Rapid bulk replacement of acceptor membrane by donor membrane during phagosome to phagoacidosome transformation in *Paramecium*

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SUMMARY

The extent to which a donor membrane will be retrieved, or if it is retrieved at all after it fuses with an acceptor membrane, is usually difficult to determine. We have studied the dynamics of membrane retrieval in the phagosome system of *Paramecium multimicronucleatum* using six monoclonal antibody markers. Our previous freeze-fracture and transmission electron microscopic studies have indicated that extensive changes take place in the membrane of the young phagosome as it progresses through its cycle. Using immunofluorescence and immunoelectron microscopy to determine the times of entry and exit of these individual antigens into the digestive vacuole system, we showed that two hydrophilic antigens, one located on the cytosolic and one on the lumenal side of the discoidal membrane (phagosome membrane precursor), were completely retrieved from the phagosome by tubulation within the first three minutes. At the same time that this membrane was retrieved, membrane from a second population of vesicles, the acidosomes, fused with the phagosome to produce the phagoacidosome. On the basis of immunogold localization on cryosections of a total of six antigens, the two specific for phagosome/discoidal vesicle membrane as well as four specific for the acidosome/phagoacidosome membrane, this replacement is total. We also showed that in the presence of the actin-active drug cytochalasin B, this replacement was essentially prevented. However, when vacuole acidification was neutralized by ammonium chloride, this replacement process continued unaffected after a lag. Consequently, acidification, per se, is not required to trigger the replacement of the phagosome membrane. We conclude, on the basis of these studies as well as our previous freeze-fracture studies that during phagoacidosome formation most of the acceptor membrane is retrieved and is replaced by the donor membrane. This shows that at least one cell type possesses the mechanisms needed to substantially replace the membrane of a phagosomal compartment when radical and rapid changes are needed to modulate the digestive and absorptive processes.

Key words: acidosome, cytochalasin B, membrane replacement, membrane tubulation, microinjection, monoclonal antibody, *Paramecium*

INTRODUCTION

That changes in molecular composition of an existing membrane can occur in cells is well established. This modulation can assume the form of co-translational or post-translational insertion of individual membrane proteins into a membrane. Alternatively, it can be achieved by the insertion of small vesicles into the membrane of membrane-bound compartments, such as the transition vesicles fusing with the cis-Golgi network, shuttle vesicles with Golgi cisternae or carrier vesicles with endosomal compartments. In many cases, the membrane of the existing compartment may not be permanently or substantially changed as the entering donor membrane is quickly retrieved in bulk from the acceptor membrane. This presumably occurs when synaptic vesicle membrane is retrieved at synapses (Zimmermann et al., 1989; von Gersdorff and Matthews, 1994) and when secretory granule membrane is retrieved and recycled following exocytosis (e.g. trichocysts in *Paramecium*, Hausmann and Allen, 1976; Allen and Fok, 1980).

One parameter that remains poorly understood in all cases of membrane fusion and retrieval is the extent of the retrieval of the donor membrane. Conversely, are there any situations where the acceptor membrane is retrieved leaving the donor membrane in place? If the latter situation occurs, it would result in the replacement of the membrane of the original acceptor compartment by the membrane of the incoming donor compartment. We are aware of only one example where this type of radical bulk membrane replacement has been suggested to occur, and this is based on our own work on the phagosomal cycle of *Paramecium*. Our previous freeze-fracture as well as thin-section electron microscopic data showed that the young digestive vacuole (DV-I) membrane is dramatically and rapidly altered within the first 3 minutes of its existence (Allen and Staehelin, 1981; Allen and Fok, 1983a,b, 1984). Initially, the E-fracture face of the DV-I membrane contains over 4000 intramembrane...
particles per µm², but within 3 minutes this same face loses almost all of these particles so that the E-fracture face of the DV-II is almost smooth, now bearing about 60 particles per µm². Secondly, a population of prominent intramembrane particles first appears on the P-fracture face during this transformation. Finally, the glycoprotein lining on the luminal side of the membrane also changes from a uniform 10 nm thick coat to a bumpy 7-10 nm thick coat. Thus, in a period of 3 minutes or less the DV-I membrane undergoes a radical transformation to become a DV-II (phagooacidosome) membrane. However, the above morphological studies could not tell us: (1) whether specific membrane components leave the acceptor membrane as a group; or (2) whether these components are sorted from one another with some components staying in the acceptor membrane while others exit the membrane.

To help provide an answer to this question, we have raised six monoclonal antibodies (mAbs), two to the membrane of DV-I and four to the acidosome and DV-II membranes. Using these mAbs at the light and electron microscopic levels, we followed the appearance and disappearance of these DV-I and DV-II antigens. We showed that, in less than 3 minutes of the release of a phagosome from the cytopharynx, the two antigens of the DV-I membrane were completely replaced by those of the acidosome membrane. This replacement was accomplished by the tabulation and fission of DV-I (acceptor) membrane concomitant with the fusion of the acidosome (donor) membrane. (Acidosomes are organelles that bind to the growing nascent DV and are carried with the DV-I as it moves along the postoral microtubules to the cell’s posterior end where these acidosomes fuse with the DV (Allen and Fok, 1983c).) Thus, in this study we demonstrate that representative antigens from the original acceptor (DV-I) membrane are removed while new antigens, not previously found in the DV-I membrane, are acquired by acquisition of the membrane of a donor (acidosome) compartment.

MATERIALS AND METHODS

Cell culture and digestive vacuole labeling

Paramecium multimicronucleatum, syngen 2, was grown axenically and harvested in mid-log phase of growth (Fok and Allen, 1979). To obtain vacuoles of known ages and stages, cells were sequentially pulsed for 3 minutes each with latex beads of increasing diameter. A 3 minute chase separated each pulse so that DVs in the same cell containing 0.1, 0.3, 0.5, 0.8 and 1.1 µm beads were 20 to 23, 15 to 18, 10 to 13, 5 to 8 and 0 to 3 minutes old, respectively. In some experiments, variations on the pulse and chase times were used.

Production of monoclonal antibodies

Monoclonal antibodies (mAbs) were obtained from a number of fusions carried out according to the procedure of Kohler and Milstein (1975) by the Monoclonal Antibody Service Facility of the University of Hawaii. Briefly, Balb/c mice were immunized using partially purified lysosomal or microsomal fractions of Paramecium with or without Freund’s adjuvant. The spleen cells were fused to a non-secreting mouse myeloma cell line P3X63-Ag8.653 (Kearney et al., 1979). The hybridomas were screened for the production of antibodies by an indirect fluorescence assay using fixed and acetone-permeabilized whole paramecia. Selected hybridomas were cloned twice by limiting dilution and, when desired, expanded for ascites production using trypan blue-primed Balb/c mice. These cell lines and their corresponding antigens are listed in Table 1.

<table>
<thead>
<tr>
<th>Hybridoma line</th>
<th>Antigen</th>
<th>M₁ of antigen (×10⁻³)</th>
<th>DV stage activity</th>
<th>IgG type</th>
</tr>
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<tr>
<td>I22F4G6</td>
<td>B2</td>
<td>140</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>B251-1-3</td>
<td>Q2</td>
<td>150</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>88B10E7</td>
<td>B3</td>
<td>29</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>E30-2-1</td>
<td>D6</td>
<td>29</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>E34-2-1</td>
<td>E9</td>
<td>130</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>M14-2-1</td>
<td>L1</td>
<td>130</td>
<td>–</td>
<td>+</td>
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Immunofluorescence and immunogold labeling on cryosections

For immunofluorescence studies, paramecia were fixed in 3% formaldehyde in 0.05 M phosphate buffer, pH 7.4, permeabilized in acetone at −20°C for 20 minutes, washed and incubated with the hybridoma supernatant followed by a secondary antibody. For electron microscopy, cells with DVs of known ages were fixed for 30 minutes in 0.25% or 0.5% glutaraldehyde in 0.05M phosphate buffer, pH 7.4, at room temperature. These cells were washed with buffer containing 50 mM glycine and pelleted in 4% gelatin. Slices of the gelled pellet were infused with 2.3 M sucrose before freezing in liquid nitrogen (Fok et al., 1988). Cryosections were obtained at −100°C with a Reichert Ultracut E fitted with an FC-4D cryo attachment. Sections were washed and incubated sequentially in hybridoma supernatants and goat anti-mouse IgG conjugated with 15 nm or 18 nm gold particles. Sections were treated with uranyl acetate/oxalate and infiltrated with a methylcellulose-uranyl acetate mixture before drying (Tokuyasu, 1984). The uranyl acetate/oxalate step was omitted in some cases.

Conventional electron microscopy

For conventional transmission electron microscopy (TEM) cells were fixed at room temperature in 1% glutaraldehyde and processed for TEM as previously described (Allen and Fok, 1980).

Microinjection to determine sidedness of the antigens

The techniques used for microinjection were as previously described (Ishida et al., 1993). To determine if the antigens were located on the luminal or cytosolic side of the membranes, cells were injected individually with 0.011 mg/ml of each of the selected mAbs that were previously conjugated to fluorescein isothiocyanate using a kit from Boehringer Mannheim Biochemicals (Indianapolis, IN). The injection volume was ~4.3% of the cell volume and cells were given a few minutes to recover from the trauma caused by the injection and to allow a complete diffusion of the antibody throughout the cell. Live cells were then observed to determine the sites of antigen-antibody reaction. These cells were then fixed and photographed immediately after a brief wash.

Biochemical characterization of the antigens

A lysosomal fraction was used for biochemical characterization of the antigens. Proteins from this fraction were separated by 10% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (Laemmli, 1970) using myosin (203 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa) and carbonic anhydrase (29 kDa) as molecular mass standards. After electrophoresis, proteins were transferred to nitrocellulose paper (Towbin et al., 1979), which was incubated sequentially with a mAb and goat anti-mouse conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA) and developed with enhanced chemiluminescence (Amersham, Arlington Heights, IL). To determine if the antigens were hydrophobic or hydrophilic polypeptides, the lysosomal fraction was solubilized in Triton X-114 and partitioned into a detergent and an aqueous phase (Bordier, 1981). These two phases were concentrated by ethanol precipitation, after which
Electrophoresis followed by immunoblotting were carried out on the polypeptides as described above.

**Cytochalasin B and NH$_4$Cl treatment**

To test the effect of cytochalasin B on antigen retrieval after acidosome-DV fusion was initiated, this drug (0.3 mM) was added to cells at the beginning of a chase after a 3 minute pulse with latex beads. To test its effect on antigen retrieval before acidosome DV fusion was initiated, this drug was added after a 15 second pre-pulse with beads. To test for reversibility, these cells were washed at 15 minutes and antigen retrieval was studied. Control cells were pulsed for 2 minutes with latex beads and chased. At various time points aliquots of cells from the different experiments were fixed in 3% formaldehyde and processed for indirect fluorescence using mAb for the B2 antigen. To test the effect of NH$_4$Cl (which raises the vacuolar pH; A. K. Fok, personal observation) on antigen removal, cells were pulsed with latex beads for 3 minutes and chased continuously in the presence of 30 mM NH$_4$Cl. Aliquots of cells were fixed at different times and processed for indirect fluorescence using mAb for Q2 antigen. Controls were treated similarly except that NH$_4$Cl was omitted.

The percentages of bead-labeled vacuoles positive for the B2 or Q2 antigens were determined for each aliquot of cells. At least 100 labeled DVs were scored for fluorescence for each point.

**RESULTS**

**Localization of antigens at the LM level**

Two groups of mAbs were used in this study. The first group consisted of two mAbs, specific for antigens referred to as B2 and Q2 that were found on the cytopharynx, nascent DV and DV-I membranes (Table 1 and Fig. 1A,B). The discoidal vesicles, which provide the membrane for DV formation, were also labeled and were seen as a veil of fluorescence extending out from the cytopharynx (arrowheads). The second group consisted of four mAbs to antigens B3, D6, E9 and L1. The E9 and L1 antigens were localized on a small number of phagoacidosomes (DV-II) in a mid-to-posterior position in the cell (Fig. 1C,D) while B3 and D6 labeled a larger number of DVs extending throughout the cell (Fig. 1E,F). In addition, there was always a moderate to heavy level of fluorescence throughout the cell’s cytosol with all mAbs. In an earlier immunofluorescence study, the mAbs to B2 and B3 were called PC1 and PC2, respectively (Fok et al., 1986). The mAbs to the E9 and L1 antigens have also been used in a recent study to follow acidosome development in *Paramecium* (Allen et al., 1993).

**Biochemical characterization of the antigens**

In immunoblots, the B2 antigen was shown to be a 140 kDa polypeptide (Fig. 2, lane 1) while the Q2 was a 150 kDa antigen (Fig. 2, lane 2). Both B2 and Q2 antigens were partitioned into the aqueous phase and were thus hydrophilic antigens (Fig. 3, lanes 1-4).

The B3 and D6 antigens had a molecular mass of 29 kDa and were probably derived from two sister clones (Fig. 2, lanes 3, 4). The mAbs to these two antigens gave relatively weak reactions on immunoblots (lanes 3 and 4 in Fig. 2 were over-exposed) and though many attempts were made to determine if these two antigens were hydrophilic or hydrophobic, the results were either negative or inconclusive. As mAb to B3 was found to give the weaker reaction on cryosections, mAb to D6 was used for most studies. Antigens E9 and L1 were both 130 kDa polypeptides and they, too, were likely derived from sister
clones (Fig. 2, lanes 5,6). The hybridoma line for L1 has since been lost. In our previous study, a blot of E9 was shown to have a strong positive band at 130 and a weak positive band at 29 kDa (Allen et al., 1993). However, recently when blots for each mAb were washed and incubated separately to insure little or no cross contamination of mAbs (Hammarback and Vallee, 1990), E9 was shown to be a single 130 kDa band. In contrast to the B2 and Q2 antigens, E9/L1 were found to be hydrophobic (Fig. 3, lanes 5-8).

**Determination of the topographic locations of the antigens**

It has been generally assumed that antibodies are normally unable to cross a membrane in the living cell. Thus to determine on which side of these cellular compartments these six epitopes or antigenic determinants are located, we microinjected four of the mAbs individually into the cytoplasm of living *Paramecium*. If the membranes in a living cell were labeled with an injected mAb, it was concluded that that antigen was located on the cytosolic side of its vacuole and vesicle membranes. If the membranes, which were previously shown to be reactive with the mAbs in formaldehyde-fixed and acetone-permeabilized cells, were not labeled in the injected cells, we assumed that the antigens were hidden from the antibody by being located in the lumens of cellular compartments. This assumption was shown to be valid in this study, as the injected mAb to an antigen known to be located on the lumenal side of the DV membrane failed to label this membrane in the microinjected cells (see below and Fig. 4). However, we found that the injected mAb could penetrate the vacuole membranes in fixed and unpermeabilized cells when some time had elapsed between fixation and observation. Thus to record the fluorescence of these injected cells, cells were photographed soon after a brief wash and fixation. The mAbs appeared to have relatively high affinities for the DV antigens, a concentration of only 0.011 mg/ml was sufficient to give strong reactions.

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**Fig. 2.** Immunoblots of B2, a p140 kDa antigen (lane 1); Q2, a p150 kDa antigen (lane 2); B3 and D6, 29 kDa antigens (lanes 3 and 4); E9 and L1, 130 kDa antigens (lanes 5 and 6).

**Fig. 3.** Immunoblots of four antigens following partitioning into aqueous (lanes 1, 3, 5 and 7) and detergent (lanes 2, 4, 6 and 8) phases. B2 (lanes 1 and 2) and Q2 (lanes 3 and 4) were partitioned into the hydrophilic aqueous phase, while E9 (lanes 5 and 6) and L1 (lanes 7 and 8) partitioned into the hydrophobic phase.

**Fig. 4.** To determine the topographic location of antigenic binding sites, FITC-conjugated mAbs were microinjected into living cells. A recovery time of 1 hour was allowed before determining the accessibility of the mAbs to the binding sites of the corresponding antigens. (A) mAb to Q2 labels the cytopharynx (arrowhead) after one hour. (B) mAb to B2 shows nonspecific diffuse fluorescence. (C) mAb to D6 strongly labeled vacuoles and vesicles. (D) mAb to E9 gave a nonspecific diffuse fluorescence. From these results it can be concluded Q2 and D6 epitopes are on the cytosolic sides of membranes, while B2 and E9 epitopes are inaccessible to microinjected mAbs as they are on the external cell surface or on the lumenal sides of the DVs and vesicles. Bar, 20 µm.
With microinjection, mAb to antigen Q2 was found to label the cytopharyngeal membrane (arrowhead, Fig. 4A), thus showing Q2 to be located on the cytosolic surface of the cytopharynx and, by extrapolation, on the discoidal vesicles as well (Fig. 4A). We also found that in microinjected cells mAb to Q2 inhibited vacuole formation so that, after a 1 hour exposure to this mAb, no DVs in these cells were labeled. Also the injected mAb appeared to inhibit the binding of the discoidal vesicles to the cytopharyngeal microtubular ribbons as the veil of fluorescence extending from the cytopharynx was absent in most injected cells. The much weaker reaction in Fig. 4A compared with the cell shown in Fig. 1A was in part the result of an excess of mAb given to the cell shown in Fig. 1A compared to only 0.011 mg/ml of mAb injected into the cell shown in Fig. 4A. As a result, the fluorescence intensity of this latter cell was very low. Injecting higher concentrations of this mAb, however, resulted in the whole cell being brightly fluorescent, thus obscuring specifically labeled structures. Antigen B2, gave no positive reaction (Fig. 4B), indicating that this antigen was located on the extracellular side of the cytopharynx and nascent DV, as well as the luminal sides of the DV-I membrane and discoidal vesicles. We also observed the reactive epitopes of D6 to be on the cytosolic side (Fig. 4C), while E9 was on the luminal side of the acidosomes and DV-II membranes (Fig. 4D).

The above observations on the sidedness of the B2 and Q2 epitopes were further confirmed by incubating live cells with mAbs to B2 and Q2 at 12°C, the temperature at which phagocytosis was stopped. These cells were then washed, fixed and labeled with a secondary antibody. Antigen B2 (Fig. 5A) but not Q2 (Fig. 5B) was shown to be located on the cytopharyngeal membrane, confirming that antigen B2 was indeed located on the extracellular surface of the cytopharynx. A similar experiment was repeated except that cells were then processed for TEM and the B2 antigen was again found to be located exclusively on the extracellular surface of the cytopharynx (Fig. 5C).

**Localization of B2 and Q2 antigens on cryosections**

On cryosections, the B2 antigen was specifically located on membranes of the cytopharynx and nascent DV, the DV-I and the large pool of discoidal vesicles (Fig. 6A). These discoidal vesicles accumulated near the cytopharynx after being transported to this locale along the cytopharyngeal microtubular ribbons. They provide the membranes for the growth and enlargement of the nascent DV (Allen, 1974). All 0 to 3-minute-old DVs (marked by the 1.1 mm latex beads) also contained this antigen, but the acidosomes next to their cytosolic sides were B2-negative (Fig. 6B). In another study, eight out of nine 0 to 4-minute-old DVs were positive for the B2 antigen and were associated with unlabeled acidosomes. Interestingly, the only DV that was B2 negative also had no acidosomes associated with its membrane, suggesting that acidosome-vacuole fusion had been completed and that this DV had already become a phagooacidosome.

In sections of cells prepared for TEM, circular profiles (small arrowheads, Fig. 7) next to a young DV were interpreted to be sections of the membrane tubules (large arrowheads, Fig. 7) that arise from the DV-I membrane at this stage. From this observation, most small circular profiles next to a DV-I in cryosections were also assumed to be cross-sections of tubular evaginations arising from the DV-I membrane. These tubules sometimes contained the B2 antigen (upper arrowhead, Fig. 6B). Fig. 6C showed a labeled indentation with unlabeled membranes on both sides. In addition, DV-I that exhibited tubulation usually showed signs of fusing with acidosomes. These fused acidosomes (arrowheads, Fig. 6D) were again mostly B2 negative, while the DV-I membrane was still B2 positive.

The distribution on cryosections of the Q2 antigen, the 150 kDa polypeptide, was nearly identical to that of the B2 antigen. This antigen was found on the discoidal vesicles along the cytopharyngeal microtubular ribbons (arrowheads) and the cytopharyngeal membrane (Fig. 8A), and on the nascent DV and the DV-I membranes (Fig. 8B). Only a few gold particles were present on the acidosomes (Fig. 8A,C). Several DV-I showed signs of membrane tubulation as indicated by membrane indentations or adjacent circular membrane profiles of ~100 nm diameter (arrowheads, Fig. 8B, C). Many of these profiles contained the Q2 antigen. Some DV-II membranes also had a little gold label (Fig. 9A) and some label was found on structures resembling the trans-Golgi network (not shown).

Lysosome membranes were entirely free of either Q2 (Fig. 9A,C) or B2 antigen (not shown). Vacuoles that had advanced to later stages (marked by 0.1 to 0.8 mm latex beads) were...
almost entirely without B2 (not shown) or Q2 (Fig. 9A-D). Out of 32 DVs that were scored as stages II to IV, only two (one 13 to 16-minute-old and one 20 to 23-minute-old) were labeled for B2. These two DVs had apparently been arrested in stage I. Other membrane systems such as the plasma membrane, the endosomal system and membranes of secretory vesicles (trichocysts) were also completely free of label (not shown).

In contrast to the highly specific locations of the B2, as stated above there was a sparse amount of Q2 on the acidosome membrane (compare Figs 6A and 8A) and DV-II (Fig. 9A). However, that Q2 was still present on the DV-II could have meant that there had been insufficient time for antigen retrieval to have been completed.

The major difference between these two antigens was their topographic locations on the membranes. From our observation of a large number of cryosections, the gold label for the Q2 antigens tended to be present more on the cytosolic side,
while much of the label for the B2 antigen was luminal. These results corresponded with data obtained from microinjection studies (Fig. 4A,B), from labeling of live cells, (Fig. 5A,B) and from labeling of fixed but unpermeabilized cells (Fig. 5C). Taken together, these results indicated that B2 and Q2 antigens were located on the luminal and cytosolic surfaces, respectively. Although located on the different sides of the DV-I membrane, both antigens were removed equally during the phagosome to phagoacidosome transformation, leaving the phagoacidosomes almost entirely devoid of both antigens.

**Localization of acidosome and DV-II membrane antigens on cryosections**

The most specific and intensely reactive of the four mAbs to the acidosomal antigens was the one raised against L1. This mAb labeled extensively the acidosomes either docked or fusing with the DV-I (Fig. 8D-G) as well as the DV-II membranes (Fig. 9E-G). Conversely, it did not label DV-I (Fig. 8F,G) and discoidal vesicles (Fig. 8D), nor did it label the cytopharynx membrane (Fig. 8D) and the nascent DV membrane (Fig. 8E). Older DVs, stages III and IV, were free of L1 (Fig. 9H-I). The localization of the E9 antigen, most likely the same antigen as L1, was equally specific but the label was less intense. Most E9-positive DVs, like L1-positive DVs, were 5 to 8 or 10 to 13 minutes old.

Antigens B3 and D6, which as noted above represent a second pair of acidosome/DV-II antigens, differed from L1/E9 in their topographical locations and to some extent in their labeling patterns at the light (Fig. 1E,F) and electron microscopic levels. Like L1, the D6 antigen was absent from the membranes of the cytopharynx, nascent DV, DV-I and discoidal vesicles and, like L1, D6 was present on the acidosomes and DV-II membranes. Thus for the first 10 minutes into the digestive cycle, little difference in distribution of the D6 membrane antigen was seen when compared with that of the L1/E9. Only at the later DV stages were some quantitative differences observed. Forty-eight of the 64 older DVs from 10 to 25 minutes old exposed to D6 or to B3 mAb retained a reduced amount of 29 kDa antigen. This agreed with the immunofluorescence results reported for the B3 antigen (refer to PC2 data of Fok et al., 1986). In line with their different topographical locations, another difference between B3/D6 and L1/E9 antigens was that there was much more of the non-membrane-associated B3/D6 antigen spread throughout the cytosol on cryosections than of the L1/E9 antigen, which was localized entirely on membranes. We suspect that only a portion of the B3/D6 antigen is localized on the cytosolic surfaces of the acidosome and DV membranes while a substantial portion remains soluble in the cytosol.

In summary, the D6/B3 and E9/L1 antigens were entirely absent from the earliest DVs. Both sets of antigens entered the DV membrane as the acidosomes fused with the phagosome to bring about the phagoacidosome formation.

**Effects of cytochalasin B and NH₄Cl on membrane replacement during phagoacidosome formation**

Previous studies in our laboratory have shown that cytochalasin B can prevent acidosomes from fusing with the phagosomes, thereby strongly inhibiting subsequent vacuole acidification (Allen and Fok, 1983c). In the present study, the effect of this drug on the replacement of antigens B2/Q2 during the transition from a phagosome to a phagoacidosome was studied. In untreated cells that had been pulsed with latex beads for 2 minutes and chased, more than 70% of these DVs precipitously lost their B2 antigen within the first 3 minutes of chase (Fig. 10). Almost all of the DVs were B2-negative after a 5-minute chase. When cytochalasin B was added 3 minutes after DV formation was initiated, i.e. after acidosome-DV fusion had commenced, the B2 antigen disappeared from the bead-containing DVs at a somewhat slower rate than that observed in untreated cells so that 30% of the DVs were still B2 positive after 15 minutes. When this drug was added to cells prepulsed for 15 seconds with latex beads so that cytochalasin B was present before DV-acidosome fusion was initiated, the loss of B2 antigen from the bead-containing DVs did not commence for the first 7 minutes and then proceeded much more slowly than in untreated cells. Over 60% of the DV-I were still B2 positive after 40 minutes. (A prepulse was necessary to allow some cells to form at least one DV for immunofluorescence study, as cytochalasin B also inhibited DV formation.) When this drug was washed out at 15 minutes, the disappearance of the B2 antigen following a 5-minute lag was precipitous. By 18 minutes after the wash-out, only about 30% of the bead-containing DVs were B2 positive (Fig. 10). By 30 minutes after wash-out (i.e. 45 minutes into the experiment) only ~10% of the DVs still had the B2 antigen. Overall, it was evident that this drug not only blocked DV-acidosome fusion as previously reported (Allen and Fok, 1983c), but inhibited the removal of the B2 antigen from the phagosome as well.

However, it was not clear at this point whether it was the incorporation of the acidosome membrane or the subsequent prevention of acidification that was responsible for the tubulation and the removal of the phagosome membrane. Subsequent incubation of cells with 30 mM NH₄Cl to alkalinize the young phagosomes did not seem to prevent this membrane retrieval, although a lag in the onset of Q2 removal was observed (Fig. 11). These results suggest that Q2/B2 removal was probably influenced more by DV-acidosome fusion than by acidification, per se.

**DISCUSSION**

It is well known that membranes need not be static molecular complexes but are frequently in a state of flux. This change is brought about in different ways. Classically, it is known that: (1) membrane proteins and phospholipids are inserted into the ER membrane as they are synthesized, which results in membrane growth; and (2) vesicles arising from the ER and Golgi system are capable of fusing with existing membranes to add new components to other membranes. More recently, it has also been shown that proteins can be inserted into existing membranes by post-translational modifications such as their acquisition of fatty acids or prenyl groups. These hydrophobic groups with their polypeptide cargo can then insert into the hydrophobic region of the membrane (reviewed by Magee and Newman, 1992). Proteins can also be inserted into the membrane via glycosylphosphatidylinositol linkages (reviewed by Brown, 1992).

Usually when a donor vesicle membrane fuses with an acceptor membrane, the membrane of the donor vesicles is thought to be retrieved more-or-less intact leaving the acceptor...
membrane as it was before fusion. In other cases, the donor membrane may become a permanent part of the acceptor membrane and sorting of donor-derived macromolecules may occur, so that only selected components remain in the acceptor membrane. In the most extreme case, the donor membrane could be completely incorporated into the acceptor membrane and the acceptor membrane itself could be completely removed. We conclude that this, in fact, is essentially happening when a phagosome is transformed into a phagoacidosome in Paramecium. As shown by data obtained with
**Fig. 8.** Comparison of Q2 (A to C) and L1 (D to G) localization on early phagosomes by immunogold labeling on cryosections. (A) Q2 is found mostly on the cytopharynx (CYX) membrane and the discoidal vesicles (d) which lie along the cytopharyngeal microtubular ribbons (arrowheads). Acidosomes (AC) have a little Q2 label. Bar, 1 µm. (B) A 0 to 3-minute-old vacuole (I, marked with 1.1 µm bead) has several tubular evaginations (arrowheads) labeled with gold. Bar, 0.5 µm. (C) A phagosome-phagoacidosome transition vacuole (I → II) shows evidence of extensive tubulation with Q2 antigen on the cross-sectioned tubules (arrowheads). AC, acidosome. Bar, 0.5 µm. (D) The cytopharynx area like that in A. L1 is found only on acidosomes (AC) and not on the discoidal vesicles (d), cytopharynx (CYX), or the cytopharyngeal microtubular ribbons (arrowheads). (E) The nascent digestive vacuole (NDV) is free of L1 but docked acidosomes (AC) are heavily labeled. (F) A 0 to 3-minute-old transition vacuole (I → II) membrane is labeled only where an acidosome (arrowhead) has fused. An unfused acidosome (AC) is also labeled. (G) A tangential section shows profiles of several docked acidosomes (AC) lying next to a transition vacuole (I → II). Acidosomes are extensively labeled for L1. The site of fusion (arrowhead) of an acidosome is also labeled but the convoluted DV-I membrane remains largely unlabeled. Bar, 1 µm.

**Fig. 9.** Continuation of the comparison of Q2 (A to D) and L1 (E to I) antigens on older vacuoles, 5 to 8 (0.8 µm bead), 10 to 13 (0.5 µm bead), 15 to 18 (0.3 µm bead) and 20 to 23 (0.1 µm bead) minutes old. (A to D) The Q2 antigen, for the most part, is not found on these older vacuoles. Lysosomes (L) are always free of this antigen. (E to G) L1 antigen is found on phagoacidosome (II) membranes but not on lysosomes (L). (H and I) The DV-III (III) and DV-IV (IV) show a second round of tubulation (small arrowheads) and possible L1 antigen removal by these tubules (H). Little or no antigenicity remains on the DV-IV. Bar, 1 µm.
freeze-fracture (Allen and Staehelin, 1981) and the quick-freeze deep-etch technique (Fok and Allen, 1990, 1993), the membrane of a phagosome, which contains a moderately particulate P-fracture face and a highly-particulate E-fracture face, is capable of changing to a membrane similar to that of the acidosomes. Acidosome membrane has a smooth E-fracture face and a P-fracture face bearing prominent intramembrane particles. At the same time tubulation of the phagosome membrane was also observed. These changes suggest that the original phagosome (acceptor) membrane is quickly retrieved and this retrieval is accomplished by membrane tubulation concomitant with the fusion of acidosomes (donor membrane) with the phagosome. This interpretation was supported by the close morphological similarities between the phagoacidosome (DV-II) and the acidosome membranes, and the total dissimilarity between the phagosome (DV-I) and the phagoacidosome membranes. However, as these were strictly morphological studies, we could not tell whether specific macromolecules within the membranes were changing or if the freeze-fracture morphology of the membrane was somehow being altered without a corresponding change in the macromolecular content. With the acquisition of not only highly specific groups of mAbs for these two membranes but also mAbs to antigenic determinants located on both sides of the same membrane, we have been able to show that the DV-I membrane antigens studied are replaced in bulk during phagosome to phagoacidosome transformation (as illustrated in Fig. 12).

Our study shows that B2 and Q2, two hydrophilic antigens located on the luminal and cytosolic side of the membrane, respectively, remain with the phagosome for only a very brief time, as immunofluorescence studies show that the half-life of B2 in the DV-I can be extrapolated to be ~2 minutes or less (Fok et al., 1986, and Fig. 10). They are being retrieved from the phagosome via membrane tubulation concomitant with acidosome fusion. Presumably these tubules immediately re-form into discoidal vesicles, as we have found no accumulated pool of B2 or Q2-labeled membranes in the cell other than the discoidal vesicles. In turn, the D6/B3, E9/L1 antigens are completely absent from the DV-I, discoidal vesicles and the retrieved tubules but are found on acidosome and DV-II membranes. As expected, antigens with epitopes located on either side of the acidosome membrane all become part of the phagoacidosome (DV-II) membrane, when the acidosomes fuse with the phagosome. These antigens are segregated from the membrane tubules that are being formed from this same phagosome. Once a part of the DV-II membrane, these topographically distinct groups of antigens, though not the topic of this study, seem to be differentially and gradually lost from the DVs as these DVs advance to stages III and IV. Thus, the exit and entrance of the various membrane antigens demonstrates conclusively that a bulk replacement of the membrane antigens, and in all likelihood the phagosome membrane itself, occurs during the transformation of the phagosome into a phagoacidosome. We are not aware of any other case where such extensive membrane replacement of an organelle’s limiting membrane is as clearly documented as is this ciliate’s phagosome membrane.

Such a system of membrane replacement raises many questions. What is the mechanism by which the phagosome membrane is retrieved? Clearly the nearly planar membrane of the phagosome becomes molded into long thin tubules, but how does this occur and what is the signal that initiates this striking change? Our experiments demonstrate that antigen retrieval is efficiently inhibited by cytochalasin B but whether this is due to preventing acidosome fusion, primarily, and membrane tubulation, secondarily, or whether cytochalasin B has some direct inhibitory effect on membrane tubulation cannot be determined. The NH4Cl experiments, on the other hand, show that inhibiting DV acidification only slightly retards rather than prevents membrane replacement.

What are the implications of bulk membrane replacement for the processes of phagocytosis and digestion in a cell? Clearly a series of different processes occurs within the confines of the DV membrane as food organisms enter, are killed, their protein
denatured and ultimately digested and the products of digestion resorbed. Changes in the DV membrane may be required to accommodate the lowering of the pH, acquisition of hydrolases by the DV and the retrieval of the useable products of digestion for the cell’s metabolism. Apparently, paramecia are capable of remodeling the boundaries of their digestive containers to bring to bear those membrane protein complexes (transporters, pumps, channels and enzymes) which are needed at specific times during an ongoing catabolic process. During remodeling a set of proteins can be replaced with a different set, which will be used to carry on subsequent physiological steps. This may occur more than once. This would be an efficient use of a cell’s resources as the different membrane pools with their transmembrane protein complexes could be made available for use over and over again. In this way, crucial membrane protein complexes in early DV stages need not be subjected to hydrolytic activity, which might be their fate if they were to stay with the DV membrane to the end of the digestive cycle. That membrane replacement is possible in single-celled eukaryotes, makes the possibility of a similar replacement in higher organisms more plausible.

This work was supported in part by National Science Foundation grants MCB 90 17455 (to A.K.F.) and MCB 92 06097 (to R.D.A.), instrumentation grants and National Institutes of Health Research Centers in Minority Institutions grant RR-03061. N.P.B. and R.F.A. were supported by NIH MBRS GM 08125 and MARC GM 07684.

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(Received 8 September 1994 - Accepted 4 November 1994)