Human Neutrophils Endocytose Multivalent Ligands from the Surface of Schistosomula of *Schistosoma mansoni* before Membrane Fusion

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**ABSTRACT** Human buffy coat cells adhering to schistosomula of *Schistosoma mansoni* that were preincubated in fluorochrome-conjugated concanavalin A (Con A), wheat germ agglutinin, lentil lectin, or purified IgG from a hyperimmunized rabbit, were examined by fluorescence and transmission electron microscopy and by freeze-fracture. All four fluorochrome-conjugated multivalent ligands were homogeneously distributed on the parasite surface after preincubation. Within 1-3 h after the addition of cells, large areas of nonfluorescence, 10-20 μm in diameter, were seen on the parasite surface. In addition, the fluorochromes were observed in granules within the cells. Electron microscope autoradiography of worms preincubated with 125I-Con A showed silver grains evenly distributed over the tegumental membrane. After the addition of cells, grains were seen over phagolysosomes in the cytoplasm of neutrophils adhering to the parasites. In addition, no grains were present over large areas of the tegumental membrane, which still retained its normal architecture, or over fusions between the neutrophil plasma membrane and the outer tegumental membrane. Rabbit IgG formed an electron-dense layer on the tegumental membrane which was endocytosed by neutrophils. Both neutrophils and eosinophils fused with the parasite in areas containing no electron-dense material on the surface. It is concluded that human neutrophils will endocytose a variety of multivalent ligands from the surface of schistosomula, which probably accounts for the failure of neutrophils to kill the parasite and acts to clear the parasite surface of both antigen and antibody. Presumably, the components of the parasite surface which have originally bound the ligands are also endocytosed since surface components labeled by galactose oxidase and NaBH4 are taken into cells when examined by light microscope autoradiography. Finally, membrane fusion occurs in areas devoid of multivalent glands, which suggests that these ligands serve to bring the cells and parasites close together, but the actual fusigens probably reside in the lipids in the outer tegumental membrane.

Schistosomula, larvae of *Schistosoma mansoni*, can be destroyed in vitro by human eosinophils—but not by neutrophils—after the parasites are preincubated in heat-inactivated sera from patients with schistosomiasis (28). Eosinophil-mediated killing is caused by exocytosis of lysosomes onto the parasite surface (3, 7, 17), and subsequent damage to the tegumental membrane is caused by major basic protein (2) and possibly peroxidases to which the worm is quite sensitive (13). The neutrophils, on the other hand, do not exocytose their lysosomes but instead fuse their plasma membranes with the outer tegumental membranes of the schistosomula over areas as large as 8 μm in diameter (3).

In these experiments, we have promoted adherence between human buffy coat cells and schistosomula with three lectins concanavalin A (Con A), wheat germ agglutinin (WGA), and lentil lectin (Len), as well as with IgG purified from a hyperimmunized rabbit (RoS). We tried to determine the distribution of the multivalent ligands during the adherence of buffy coat cells to the parasite and, in particular, within the large membrane fusions. To this end, we used fluorescein- or rhodamine-
conjugated lectins and examined the preparations in a fluorescence microscope. In addition, we used $^{125}$I-Con A (20) to examine the distribution of that lectin ultrastructurally by electron microscope autoradiography (EMARG). Finally, the membrane fusions were examined by freeze-fracture and transmission electron microscopy (TEM).

MATERIALS AND METHODS

Lectin-mediated Adherence Reactions

Mechanical or skin schistosomula were prepared as previously described (3, 20) and stored overnight at 4°C in RPMI-1640. In the morning they were washed three times with Eagle's minimal essential medium containing 0.1 M HEPES buffer, pH 7.2 (MEM). The worms were then preincubated with fluorescein- or rhodamine-conjugated Con A, WGA, or Len (Vector Laboratories, Burlingame, Cal., and Sigma Chemical Co., St. Louis, MO) at a concentration of 50 μg of lectin/ml and in a volume of 200 μl/1,000 schistosomula for 45 min at room temperature. After preincubation the parasites were washed three times in MEM. Human buffy coat cells, prepared as previously described, were added to the worms, so that typically $3 \times 10^6$ cells and $3 \times 10^6$ worms in a final volume of 400 μl of MEM were incubated in a 7 x 38-mm round bottom tube for 1 to 3 h at 37°C (3). For the autoradiography experiments, we used $^{125}$I-Con A (20) instead of the fluorescent-conjugated lectins. Viability of the parasites was assessed by their ability to move and to exclude toluidine blue. The number of times the experiments were done is given in Table 1.

Rabbit Antischistosomal IgG (RaS)-mediated Adherence Reactions

The IgG fraction of sera from a New Zealand White (NZW) rabbit hyperimmunized by repeated injections of a schistosome homogenate in Freund's adjuvant was prepared by ammonium sulfate precipitation and DEAE-cellulose chromatography and generously given to us by Dr. Donald Harn (Harvard Medical School). Fluorescein- and rhodamine-isothiocyanate were conjugated to these antischistosomal antibodies by overnight incubations at 4°C in 0.5 M bicarbonate buffer, pH 9 (12). Unreacted fluorescein was removed by exhaustive dialysis against PBS, and the molar ratio of fluorescein to RaS was 1.1 Rhodamine-antibody conjugates with a molar ratio of 1.5 were eluted with increasing concentrations of phosphate from a DE-52 column (12). Both antibody conjugates did not contain unreacted fluorochromes by chromatography either on a layer of Sephadex G-25 or on silica gel plates with chloroform:methanol:water solvent. Both preparations of fluorochrome-conjugated RaS contained ~3 mg/ml protein and were stored as aliquots in PBS at -20°C. Schistosomula were labeled for 45 min at 4°C in 1.5 to 1.20 dilutions of RaS in MEM and washed three times. RaS bound to the surface of schistosomula and to cercarial bodies and tails when examined by immunofluorescence microscopy. Buffy coat cells were cultured with RaS-preincubated worms, as described above for lectin-preincubated worms.

Microscopy

Adherence reactions were examined in a Leitz Orthomat microscope equipped with a Ploem illumination system equipped with an H-2 cube and a 450-nm filter for viewing fluorescein and an N-2 cube for viewing rhodamine. Preparations were photographed with a Leitz Orthomat camera on Kodak Tri-X film. Before photography, the worms were immobilized with 0.01 M eserine sulphate (20).

TEM and freeze-fracture were performed as previously described (3). Light and electron microscope radioautography were carried out by the method of Salpeter (18) with Ilford L4.4 emulsion.

| Table 1. Number of Experiments |
|-------------------------------|
|                              | RaS | Con A | WGA | Lentil |
| Fluorescence                  | 5   | 22    | 13  | 3     |
| TEM                           | 3   | 8     | 4   | 2     |
| Freeze-fracture               |     | 5     | 6   | 2     |
| EMARG                         |     | 3     |     |       |

* Each number represents an experiment done on a different day with different preparations of cells and parasites. In most cases, the fluorescence experiments were run in triplicate and examined at various time points.

RESULTS

Lectin-mediated Adherence—Light Microscopy

Buffy coat cells begin to adhere to schistosomula preincubated with lectins within 5 min and large numbers of cells (~20/organism) are attached to the parasites within 1 to 3 h (1). All three lectins, WGA, Con A, and Len, induce similar numbers of buffy coat cells to adhere at the same rate to the worms. Cell adherence is inhibited by the addition of either 0.1 M α-methyl mannoside during preincubation with Con A and Len or 0.1 M N-acetyl glucosamine during preincubation with WGA. Addition of the appropriate sugars after lectin preincubation causes little or no decrease in the number of cells adhering to the schistosomula. Fluorochrome-conjugated lectins are homogeneously distributed on the surface of the parasite immediately after incubation and when the parasites are cultured in the absence of cells (20) (Fig. 1). The anterior portion of the worm appears brighter than the posterior portion because the anterior portion has a greater density of spines and, consequently, a greater surface area than the posterior portion (Fig. 1). After 1–3 h of incubation with buffy coat cells, the lectins are no longer homogeneously distributed on the parasite surface (Fig. 2). Instead, large (10–20 μm in diameter), nonfluorescent, dark areas are present (Fig. 2). There is no preferred distribution of these dark areas on the parasite surface or association of these areas with specialized surface structures, e.g., the tail socket, acetabulum, and openings of the acetalabular and preacetabular glands at the anterior end (Fig. 2). Comparison of phase-contrast and fluorescent images of the same parasite shows that spines are still present in the areas that do not contain the fluorescent lectin (Fig. 3 and 4). In addition, the fluorochrome can be seen in 0.2- to 0.4-μm granules within the buffy coat cells adhering to the worm (Figs. 2 and 4). After overnight culture at 37°C, >80% of the worms are alive the next day in preparations containing either worms or worms and cells, with or without lectin preincubation.

Lectin-mediated Adherence—Electron Microscopy

To determine the distribution of Con A on the parasite and the cell type(s) involved in the adherence reaction, parasites were preincubated in $^{125}$I-Con A, buffy coat cells were added, and EMARG was performed. Worms that are freshly labeled with $^{125}$I-Con A and that have not been incubated with buffy coat cells have silver grains located over the tegumental membrane of the entire organism (Fig. 5). After 1–3 h of incubation with cells the silver grains are no longer present over the entire tegument. Areas, 10–20 μm in length, that contain no overlying grains alternate with areas that have the same overlying grain density as controls (Figs. 6–9). The tegumental membrane which has no overlying grains is pentalaminar which is the normal structure of this membrane (see Fig. 8 and references 3, 11, 26).

In general, PMNs are the only cell type seen on the parasite surface. Silver grains appear in clusters over the cytoplasm of the PMNs and appear to be associated with phagolysosomes (Figs. 6 and 9). Silver grains are rarely present over cells that are not attached to the parasite. PMNs also fuse with the tegumental membrane of the lectin-preincubated schistosomula, as has been previously described in antibody and complement dependent adherence reactions (3). Less than 10% of the adherent PMNs fuse and the fusions occur in areas over which very few silver grains are seen (Figs. 7–9). The fused PMNs are...
broadly attached to the worm over a distance of 7-10 μm (Figs. 7 and 9). The fused membranes are pentalaminar rather than 7- or 9-layered which would be seen if fusion did not occur (Fig. 8).

Freeze-fracture of the lectin-mediated adherence reactions show fusion areas similar to those seen in antibody and complement-mediated adherence reactions (3). In brief, the fracture plane passes through the outer membrane but at the edge of a fusion area it skips into the inner membrane (Fig. 10). Within the fusion area, which is 5-8 μm in diameter, the fracture plane returns to the outer membrane. Here, there are two areas one of which is rich in intramembrane particles (IMPs) and the

FIGURES 5 and 6  Fig. 5: Electron microscope autoradiography of mechanically prepared schistosomula (S) incubated with 125I-Con A. The silver grains lie over the tegumental membrane. t, tegument. x7,400. Fig. 6: Electron microscope autoradiograph of a mechanically prepared schistosomulum preincubated with 125I-Con A and human buffy coat cells for 1 h. On the right side of the picture silver grains, with a distribution and density similar to those in the control shown in Fig. 5, lie over the tegument (t). On the left, there are no silver grains lying over the tegument. Silver grains are also present over the cytoplasm of the neutrophil (N) attached to the schistosomulum (S). x10,000.

FIGURES 1-4  Fig. 1: Skin schistosomulum at the end of the preincubation with fluorescein-Con A. The label is evenly distributed over the parasite. The anterior end, at the top, is brighter because there is a higher density of spines on this portion. The bright area on the body (arrowhead) is due to staining of the acetabulum or ventral sucker located on the other side of the worm. x680. Fig. 2: Mechanically prepared schistosomulum preincubated in fluorescein-Con A and incubated with buffy coat cells for 3 h. The label is no longer uniformly distributed on the worm. Dark, nonfluorescent areas are present on the parasite (arrows). The fluorescein is also present in granules in the cells (arrowheads). x680. Figs. 3 and 4: Phase-contrast and fluorescence micrographs of the same schistosomulum preincubated with fluorescein-Con A and incubated with buffy coat cells for 3 h. Most of the cells attached to the worm contain fluorescein in their granules. In addition, in one region (circle) a dark area is seen on the surface although spines are still present in the phase-contrast micrograph. x680.
other poor (Fig. 10). The IMP-rich areas are unattached PMN plasma membranes. The IMP-poor areas are hybrid membranes derived from the worm outer tegumental membrane and the PMN plasma membrane and containing an IMP concentration intermediate between those of the membranes from which it was formed (3).

In an attempt to test whether surface components are endocytosed along with Con A, the parasite surface was labeled by the galactose oxidase, NaB3H4 technique described in our accompanying paper (19), preincubated in fluoresceinated Con A and incubated with buffy coat cells. After 1–3 h when cell adherence and endocytosis of Con A were prominent, the samples were fixed and examined by light microscope autoradiography (LMARG). Worms that had not been incubated with cells have grains confined mainly to the tegument, with a few grains scattered internally (See Fig. 2 in reference 19). When worms are incubated with cells, the grain distribution on the surface is irregular, with areas containing grains alter-

![Figure 10](image)

**Figure 10** Freeze-fracture micrograph of a mechanically prepared schistosomulum preincubated with Con A and incubated with buffy coat cells for 3 h. Normal schistosomulum inner (E₁) and outer (E₂) membranes are seen at the lower right and are separated from the fusion area by a zone of skip fracture into the inner membrane (arrows). The fusion area itself is composed of both the fused membrane, seen as an IMP-poor area, (pp), and unfused PMN membrane, seen as an IMP-rich area (pr) which is lifted from the surface. × 63,000.

**Figures 7–9** Figs. 7 and 8: Electron microscope autoradiographs of a mechanically prepared schistosomulum preincubated with ¹²⁵I-Con A and incubated with buffy coat cells for 3 h. A neutrophil (N) has fused to the schistosomulum (S) to which it is attached. Silver grains are not present over the fused membrane or over the adjacent tegumental membrane. Other worms on this grid were as heavily labeled as the controls shown in Fig. 5. Fig. 8 is a high-power view of the area in the rectangle in Fig. 7. The normal tegumental membrane on the left (arrow) is pentalaminar as is the fused membrane between the parasite and the cell (arrowheads). ¹, tegument. Fig. 7: ×15,000. Fig. 8: ×110,000. Fig. 9: Electron microscope autoradiograph of human neutrophil (N) fused to a schistosomulum (S) preincubated with ¹²⁵I-Con A and incubated with buffy coat cells for 3 h. The broad fusion area has virtually no overlying silver grains. The silver grains over the cytoplasm appear to be associated with phagolysosomes (p). × 24,000.
nating with areas devoid of grains (Fig. 11). In addition, grains are seen over the cytoplasm of some of the cells adherent to the parasite (Fig. 11).

**RaS-mediated Adherence**

Skin schistosomula incubated in fluorochrome-conjugated RaS have a uniform distribution of fluorescence on their surface similar to that of Con A (see Fig. 1). After 1-3 h of incubation with human buffy coat cells, the fluorochrome is seen in the granules of the cells and is absent from patches on the surface (Fig. 12). Electron microscopy of parasites incubated in 1:5 to 1:20 dilutions of RaS reveals an electron-dense coat, ~1 μm thick, on the tegumental membrane (Fig. 13). Buffy coat cells endocytose this coat material from the parasite surface (Fig. 14). Fusions between neutrophils and the parasites are seen in <10% of the adherent cells. Fusions are also seen between eosinophils and the parasite (Fig. 15). With both neutrophils and eosinophils, the fusions occur in areas which are devoid of the electron-dense coat (Fig. 15).

**DISCUSSION**

Two classes of multivalent ligands, lectins and RaS IgG, have been used to promote adherence between human buffy coat cells and schistosomula. The resulting adherence resembles that promoted by sera from patients infected with schistosomiasis (3). These multivalent ligands, therefore, serve as a model for human IgG and we have determined their distribution in the adherence reactions by both light and electron microscopy. There are two major results. First, neutrophils endocytose both types of ligands from the parasite surface but the tegumental membrane retains its configuration as a double lipid bilayer. Second, small numbers of neutrophils and eosinophils fuse their plasma membranes with the outer tegumental membrane of the parasite in areas where the multivalent ligands are absent.

Adherence between cells and the parasites is apparently caused by the binding of the various lectins to the appropriate sugars on the parasite and cell surfaces. In the case of RaS, adherence is caused by the binding of IgG to antigenic determinants on the parasite surface and the binding of the Fc
portion of the molecule to the appropriate Fc receptor on the cell. Despite the different modes of binding of lectins and IgG, human neutrophils endocytose both ligands from the parasite surface into phagolysosomes. Presumably, the components of the parasite surface which have originally bound the ligands are also endocytosed since labeled surface components (19) are taken into cells by LMARG. However, although surface components are removed, the two lipid bilayers which compose the tegumental membrane covering the parasite are structurally unaltered, as seen by freeze-fracture or thin-section microscopy. This observation suggests that the surface components are removed as micelles or very small quantities, as previously concluded (19). Thus, the cells may be regarded as simply accelerating the sloughing or surface shedding which naturally occurs in this parasite (19, 20).

A somewhat different view of the parasite surface is provided by RoS which demonstrates a 1-μm thick, electron-dense coat on the outer membrane. This coat is not seen on schistosomula preincubated with 1:20 dilutions of human antisera (see Fig. 2 in reference 3) and does not appear to be cercarial glycocalyx which is electron-dense in the absence of antibody (10, 14). On the other hand, there is a resemblance to the cercarial surface in that an increase in the thickness of the cercarial glycocalyx is observed in the presence of antischistosomal antibodies, the so-called "cercarienhüllen reaktion" (14, 15, 29). The electron-dense material seen on cercariae and RoS-preincubated schistosomula also resembles coats seen on rickettsia (24) and bacteria (21, 23) after incubated in antibody or complement. These coats do not appear to represent a form of "staining" of preexisting structure because hemocyanin (20) touches the outer membrane in the absence of antibody. This suggests that there may be an unfolding or conformation change of surface molecules in the presence of antibody or complement that accounts for the appearance of these coats.

The fact that membrane fusion between cells and schistosomula can be promoted by three lectins and/or a heterologous antibody, as well as homologous antisera and complement (3), suggests that these multivalent ligands are not themselves the cause of the fusions. Instead, they appear to bring the cell and parasite membranes close together (3). Fusion is possibly induced by some component, which is present in the outer tegumental membrane. This interpretation is favored by the fact that the parasite can fuse with both neutrophils and eosinophils.

These studies also suggest that the multivalent ligand, as well as the parasite surface components to which the multiva-

**Figures 13-15**

Fig. 13: Electron micrograph of the tegument (t) of a schistosomulum incubated with a 1:5 dilution of RoS for 45 min. Note the electron-dense layer (d) on the tegumental membrane. This material is not seen on worms incubated with 50 μg/ml of Con A (20), on worms incubated in 1:20 dilutions of human anti-schistosomular sera (3), or on worms that are freshly transformed (3). Fig. 14: Electron micrograph of a schistosomulum preincubated with RoS and incubated with buffy coat cells for 15 min. Neutrophil (N) adhering to the tegument (t) is endocytosing the dense material (d) from the surface into phagolysosomes (p). x 49,000. Fig. 15: Electron micrograph of a schistosomulum (S) preincubated in RoS and incubated with buffy coat cells for 1 h. An eosinophil (Eo) is fused to the parasite (arrows). No electron-dense material such is shown in Figs. 13 and 14 is present on the worm. x 31,000.
lent ligand was bound, are absent from the fused membranes. Con A, as seen by autoradiography, and the RoS, as seen in the electron-dense layer, are not present in the areas of membrane fusion. The surface components to which the multivalent ligands were bound are also presumably absent from the fusions, since the NaB³H₄-labeled Con A binding sites appeared to be endocytosed along with Con A. These results are in agreement with other studies of membrane fusion that suggest that membrane proteins are cleared from fusing membranes and that membrane fusion is a lipid-mediated event (16, 25), although this has been questioned (5). Finally, the endocytosis of multivalent ligands and surface components may explain the paradox of how the parasite membrane can form such large fusions, up to 8 µm in diameter, without evidence of superfluous membrane, e.g., blebs or villi, on its surface.

What are the implications of these experiments in regard to the ability of the immune system to kill schistosomula? Schistosomula preincubated with antischistosomular serum under the conditions used in these experiments can be killed by human eosinophils but not by neutrophils (28). The eosinophils discharge their lysosomes onto the parasite surface, whereas the neutrophils do not exocytose (3, 7). We suggest that the neutrophils do not exocytose because they have endocytosed the “signal” or multivalent ligand that should cause exocytosis. The neutrophil apparently sees a nonphagocytosable substrate (8) that is >10 times the size of the cell, as an endocytosable complex, akin to an antigen-antibody complex. The endocytosis of antibody and parasite surface components not only prevents the neutrophils from discharging but also removes antibody which is the signal for the eosinophil to exocytose. Therefore, neutrophils, under appropriate conditions, should inhibit eosinophil-mediated antibody-dependent killing. The specific killing ability of the eosinophil itself must reside in its Fc receptors, either in their ability to recognize different classes of immunoglobulin than the neutrophil or in the nature of the receptor itself. In addition, the clearance of antibody by neutrophils would account for the decreasing numbers of cells that adhere to the parasite over time in in vitro adherence reactions (27).

Finally, these experiments suggest that the membrane fusions are not limited to the narrow experimental conditions under which they were originally produced (3, 4). Fusions could conceivably occur with any membrane that is close enough to the parasite. Since the parasite spends most of its lifespan in the vascular system, it may be mechanically close enough to either circulating blood cells or the endothelium to permit such fusions. The fusions, themselves, may in turn explain how schistosomes can acquire such potentially protective host antigens as the major histocompatibility complex (22), the Forssman antigen (6), and the ABO blood group glycolipids (9).

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