes from culture were performed using culture medium instead of PSG.
Antiserum

Infection serum. Serum was taken 1 day after remission of parasitaemia (usually day 7 post-infection) from rats (Hooded Lister, 300 g) infected intraperitoneally with 10⁶ trypanosomes of one of the VATs ETat 1, 2, 5 or GUTat 1. Such sera were considered to be natural in that they closely represented the situation occurring in the host during infection. It is likely that this early anti-trypanosome activity consisted mainly of IgM (Seed, 1972). All sera were inactivated by being incubated at 56 °C for 30 min. Control sera were taken either from uninfected rats or by taking blood samples from rats prior to immunization. Infection sera were thoroughly absorbed with homogenate of culture forms of T. brucei as described in Barry & Vickerman (1979).

Rabbit hyperimmune serum (HIS) anti-T. brucei culture forms. This antiserum was raised in a rabbit by repeated injection of lyophilized culture forms of T. brucei emulsified in Freund's Incomplete Adjuvant. Details are given in Barry & Vickerman (1979).

Rabbit HIS anti-rat plasma. Rat (Hooded Lister) plasma was obtained by centrifuging heparinated blood at 1600 g for 10 min and lyophilizing the supernatant. Two milligrams of this material were reconstituted in 0.5 ml of distilled water and emulsified with 0.5 ml of Freund's Complete Adjuvant. The emulsion was injected by the subcapsular route. This procedure was repeated 7 times at weekly intervals, injecting alternate shoulders of the rabbit. Blood was collected from the marginal ear vein 14 days after the last injection. An immunoelectrophoretic analysis was performed on this antiserum against freshly collected rat plasma, and precipitating activity against at least 15 components was detected.

Rabbit HIS anti-rat blood cells. The cell fraction of the rat blood used to hyperimmunize the rabbit was obtained by centrifuging heparinated blood at 1600 g for 10 min and lyophilizing the supernatant. Two milligrams of this material were reconstituted in 0.5 ml of distilled water and emulsified with 0.5 ml of Freund's Complete Adjuvant and injected subcapsularly. This procedure was repeated 7 times at weekly intervals, injecting alternate sides of the rabbit. Blood was collected from the marginal ear vein 14 days after the last injection. In an indirect immunofluorescence reaction against rat blood cells this antiserum had a titre of 1:256.

IgG anti-ETat 1 soluble extract. A soluble extract of homogenate of ETat 1 trypanosomes, ruptured by mortar and pestle, was prepared as described by Le Ray (1975) and lyophilized. For immunization 0.1 mg of the lyophilizate was reconstituted in 2 ml of Freund's Complete Adjuvant and administered via 20 intradermal injections on each flank of a rabbit. Serum was collected by day 21. IgG was fractionated from this serum by column chromatography on DEAE cellulose (Reif, 1969) using 0.1 M phosphate buffer, pH 7.6, and further purified from contaminative haemoglobin by precipitation in 50 % ammonium sulphate. The purity of the IgG was checked by immunoelectrophoresis against goat anti-rabbit serum and the specificity by immunoelectrophoresis against ETat 1 soluble extract; a strong precipitation reaction with the variable antigen component (Le Ray, 1975) was revealed. The IgG was conjugated to rhodamine isothiocyanate (RTTC; mixed isomers, Sigma Chemical Co.) by the method of Cebra & Goldstein (1965). The fluorochrome:protein molar ratio was 0.36.

Capping experiments

Trypanosomes were suspended at a concentration of 10⁵/ml in PSG containing 10 % foetal calf serum on ice. All subsequent operations, unless otherwise stated, were performed on ice or at 4 °C in a refrigerated centrifuge (Mistral 4E, M.S.E.). 0.1-ml volumes were dispensed into 15-ml glass centrifuge tubes (M.S.E.) and an appropriate volume of the antiserum added. After incubation for 15 min at low temperature, the trypanosomes were washed twice in 10 ml of fresh PSG:10 % foetal calf serum. For indirect immunofluorescence studies, the trypanosomes were resuspended to the original volume and 5 μl of the previously titrated conjugate (fluorescein isothiocyanate, FITC, conjugated rabbit anti-rat immunoglobulins, Wellcome) were mixed with the cells, and the samples incubated and washed as before.
Immuno-fluorescence

For immuno-fluorescence studies on residual trypanosome antigens after capping of the variable antigen, antisera were applied at dilutions of 1:2 and 1:20 for 30 min at 0–4 °C, the cells washed twice by centrifugation in PSG before exposure to the appropriate conjugate; FITC-conjugated goat anti-rabbit immunoglobulins or FITC-conjugated goat anti-rat immunoglobulins (Pasteur Institute, Paris) were applied at a dilution of 1:200 for 30 min at 0–4 °C. After 2 further washes the living trypanosomes were examined microscopically.

Microscopy

Preparations were examined wet with a Leitz Ortholux II microscope with transmitted-light phase-contrast and incident-light fluorescence using an HBO 50 high-pressure mercury lamp. For FITC the filters used were 2 × KP490 (exciting), TK510 (dichroic mirror) and K515 (suppressing), and for RITC 2-mm BG 36 + S 546 (exciting), TK580 (dichroic mirror) and K580 (suppressing). Photographs were taken with a Leitz Orthomat automatic camera using 400 ASA HP4 (Ilford) black-and-white film.

RESULTS

Indirect immuno-fluorescence

Titration of antiserum, and pattern of capping. The effect on ETat 1 trypanosomes of different dilutions of VAT-specific antiserum from each rat was investigated.

Table 1. Effect of concentration of antiserum on trypanosomes incubated under capping conditions

<table>
<thead>
<tr>
<th>Volume of 1:10 diluted antiserum added per 10⁶ cells, µl</th>
<th>Effective dilution</th>
<th>Effect on trypanosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>—</td>
<td>100% alive, unlabelled</td>
</tr>
<tr>
<td>1</td>
<td>1:100</td>
<td>85% capped</td>
</tr>
<tr>
<td>2.5</td>
<td>1:400</td>
<td>90% capped</td>
</tr>
<tr>
<td>5</td>
<td>1:200</td>
<td>&gt; 90% dead, 5% overall label</td>
</tr>
<tr>
<td>10</td>
<td>1:110</td>
<td>100% dead, agglutinated</td>
</tr>
<tr>
<td>20</td>
<td>1:60</td>
<td>100% dead, agglutinated</td>
</tr>
</tbody>
</table>

100 trypanosomes counted per sample.

Parasites labelled in the cold displayed overall fluorescence (Fig. 1). Those labelled at low temperature and then incubated at 37°C for 15 min showed various effects. Results for a typical antiserum are shown in Table 1. Higher concentrations of homologous antiserum (≥ 1:200) resulted in massive agglutination and cell death, although some actively moving trypanosomes displayed overall diffuse fluorescence, while at lower concentrations the trypanosomes retained full motility and a high proportion became capped. In all cases, the fluorescence cap was at the posterior end of the flagellate, around the flagellar pocket (Figs. 2–4), and capping had no apparent effect on motility. Other labelling patterns were observed on slowly
Capping on Trypanosoma brucei

moving or dead trypanosomes at higher antiserum concentrations; these included overall labelling, and patches on the flagellum and the anterior end of the cell, usually associated with adhesion to the glass slide or coverslip. As trypanosome motility was impaired in these cases, such patterns were considered abnormal. In the absence of foetal calf serum from the PSG these labelling patterns were apparent at all antiserum dilutions, as was the production of streamer-like processes from the trypanosome body (Wright, Lumsden & Hales, 1970).

For subsequent experiments the 1:400 dilution of this antiserum was used as this had allowed optimal capping effect and preservation of motility. Antisera from other

Figs. 1–4. Capping of variable antigen on ETat 1 trypanosomes. × 2100.

Fig. 1. Specific immunofluorescent labelling of surface variable antigen on ETat 1 trypanosome at 0–4 °C. The label is distributed over the entire surface of the parasite, with no trace of patching. Blue epi-illumination.

Fig. 2. After 5-min incubation at 37 °C the label has begun to move to the posterior of the cell. Simultaneous blue epi-illumination and transmitted phase-contrast.

Fig. 3. On some trypanosomes a non-fluorescent line (arrowed) could be observed traversing the capping mass. Simultaneous blue epi-illumination and transmitted phase-contrast.

Fig. 4. After 10 min incubation at 37 °C the label has capped to the flagellar pocket region. Simultaneous blue epi-illumination and transmitted phase-contrast.
rats gave comparable results over the same dilution ranges. All controls (normal rat serum + conjugate, or conjugate alone) gave no labelling of the trypanosomes.

**Time course of capping**

Trypanosomes were labelled at low temperature and then incubated in a water bath at 37 °C, samples being taken at given intervals and fixed in 1-6% (final conc.) formaldehyde, with 1 drop being examined first to check trypanosome motility. In each case > 99% of the parasites were fully motile.

Table 2. Development of capping with time on trypanosomes labelled at low temperature then incubated at 37 °C

<table>
<thead>
<tr>
<th>Time incubated at 37 °C, min</th>
<th>Labelling pattern</th>
<th>Overall</th>
<th>Capped</th>
<th>No label</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>19</td>
<td>81</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>10</td>
<td>89</td>
<td>1</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>4</td>
<td>94</td>
<td>2</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>2</td>
<td>52</td>
<td>46</td>
</tr>
<tr>
<td>60</td>
<td></td>
<td>2</td>
<td>44</td>
<td>54</td>
</tr>
</tbody>
</table>

100 trypanosomes counted per sample.

The capping process was well in progress within 5 min (Table 2), the label moving to the posterior end of the cell. It was noticeable that patching of the surface marker did not occur as a prelude to capping. By 30 min 46% of the trypanosomes were devoid of label, and this proportion rose within the next 30 min (Table 2). It was not possible to resolve at the level of the light microscope the fate of the label, i.e. whether it was ingested or shed.

**Identity of residual antigens on the cell surface**

Antisera against a variety of different antigens (ETat 1, culture forms of *T. brucei*, host blood cells, host plasma) were applied in indirect immunofluorescence at low temperatures to both normal and capped ETat 1 trypanosomes. Of these antisera only 2 labelled non-capped trypanosomes; rat anti-ETat 1 and the same serum absorbed with culture forms of *T. brucei* (Table 3). Rat anti-ETat 1 and hyperimmune serum against culture forms of *T. brucei* were the only two which showed activity against capped ETat 1 trypanosomes. Thus, while non-capped ETat 1 parasites displayed labelling of only variable antigen, the capped trypanosomes showed strongest reaction with anti-culture forms antiserum, which in effect is an antiserum against common membrane antigens (see Barry & Vickerman, 1979), and a weaker reaction with rat anti-ETat 1 (Table 3). On absorption of this last antiserum with homogenate of culture forms, a process designed to remove antibodies against common antigens, the capacity to label capped trypanosomes disappeared. It would appear that capping leaves no detectable variable antigen on the cell surface (outside the
Capping on *Trypanosoma brucei* cap) and results in the exposure of trypanosome common antigens, with no trace of host-like antigens.

**Identity of antigen on the cell surface 3 h after capping**

To investigate the role of capping in antigenic variation an experiment was set up to identify the antigen on the cell surface 3 h after capping; *Leishmania* exhibits reappearance of at least some surface antigen over a similar period after capping (Doyle *et al.* 1974; Dwyer, 1976).

**Table 3. Indirect immunofluorescence analysis of antigens on surface of trypanosomes before and after capping of variable antigen**

<table>
<thead>
<tr>
<th>Serum applied</th>
<th>Intensity of fluorescence</th>
<th>Normal ETat 1</th>
<th>Capped ETat 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-trypanosome</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat anti-ETat 1</td>
<td>+ + +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Rat anti-ETat 1, absorbed</td>
<td>+ + +</td>
<td>-</td>
<td>+ + +</td>
</tr>
<tr>
<td>with culture forms</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit hyperimmune</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>anti-culture forms</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-host</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit hyperimmune</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-rat blood cells</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rabbit hyperimmune</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-rat plasma</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal rat</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Normal rabbit</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

+ + +, very bright fluorescence; +, weak fluorescence; -, no fluorescence.

ETat 1 trypanosomes in a series of samples were labelled at low temperature and transferred to a waterbath at 37 °C. It was found necessary to increase the foetal calf serum content of the PSG from 10 to 40% to maintain the trypanosomes in a motile condition over 3 h and throughout the increased number of centrifugations required in this experiment. At given times samples of trypanosomes were examined for labelling patterns and also subjected to indirect immunofluorescence at low temperature with a variety of rat antisera. These included anti-ETat 1, anti-ETat 2 and anti-ETat 5. Each antiserum had been absorbed with homogenate of culture forms of *T. brucei*, to abolish reactivity with trypanosome membrane common antigens. The VATs ETat 2 and ETat 5 are major relapse variants of ETat 1 (McNeilage *et al.* 1969) so it could be expected that induction of antigenic variation would produce one or both of these VATs within the samples. The results of this experiment are shown in Table 4.

The original population of trypanosomes contained no ETat 2 or ETat 5 out of 100 counted, whereas 98 out of 100 were labelled with anti-ETat 1. After 15 min
under appropriate conditions 95% of the trypanosomes were capped, with 5% unlabelled, and no ETat 1, 2 or 5 antigen could be detected by immunofluorescence on the cell surface. After 3 h of capping conditions the label had disappeared from the surface of more than 90% of the parasites while a few retained small caps. Further immunofluorescence of these trypanosomes revealed the presence, over the whole cell surface, of ETat 1 antigen, but no ETat 2 or 5 (Table 4). Trypanosomes in another sample maintained at low temperature for 3 h exhibited overall labelling. Controls (normal rat serum or serum omitted in the first stage of indirect immunofluorescence) gave no staining of trypanosomes at any stage in the experiment.

Table 4. *Indirect immunofluorescence identification of antigen reappearing on ETat 1 trypanosome surface after capping of variable antigen*  

<table>
<thead>
<tr>
<th>Time trypanosomes used</th>
<th>Label distribution</th>
<th>% of trypanosomes with given label distribution after application of immunofluorescence with serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Overall</td>
<td>Anti-ETat 1</td>
</tr>
<tr>
<td>Before capping</td>
<td>Overall</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>Cap</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>2</td>
</tr>
<tr>
<td>15 min capping conditions</td>
<td>Overall</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Cap</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>5</td>
</tr>
<tr>
<td>3 h capping conditions</td>
<td>Overall</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Cap</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>95</td>
</tr>
</tbody>
</table>

100 trypanosomes counted per sample. –, indistinguishable; ND, not done.

*Influence of fluorescent conjugate on the process of capping*

In the original report on capping Taylor *et al.* (1971) noticed that redistribution of some antigens required the presence of the second layer of antibody in indirect immunofluorescence, and indeed this second layer is necessary for capping of most antigens on lymphoid cells.

It was possible, by manipulation of temperature of incubation, to ascertain whether trypanosome capping could operate independently of the second layer. Thus, conditions were set up as shown in Table 5, each layer of antiserum having the opportunity to induce capping for 15 min at the permissive temperature, 37 °C, the other being applied for 15 min at 0–4 °C, the non-permissive range. Clearly, capping occurred only when the second antibody layer was applied at the permissive temperature, indicating that capping of trypanosome variable antigen by host antibody alone is unlikely to occur in nature.
Direct immunofluorescence

To investigate further the finding that an infection serum could not induce capping on its own, RITC-conjugated IgG against ETat 1 soluble extract was applied to ETat 1 trypanosomes in the direct immunofluorescent technique.

The conjugate was titrated at 37 °C against the parasites, as described above, to obtain the optimal dilutions for any possible capping. At high concentrations (> 1:100) some agglutination occurred, but labelling was over the entire surface. At the lower concentrations (< 1:100) the trypanosomes showed very faint overall fluorescence, with brighter fluorescence in the flagellar pocket region, but there was no evidence of patching or capping. Trypanosomes of one sample were incubated in the conjugate for 30 min, washed at low temperature and then observed over a period of 1 h at room temperature (21 °C). No alteration of labelling distribution was evident. The flagellar pocket staining was VAT-specific, as GUTat 1 trypanosomes did not show any staining on incubation in the conjugate.

Table 5. Influence of type of immunofluorescence reaction on the process of capping

<table>
<thead>
<tr>
<th>Type of immunofluorescence reaction</th>
<th>Temperature of application of given antiserum, °C</th>
<th>Labelling pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rat anti-ETat 1</td>
<td>FITC-anti-rat Ig</td>
</tr>
<tr>
<td>Indirect</td>
<td>0-4</td>
<td>0-4</td>
</tr>
<tr>
<td></td>
<td>0-4</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>0-4</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>Direct</td>
<td>RITC-anti ETat 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0-4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>37</td>
<td></td>
</tr>
</tbody>
</table>

100 trypanosomes counted per sample.
FITC-anti rat Ig, fluorescein isothiocyanate-conjugated rabbit anti-rat Ig.
RITC-anti ETat 1, rhodamine isothiocyanate-conjugated rabbit IgG anti-ETat 1 trypanosome homogenate.

Attempted capping of variable antigen on trypanosomes transforming in culture

To study the effect of packing of surface variable antigen on capping (see Discussion) bloodstream trypanosomes were inoculated into Pittam's Medium and samples removed after 12 and 24 h for capping experiments. The surface coat and variable antigen of bloodstream form T. brucei become less densely packed in this period, during which transformation to the procyclic stage proceeds (Brown, Evans & Vickerman, 1973; Barry & Vickerman, 1979). As the ETat line of trypanosomes used in these experiments was monomorphic and had thus lost the capacity to transform to the procyclic stage in vitro, a clone from a pleomorphic line of trypanosomes was used. This clone was of the VAT GUTat 1.

Rat anti-GUTat 1, absorbed with homogenate of T. brucei culture forms, was
titrated against bloodstream forms for capping capacity and found to have optimum activity at the 1:200 dilution. The same antiserum was also titrated against 12- and 24-h forms from culture and a different pattern of labelling was observed over the same range. Whereas the label moved from an overall fluorescence directly to a cap on bloodstream forms, on the living transforming trypanosomes extensive patching was evident, even at low temperature. Observation on further fate of the label was not possible, however, as these trypanosomes proved to be highly susceptible to the washing procedure. This susceptibility existed in both PSG and culture medium, and at 0-4 °C and 37 °C.

DISCUSSION

Antigenic variation

There are several reasons for supposing that capping might be involved in the process of antigenic variation of *Trypanosoma brucei*. Firstly, it has long been speculated that this variation results from the presence of specific antibodies (Ehrlich, 1909), mainly from experiments in which it appeared that antibody-mediated neutralization of a population was followed by appearance of new variants (Inoki, Osaki & Nakabayashi, 1956; Gray, 1962; Takayanagi & Enriquez, 1973), although the possibility of the initial presence of a mixture of variable antigen types (VATs) was not ruled out in these experiments. Secondly, these trypanosomes, unlike most other cells which have been subjected to capping studies, regularly encounter surface-specific antibodies as a natural consequence of their parasitism. Thirdly, capping has been demonstrated on several protozoa including species of *Entamoeba* (Pinto da Silva et al. 1975), *Naegleria* (Preston et al. 1975) and *Leishmania* (Doyle et al. 1974; Dwyer, 1976); this last organism also displayed subsequent reappearance of surface antigen. Recently there have been descriptions of capping on *Trypanosoma lewisi* (Cherian & Dusanic, 1978) and *Trypanosoma cruzi* (Schmunis, Szarfman, Langembach & De Souza, 1978) which are distantly related to *T. brucei* but have different surface properties.

My results suggest that, under the conditions and time period in these experiments, capping does not play an important role in the phenomenon of antigenic variation. Redistribution of variable antigen on ETat 1 trypanosomes was induced only by indirect immunofluorescence – a single layer of antibody such as would be available in nature was ineffective. Moreover, even if some mechanism were available for the capping of variable antigen in vivo, the replacing antigen appears to be of the same rather than a different VAT. It seems likely that this replacement emanates from an intracellular pool of variable antigen or a precursor, as previously postulated (Barry & Vickerman, 1979). The possibility that a heterologous variable antigen may appear over a longer period would seem to be of little value to the parasite, as immune-mediated destruction would probably occur in the meantime. The role of antibodies in the induction of antigenic variation has been questioned recently (reviewed by Vickerman, 1978). Thus, Van Meirvenne et al. (1975) showed that an unrelapsed clone infection contains a mixture of VATs, while studies on trypanosomes derived
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from the tsetse fly vector (Le Ray, Barry, Easton & Vickerman, 1977) and those present in the salivary glands of the fly (Le Ray, Barry & Vickerman, 1978; Barry, Hajduk, Vickerman & Le Ray, 1979) revealed a high level of VAT heterogeneity in the apparent absence of antibodies. The results reported here are in general agreement with this.

It would also appear that capping of variable antigen on *T. brucei* does not occur in vivo. Trypanosomes taken from a mouse at the onset of the first parasitaemic remission label overall with FITC-conjugated anti-mouse Ig (unpublished observation), suggesting that host antibodies do not induce redistribution of the surface variable antigen.

It is significant that, on capping of the variable antigen in vitro, only trypanosome common antigens, and not heterologous variable antigen, became exposed, implying that variable antigens are not arranged as layers within the surface coat (see discussion following Vickerman, 1974).

**Pattern of capping**

The observed redistribution of variable antigen on *T. brucei* is similar in some aspects, although different in others (see below), to the general pattern of capping seen on other cells (reviewed by Nicolson, 1976; Loor, 1977). For instance, the cap usually moves to that area of the plasma membrane overlying the Golgi apparatus, which on trypanosomes is the flagellar pocket region (Vickerman, 1969b), the observed area of formation of the variable antigen cap. A prozone effect was also observed on living trypanosomes at high concentrations of antibody: overall labelling persisted at 37 °C. At higher dilutions of antiserum, capping occurred, presumably due to formation of a lattice of the antigen molecules.

Capped material is usually either endocytosed or shed, but for *T. brucei* it was not possible to resolve the fate of the capped variable antigen, although preliminary electron-microscopic studies (unpublished results) have shown a piling up of material around the flagellar pocket. Cherian & Dusanic (1978) have shown that capped antigen on *T. lewisi* is internalized.

Trypanosomes have been observed to release long fingerlike streamers of cytoplasm, enclosed in plasma membrane and surface coat but without organelles (Wright et al. 1970). These structures are known as plasmanemes (Vickerman & Luckins, 1969) and it has been suggested that they provide a means of escaping the immune response by shedding surface membrane bearing variable antigen with attached antibody (reviewed by Vickerman, 1974), although there is also evidence that they are artifacts caused by in vitro manipulation of the parasites (Ellis, Ormerod & Lumsden, 1976). My experiments tend to confirm this latter view. Plasmanemes became apparent only after incubation in vitro for 3 h in buffer without serum supplement, and at least 6 centrifugations in the presence of bound antibodies, at a time when the trypanosomes were losing motility and presumably dying.
The ability of the variable antigens on the surface of *T. brucei* to be capped suggests that the surface of *T. brucei* is fluid (in a suitable buffer at 37 °C) and permits lateral movement of variable antigen molecules; it appears to resemble the surface of other cells in this property. However, some aspects of the trypanosome surface are very different from what has been observed on other cells.

The necessity for a second layer of antibody to induce capping is usually interpreted as meaning that the given cell surface antigen molecules are so dilute that they are further apart than the span of the combining sites of the antibodies of the first layer (12 nm for IgG) but within the effective span of those of the second layer (~ 36 nm) (Unanue & Karnovsky, 1972). The immediate conclusion then would be that the individual surface coat molecules are more than 12 nm apart. Indeed Cherian & Dusanic (1978) have recently speculated that this is the case for *T. lewisi*. But for *T. brucei* this idea is patently untrue, for the following reasons.

Firstly, there is much evidence that the coat is composed of a densely packed layer of glycoprotein antigen molecules. This evidence is based on electron microscopy of thin sections of trypanosomes (Vickerman, 1969a), whole cell electrophoresis, where these protozoa behave as if they have a proteinaceous and not a phospholipid surface (Hollingshead, Pethica & Ryley, 1963), and cytochemistry and lectin binding (Seed, Seed & Brindley, 1976). Also, serological tests are VAT-specific and membrane common antigens are inaccessible to antibodies, suggesting a shielding role for the coat. Cross (1975) has calculated that there may be $7 \times 10^6$ molecules of variable antigen (molecular weight 65,000) on the surface of each trypanosome. When it is considered that an individual B lymphocyte, which is larger than a trypanosome, possesses only $5 \times 10^4 - 10 \times 10^4$ surface molecules of Ig (Unanue & Karnovsky, 1972), and these can be formed into a lattice and capped under the influence of a single antibody layer, then clearly it is not a low concentration of trypanosome variable antigen molecules which prevents single-layer capping. Dense packing of the surface glycoprotein molecules could also account for the absence of patching prior to capping; lattice formation by antibodies could not draw the molecules more closely together. Thus, the requirement for 2 layers of antibody for capping of *T. brucei* variable antigen demands another explanation.

Such an explanation is suggested by the model of the surface coat tentatively proposed by Cross (1975), where each molecule may present a face of $4 \times 4$ nm at the exterior of the coat. Such dimensions are in general agreement with the molecular weight of the isolated glycoprotein and the approximate thickness of the surface coat (12-15 nm) as judged by electron microscopy (Vickerman, 1969a). X-ray crystallography studies indicate that each antigen-binding site on an antibody molecule is a relatively flat area of approximate dimension $2.0 \times 2.5$ nm and is contained within an area of $4.0 \times 5.0$ nm, the end of the Fab arm (Poljak, 1975). It can be seen then that, regardless of how many antigenic sites are exposed on the surface of each molecule of the coat, it would be impossible sterically for more than one Ig molecule to bind to any one molecule of variable antigen. Such inhibition has been observed.
Capping on Trypanosoma brucei on sperm whale myoglobin, which possesses 6 antigenic determinant sites but can bind only 3 antibody molecules at one time (Crumpton & Wilkinson, 1965). The result then would be failure of the antibodies to produce the cross-linked lattice necessary for capping; such would become available indirectly, only on addition of the second antibody layer. Another possible reason for lack of cap formation by the first layer of antibody may be that all but one of the antigenic determinants is ‘hidden’ as a result of the close proximity of adjacent molecules.

To test the hypothesis of steric inhibition, attempts were made to cap variable antigen on trypanosomes transforming to the coatless procyclic stage in vitro. The surface variable antigen becomes much less densely packed during this transformation (Brown et al. 1973; Barry & Vickerman, 1979) thereby possibly exposing more determinants and allowing more than one antibody molecule to bind per antigen molecule. Patching was observed, showing that cross-linking did occur, but these trypanosomes could not withstand the handling procedures long enough to permit any capping to occur.

Capping and the structural organization of variable antigen on Trypanosoma brucei would appear to involve several interesting features, some of them peculiar to this organism.

Firstly, patching does not occur prior to capping on bloodstream forms, for the possible reasons given above. Secondly the densely packed surface coat protein molecules require the indirect immunofluorescence method for detection of capping, once again possibly for these reasons. Thirdly, it seems that the surface coat glycoprotein cannot be classified as being wholly integral or peripheral; on disruption of surface-labelled bloodstream form trypanosomes by mechanical means not all of the label is released with the aqueous-soluble protein. Cross (1975) found that around 20% of the protein-binding non-penetrating surface reagent formylmethionine-sulphone-methyl-phosphate remained aqueous-insoluble in homogenates of T. brucei, and Rovis, Barbet & Williams (1978), working with T. congolense labelled by the lactoperoxidase-iodination method, demonstrated that about 60–80% of the label was released with aqueous-soluble variable antigen, whereas the remainder was solubilized with variable antigen in the non-ionic detergent NP-40. The possibility, however, that the aqueous-insoluble fractions were sequestered in membrane vesicles has not been ruled out. Studies on the purification of plasma membranes from T. brucei also have suggested that some surface variable antigen remains attached to the membrane as integral glycoprotein (L. Rovis, personal communication).

A fourth interesting feature of capping on T. brucei is the apparent lack of microfilaments underlying the plasma membrane. These structures have long been associated theoretically with the process of capping, mainly due to the inhibitory effect of cytochalasin B on surface marker movement (Nicolson, 1976) and indeed recent evidence shows them to be closely involved with capping of Ig on lymphocytes (Flanagan & Koch, 1978). In T. brucei, however, microfilaments have not been detected under the plasma membrane except at the attachment region of the flagellum along the length of the trypanosome (K. Vickerman, personal communication), where there is a line of macular desmosomes, each with its attached microfilaments (Vickerman,
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1969a). If capping does require the functioning of cytoskeletal elements there are several possible ways in which T. brucei may be equipped for this. Surface movement could be controlled by dynein-like arms linking pellicular microtubules to the plasma membrane; such arm-like projections have been observed in trypanosomes (Vickerman, 1969a). On the other hand, the pellicular microtubules may exert a metabolic rather than structural control over the functions of the plasma membrane (J. Lagnado, personal communication). Alternatively, microfilaments may be absent normally but could be stimulated to polymerize on cross-linking of the surface coat molecules. Another way could be that surface is under control of the relatively few microfilaments associated with the macular desmosomes, and indeed there is some indirect evidence for this. During capping of variable antigen on T. brucei a non-labelled region possibly corresponding to the line of flagellar adhesion was seen traversing the fluorescent mass on some individuals (Fig. 3), indicating a different property on part of the trypanosome surface, and Cherian & Dusanic (1978), working on the related T. lewisi, observed that ferritin-conjugated antibody was redistributed from the whole cell surface to the flagellar pocket via this line of flagellar adhesion. The flagellum of another protozoan, Chlamydomonas, exhibits surface movement unlike that on the rest of the cell, suggesting that this part has a special role with respect to overall cell surface mobility (Bloodgood, 1977). Lastly, a membrane-flow mechanism such as that proposed by Bretscher (1976) may operate on T. brucei.

Clearly, until more is known of the molecular structure of the surface of this trypanosome, it is not possible to determine what actually controls and is involved in capping, but further elucidation should give us valuable information on both membrane fluidity and trypanosome biology.

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REFERENCES


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