The Adherence of Human Neutrophils and Eosinophils to Schistosomula: Evidence for Membrane Fusion between Cells and Parasites

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ABSTRACT   Human neutrophils and eosinophils adhere to the surface of schistosomula of *Schistosoma mansoni* that have been preincubated with antischistosomular sera with or without complement. Neutrophils are seen to form small (<0.5 μm), heptalaminar and large (5–8 μm), pentalaminar fusions with the normal pentalaminar parasite surface membrane. By freeze-fracture techniques, attachment areas 5–8 μm in diameter are seen to form between neutrophils and schistosomula. These areas have three zones—an edge and two centrally located areas, one of which is rich and one of which is poor in intramembrane particles (IMPs). The edge zone is continuous around the attachment areas and is usually composed of a skip-fracture that passes out of the schistosomular outer membrane into the inner membrane. In some cases, the edge zone is made up of a string of IMPs. The IMP-rich central areas have an IMP concentration similar to that of unattached neutrophil membranes, are raised off of the surface of the schistosomulum, and have two normal schistosomular membranes underneath, indicating that they are indeed unattached. The IMP-poor central areas are composed of a fused or hybrid membrane that is continuous with the neutrophil plasma membrane but that bears the same spatial relationship to the schistosomular inner membrane that the normal outer membrane does. Similar changes are seen in samples prepared without glycerination. Eosinophils generally do not fuse with the schistosomular outer membrane but, instead, discharge their granular contents onto the surface of the schistosomula and appear to adhere to the parasite through this discharged material. It is suggested that schistosomula have a capability to fuse with mammalian cells and that this fusion proceeds from a fusion of the outer leaflets to a fusion of the bilayers, as appears also to be the case in other systems.

Schistosoma mansoni presents a paradox in that adult parasites are able to survive in the bloodstream for long periods of time (years in some cases), whereas schistosomula, the larvae that develop from cercariae that have penetrated the skin, are destroyed by an antibody-dependent granulocyte reaction (31). Two questions arise from this paradox. First, how do adult parasites avoid the immune response? One answer that has been given is that the parasites acquire host antigens, namely ABH blood group glycolipids (12), Forssman antigen (7), and components of the major histocompatibility complex (30),

which possibly prevent the host's immune system from recognizing the parasite as foreign. However, the mechanism by which these host molecules are acquired by the parasite is unclear. Second, how do granulocytes interact with the surface of the schistosomulum and, in particular, what are the differences between eosinophils, which appear to kill the schistosomula, and neutrophils, which, in our experiments, do not (35)? Previous thin-section studies have focused on the eosinophil and found that it discharges its granules onto the parasite surface (11, 22). In the present study, we examined with thin-

section and freeze-fracture techniques the adherence of human neutrophils and eosinophils to schistosomula that had been preincubated with schistosomular sera and complement. More specifically, we looked for membrane interactions between these cells and the parasite.

MATERIALS AND METHODS

S. mansoni Life Cycle and Preparation of Schistosomula

A Puerto Rican strain of *S. mansoni* was maintained by routine passage through outbred mice and *Biomphalaria glabrata* snails. Schistosomula were prepared by allowing infective cercariae to penetrate through a shaved abdominal rat skin clamped between an upper and a lower chamber (6, 32). The lower chamber was filled with Earle's balanced salt solution containing 0.5% lactalbumin hydrolysate (Flow Laboratories, Inc., Rockville, Md.), penicillin (100 µg/ml) and 10% fetal calf serum (Flow Laboratories) that had been inactivated at 56°C for 1 h (E LAC/FCS). The upper chamber contained cercariae shed from infected snails over the preceding 3 h. The entire assembly was incubated for 3 h, with the lower chamber immersed in a water bath at 37°C. During this period, the cercariae penetrated the skin and transformed into schistosomula, which fell to the bottom of the lower chamber. At the end of the incubation period, the assembly was dismantled, and the pellet of schistosomula in the lower chamber was recovered and washed twice in E LAC/FCS by centrifugation at 250 g for 30 s at ambient temperature. These schistosomula, which were routinely >90% viable as judged by their ability to exclude toluidine blue (35), and which were contaminated with <10% residual cercariae, were stored overnight at 4°C in E LAC/FCS for use on the following day.

Preparation of Human Leukocytes

Human leukocytes were prepared from samples of heparinized peripheral blood from normal donors by methods described in detail elsewhere (35). Briefly, 5 vol of peripheral blood containing 10 U/ml heparin (Panheparin, Abbott Laboratories, North Chicago, Ill.) were allowed to sediment with 1 vol of 4.5% dextran (Dextran 250, Sigma Chemical Co., St. Louis, Mo.) in phosphate-buffered saline, pH 7.4, for 30 min at 37°C. The leukocyte-rich supernate was withdrawn, and the leukocytes were washed twice by centrifugation at 250 g for 10 min at ambient temperature in Eagle's Minimal Essential Medium (MEM, Grand Island Biological Co., Grand Island, N. Y.) containing 100 U/ml penicillin and 100 µg/ml streptomycin, buffered with 25 mM N-2-hydroxyethlpiperazine-N'-2-ethanesulfonic acid (HEPES). These cells, resuspended in MEM supplemented in 10% heat-inactivated fetal calf serum (MEM/FCS), were used as mixed leukocytes. The mixture contained 3–10% eosinophils, 50–80% neutrophils, and 15–50% mononuclear cells.

Further purification was achieved by centrifugation of mixed leukocytes over discontinuous gradients of metrizamide (Nyegaard, Oslo). Metrizamide was dissolved in Tyrode's solution containing 0.1% gelatin and 30 mg/liter DNAse (Worthington Biochemical Corp., Freehold, N. J.; 250 U/mg). Each gradient consisted of 2-ml steps of 18, 20, 22, 23, 24, and 25% metrizamide in a conical polystyrene tube. Aliquots of not more than 7 × 10⁷ mixed leukocytes were applied to each gradient, which was then centrifuged at 1,200 g for 45 min at 20°C. The cells at each interface were withdrawn and washed twice in MEM/FCS. Fractions were counted with a Coulter counter (Coulter Electronics Inc., Hialeah, Fla.), and cytocentrifuge preparations were made and stained with Wright's stain for immediate examination. Neutrophils were characteristically enriched in the 22–23% interface, and eosinophils in the 23–24% interface. Fractions were pooled, in different experiments, to yield preparations that contained 85–98% eosinophils or 94–95% neutrophils. The cells were resuspended in MEM/FCS at 10 or 20 × 10⁶ cells per ml.

Human and Guinea Pig Sera

Sera from patients with active, untreated *Schistosoma mansoni* infection were used as sources of antischistosomular antibody. The sera were heat-inactivated at 56°C for 1 h, and dilutions that cause high levels of eosinophil-mediated adherence and damage to schistosomula (35) were chosen. Such sera were not directly toxic to the organisms. Eight separate sera were used in the experiments reported here, with qualitatively indistinguishable results.

Sera from uninfected human subjects or from guinea pigs were used as a source of complement. Aliquots of fresh serum were stored at −70°C for not more than 2 mo. Each aliquot was thawed only once and was diluted to a 1:30 or 1:40 final concentration in MEM/FCS. Both human and guinea pig complement

enhanced the antibody-dependent adherence of leukocytes, but guinea pig complement had a more marked effect and was used in most experiments.

Adherence Reactions between Cells and Schistosomula

The basic reaction under investigation was the antibody- and complement-dependent adherence of various leukocytes to schistosomula. The number of experiments is shown in Table I.

In the basic experiment, schistosomula prepared the previous day were washed twice in MEM/FCS and resuspended at 10,000 organisms per ml. Aliquots of 0.1 ml, containing 1,000 organisms, were then dispensed into 7 × 38-mm round-bottomed polystyrene tubes (Luckham Ltd., Burgess Hill, England). Aliquots of 0.1 ml of an appropriate dilution of antischistosomular serum, or of MEM/FCS as control, were then added, and the preparations were allowed to stand at ambient temperature for 30 min.

Aliquots of 1.0 ml of the appropriate leukocyte preparations at a concentration of 20 × 10⁶ cells per ml (2,000 cells per schistosomulum) were added, and the preparations were incubated in humidified plastic boxes at 37°C for 30 min to allow fixation of complement. Finally, the leukocytes were added, and the preparations were incubated as described above.

Fixation Methods for Transmission Microscopy and Freeze-fracture

For both thin-section and freeze-fracture studies, cells and schistosomula were fixed in suspension in an equal volume of Karnovsky's aldehyde fixative (17) for 15–30 min. Fixation was carried out at 4°C for thin-section and at 25°C for freeze-fracture studies. For thin-section studies, the fixed suspension was centrifuged in a Beckman Microfuge B (Beckman Instruments, Inc., Spinc. Div., Palo Alto, Calif.). The pellets were removed from the tubes, postfixed in acetate veronal-buffered 1% OsO₄ for 90 min at 4°C, and stained in block in 0.5% uranyl acetate for 2 h at 25°C (9). Dehydration and Epon embedding were routine. For freeze-fracture studies, the fixed suspension was centrifuged for 10 min in the microfuge. Each pellet consisted of 2,000 schistosomula and 2 × 10⁶ cells. The pellet was rinsed in 0.1 M cacodylate, pH 7.4, incubated overnight at 4°C in 25% glycerol in the same buffer, frozen in freon slush, and stored in liquid nitrogen. In some experiments, unglycerinated samples were frozen within 1 h of fixation after being rinsed in 0.1 M cacodylate, pH 7.4. Samples were fractured in a Balzers BA 360 M freeze-fracture device (Balzers Corp., Nashua, N. H.) at a stage temperature of −115°C. Carbon-platinum replicas were made of the fractured surface, and the underlying tissue was dissolved away with household bleach and 10% KOH for 3 h. The replicas were washed with water and picked up on naked copper grids.

Microscopy

Thick sections (0.3 µm) were cut with glass knives and stained with azure II-methylene blue. Thin sections, with silver interference colors, were cut with a

Table I

Number of Experiments

	Thin-section	Freeze-fracture
Schistosomula + NP + Ab	3	—
Schistosomula + NP + C	1	—
Schistosomula + NP + Ab + C	1 (3)* (4)‡	8 (3)§
Schistosomula + Eo + Ab	2	5
Schistosomula + Neut. + Ab + C	—	4‡

NP, nonpurified buffy coat cells; Ab, antischistosomal antibody preincubation; C, complement preincubation; Eo, purified eosinophils; Neut., purified neutrophils.

Each number represents a different day on which the experiment was performed. Two blocks were prepared for each thin-section experiment. An average of six pellets were prepared for each freeze-fracture experiment. An average of two to four replicas were obtained from them, but as many as six to nine replicas were examined in some experiments.

* Number in parentheses represents sucrose stress experiments, each of which was done at concentrations of 0.25, 0.37, and 0.5 M sucrose (2).

‡ These experiments also served as the controls for the mechanical stress experiments (2).

§ Number in parentheses represents experiments in which glycerol was omitted. These experiments were run with glycerinated controls which are not included in the number on the left.
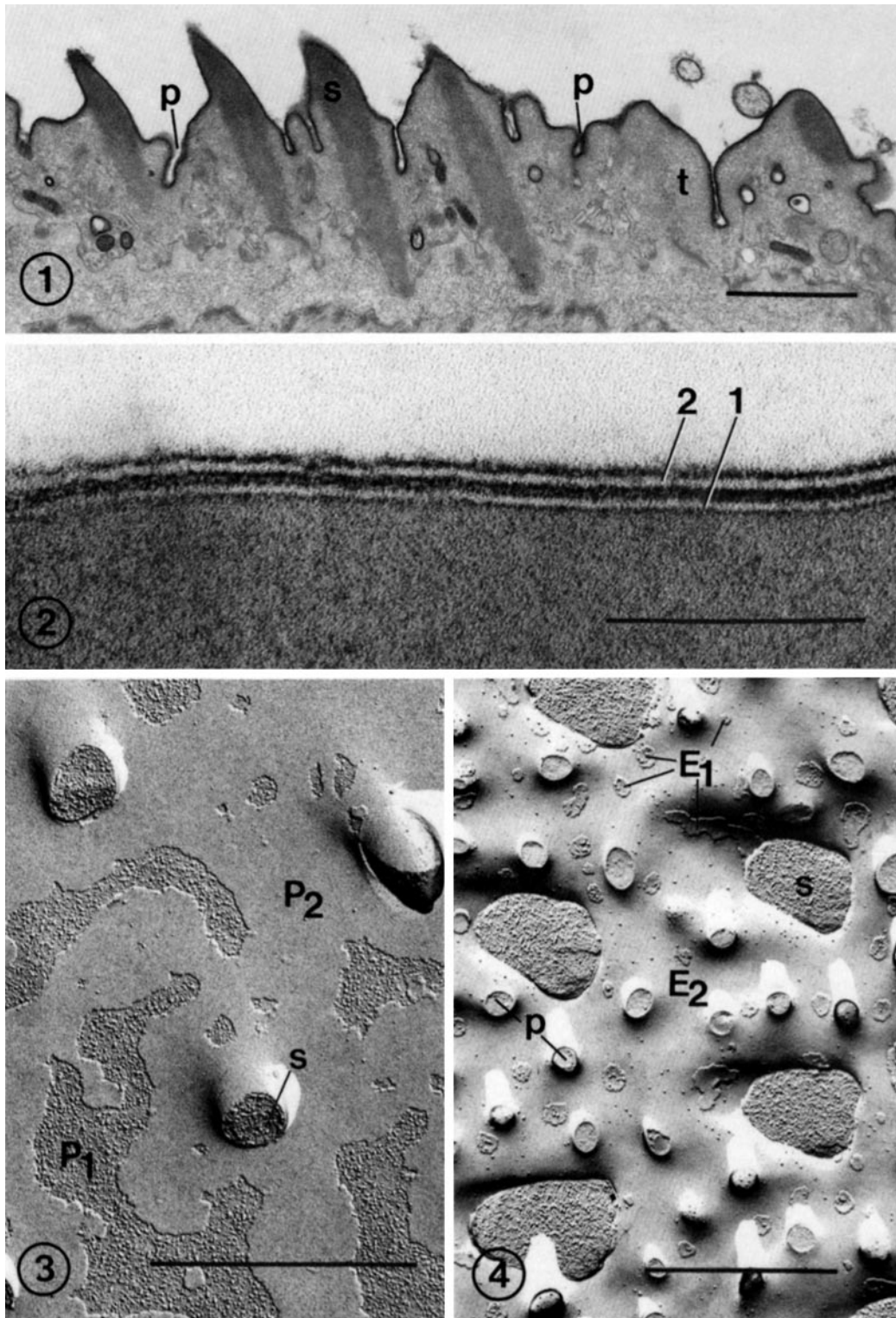
FIGURE 1   Low-power view of the tegument (*t*) of a schistosomulum. Note that the surface is covered by a continuous electron-dense line. *s*, spines. *p*, pits. Bar, 1.0 µm. × 19,500.

FIGURE 2   High-power view of tegumental membrane. The membrane is pentalaminar and composed of two closely apposed trilaminar membranes. The inner membrane is designated *1* and the outer *2*. Bar, 0.1 µm. × 400,000.

FIGURE 3   Freeze-fracture of tegumental membrane, P-face view, showing large areas of the IMP-rich inner membrane (P_1) and the IMP-poor outer membrane (P_2). The spines (*s*) are cross-fractured. Bar, 1 µm. × 41,500.

FIGURE 4   Freeze-fracture of tegumental membrane, E-face view, showing primarily the IMP-poor outer membrane (E_2), with small areas of the inner membrane (E_1). The spines (*s*) and pits (*p*) are cross-fractured. Bar, 1 µm. × 31,500.

diamond knife, picked up on formvar-carbon-coated copper grids, and stained on grid for 5 min in uranyl acetate and for 1 min in lead citrate (28). Grids with both thin sections and freeze-fracture replicas were examined in a JEOL 100C electron microscope equipped with a tilting stage at an accelerating voltage of 80 kV.

Scanning Electron Microscopy

Preparations were washed in buffer, fixed in Karnovsky's fixative (17) for 1 h at 4°C, postfixed in OsO_4 for 1 hr at 4°C, dehydrated in graded ethanols and acetone, critical point dried in liquid CO_2, sputter coated with gold-palladium, and examined in a JEOL 100C ASID.

RESULTS

Normal Schistosomulum Tegumental Membrane

The surface of schistosomula of *S. mansoni* has been described by others (14, 23) and will be briefly summarized here. The organism is ~100 μm long and 25 μm in diameter. Its surface is covered by large (1.0-μm-long) spines, and there are a few specialized areas, including the opening of the acetabular glands, located on the anterior tip, the ventral sucker, located in the midportion, and the tail socket, located at the posterior tip. The entire surface is covered by an anucleate syncytium, the tegument (Fig. 1), which is ~2 μm thick and is connected to cell bodies deep inside the organism. The tegumental cytoplasm contains a few mitochondria, membranous bodies, and crystalline material that makes up the spines (Fig. 1). Between the spines are small surface invaginations, the pits, that are ~25 nm in diameter and ~100 nm deep (Fig. 1). The tegumental membrane covers the entire surface of the schistosomulum. In thin section, the membrane is seen as a pentalaminar structure composed of two outer 2.5-nm electron-dense layers, a central 5.0-nm electron-dense layer, and two 2.5-3.5-nm light layers, which separate the central and outer layers (Fig. 2). Freeze-fracture techniques result in two fracture planes and four faces, indicating that the tegumental membrane is composed of two closely apposed unit membranes, each containing a lipid bilayer (15, 21, 34). The fracture plane passes preferentially through the outer, or 2, membrane and variably skips into the inner, or 1, membrane (Figs. 3 and 4). Usually, the inner membrane is seen in 50-nm patches (Fig. 4), but occasionally it occupies up to half of the fractured surface area (Fig. 3). The inner membrane P face (P_1) has a very high concentration of intramembrane particles (IMPs) (Fig. 3), and the inner membrane E face (E_2) has fewer IMPs than P_1 (Fig. 4). The outer membrane, both P (P_2) and E (E_2) faces, contains either no or very few IMPs (Figs. 3 and 4). In addition to revealing the two membrane faces, the fracture plane usually cross-fractures the spines and tends to cross the neck of the pits so that ice is seen in the center of the pits (Fig. 4).

Neutrophil Adherence to Schistosomula

Neutrophils that have been induced to adhere to schistosomula with schistosomular serum alone or together with complement appear qualitatively similar, although more cells adhere when complement is present. By examination with light or scanning electron microscopy, cells can be seen adhering to schistosomula singly or in groups (Fig. 5). There is a large variation in the number of cells adhering to schistosomula. In a typical experiment, ~10-15% of the schistosomula have no cells adhering to them, and 50% have more than 20 (2). By thin-section microscopy, the cells are seen to orient themselves with respect to the surface of the schistosomulum in such a way that the nucleus is farthest from the schistosomulum, the granules are between the nucleus and the schistosomulum, and a layer of disrupted filaments (20) is beneath the plasma membrane closest to the schistosomulum (Fig. 6). In groups of neutrophils or of neutrophils and eosinophils adhering to schistosomula, the plasma membranes of the cells become tightly apposed (Fig. 6) but their outer leaflets do not appear to fuse. Few neutrophils (<5%) are seen discharging their granules. Higher power examination shows that two types of interaction occur between the plasma membrane of the neutrophil and the
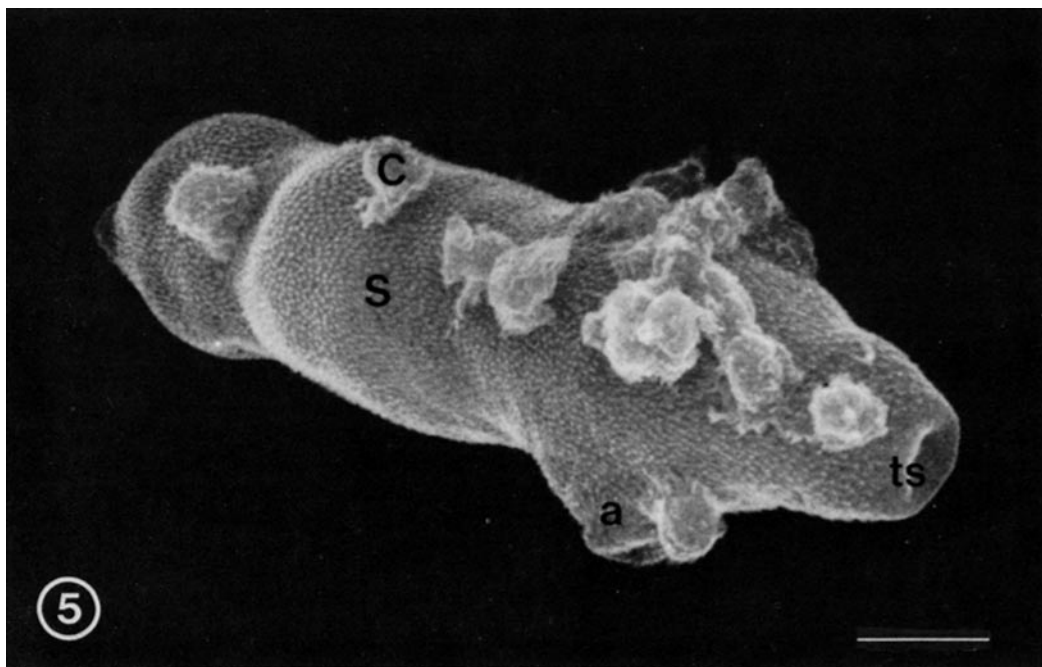

FIGURE 5   Scanning micrograph of buffy coat cells (C) adhering to a schistosomulum (S) that was preincubated in antibody and complement. Some cells are adhering singly and others in a group to various parts of the schistosomular surface, including the ventral sucker or acetabulum (a). ts, tail socket. Bar, 1 μm. × 1,750.
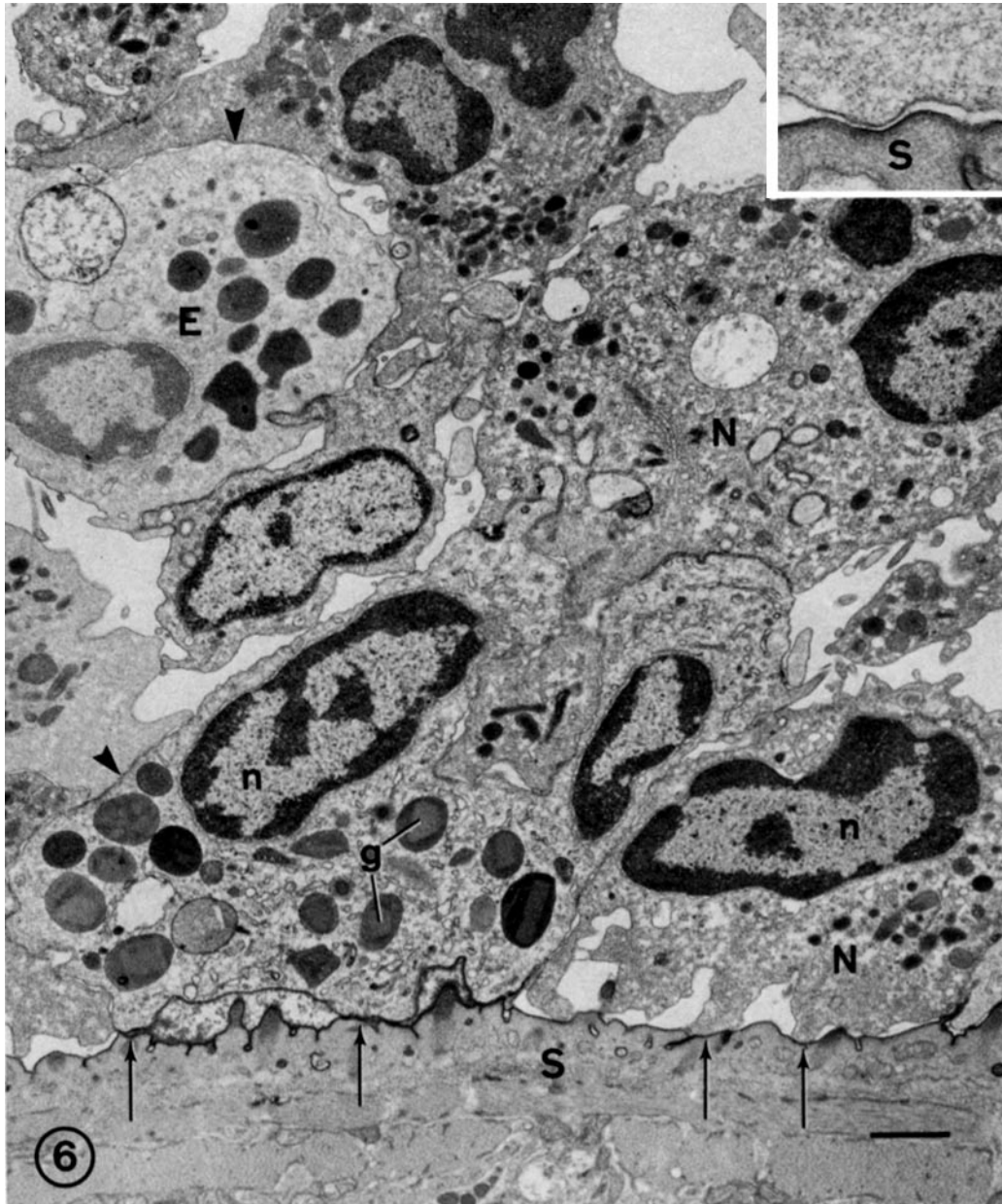
FIGURE 6 Low-power micrograph of buffy coat cells adhering to the surface of a schistosomulum (S) preincubated with antibody. Both eosinophils (E) and neutrophils (N) are adhering in a large group in which membranes of the cells are tightly apposed (arrowheads). The cells are oriented against the surface of the schistosomulum so that the granules (g) are between the nucleus (n) and the schistosomular surface. The cells are attached to the tegument (t) in only a few places (arrows). Electron-dense material is present between the eosinophil and the schistosomulum but not between the neutrophil and the schistosomulum. Inset shows disrupted filaments in the cytoplasm of the neutrophil adjacent to the schistosomulum (S), similar to those seen in OsO₄-fixed actin gels (20). Bar, 1 µm. X 10,400.

tegumental membrane of the schistosomulum. First, most cells are attached so that the two membranes contact each other only at a few points (Fig. 6). In properly oriented sections and in sections oriented by tilting with a eucentric goniometer, these contact points appear as heptalaminar structures consisting of a fusion[1] of the outer leaflets of the neutrophil and

tegumental membranes (Figs. 7 and 8). These fused leaflets are 2.5-3.5 nm thick and extend for 10 to 100 nm (Figs. 7 and 8). The second type of interaction is seen in cells that are flattened and tightly attached to schistosomula over a distance of 5-8

[1] The word fusion has at least three usages. First, it has been used to describe the fusion of the outer leaflets of trilaminar membranes that form the pentalaminar structures that occur in occluding junctions (8) or in the early stages of vesicle fusion (26) or secretion (25, 27). In this paper, analogous fusions are called "heptalaminar fusions" because the fusions occur between pentalaminar and trilaminar membranes. Second, fusion has been used to describe the formation of a hybrid

membrane from two trilaminar membranes resulting in a trilaminar structure (diaphragm) that is seen during secretion (25, 27) and vesicular transport (26). In this paper, such fusions are simply called "fusions" or "pentalaminar fusions." Third, fusion denotes the process by which two cells become one, i.e., viral- or polyethylene glycol-induced cell fusion. Fusion is never used in this sense in this paper because continuity or admixing of the neutrophil and schistosomular cytoplasm has not been seen.
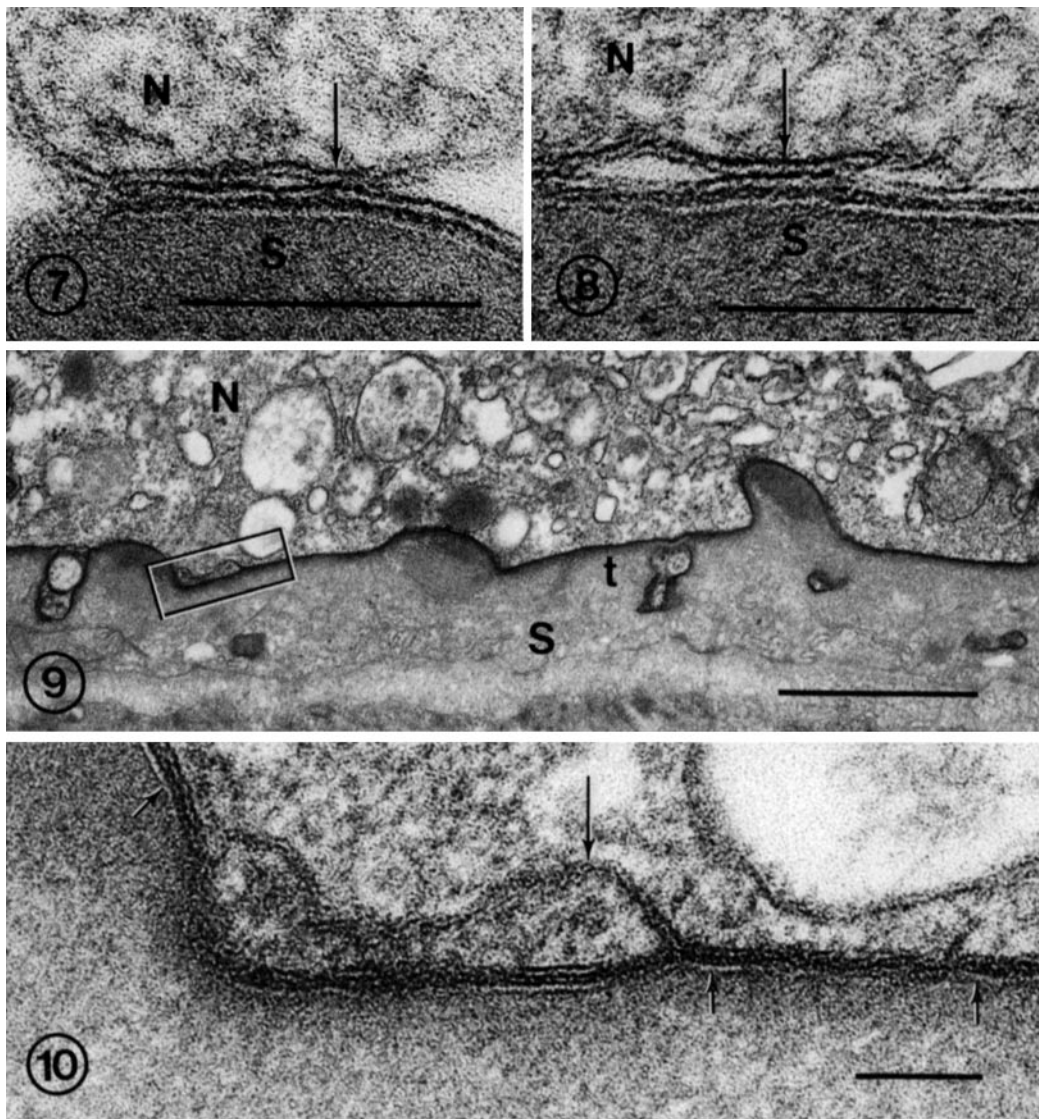
FIGURES 7 AND 8 High-power views of heptalaminar fusions (arrows) between neutrophils (N) and schistosomula (S) preincubated in antibody. Bars, 0.1 μm. (Fig. 7) × 400,000. (Fig. 8) × 330,000.

FIGURES 9 AND 10 Neutrophil (N) adhering tightly to the surface of a schistosomulum (S) preincubated in antibody and complement. Note that the cell is flattened and tightly adherent to the tegument (t). Fig. 10 is a high-power view of the area in rectangle in Fig. 9. Note that the membrane between the cell and the schistosomulum is pentalaminar (short arrows). Some membrane appears to be lifted off the surface (long arrow). (Fig. 9) Bar, 1 μm. × 26,500. (Fig. 10) Bar, 0.1 μm. × 171,600.

μm (Figs. 9 and 10). Pentalaminar fusions between the cell and schistosomular membranes extend the length of this attachment, i.e., 5–8 μm, except where the cell membrane crosses the pits and in those areas where the neutrophil membrane is unattached (Figs. 9 and 10).

By examination with freeze-fracture techniques, alterations are seen in the structure of the normal schistosomular membranes that correspond to areas where the cells are attached to the schistosomula. These alterations are not seen in schistosomula incubated with antibody and complement but without cells, in schistosomula that are fixed before the cells are added, or in schistosomula incubated with cells but without antibody or complement. In a typical replica, attachment areas are present on 30% of the schistosomula profiles. The attachment areas are large (5–8 μm in diameter) and can be divided into three zones—an edge and two centrally located areas, one of which is rich in IMPs and one of which is poor (see below and Fig.

11). In replicas in which the fracture plane cross-fractures the cell and passes onto the surface of the schistosomulum, the edge of the cell is continuous with the edge of the area of attachment on the schistosomulum, and the cell cytoplasm is adjoined to the centrally located areas (Figs. 12 and 13).

The edges of the attachment areas are continuous and relatively curvilinear, with occasional fingerlike projections jutting out onto the normal schistosomular membrane (Figs. 12–15). At higher power, the edge of the attachment area has three distinct appearances. First, in replicas in which the fracture plane passes from the normal schistosomular membrane into the area of attachment (Fig. 11 B), the plane almost invariably (98%) skips from the 2 membrane into the 1 membrane for a distance of 20–30 nm at the edge of the area of attachment (Figs. 13–18). This skip-fracture reveals a zone of the 1 membrane face, either a trench in the P face (Figs. 13 and 17) or a ridge in the E face (Figs. 14–16), around the area of attachment.
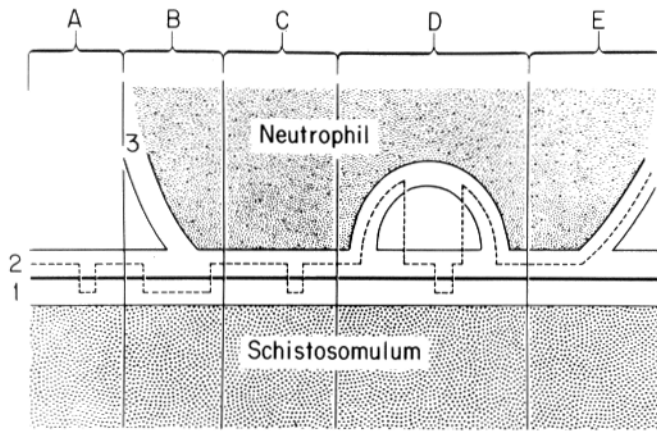
FIGURE 11 Schematic diagram of the fracture planes through the area of fusion between a neutrophil and a schistosomulum. The schistosomula inner membrane is designated 1, the outer membrane 2, and the neutrophil plasma membrane 3. (A) A normal schistosomular membrane, the fracture plane (dotted line), usually passes through the outer membrane and occasionally skips into the inner membrane. (B) At the edge of an attachment area, the fracture plane skips out of the outer membrane into the inner membrane over a broad area. (C) In a particle-poor area, the fracture plane passes through the fused membrane and occasionally skips into the inner membrane. (D) In a particle-rich area, the plane passes onto the raised particle-rich membrane, but jumps down into the two normal parasite membranes underneath. (E) The fracture plane passes out of the particle-poor fused membrane up onto the particle-rich unattached cell membrane.

The edges of the two membranes that abut the edge zone appear equidistant from the inner membrane (Figs. 13–16). The second appearance is present in only a few cases (2–5%), and is seen as a string of large IMPs rather than the zone just described (Figs. 15 and 16). The third appearance occurs when the fracture plane passes through the cell membrane onto the area of attachment (Fig. 11 E). The fracture plane does not leave the membrane, but there is a precipitous drop in the concentration of IMPs where the cell membrane attaches to the schistosomulum (Fig. 13).

The central portion of the attachment area, which is composed of IMP-rich and IMP-poor regions, is the same whether the fracture plane passes onto it from the cell or from the schistosomular membrane. The relative distribution of these two regions varies within different attachment areas in the same preparation. The central membrane can be entirely IMP poor (Fig. 13), mainly IMP poor (Fig. 15), or a reticulum composed equally of rich and poor regions (Figs. 12, 14, and 17). The IMP-rich regions appear raised in the P-face view (Figs. 12, 17, and 18) and depressed in the E-face view (Figs. 14 and 15). The concentration of IMPs in the IMP-rich areas is comparable to the concentration in neutrophil membranes not attached to schistosomula (Fig. 17). When the fracture plane skips out of the IMP-rich areas (Fig. 11 D), normal schistosomular P_1 and P_2 faces (Figs. 17 and 18) or E_1 and E_2 faces (Fig. 14) are seen.

The IMP-poor membrane is flat and conforms to the shape of the surface of the schistosomulum (Figs. 12–18). The IMP-poor membrane extends over the pits so that ice is no longer seen in their centers (Figs. 15 and 16). The concentration of IMPs is less than that seen on unattached neutrophil membranes and greater than that seen on the normal outer membranes (Figs. 12 and 17). When the fracture plane skips out of the IMP-poor membrane, only E_1 (Figs. 14 and 15) or P_1 (Fig.

13) faces are seen. These 1 membrane faces appear in the same spatial relationship to the IMP-poor membrane that they do to the normal 2 membrane.

To test the effect of glycerination on the freeze-fracture appearance described above, preparations were fixed and frozen without cryoprotection. In areas of schistosomula where cells are not attached, the fracture faces of the schistosomular outer membrane are smooth and similar in appearance to the faces seen in cryoprotected preparations (Figs. 19 and 20). Neutrophil plasma membranes, on the other hand, are markedly different because the fracture face is no longer smooth (Fig. 19). At the edge of areas of attachment, the zone of fracture into the inner membrane is still seen, as are IMP-rich and IMP-poor areas (Figs. 19 and 20). However, in the IMP-poor areas, the fracture is not smooth but, instead, is intermediate between that of the schistosomulum and that of the cell (Fig. 19). In addition, the fracture plane passes into the inner membrane faces under the IMP-poor areas more frequently than in the glycerinated tissue. In the IMP-rich areas, the number of IMPs is lower than in the glycerinated samples, and the borders of these areas are less well defined (Fig. 20).

Eosinophils

Eosinophils orient against the surface of the schistosomulum in a fashion similar to neutrophils (Fig. 6). However, unlike neutrophils, many eosinophils discharge their granules against the schistosomular surface (Fig. 21). This occurs by multiple fusions of granular membranes with the plasma membrane in one or two areas so that the membranes of the discharged granules form large invaginations within the cytoplasm (Fig. 21). The discharged granule contents adhere to the schistosomular surface (Fig. 21). High power examination of most eosinophils adhering to schistosomula shows that the cells are adhering to this layer of discharged granular material on the schistosomula (Fig. 21, inset). Occasionally, heptalaminar fusions similar to those seen in neutrophil adherence are present, but the pentalaminar fusions are very rarely observed. By freeze-fracture techniques, the eosinophil plasma membrane can be seen closely apposed to the surface of the schistosomulum, but the attachment areas described above for the neutrophil are not seen (Fig. 22).

DISCUSSION

We have examined the adherence of neutrophils and eosinophils to the surface membranes of schistosomula. The neutrophil plasma membrane fuses with the outer tegumental membrane of schistosomula preincubated with antischistosomular serum and complement. The evidence for this fusion comes from both freeze-fracture and thin-section images. In thin section, pentalaminar fusions are seen between the trilaminar neutrophil plasma membrane and the normally pentalaminar schistosomular membrane. This finding indicates that there has been a fusion resulting in the elimination of one bilayer. Freeze-fracture images show that the neutrophil is attached to the schistosomulum in areas where there are only two membrane faces instead of the normal three, i.e., the cell, and the schistosomular inner and outer membrane. These two faces in the attachment area are composed of the schistosomular inner membrane and a fused membrane that shares the characteristics of both the neutrophil plasma membrane and the parasite's outer membrane. The fused membrane is continuous with the neutrophil plasma membrane, and it holds the same spatial
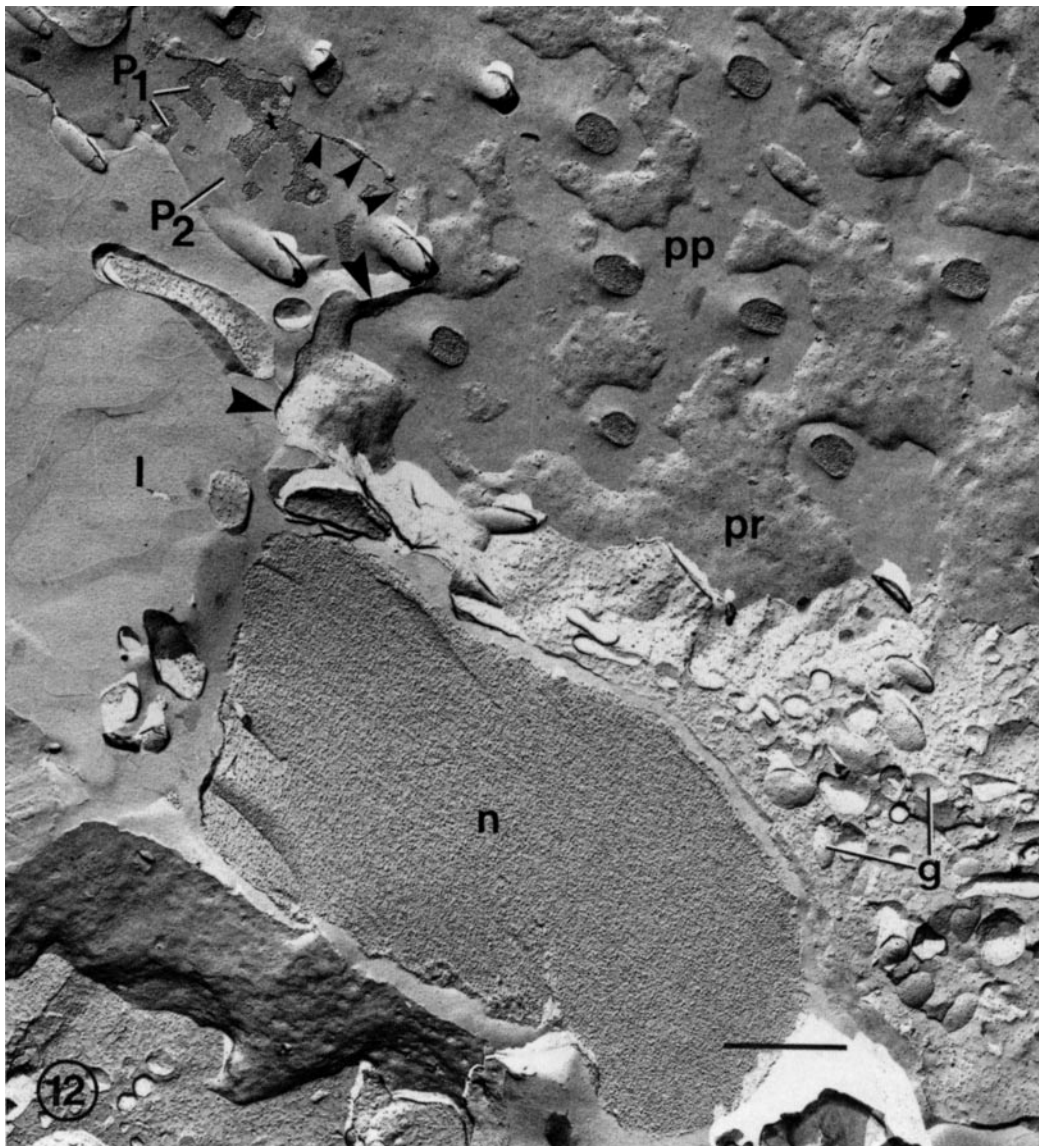
FIGURE 12 Low-power, freeze-fracture micrograph of a neutrophil adhering to a schistosomulum preincubated with antibody and complement. Normal P_1 and P_2 faces of the schistosomular membrane are seen at the upper left. On the right side of the picture, the schistosomular membrane is modified so that there are raised areas with IMPs (pr) and flat areas with very few IMPs (pp). The particle-rich areas occupy a larger portion of the right side of the modified membrane, where they form a reticulum. At the bottom of the picture there is a cross-fracture through the cytoplasm of a cell with its nucleus (n) and granules (g), which is adjoined to the modified surface membrane of the schistosomulum. The edge of the cell (large arrowhead) is continuous with the edge of the modified surface membrane (small arrowheads). I, ice. Bar, 1 µm. × 16,500.

relationship to the schistosomular inner membrane as does the normal outer membrane. The hybrid nature of this membrane is further indicated by the fracture face of the fused membrane, which is intermediate between the very smooth face of the schistosomular outer membrane and the rough face of the cell membrane in specimens that were not glycerinated.

We can only speculate on the sequential development of these fusion areas in that we have examined samples at a time in which cells are in various stages of the fusion process and are, therefore, unsynchronized. It appears that the neutrophils and schistosomula are brought very close together by the interaction of the Fc and C3 receptors on the cell membrane with the IgG and C3 bound to the surface of the parasite. The second event is the formation of the heptalaminar structures that appear as fusions of the outer leaflets of the cell and schistosomular membranes. It is suggested that they precede

pentalaminar fusion of the membranes because they are much smaller than the final fusions, <0.5 µm vs. 5–8 µm. The heptalaminar structures also appear to be weaker than the pentalaminar fusions because the heptalaminar fusions are not seen in samples hypertonically stressed with sucrose, but pentalaminar fusions are (2). In addition, it has been observed that fusion of the outer leaflets precedes fusion of the membranes in other systems, e.g., vesicle fusion in capillary endothelia (26), secretion during encystment of *Phytophthora palmivora* (27), mast cell secretion (19), and acrosomal vesicle fusion of *Limulus* sperm (33). The schistosomular membrane then presumbly induces complete fusion with the neutrophil membrane. This is suggested because the outer membrane is virtually devoid of IMPs, as are other membrane loci at the point where fusion will occur (5, 10, 16, 19, 24, 27, 33; see reference 33 for summary and discussion). That the schistosomulum is providing the
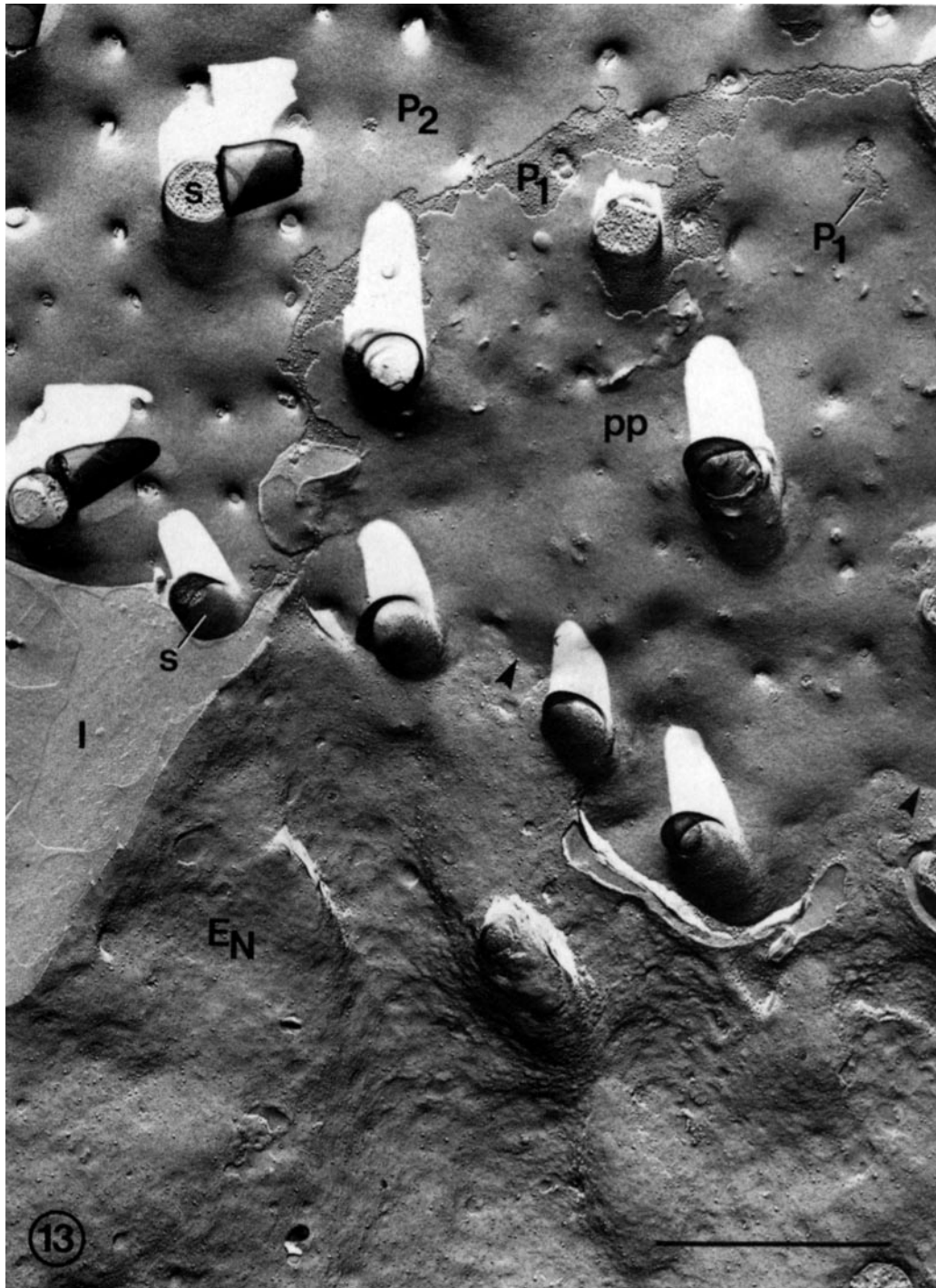
FIGURE 13 Neutrophil adhering to a schistosomulum preincubated in antibody and complement. Unattached neutrophil membrane, E-face view (E_N) is seen at the bottom of the picture. At the upper left, a normal schistosomular P_2 face that is practically devoid of IMPs is seen. In the middle right-hand part of the picture, an IMP-poor area (pp) that is separated from the normal P_2 face by a zone of P_1 is seen. Note the area of P_1 within the IMP-poor membrane. Note also that the IMP-poor membrane is continuous with the unattached neutrophil membrane, and that there is an abrupt transition in the concentration of the IMPs where the membrane leaves the schistosomular surface (arrowheads). Note the groove or depression 4 mm to the left of the arrowhead on the right. I, ice. s, spines. Bar, 1 μm. X 34,000.

fusigen(s) is also supported by the fact that schistosomula can form pentalaminar structures with erythrocytes (unpublished observation), whereas neutrophils, to our knowledge, have not been reported to fuse with other cells or organisms. Finally, phospholipids (phosphatidyl inositol and phosphatidyl serine) have been extracted from schistosomula and have been used to induce "agglutination" in erythrocytes (3). Although the location of these lipids in the schistosomulum is unknown, it is reasonable to postulate that some are in the IMP-poor outer membrane, which appears to be composed largely of lipids or is at least similar in appearance to other protein-poor lipid bilayers (1).
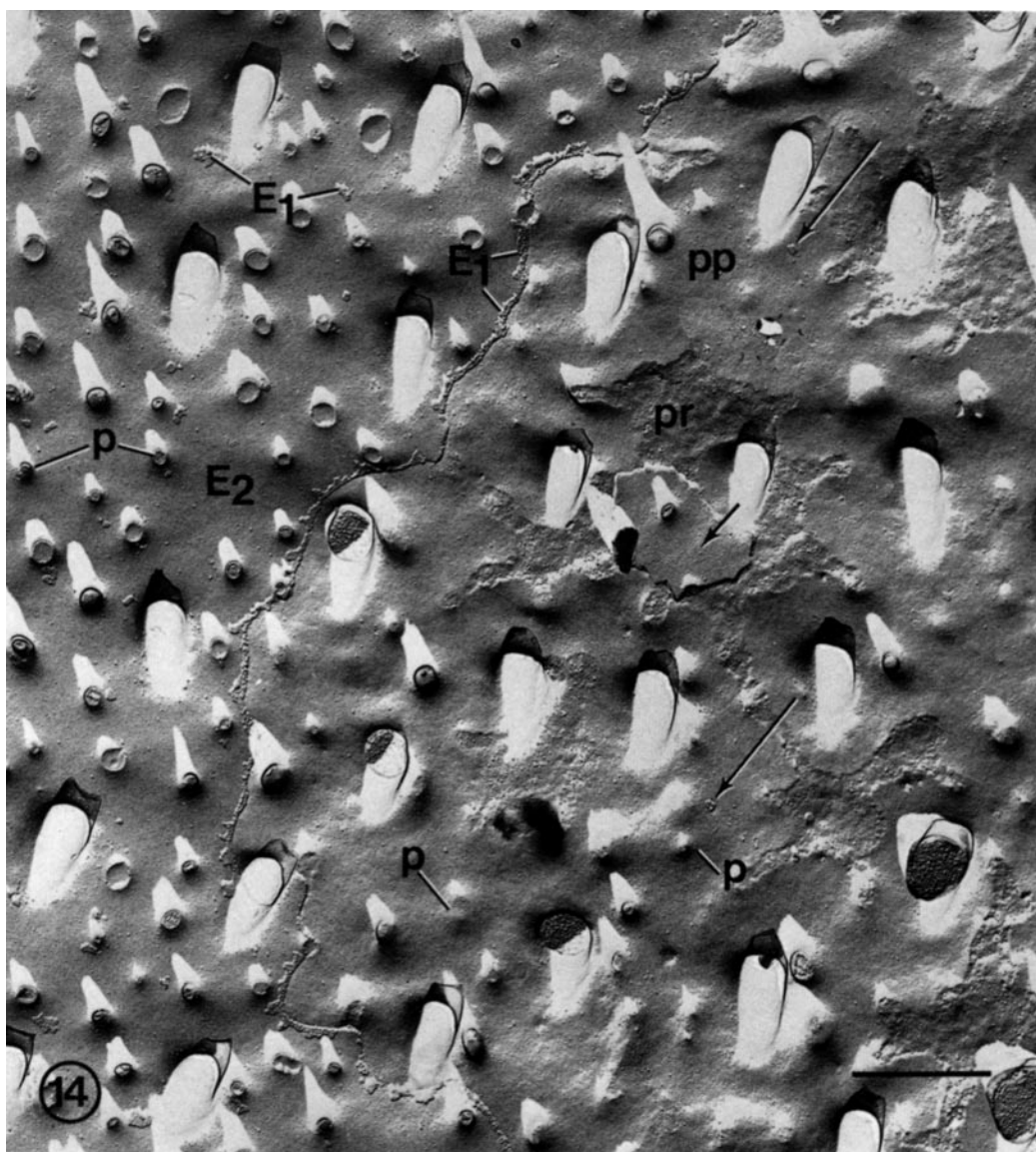
FIGURE 14 Low-power, freeze-fracture micrograph showing an area of attachment of a neutrophil to a schistosomulum preincubated in antibody and complement. On the left, a normal E-face view of the schistosomular membrane is seen with E_2 and a few E_1 faces and numerous cross-fractured pits (p). On the right is a modified membrane that can be divided into flat, particle-poor (pp) and sunken, particle-rich (pr) areas. E_1 faces can be seen on the particle-poor areas (long arrows). An E_2 face can be seen overlying the particle-rich areas (short arrow). Note that the normal and modified membranes are separated by a curvilinear border formed by a ridge of the E_1 membrane, and that the pits under the modified membrane are covered by the particle-poor membrane. Bar, 1 μm. × 21,000.

The net result of the fusion process is the formation of the pentalaminar fusions seen in thin section and the attachment areas seen in freeze-fracture. The attachment area is demarcated from the normal schistosomular outer membrane by a zone in which the fracture plane has skipped from the outer into the inner membrane or by a string of IMPs. The string of IMPs, which can be seen more clearly if the cells are removed, as well as the segregation of the fused membrane from both the schistosomular and the neutrophil unfused membranes suggest that the edge zone is similar to an occluding junction (see reference 2 for discussion). However, the fact that the step-fractures into the inner membrane are larger than the IMPs suggests that there is a component of the edge zone in addition to the IMPs, possibly turbulence in the lipid bilayers (27). A second possible function for the edge zone IMPs may be that they are a specific set of proteins that aid in the induction of

fusion, similar to IMPs in other systems (29, 33, 36). The division of the central portion of the attachment areas into fused and unfused zones is very similar to the fused membrane seen during encystment of *Phytophthora palmivora* (27). It is not clear whether the porportion of fused to unfused membrane is caused by multiple fusion sites (27), or whether it represents different stages in the evolution of fusion, perhaps necessitated by the constraints of packing two membranes into one. The fused or IMP-poor membrane generally contains more IMPs than are seen on fused membranes in other systems (27, 29) and is intermediate between the high IMP concentration of the neutrophil membrane and the low IMP concentration of the schistosomular outer membrane. We favor the interpretation that these IMPs are originally derived from the neutrophil, but we have no proof.

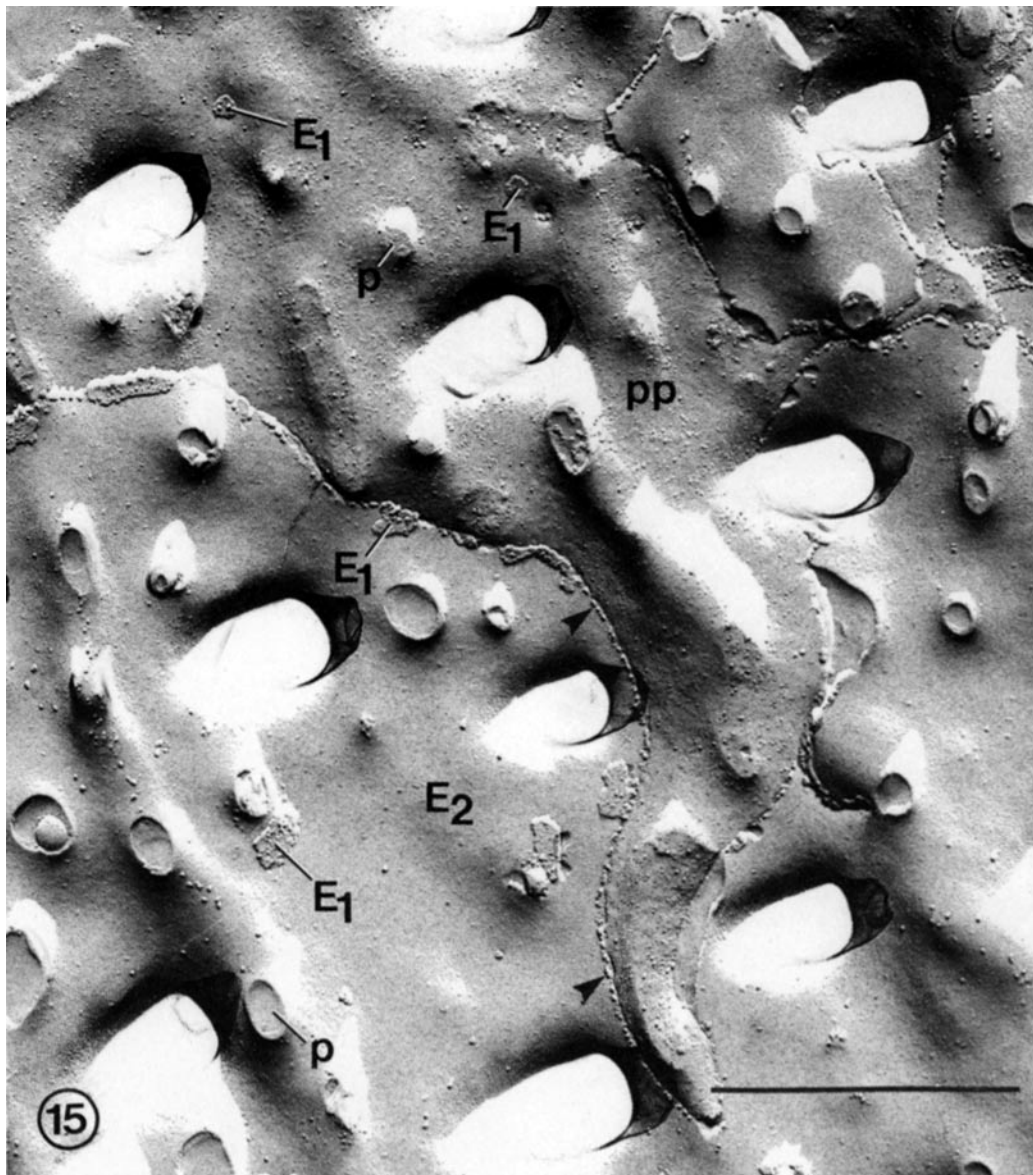There is a great deal of concern as to whether the morphology

FIGURE 15 Neutrophil adhering to a schistosomulum preincubated in antibody and complement. Normal schistosomular E_1 and E_2 faces along with cross-fractured pits (p) are seen at the bottom of the picture. An attachment area is seen at the top. An IMP-poor (pp) membrane has a few E_1 fragments on it, and the membrane covers the pits (p). The attachment area forms a finger projecting into the normal membrane. The edge of the attachment area is composed of both step-fractures into the E_1 face and strings of IMPs (arrowheads). Bar, 1 μm. × 40,500.

of fusion represents an artifact of preparation (4, 15, 18, 19). We accept this possibility. The major culprit thus far implicated in the induction of artifacts has been glycerination (4, 18). We have controlled for this by using long-term glycerination, which prevents glycerol-induced artifacts in myelin (18) and by fracturing unglycerinated preparations. The agreement between our freeze-fracture images obtained with and without glycerination and our thin-section images suggests that our observations are not artifacts. The only elements common to all our preparations are formaldehyde, glutaraldehyde, and cacodylate. Perhaps these substances do something to change the morphology (13). However, although artifacts may explain some of the findings in fused membranes, the differences in the findings in various systems may be a reflection of basic differences in the biology of those systems. The most likely parameters to produce such differences are the geometry of the fusion event, the kinetics of the fusion event, the area over

which fusion occurs, and the stability of the fused membrane. The last is an important point because in this system the fused membrane appears to be relatively stable (2), whereas, during secretion, membrane diaphragms have an extremely short life-span (<1 s) (4).

The contrast between the adherence of eosinophils to schistosomula and that of neutrophils to schistosomula is striking. Eosinophils form heptalaminar fusions occasionally and pentalaminar fusions rarely. However, eosinophils do discharge their granule contents onto the surfaces of the schistosomula (11, 22) and appear to adhere to the parasite through this layer of discharged material. This is shown in experiments in which the eosinophils are torn from the parasite's surface but their plasma membranes are left attached to the electron-dense material (see Fig. 7 of reference 2).

The fusions between the neutrophil and schistosomular membranes may help the parasite avoid the immune response
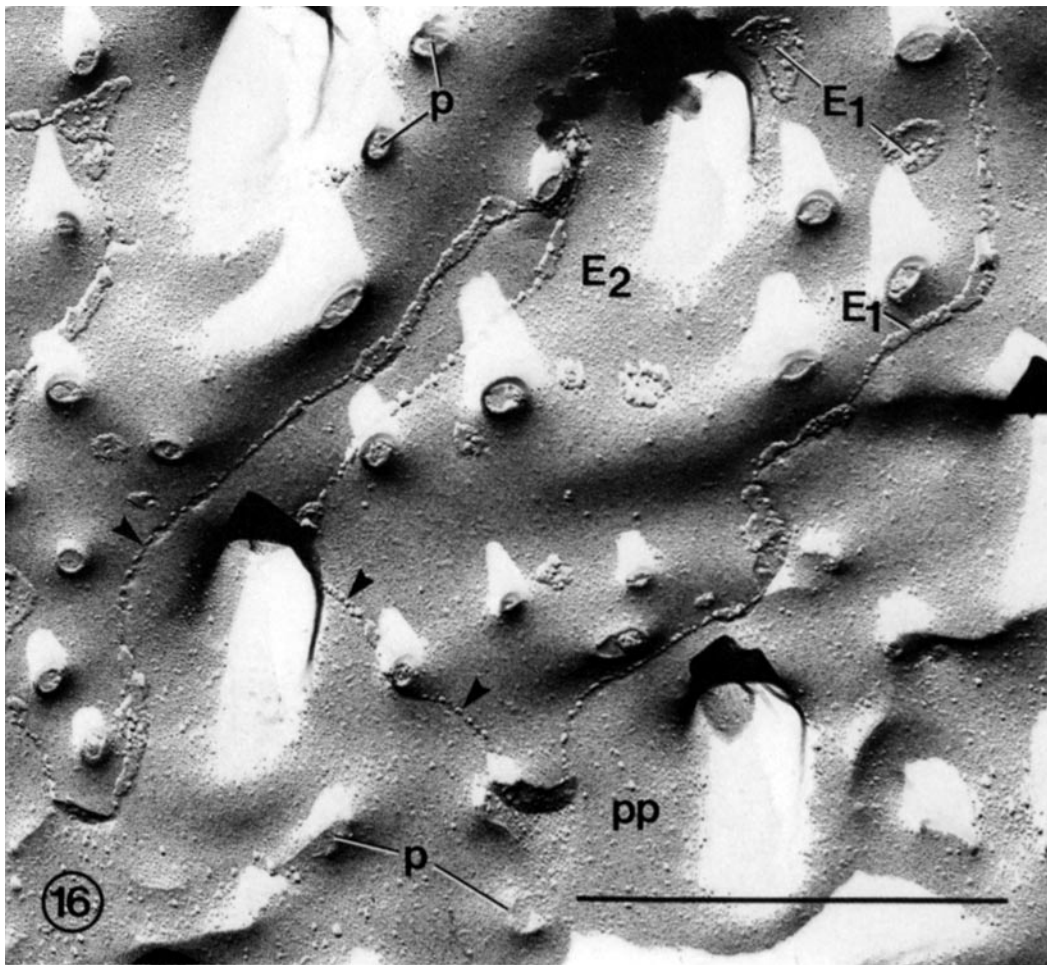
FIGURE 16 Neutrophil adhering to a schistosomulum preincubated with antibody and complement. Normal schistosomular E_1 and E_2 faces and cross-fractured pits (p) are seen at the top. An attachment area composed of IMP-poor membrane (pp) is seen covering the pits at the bottom. The two areas are separated by a border composed of strings of IMPs (arrowheads) and the E_1 membrane face. Bar, 1 μm. $\times$ 57,000.
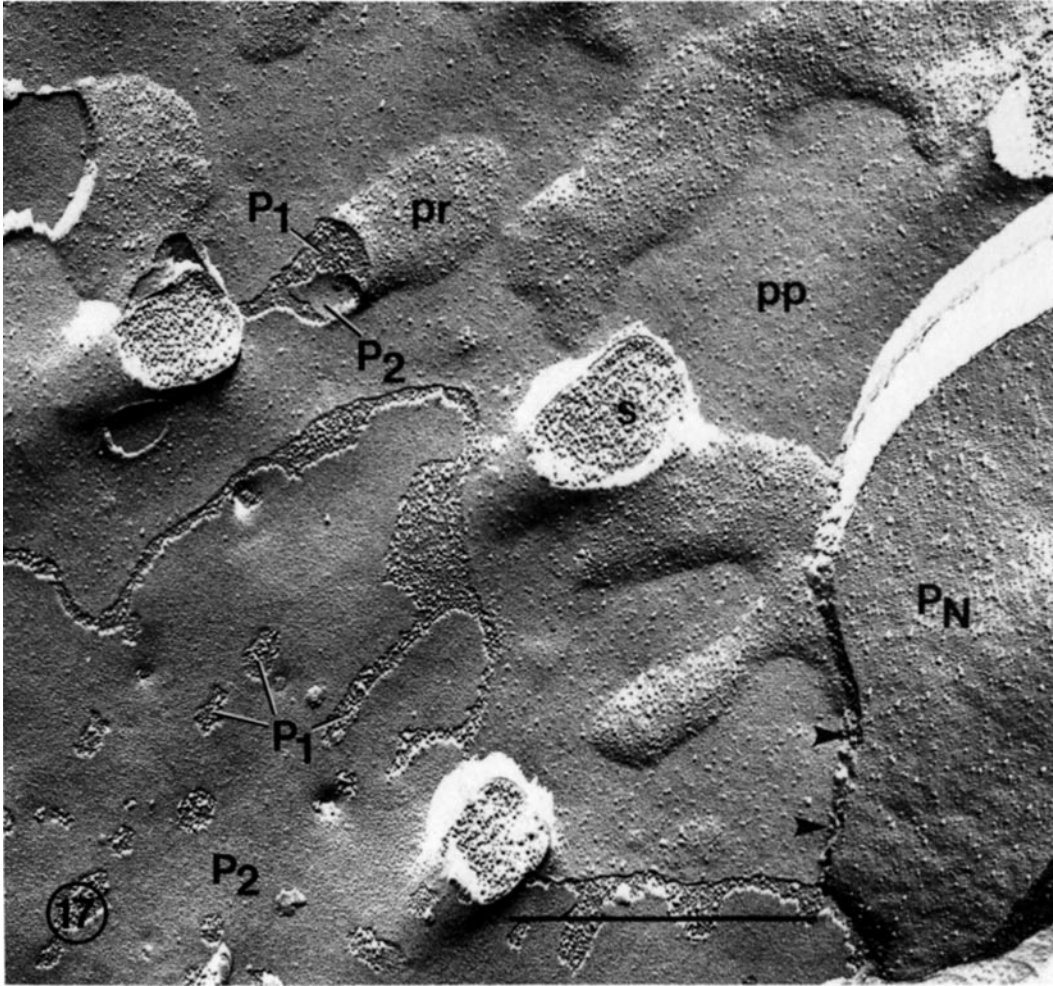
FIGURE 17 High-power view of neutrophil adhering to a schistosomulum preincubated with antibody and complement. Normal schistosomular P_1 and P_2 faces are seen at the bottom of the picture. An area of attachment with raised IMP-rich (pr) and IMP-poor (pp) areas is seen at the top and is separated from the normal membrane by a zone of P_1. The IMP-poor membrane and the P_2 face are the same distance from the P_1 membrane. In the IMP-rich area where the fracture steps out of the membrane, normal P_2 and P_1 membrane faces are seen. Note that the concentration of IMPs in the rich area is approximately equal to that of the P face of the neutrophil (P_N) on the right. The P_N face is on the side of the cell not attached to the schistosomulum and is separated from the fused membrane by the cytoplasm (arrowheads). s, spine. Bar, 1 μm. X 41,000.
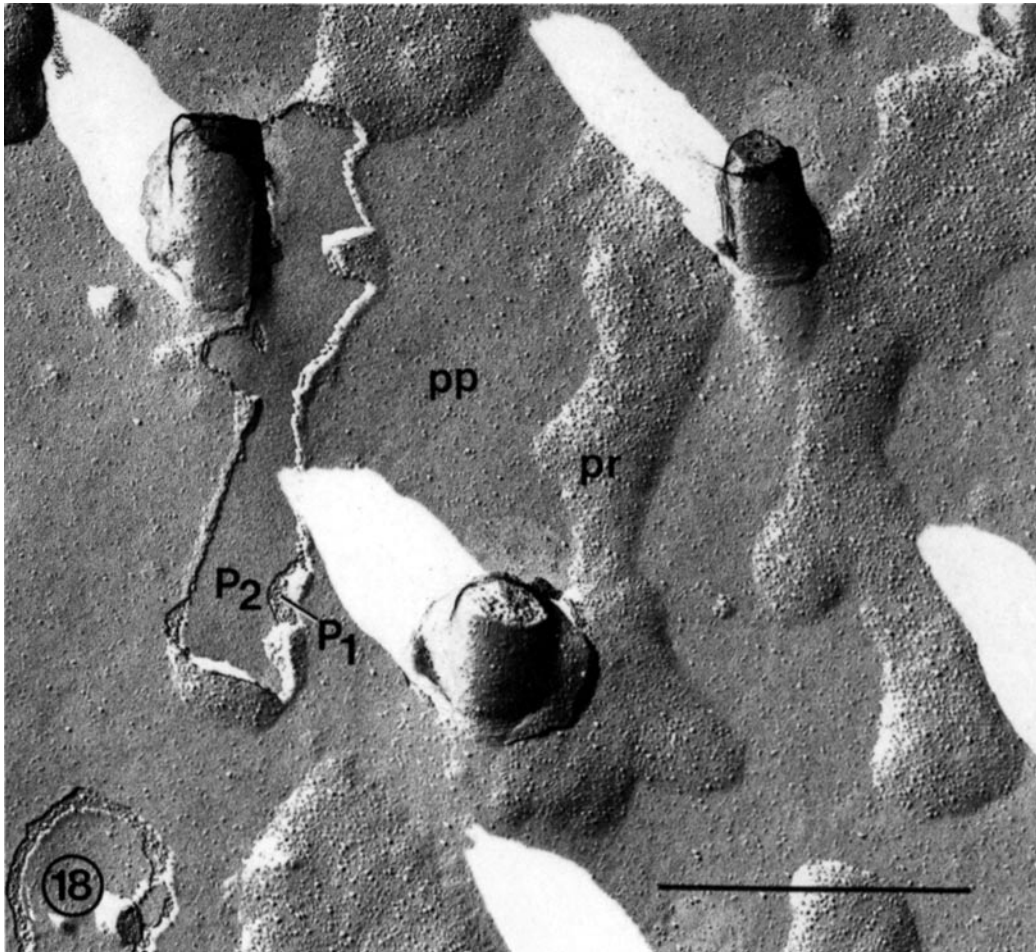
FIGURE 18   A central portion of an area of attachment of a neutrophil to a schistosomulum preincubated in antibody and complement. Note the IMP-rich (*pr*) and IMP-poor (*pp*) areas. The IMP-rich area has normal P_1 and P_2 faces underneath. Bar, 1 μm. × 42,000.
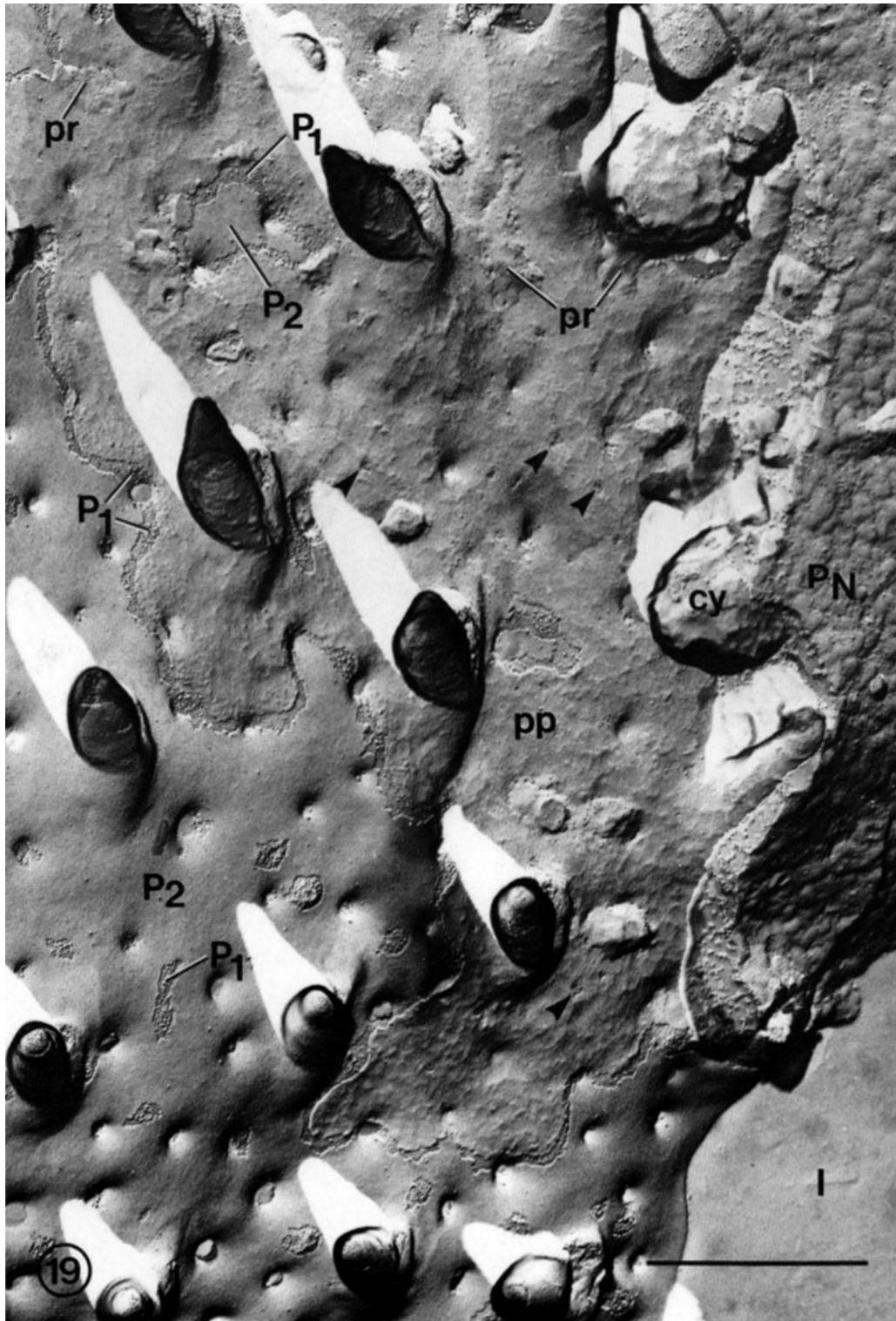
FIGURE 19 Neutrophil adhering to a schistosomulum preincubated in antibody and complement from a preparation from which glycerol was omitted. On the left, there are normal schistosomular P_1 and P_2 faces that are similar to those seen in glycerinated samples. On the right, a cell is attached to the schistosomulum. The neutrophil P face (P_N) is cobblestoned and is separated from the schistosomular membrane by cytoplasm (cy). The attachment area is located in the middle of the picture and is separated from the normal membrane by a trench of the P_1 membrane. The attachment area is mainly IMP-poor (pp) with a few areas of IMP-rich (pr). The IMP-rich areas have normal P_1 and P_2 faces underneath, and the IMP-poor area has small skip-fractures into the P_1 membrane (arrowheads). The roughness of the IMP-poor membrane is intermediate between that of the cell P_N face and the schistosomular P_2 face. I, ice. Bar, 1 μm. × 30,500.
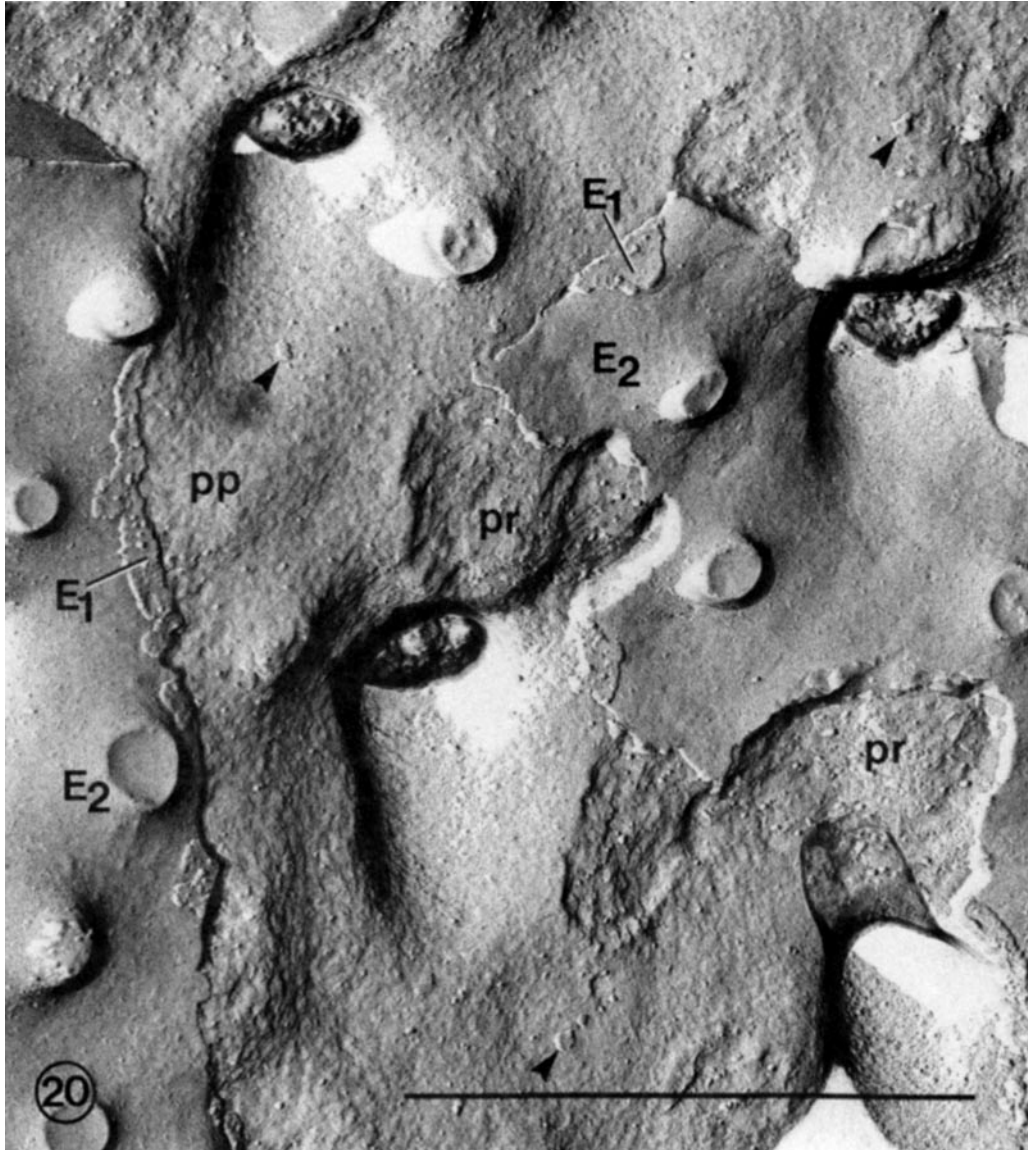
FIGURE 20 Neutrophil adhering to a schistosomulum preincubated with antibody and complement from a preparation from which glycerol was omitted. The normal schistosomular E₂ face on the left is separated from an attachment area by a ridge of E₁ face. The attachment area contains both IMP-rich (pr) and IMP-poor (pp) faces. Where the fracture plane has skipped out of the IMP-rich membrane, normal E₁ and E₂ faces are seen. Where it has left the IMP-poor membrane, fragments of E₁ are seen (arrowheads). Bar, 1 μm. × 74,400.
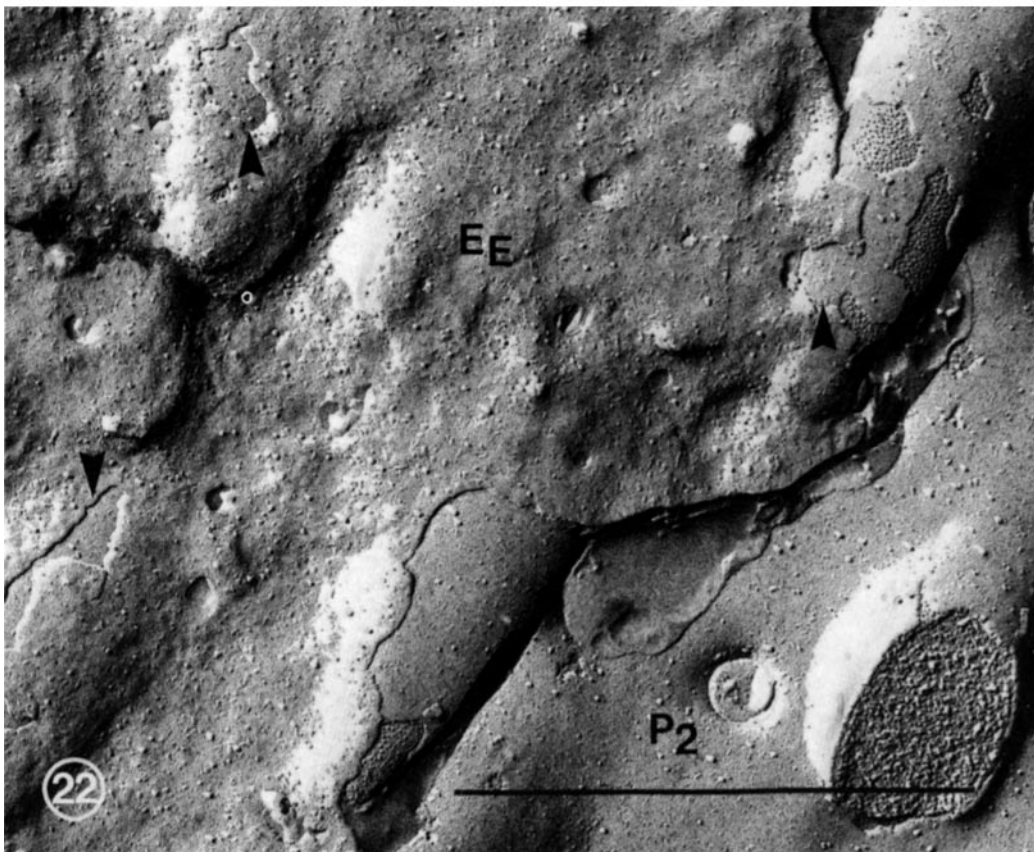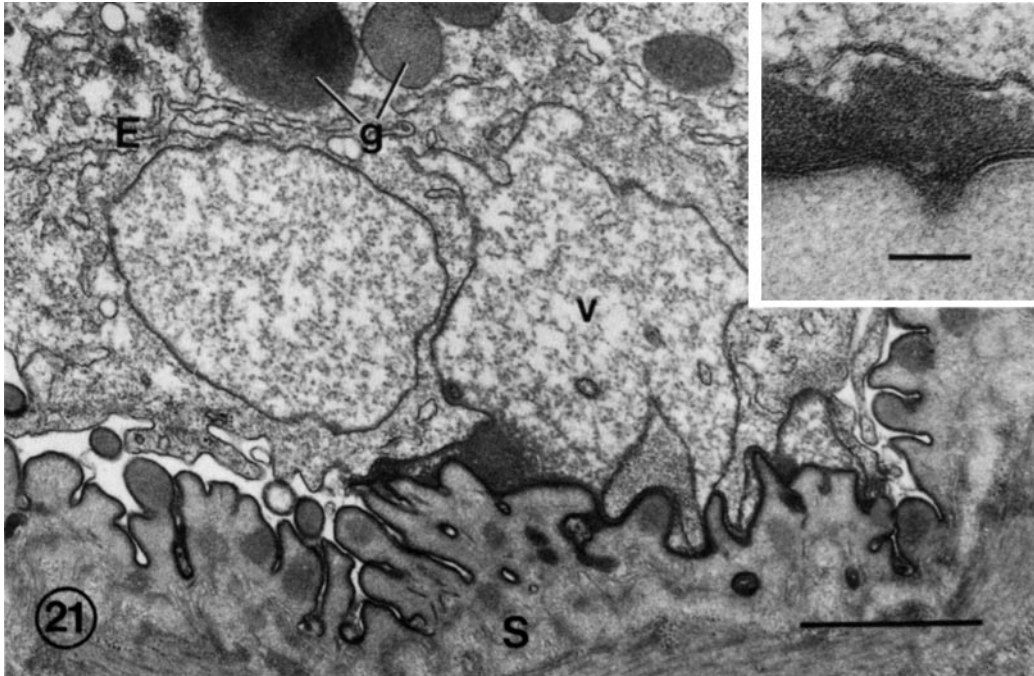
FIGURE 21 Eosinophil (*E*) discharging granule (*g*) contents onto the surface of a schistosomulum (*S*) preincubated with antibody. Note the large invagination (*v*) into the eosinophil cytoplasm. These invaginations are formed by multiple granules discharging at the same site. Electron-dense material adheres to the surface of the schistosomulum and has penetrated into the pits. The *inset* shows an eosinophil adhering to the electron-dense material on the parasite surface. Bar, 1 μm. × 24,000. (*inset*) Bar, 0.1 μm. × 110,500.

FIGURE 22 Freeze-fracture micrograph of an eosinophil adhering to a schistosomulum preincubated in antibody. Note that the eosinophil E face (*E*ᴇ) is closely apposed to the schistosomular membrane (arrowheads). The eosinophil membrane does not form an attachment area. Bar, 1 μm. × 68,600.

in two ways. First, the fusion may prevent exocytosis of the neutrophil granule contents by preventing fusion of the perigranular membrane with the plasma membrane. Consequently, the parasite surface would not be exposed to the toxic lysosomal constituents. Second, the fusion process may be the mechanism by which the parasite acquires host antigens (12, 30, 31). These antigens may block recognition of the parasite as a foreign entity by the immune system (see reference 2 for discussion).

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