

ANTIBODY-INDUCED MOVEMENT OF MEMBRANE COMPONENTS
OF *LEISHMANIA ENRIETTII*

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(Received for publication 10 December 1974)

Protozoal parasites of the genus *Leishmania* have a unit trilaminar surface membrane (1). Such membranes in mammalian cells are considered to be lipid bilayers in which the integrated membrane proteins are capable of translational diffusion (2). Movement of surface membrane components of mammalian cells into discrete aggregates or a single aggregate, a "cap", has been observed after reaction with ligands such as specific antibody and Concanavalin A (3-8). These aggregates may subsequently be endocytosed or shed. Such changes in membrane components may constitute the initial step in important phenomena such as antigenic modulation, lymphocyte activation, and immunological tolerance (3-5, 8).

Here we describe the effects of antibodies on surface membrane antigens of the amastigote and promastigote forms (9) of *Leishmania enriettii*. These include aggregation and distinctive forms of capping with at least temporary loss of antigens from the surface of the parasite.

Materials and Methods

Parasites.—Viable amastigotes were obtained from infected macrophages by excision and maceration of lesions produced in guinea pigs by intradermal inoculation of *L. enriettii* 3-6 wk previously (10). Parasites were recovered by centrifugation, washed, and resuspended in Dulbecco's modification of Eagle's medium (11), supplemented with 10% fetal calf serum. Promastigotes were cultured at room temperature in the same medium supplemented with 5% fetal calf serum and 5% of a solution of human hemoglobin (growth medium).

Antibodies to Leishmania.—Guinea pigs were infected intradermally with *L. enriettii* and sera were obtained either during the course of disease (3-6 wk after infection) or after challenge of immune animals with healed lesions. An indirect fluorescent antibody test on formalin-fixed parasites showed antibody titers from 1:64 to 1:1,024 in these sera.

γ_2 -globulins isolated from immune sera by DEAE-cellulose chromatography (12) gave similar results to those described for unfractionated sera in immunofluorescence of viable parasites. Immune sera were heated to 56°C for 30 min before use in immunofluorescence.

* Recipient of a fellowship from the Wellcome Trust.

† Supported by the Unit of Malaria and Parasitic Diseases of the World Health Organization.

§ Supported by the Wellcome Trust.

Immunofluorescence.—

Fluorescein conjugation: The globulin fraction of a rabbit antiguinea pig IgG (Miles Laboratories, Inc., Miles Research Div., Kankakee, Ill.) was conjugated to fluorescein isothiocyanate isomer I (Becton-Dickinson & Co., Cockeysville, Md.) by dialysis (13), and subsequently fractionated on DEAE-cellulose (14) to provide material with molar fluorescein: protein ratios of 3–4:1.

Fluorescent labeling of parasite membrane antigens: Viable parasites ($10^6/100 \mu\text{l}$) were incubated with guinea pig serum for 30 min, washed three times in medium, incubated with $100 \mu\text{l}$ of fluorescein conjugate for 30 min, again washed three times in medium, and finally resuspended for microscopy in medium with 5% fetal calf serum (amastigotes) or growth medium (promastigotes). All manipulations were at 0°C unless indicated otherwise. The fluorescein conjugate was diluted in medium and used at the dilutions found to be optimal in the indirect fluorescent antibody test on fixed parasites. Preparations were examined immediately after removal from 0°C by phase contrast and for fluorescence by UV epi-illumination using a Zeiss Universal microscope (Carl Zeiss, Inc., New York). A minimum of 100 viable parasites were assessed in each test.

RESULTS

Membrane Fluorescence of Amastigotes.— 10^6 amastigotes sequentially incubated at 0°C with $10 \mu\text{l}$ of guinea pig sera and fluorescein conjugate showed a diffuse microgranular staining pattern (Fig. 1 A). When incubation with serum and conjugate was carried out at 23°C or 37°C , many parasites showed localization of fluorescence into a cap over the anterior pole (Fig. 1 B). Controls in which guinea pig serum was omitted showed only occasional parasites having a small spot of fluorescence in the region of the flagellar pocket. To exclude the possibility that the changes seen at the higher temperatures represented an early stage of transformation to the promastigote form, amastigotes were stained after incubation for 6 h at 23°C in medium supplemented with 5% fetal calf serum. The pattern of fluorescent labeling at 0°C remained diffuse.

Membrane Fluorescence of Promastigotes.— 10^6 promastigotes similarly incubated with guinea pig serum and fluorescein conjugate at 0°C showed a diffuse granular staining pattern (Fig. 1 D). Most parasites labeled at 23°C showed discrete areas of fluorescence over both ends of the parasite (Fig. 1 E and F). Observation of parasites stained at 0°C and then held at room temperature showed that the fluorescence over the anterior half of the parasite coalesced towards the flagellar end of the parasite. The remaining fluorescence moved towards the tail of the parasite where on occasions it became concentrated into a filamentous process. Movement of fluorescence towards the anterior end of the parasite occurred more rapidly than that towards the tail, but in highly motile parasites the whole process was complete in under 15 min. Controls omitting guinea pig serum did not stain.

Capping and the Amount of Immune Serum Present.—Amastigotes and promastigotes were incubated at 0°C with different amounts of guinea pig serum followed by fluorescein conjugate, then incubated for 30 min at 37°C (amastigotes) or 23°C (promastigotes) before being returned to 0°C and scored for patterns of fluorescence.

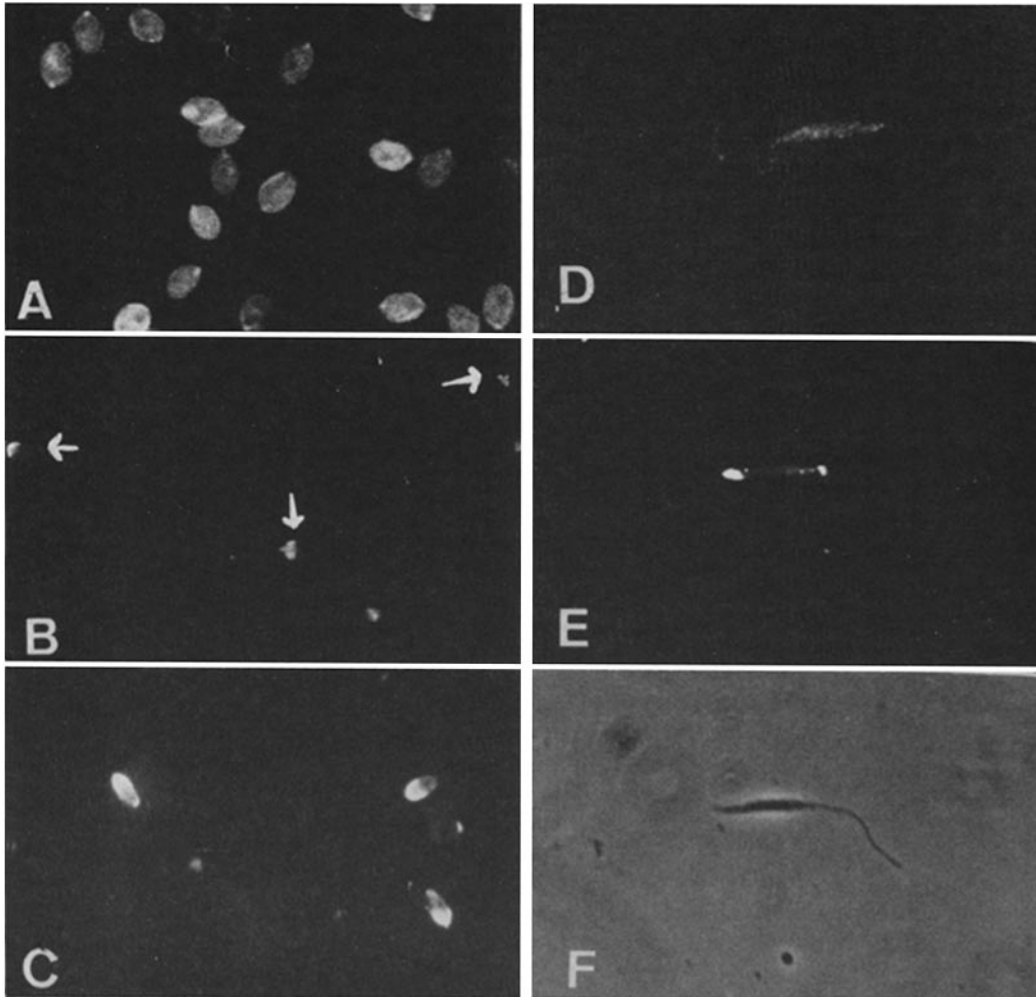


FIG. 1. (A) Diffuse microgranular membrane fluorescence of amastigotes incubated with immune guinea pig serum and then with FITC-labeled antiguinea pig Ig at 0°C. (B) Fluorescent caps of amastigotes incubated with the same reagents but at 37°C. Fluorescence is localized to the anterior pole as defined by the presence of the residual flagellum (arrows). (C) Fluorescent pattern of amastigotes after incubation with immune guinea pig serum under capping conditions, and subsequent incubation with FITC-labeled antiguinea pig Ig under noncapping conditions. There is a bright cap of fluorescence towards the anterior pole of the parasite, and the remainder of the parasite shows a diffuse microgranular stain. (D) Diffuse granular membrane fluorescence of promastigotes incubated with immune guinea pig serum and a FITC-labeled antiguinea pig Ig at 0°C. (E and F) Fluorescent caps over both poles of a promastigote after incubation with the same reagents but at 23°C (E). The larger wedge-shaped area of fluorescence is over the posterior pole and the smaller spot of fluorescence over the anterior pole of the parasite, as can be seen from the phase-contrast appearance of the parasite (F). All photomicrographs are reproduced at $\times 1,200$.

There was an optimum amount of each serum required to produce maximum capping (Fig. 2). The higher the titer of a given serum in the fluorescent antibody test, the smaller the volume required for maximum capping.

The Time Required for Capping.—Parasites were incubated at 0°C with the amounts of guinea pig sera optimum for maximum capping and then with the fluorescein conjugate. Aliquots of parasites were then resuspended in medium at 23°C or 37°C for 5, 10, 15, 30, or 60 min before being returned to 0°C and scored for patterns of fluorescence. Representative experiments are shown in

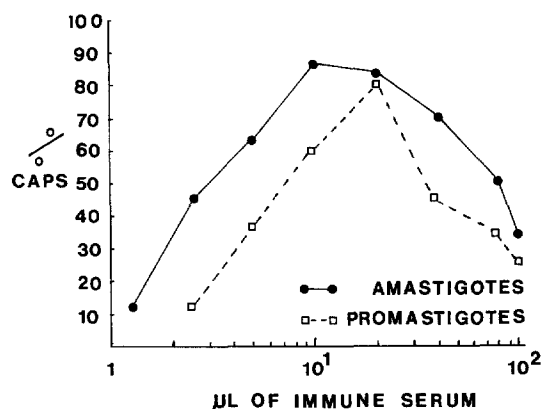


FIG. 2. The percentage of capped parasites found after 30 min of incubation at 37°C (amastigotes) or 23°C (promastigotes) of 10^6 parasites previously reacted at 0°C with different volumes of immune guinea pig Ig, and then with 100 μ l of fluorescein antiginea pig Ig.

Fig. 3. The time required for capping appeared similar to that previously described for lymphocytes (3).

The Effect of Metabolic Inhibitors.—Sodium azide and iodoacetamide were studied. Parasites were incubated for 30 min at 23°C with inhibitor in the medium and subsequently incubated at 0°C with optimum amounts of guinea pig serum and conjugate. They were then held for 30 min at a temperature appropriate for capping (23°C for promastigotes, 37°C for amastigotes). Inhibitor was present at the same concentration throughout the experiment. The results are shown in Table I. Capping of amastigotes was inhibited at the higher concentrations of both reagents. The inhibition due to sodium azide was found to be partially reversible after washing to remove the inhibitor. Capping of promastigotes was completely and irreversibly inhibited at all concentrations of both reagents.

The Role of the Second Ligand in Capping.—Direct experiments using conjugated guinea pig immunoglobulins were carried out to determine if guinea-pig antibodies alone could induce membrane antigen movement. These, however, showed only weak fluorescence, probably due to loss of antibody activity

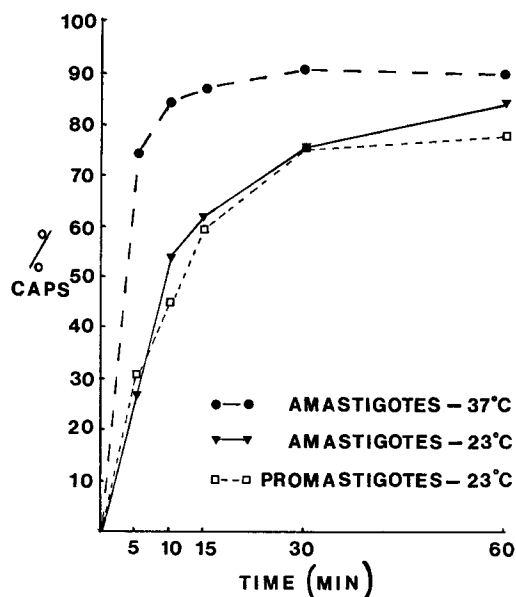


FIG. 3. Rate of formation of caps in amastigotes and promastigotes reacted with optimum amounts of immune guinea pig serum and fluorescein-labeled antiguinea pig Ig at 0°C, and subsequently incubated for various periods at 37°C or 23°C.

TABLE I

Inhibition of Capping of Amastigotes of L. Enriettii by Sodium Azide and Iodoacetamide

Concentration of inhibitor (Molarity)	Percentage inhibition of cap formation*	
	Sodium azide	Iodoacetamide
10^{-1}	98	100
5×10^{-2}	66	100
10^{-2}	50	100
5×10^{-3}	35	68
10^{-3}	24	9
5×10^{-4}	0	0

* Percentage of inhibition compared to the percentage of caps (80–90%) found in control parasites that were incubated under similar conditions without inhibitor.

during isolation and conjugation. Experiments were therefore carried out under conditions which would permit antigen movement in the presence of guinea pig antibodies, but which would prevent any subsequent movement due to the antiguinea pig Ig conjugate. Amastigotes were exposed to guinea pig serum for 30 min at 37°C. Further processing was carried out at 0°C either in the presence of 5×10^{-2} M sodium azide or after fixation of surface antigens by 2% formalin at 0°C (6); both methods gave similar results.

Under these conditions parasites exposed to certain sera showed both a fluorescent cap and a diffuse granular fluorescence (Fig. 1 C). As in the previous experiments, each guinea pig serum could be titrated to find the optimum amount required to give the maximum number of these partially capped cells. When partially capped parasites that were prepared by incubation in sodium azide were washed in fresh medium and incubated at 23°C, they developed complete caps. Parasites fixed in formalin before incubation with immune serum showed only a diffuse fluorescence. These findings suggested that certain membrane antigens were capped by some guinea pig sera alone, but that capping of the remaining antigens was dependent on the action of the second ligand.

Reappearance of Membrane Antigens after Capping.—Amastigotes and promastigotes were incubated at 0°C with quantities of guinea pig serum optimum for capping, and then with fluorescein conjugate. They were then incubated at 23°C and examined at various times.

After 30 min of incubation parasites showed caps, and immediate re-exposure to guinea pig serum and conjugate at 0°C did not alter this staining pattern. After 3 h, only 20–25% of the parasites still showed definite caps, the remainder showed only small amounts of fluorescence or were unstained. After re-exposure to the guinea pig serum and conjugate at 0°C, all parasites showed a diffuse granular pattern of fluorescence, including those with the residual cap. Parasites re-exposed to fluorescein conjugate alone showed no diffuse staining.

DISCUSSION

Guinea pigs infected with *L. enriettii* were found to produce antibodies reactive with parasite surface antigens. This reaction could occur between amastigotes and serum which were simultaneously obtained from the same animal. Capping of antigens was induced by exposure of amastigotes or promastigotes to guinea pig antibodies and a second ligand, fluorescein-conjugated anti-guinea pig Ig. Guinea pig serum alone could produce partial capping.

These findings were similar in many respects to those previously described for mammalian cells. Capping required viable parasites and optimum antibody concentrations. The rate of capping was temperature dependent; caps were not formed at 0°C, and at 23°C and 37°C the rate was similar to that described for lymphocyte immunoglobulins (3). Sodium azide and iodoacetamide inhibited cap formation by amastigotes at concentrations similar to those required to inhibit capping of lymphocyte immunoglobulin (3, 7). Promastigotes were more susceptible to these inhibitors, perhaps reflecting their largely aerobic metabolism (15). Thus, concepts of membrane antigen mobility previously developed from studies on mammalian cells are generally applicable to this protozoan.

Capping of amastigotes showed a single polarity towards the anterior pole of the parasite which contains the flagellum and the Golgi apparatus (1).

A similar polarity of capping towards the region of the Golgi apparatus has been observed in lymphocytes (3, 7, 8). Capping of promastigotes invariably showed a double polarity, a phenomenon not so far described for mammalian cells. The differences in the movement of aggregated antigens in different regions of the promastigote were perhaps due to the very active movements of the organism. This finding is now being studied by electron microscopy.

Capped antigens disappeared from the parasite membrane. Their fate was not certain, but since endocytosis was not observed it seems likely that the bulk of the complexes were shed. Antigens reappeared diffusely over the parasite membrane within a few hours of capping. Since the guinea pig antisera were likely to show specificity for several different antigens, it is not clear whether these new antigens were in all respects identical to those previously present.

These phenomena described for *Leishmania* are likely to be a general occurrence after the reaction of antibodies with living protozoal parasites, and they imply that parasite membrane modulation by antibody may be an early and fundamental effect of the immune response. This may provide an explanation for the antigenic variation during the course of infection which is a prominent feature of African trypanosomiasis (16). Even temporary clearance or alteration in the pattern of the distribution of parasite membrane receptors could be important. The effect, for example, of intact antimalarial antibody, of (Fab)₂ but not of Fab, in preventing in vitro infection of erythrocytes by malarial merozoites could be ascribed to antibody-induced loss or redistribution of parasite membrane receptors that are specific for sites on erythrocytes (17, 18).

Guinea pigs develop a high degree of protective immunity to reinfection after an infection with *L. enriettii*, but the immune mechanisms of protection are unknown (19). It now appears that in this and other protozoal infections the role of antibodies must be considered not only in terms of classical functions such as lysis and opsonization but also as acting by modifying the membrane structure of the living organism.

SUMMARY

Incubation in vitro of viable *Leishmania enriettii* with antibodies from infected or immune guinea pigs and a fluorescein-labeled antiguinea pig Ig conjugate induced aggregation of surface antigens to form a "cap" over the anterior pole of the amastigote and over both the anterior and posterior poles of the promastigote form of the parasite. Cap formation occurred only with optimum quantities of guinea pig antibodies and was inhibited by low temperature and the metabolic inhibitors, sodium azide and iodoacetamide. The aggregated antigens were rapidly lost from the surface of the parasite but reappeared after 3 h of incubation at 23°C. This phenomenon of ligand-induced membrane antigen movement is apparently similar to that described in mam-

malian cells, and may represent the first stage of the interaction between host antibodies and the surface membrane of protozoal parasites.

We gratefully acknowledge the assistance of Mr. P. Dubied in the preparation of the photomicrographs.

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