Rat Retinal Pigment Epithelial Cells Show Specificity of Phagocytosis In Vitro

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Abstract. The retinal pigment epithelial (RPE) cell of the eye normally phagocytizes only retinal rod outer segments (ROS). The specificity of this phagocytic process was examined by incubating RPE cells with a variety of particle types. Confluent RPE cell cultures were incubated for 3 h at 37°C in the presence of rat ROS, rat red blood cells (RBC), algae, bacteria, or yeast. Other cell cultures were incubated with equal numbers of ROS and one other particle type. Quantitative scanning electron microscopy was used to determine the numbers and morphology of particles bound to RPE cells, while double immunofluorescence labeling (Chaitin, M. H., and M. O. Hall, 1983, *Invest. Ophthalmol. Vis. Sci.*, 24:812–820) was used to quantitate particle binding and ingestion. Both assays demonstrated phagocytosis to be a highly specific process. RPE cells bound 40–250× more ROS than RBC, 30× more ROS than algae, and 5× more ROS than bacteria or yeast. Ingestion was more specific than binding; RPE cells ingested 970× more ROS than RBC, 140× more ROS than bacteria, and 35× more ROS than yeast. The phagocytic preference for ROS was maintained in competition experiments with other particle types. Serum was found to be essential for phagocytosis. This study demonstrates that both the binding and ingestion phases of phagocytosis are highly specific processes.

THE vertebrate eye consists of two layers of neuronally derived cells, the neural retina and the retinal pigment epithelium (RPE).[1] Rods and cones, the photoreceptive cells of the neural retina, grow continuously at their base while shedding their tips once each day (36, 37, 59). Rod outer segment (ROS) shedding is circadian in many vertebrates (2, 37, 38) and has been shown to occur 30–120 min after the onset of illumination in rats (37). Shed ROS are readily phagocytized by adjacent RPE cells (60), a single RPE cell ingesting as many as 30,000 ROS in a day (4). The phagocytic RPE cell differs from the better studied phagocytic macrophage in that it normally ingests only one particle type in vivo. Macrophages, by contrast, bind and ingest damaged cells and cells or particles with antibody or complement coating their surfaces (52).

ROS phagocytosis by RPE cells has been studied both in vivo and in vitro. In vivo, RPE cell microvilli interdigitate between and surround ROS before shedding, thereby facilitating the subsequent engulfment of ROS tips (4, 54, 60). In vitro, confluent cultures of rat RPE cells phagocytize large numbers of freshly isolated rat ROS (9, 13). In addition to ingesting ROS, the RPE cell has been reported to bind and/or ingest large numbers of latex beads (13, 15, 16, 28, 32, 33, 51), many carbon particles (11), and a few red blood cells (RBC) (35), but no bacteria (32, 34). These studies suggest that the RPE cell is selective in what it phagocytizes. Philp and Bernstein (50, 51) have suggestive evidence that specific phagocytosis of ROS and nonspecific phagocytosis of latex beads by RPE cells involve different mechanisms. The nonspecific process is predominant in early retinal development (51), while the specific process is predominant after ROS differentiate (51, 57). Thus the RPE cell, like the macrophage (18, 52), shows both specific and nonspecific phagocytosis.

The current investigation is the first quantitative study of the phagocytic specificity of RPE cells. An improved method of RPE cell culture, developed in our laboratory (40), provided a controlled environment in which both particle binding and ingestion were quantitated. Two assays, scanning electron microscopy (SEM) and double immunofluorescence labeling (9), were used to quantitate particle binding and ingestion. We found that rat RPE cells show a strong preference for the binding and ingestion of ROS over all other particles tested. Thus, both the binding and ingestion phases of RPE cell phagocytosis appear to be highly specific processes.

Materials and Methods

Monolayer Culture

RPE cell sheets were isolated from 10-d-old Long Evans rats (40), dissociated into a single cell suspension and cultured in growth medium containing Eagle's minimum essential medium with antibiotics and 20% fetal

1. *Abbreviations used in this paper*: FCS, fetal calf serum; GARG, goat anti-rabbit IgG; RBC, red blood cell(s); ROS, rod outer segment(s); RPE, retinal pigment epithelium; SEM, scanning electron microscopy.

calf serum (FCS) (Gibco, Grand Island, NY). A detailed description of this technique is given in a separate publication (40). 20,000 RPE cells were plated in 0.06 ml medium on 13-mm thermanox coverslips (Lux, New York, NY), for SEM, or at 30,000–35,000 RPE cells in 0.1 ml on 18-mm glass coverslips, for double immunofluorescent microscopy. Cells were used for phagocytic assay at 4–6 d of culture, just after reaching confluence.

Isolation of Rat ROS

Adult Long Evans rats were killed 30 min after the onset of illumination. ROS were isolated on a 27/50% sucrose gradient, according to the method developed by Godchaux and Zimmerman (17) as modified by Chaitin and Hall (9). This technique yields ~20 × 10^6 ROS per eye. The pellet of isolated ROS was kept at 4°C and resuspended in sterile growth medium containing 2.5% sucrose at a concentration of 20 × 10^6 ROS/ml just before feeding.

Isolation of Other Particles

The following procedures were used for particle preparation: (*a*) Baker's yeast (*Saccharomyces cerevisiae*) was grown overnight in 0.1 M phosphate-buffered saline (PBS), pH 7.4, at room temperature, fixed in freshly prepared 4% formaldehyde in phosphate buffer for 15 min at 4°C, and rinsed three times with PBS by centrifugation at 1,500 rpm for 5 min. (*b*) Bacteria (*Staphylococcus aureus*) were grown for 24 h in tryptic soy broth at 37.5°C, killed by ultraviolet irradiation overnight, and rinsed in PBS by centrifugation at 4,500 rpm for 15 min. (*c*) Algae (*Botryococcus brauni*) were rinsed twice just before use, by centrifugation in PBS at 1,000 rpm for 2.5 min. (*d*) Rat RBC were freshly isolated from the same rats used for ROS isolation. Blood was removed from the heart into heparinized tubes containing PBS. An RBC pellet was then obtained by centrifugation at 2,000 rpm for 5 min. All particles were rinsed, counted, and resuspended at 20 × 10^6 particles/ml in growth medium containing 2.5% sucrose.

Coating RBC with Specific Antiserum

Freshly isolated rat RBC were pelleted and resuspended in 20 times their cellular volume with PBS containing 1% bovine serum albumin (BSA) (43). A one-half agglutinating titer (1:20,000) of rabbit anti-rat RBC antiserum (Cappel Laboratories, Cochranville, PA) was added to half of the RBC suspension, and both treated and untreated RBC were incubated for 30 min at 37°C, according to Michl, Ohlbaum, and Silverstein (43). RBC were rinsed three times by centrifugation with PBS + 1% BSA and resuspended at 20 × 10^6 cells/ml in growth medium containing 2.5% sucrose.

Coating ROS with Specific Antiserum

Freshly isolated rat ROS were incubated in PBS containing 1% BSA for 30 min at 4°C in the presence or absence of rabbit anti-bovine ROS antiserum (provided by Dr. V. M. Clark). This antiserum specifically stains ROS (49) and has been shown on transblots to stain rhodopsin and a number of unidentified ROS proteins (Clark, V. M., unpublished observations). The antiserum was used at a 1:5 dilution. ROS were then rinsed once by centrifugation and resuspended in complete growth medium containing 2.5% sucrose for immunofluorescent phagocytic assay.

Incubation of Rat ROS or Other Particles with Cultured RPE Cells

Coverslips containing confluent RPE cell colonies were placed in multiwell (Costar, Cambridge, MA) dishes for particle feeding (10). RPE cells grown on small (13 mm) thermanox coverslips were placed in 24-well dishes and fed 20 × 10^6 of one of the following particles: rat RBC, yeast, bacteria, algae, or rat ROS. RPE cells grown on large (18 mm) glass coverslips were placed in 12-well dishes and fed rat RBC, yeast, bacteria, or rat ROS. 1 ml of particle suspension was added to each well with an equal volume of ROS suspension or growth medium, yielding a final concentration of 10 × 10^6 particles, of each type, per milliliter. All particle incubations were for 3 h at 37°C. The effects of sucrose on phagocytosis were tested by adding ROS in growth medium containing 0%, 0.25%, 0.5%, 1.25%, 2.5%, 5%, 10%, or 20% sucrose. After particle incubations, all unbound particles were removed by vigorously shaking each coverslip three times for 5 s each in PBS containing 0.81 mM Ca^{++} and 1.27 mM Mg^{++} at 37°C, just before fixation.

Quantitative Immunofluorescent Assay of Particle Binding to Rat RPE Cells In Vitro

After particle feeding and rinsing, cells on glass coverslips were fixed in 2 ml of 3.7% formaldehyde in 0.1 M PBS, pH 7.4, for 30 min at 37°C (9). To reduce breakage of attached ROS, the formaldehyde concentration was gradually decreased by serial dilution with PBS, before a final 30-min rinse in PBS. ROS and yeast particles, bound to the RPE cell surface, were labeled with rabbit anti-bovine ROS antiserum for 10 min at 37°C, according to Chaitin and Hall (9). While this antibody is specific for ROS (49), we found that it fortuitously also stains yeast, thus allowing the immunofluorescent detection of this particle. Bound RBC were reacted with 50 μl of a 1:10 dilution of rabbit anti-rat RBC antiserum for 10 min at 37°C. *Staphylococcus aureus* bound to the RPE cell surface were not treated during this step but were left in PBS. After reaction with the first antibody, all cell cultures were rinsed in PBS containing 0.1% BSA (Boehringer Mannheim, Indianapolis, IN) for 1.5 h and then stained with fluorescein-labeled goat anti-rabbit IgG (fluorescein-GARG, Cappel Laboratories) according to Chaitin and Hall (9). Because of the high affinity of protein A in the cell coat of *S. aureus* for IgG molecules, this step stains all attached *S. aureus* particles. Coverslips were left overnight to rinse in PBS with 0.1% BSA at 4°C. Cell membranes were then permeabilized by an aqueous acetone series (3 × 5 min) and rehydrated for 30 min in PBS with 0.1% BSA. The cell monolayers were then incubated with a 50-μl aliquot of antiserum for 1 h at 37°C. Yeast and ROS were stained with anti-ROS (1:50), rat erythrocytes with anti-RBC (1:10), and *S. aureus* were incubated in PBS. Coverslips were washed for 1.5 h in PBS and incubated with rhodamine-conjugated goat anti-rabbit IgG (rhodamine-GARG) (1:15) for 1 h at 37°C (9). As described above, this step stains all ingested *S. aureus* particles. Stained coverslips were rinsed, mounted, and examined at 390× with a Zeiss photomicroscope equipped with an HBO 50-W AC mercury arc lamp and a III RS epifluorescent condenser. Photographs were taken with Ektachrome 400 film under water immersion. All visible particles >1-μm diam were counted in confluent areas of RPE cultures using a 1-cm^2 grid (9). At least 10 separate grid areas were counted per glass coverslip. Particles that bind to the outside of cells label bright green with fluorescein-GARG, while rhodamine-GARG labels total particles (ingested + bound) bright red. The number of ingested particles was determined by subtracting the number of bound particles from the total number of particles for each grid area (0.083 mm^2) counted. At least two coverslips containing RPE cell colonies were counted for each experimental time point.

SEM

Immunofluorescence can only be used to study the binding and ingestion of particles to which specific antibodies are available. For this reason, immunofluorescence cannot be used to quantitate the binding of the algae used, for which no antibodies are available. Thus, SEM was used to study the binding of this particle to RPE cells, as well as the binding of yeast, RBC, bacteria, and rat ROS. SEM studies also revealed the typical surface morphologies during binding of ROS and other particles to rat RPE cells.

ROS or other particles were fed to confluent RPE cell cultures for 3 h at 37°C. Excess particles were rinsed off by shaking coverslips in PBS with 1.27 mM $CaCl_2$ and 0.81 mM $MgSO_4$, and cells were fixed in situ with 1.25% EM grade glutaraldehyde (Pelco, Tustin, CA) plus freshly prepared 4% formaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Postfixation was in 1% OsO_4 in 0.1 M sodium cacodylate buffer with 1 mM Ca^{++}, pH 6.0, for 30 min at 4°C. $CaCl_2$ in cacodylate buffer was used to minimize swelling of ROS (29), while low temperature (4°C) and low pH (6.0) were used to minimize actin microfilament destruction by osmium tetroxide (39). Fixed cell colonies were stored overnight in 0.2 M cacodylate buffer containing 7% sucrose.

Coverslips were rinsed 2 × 5 min with water, dehydrated rapidly through 100% ethanol, rinsed 3 × 10 min in 100% ethanol, placed on a wire mesh holder, and critical point dried with CO_2. Dried coverslips were mounted on aluminum stubs and sputter-coated with gold-palladium for a total time of 2 min (8 × 15 s of coating + 30 s of cooling) on a Technics coater in an argon atmosphere at 120 millitorr. Specimens were stored desiccated until ready to examine on an ETEC scanning electron microscope. Photographs were taken with Polaroid PN-55 black and white film at 1,600×. This magnification is sufficient to count both the smallest (bacteria) and the largest (algae) particles. 100–200 fields of view at 1,600× were counted from each coverslip, and 4–10 coverslips were counted for each particle type in six independent experiments.

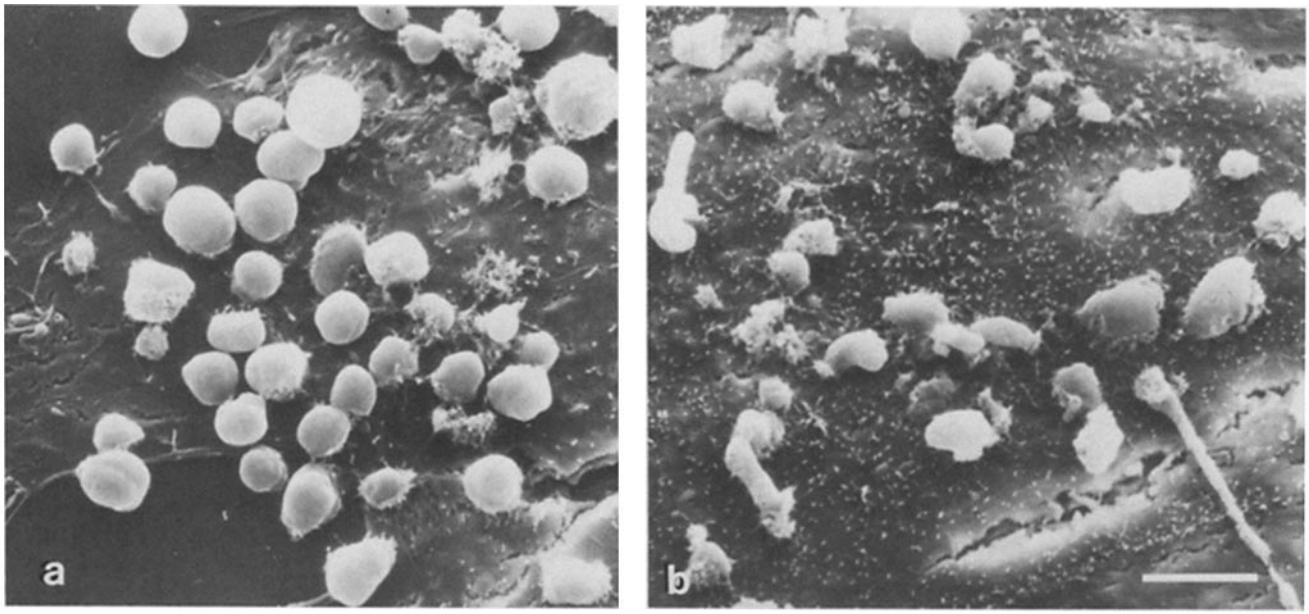

Figure 1. The SEM morphologies of ROS after a 3-h incubation with RPE cells in vitro. (*a*) ROS swell and often burst in growth medium lacking sucrose. (*b*) The addition of 2.5% sucrose prevents excessive ROS swelling. Bar, 5 μm.

Variation of Serum Concentration during Phagocytosis

The role of serum in phagocytosis was determined by varying the concentration of FCS present during a 3-h incubation of ROS with RPE cells. NuSerum (Collaborative Research, Inc., Waltham, MA), a serum substitute which contains 25% FCS, was used to make all media. RPE cells were grown to confluence in growth medium (20% FCS), rinsed three times in medium that lacked serum, and placed in medium containing 0–20% FCS for phagocytic assay. The data were fit to an exponential curve of the form $n_t = n_\infty - ae^{-kt}$ using BMDP statistical software, as described in a previous publication (40).

Results

Morphology of ROS Phagocytosis

Preliminary morphological studies reveal that isolated ROS can be stored in 20% sucrose in PBS at 4°C for several hours without adversely affecting ROS morphology. ROS transferred to growth medium that lacked sucrose swelled rapidly, becoming spherical and sometimes bursting during a 3-h incubation at 37°C with RPE cells (Fig. 1 *a*). Addition of low concentrations (2.5%) of sucrose caused a marked decrease in ROS swelling (Fig. 1 *b*), while higher sucrose concentrations resulted in better preservation of ROS morphology. Fig. 2 shows that phagocytosis, as assayed by double immunofluorescence, was not adversely affected by the presence of 2.5% sucrose, while higher sucrose concentrations reduced ROS binding and ingestion by RPE cells. Therefore, 2.5% sucrose was included in the growth medium during ROS binding and ingestion in all subsequent experiments.

Fig. 3 demonstrates the postulated sequential morphologies of ROS binding and ingestion using SEM. RPE cells respond to ROS attachment by elaborating cell surface processes which surround and engulf the ROS. Fig. 3 *a* shows that RPE cells send out projections that make initial contact with ROS attached to their surface. These cell surface projections then expand into sheet-like processes (Fig. 3 *b*) that surround (Fig. 3 *c*) and engulf (Fig. 3 *d*) adherent ROS. Fig. 3 *d* shows the outline of an ingested ROS. Thus, ROS attachment requires only that ROS adhere to the RPE cell surface, while ROS ingestion requires extensive interactions between RPE and ROS cell surfaces, leading to ROS engulfment.

Kinetics of ROS Phagocytosis

When ROS are layered over an RPE cell monolayer, they rapidly settle onto the surface of the cells. ROS are bound to the cell surface within 15 min, while significant ingestion of ROS is seen by 30 min of incubation (Fig. 4). During the first 30 min, more ROS are bound than are ingested. Thereafter, the ingestion of ROS is essentially linear up to 2 h and reaches a plateau after 3 h of incubation. (In different experiments,

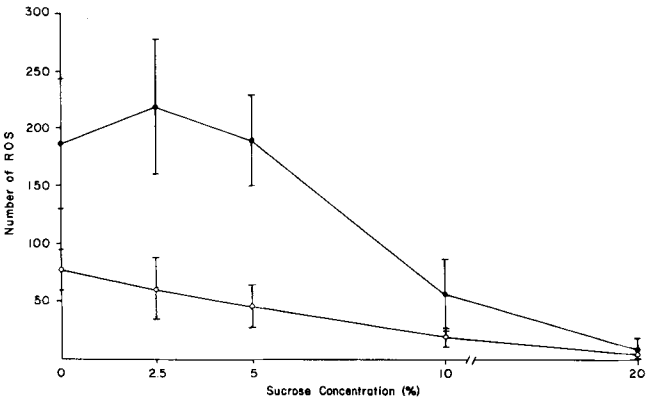

Figure 2. The effect of sucrose on the binding (○) and ingestion (●) of ROS by RPE cells in vitro. Freshly isolated rat ROS are incubated for 3 h with confluent RPE cell monolayers in the presence of different concentrations of sucrose. Bound and ingested particles are counted per field of view at 390× (0.083 mm²). Error bars indicate ± 1 SD.

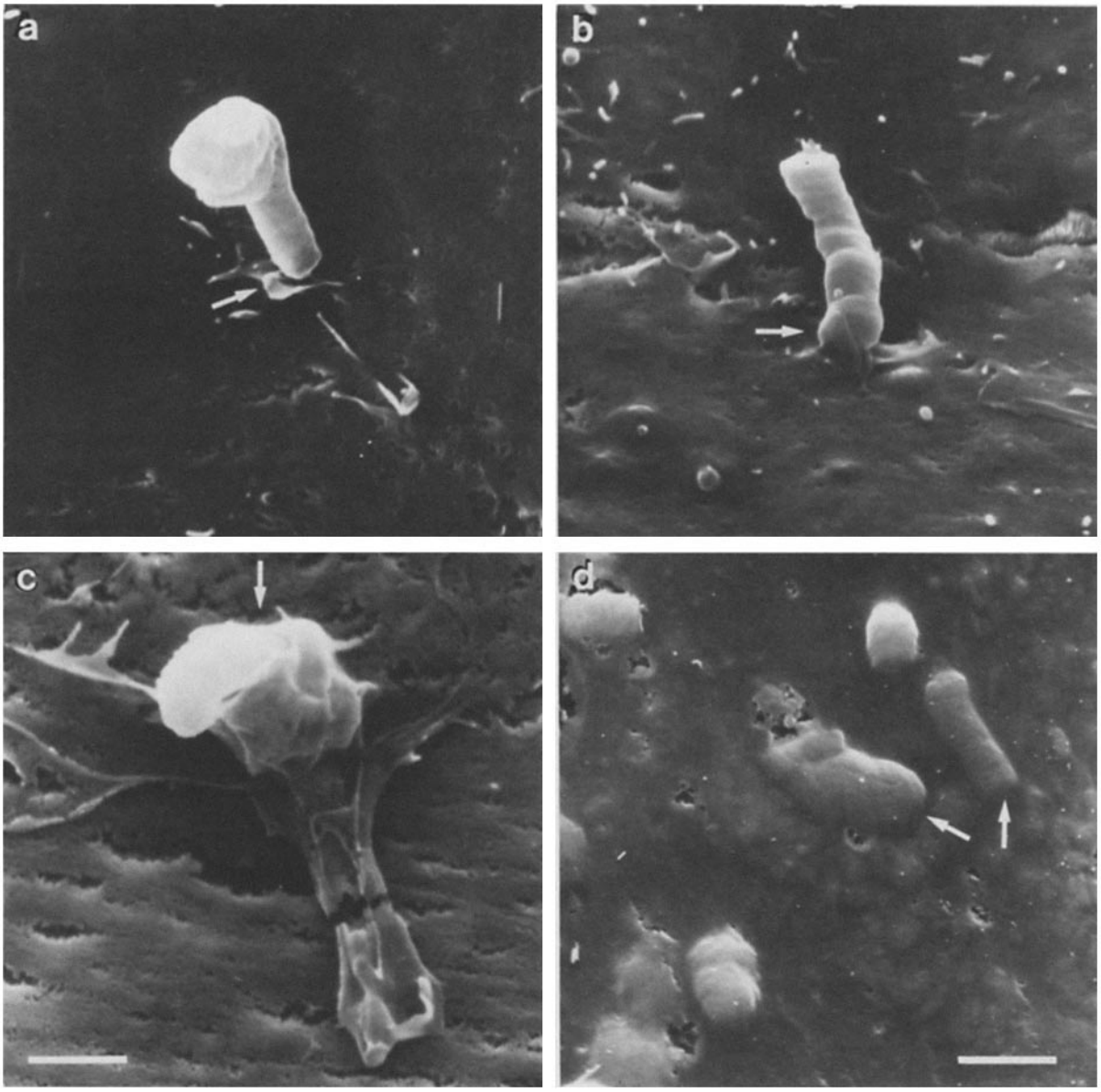

Figure 3. SEM images of the sequential events in the phagocytosis of rat ROS by rat RPE cells. (*a*) A lip of RPE cell membrane underlies a ROS at its site of attachment to the RPE cell surface (*arrow*). (*b*) A small sheet-like process of RPE cell membrane surrounds the base of an attached ROS (*arrow*). (*c*) Sheets of RPE cell membrane ensheath the bottom half of a ROS (*arrow*). Bar (for *a–c*), 2 μm. (*d*) The outlines of engulfed ROS appear submerged beneath the surface of an RPE cell (*arrows*). Bar, 4 μm.

this plateau is reached between 3 and 4 h, which appears to be due to slight differences in the phagocytic ability of different preparations of RPE cells.) ROS binding increases slowly, in a linear fashion, for the duration of the 3-h incubation. Since maximal ROS ingestion has occurred at 3 h of incubation, this was the incubation time used for all of the particle feeding experiments reported. Of the different particles tested, yeast and algae settled onto the monolayer faster than ROS; RBC and ROS settled at the same rate, while bacteria settled more slowly than ROS. However, at least 55% of the bacteria had settled onto the monolayer after 1.5 h of incubation, as determined by counting the number of bacteria in the supernatant at various times during the 3-h incubation. Thus, sufficient time was available during a standard incubation for the RPE cells to bind and ingest significant numbers of the less dense bacteria, if they were phagocytized at the same rate as ROS.

Specificity of Phagocytosis: Quantitation of Particle Binding and Ingestion by SEM

SEM permits the visualization and quantitation of particle binding for a variety of particle types, including those for which specific antibodies are not readily available. The specificity of the phagocytic process was examined by providing RPE cells with a choice between ROS and other particles. Fig. 5 shows the typical SEM morphologies of each particle type after incubation with RPE cells. The particles tested show the following average diameters: algae (8.3 μm) are the largest particles; RBC (4.7 μm), yeast (4.6 μm), and ROS (4.2 μm when spherical) are intermediate in size;

and bacteria (1 μm) are smallest. Fixed isolated rat ROS are 1 μm in diameter and have an average length of 5 μm.

Particle binding was quantitatively analyzed after feeding particles alone or in the presence of equal numbers of ROS (see Materials and Methods). Fig. 6 shows the average number of bound particles when only one type of particle was fed to each RPE cell monolayer. These data clearly demonstrate that while RPE cells bound all of the particles tested, they showed a marked preference for ROS. RPE cells bound 4–5× more ROS than did yeast or bacteria, 30× more ROS than did algae, and 250× more ROS than did rat RBC. Thus, cultured rat RPE cells bound more ROS than did any of the other particles tested.

Fig. 7 shows the average number of particles bound per field of view when each RPE cell colony was fed 10 × 10^6 ROS plus 10 × 10^6 of one other particle type in 2 ml of medium. This figure demonstrates that when given a choice between ROS and other particle types, RPE cells preferentially bound ROS. RPE cells bound 150× more ROS than did homologous RBC, 4–5× more ROS than did bacteria or algae, and 1.7× more ROS than did yeast. Thus, RPE cells showed the following order of preference for the binding of various particles, regardless of the presence (Fig. 7) or absence (Fig. 6) of ROS: ROS > yeast > bacteria > algae > RBC. ROS are, in all cases, the most preferred particle.

The number of ROS bound is not significantly altered by the presence of equal numbers of bacteria; however, RBC and algae do significantly decrease ROS binding. This decrease is probably due to the density of RBC and algae, which coat the RPE cell surface like a carpet and physically block RPE cell contact with ROS. Despite this density effect, RPE cells still bound significantly more ROS than did RBC or algae (Fig. 7). Likewise, ROS may reduce the contact with the cell surface of the lighter bacteria when these are fed together (Fig. 7). However, the binding of bacteria is still much lower than ROS whether the bacteria are fed alone (Fig. 6) or together with ROS (Fig. 7). Additionally, the number of bacteria bound is very similar in both cases. When yeast and ROS are added together (Fig. 7), the binding of ROS is also significantly decreased. Additionally, under these conditions, RPE cells did not bind significantly more ROS than did yeast. Thus, yeast may competitively inhibit ROS binding in addition to physically blocking ROS contact with RPE cells. However, RPE cells do bind significantly more ROS than do yeast when these two particles are added separately (Fig. 6).

Specificity of Phagocytosis: Immunofluorescence Microscopy of Particle Binding and Ingestion

The technique of double immunofluorescence microscopy permits visualization of both bound and ingested particles for any particle to which specific antibodies are available. RPE cells were incubated for 3 h at 37°C in the presence of yeast, bacteria, algae, rat RBC, or rat ROS at a concentration of 10 × 10^6 particles/ml. These particles were fed alone or in the presence of an equal number of rat ROS. Fig. 8 shows the typical appearance of immunofluorescently labeled ROS after a 3-h incubation with RPE cells. For quantitation, a confluent central area of RPE cells is located under phase optics (Fig. 8 *a*). Fluorescein-labeled ROS bound to the outside of cells are counted (Fig. 8 *b*) after which

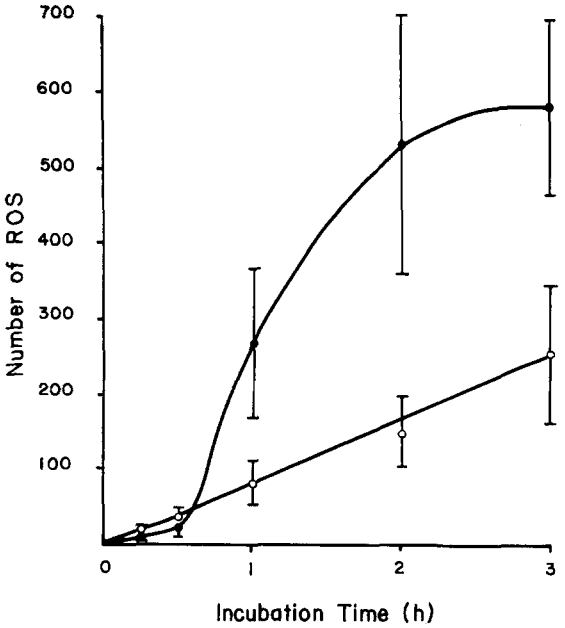

Figure 4. The time course of ROS binding (○) and ingestion (●) at 37°C, measured by double immunofluorescence labeling. Each point represents the mean ± 1 SD.

rhodamine-labeled total ROS (bound + ingested) are counted (Fig. 8 *c*). The number of ingested particles was determined by subtraction. Fig. 8 demonstrates that after 3 h of incubation, the average RPE cell has many more ROS internalized than are bound to its surface.

While all of the bound ROS stain with equal intensity using fluorescein-GARG, ingested ROS show a range of staining intensities with rhodamine-GARG, from very bright to very dim. Separate studies (22) have shown that this is due to the rapid degradation of the ROS particles after ingestion, presumably resulting in a decrease in the number of antigenic sites to which the primary ROS antibody can bind. Although all visible particles >1-μm diam are counted, some very dim particles would be invisible, and thus would not be counted. Thus the number of ingested ROS is almost certainly slightly greater than the number that can be seen. However, this variation in staining intensity of ingested particles was not seen with yeast, bacteria, or RBC. All of these particles stained with an approximately equal intensity after ingestion, as did those fluorescein-GARG stained particles bound to the RPE cell surface. Presumably, digestion of these particles was not as rapid as the digestion of ROS.

Table I shows the average number of particles bound to or ingested by RPE cells per field of view. The top part of Table I shows particle counts when one particle type (RBC, bacteria, yeast, or ROS) was fed to each monolayer culture. A comparison of different particle types demonstrates that RPE cells bound 5–44× more ROS and ingested 35–970× more ROS than did any other particle type. Yeast was the second most preferred particle, being ingested significantly more than bacteria or rat RBC, but significantly less than ROS. The bottom part of Table I shows particle counts when each RPE cell culture was fed two particle types (ROS plus either RBC, bacteria, or yeast). The results clearly demonstrate that RPE cells prefer ROS, even in the presence of equal

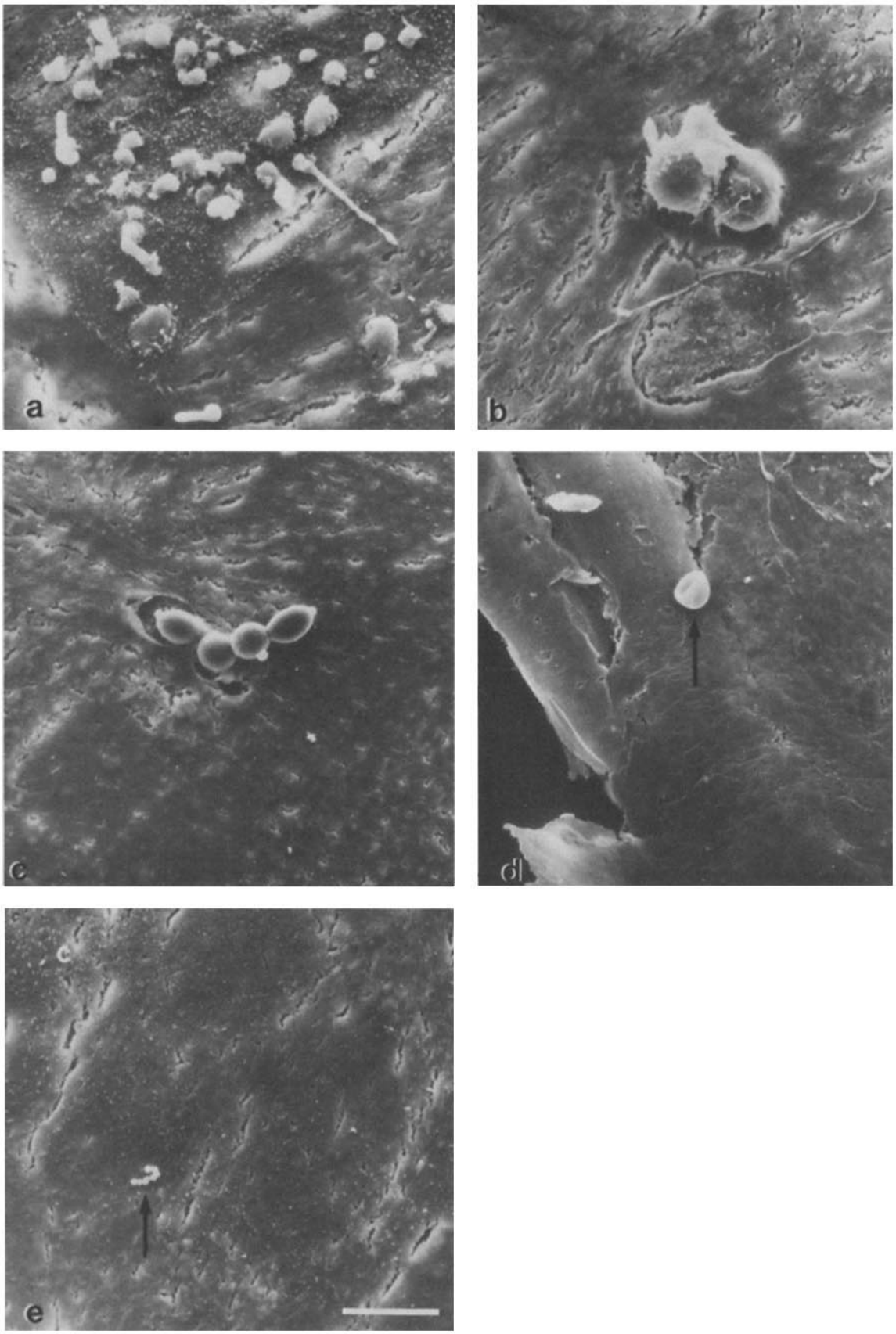

Figure 5. SEM showing the comparable sizes of various particles incubated with RPE cells. (*a*) Rat ROS; (*b*) algae (*Botryococcus brauni*); (*c*) yeast (*Saccharomyces cerevisiae*); (*d*) rat red blood cell; (*e*) bacteria (*Staphylococcus aureus*) (*arrow*). Bar, 10 μm.

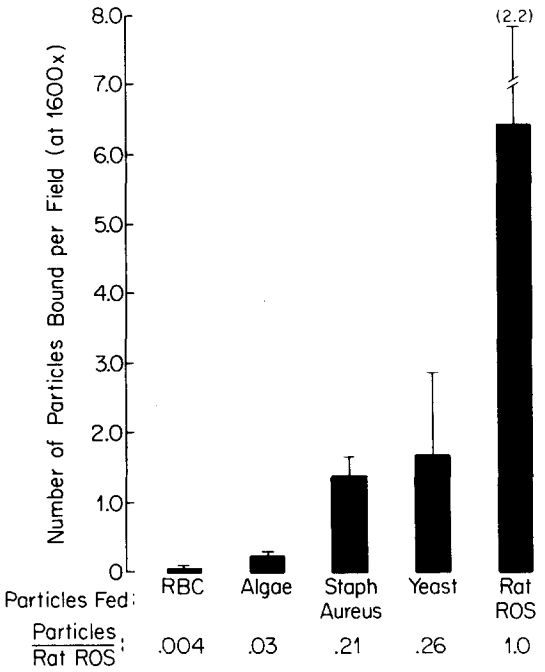

Figure 6. Quantitative analysis of particle binding to RPE cells when a single particle type is added at 10 × 10^6 particles/ml to each RPE cell monolayer. Binding is assayed using SEM. Each bar represents the average number of particles bound per field of view at 1,600×. RPE cells bind all of the particle types tested, but show a strong preference for ROS. Error bars indicate ± 1 SD.

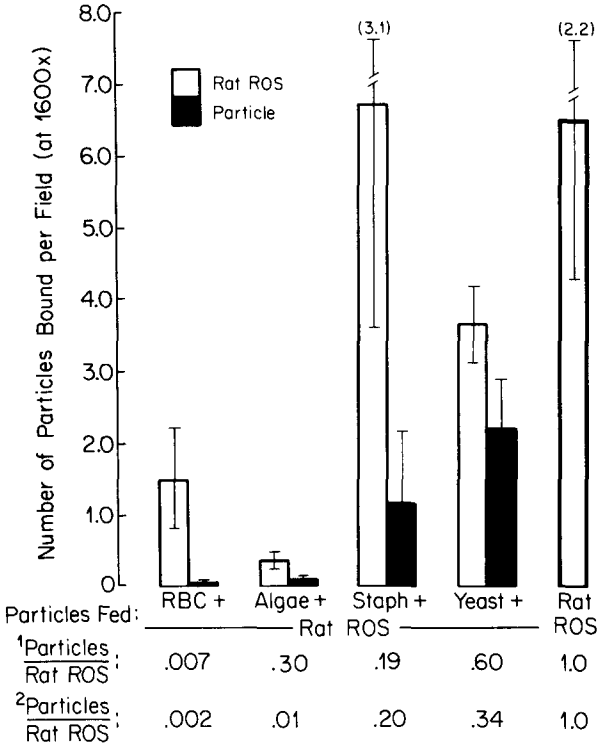

Figure 7. Quantitative analysis of particle binding by SEM. Each RPE cell colony is incubated with equal numbers of ROS plus one other particle type. Each bar represents the average number of ROS or other particle bound per field of view at 1,600×. Two ratios are shown for the number of particles bound/number of ROS bound: [1]ROS, number of ROS bound when ROS are added together with one other particle type; [2]ROS, number of ROS bound when ROS are added alone. These ratios demonstrate that RPE cells show a strong preference for ROS, even in the presence of equal numbers of another particle type. Error bars indicate ± 1 SD.

numbers of one other particle type. In every case, significantly more (17–162×) ROS than other particles were phagocytized by RPE cells. Thus, both the binding and ingestion phase of phagocytosis are highly specific processes.

Table II shows the ratio of particles/ROS for both binding and ingestion by RPE cells. The first two columns show the relative binding of different particles as determined by SEM and immunofluorescence microscopy, respectively. Both methods of analysis demonstrate that RPE cells show a strong preference for the binding of ROS over all other particle types tested. The two methods of analysis yield similar data, except in the case of RBC, which show significantly lower counts by SEM, most probably due to particle loss during critical point drying. The third column shows normalized particle counts for ingestion as assayed by immunofluorescence microscopy. A comparison of binding and ingestion data (columns 2 and 3) shows that RPE cells bind 5–44× more ROS and ingest 35–967× more ROS than do other particle types. The fourth column shows percent particle ingestion. Rat ROS showed a higher percent ingestion than any other particle type, 84% of total ROS counts being due to internalized ROS, compared with 45% for yeast, 26% for RBC, and 21% for bacteria. Thus, the two techniques of SEM and immunofluorescence microscopy show binding and ingestion to be highly specific processes, with ingestion being a more highly specific process than binding. These results suggest that binding and ingestion may be separable processes.

Role of Serum Factors in RPE Cell Phagocytosis

Fig. 9 clearly demonstrates that serum factors are essential for RPE cell phagocytosis. ROS binding and ingestion were inversely affected by lowering the serum concentration in the medium. At FCS concentrations below 2.0%, ROS binding was significantly enhanced, while ROS ingestion was significantly decreased. A decrease in the concentration of FCS from 20% to 0% resulted in a twofold increase in ROS binding and a 10-fold decrease in ROS ingestion. The effect of serum on ROS binding may be secondary to the failure of ROS ingestion at low serum concentrations. Serum concentrations of 2.5% to 20% resulted in normal ROS phagocytosis.

IgG and complement are essential serum components for particle binding and ingestion by macrophages (52). Similarly, canine (46) and monkey (14) RPE cells have been reported to preferentially bind erythrocytes pre-coated with IgG. We decided to investigate the role of IgG in the phagocytosis of ROS and RBC by rat RPE cells. Confluent RPE cell cultures were fed ROS or RBC, which were untreated or pre-coated with their specific antisera. Particle binding and ingestion were quantitatively analyzed by double immunofluorescence. Table III shows that antibody pre-coating did not significantly alter either binding or ingestion of RBC or ROS by RPE cells. Thus, while IgG receptors may be present on rat RPE cells, they do not appear to play any significant role in the recognition of either specific (ROS) or nonspecific (RBC) particles. Since serum is essential for normal ROS phagocytosis (Fig. 9), serum factors other than IgG are implicated in particle binding and ingestion by RPE cells.

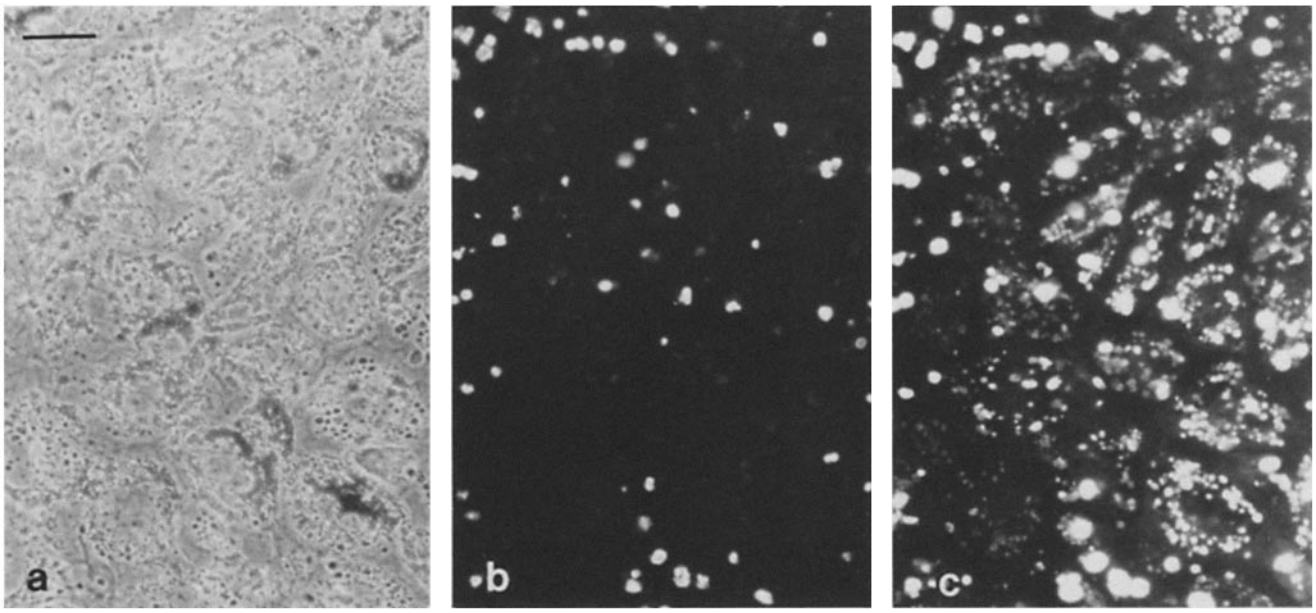

Figure 8. Typical fields of view, by immunofluorescent microscopy of RPE cells with bound and ingested ROS. (*a*) Phase image of RPE cell monolayer. (*b*) Fluorescein fluorescence showing bound ROS. The faint images are ingested, rhodamine-stained ROS which fluoresce faintly under the fluorescein illumination. (*c*) Rhodamine fluorescence showing total ROS (bound and ingested). Particles in various stages of digestion stain with different intensities. Bar, 10 μm.

Discussion

This study clearly demonstrates that RPE cells show a high degree of phagocytic specificity. These cells preferentially phagocytize congenic ROS when ROS are fed alone or in the presence of equal numbers of one other particle type. Both the binding and ingestion phases of phagocytosis are specific processes, with ingestion being more highly specific than binding. RPE cells do bind and ingest non-ROS particles, but in much lower numbers than ROS.

The RPE cell resembles the better known phagocytic macrophage in its ability to differentiate between specific and nonspecific particle types. Macrophages show specific phagocytosis of particles containing IgG (Fc portion) and complement (C3b fragment) on their surfaces as well as the nonspecific phagocytosis of latex, yeast, and non-opsinized RBC (19, 44, 52). Binding and ingestion appear to be separable, independent processes in both the macrophage (19, 20, 44, 58) and the RPE cell, for in both cell types, binding can occur without subsequent ingestion. For example, particle ingestion is prevented, without affecting binding, at 17°C in both the macrophage (53) and the RPE cell (22). The two processes are also separable by genetic mutation, for RPE cells from the retinal dystrophic RCS rat show normal binding but very little ingestion of ROS (9).

Rat RPE cells, unlike canine (46) and monkey (14) RPE cells, do not show preferential phagocytosis of RBC or ROS, which are precoated with their specific antisera. These results suggest that Fc receptors of IgG are not involved in particle binding or ingestion by rat RPE cells, and that a separate, highly specific receptor is probably required for the phagocytosis of ROS. Our results also suggest that serum factors other than IgG are essential to ROS phagocytosis by RPE cells, for RPE cells show little ingestion of ROS in the absence of serum.

Mannose residues have been postulated to play a role in the recognition, binding, and ingestion of particles by macrophages. Mannose residues and their analogues compete

Table I. Quantitative Analysis of Particle Binding and Ingestion Using Double Immunofluorescence Labeling

Particle(s)	Bound	Ingested	% Ingestion‡
RBC	3.1 ± 1.9	0.8 ± 0.6	26
Bacteria	27.1 ± 0.6	5.6 ± 2.8	21
Yeast	28.2 ± 12.7	22.7 ± 5.0	45
ROS	135.6 ± 125.1	802.6 ± 300.5	84
RBC* + ROS	3.7 ± 2.3	1.6 ± 1.2	30
RBC + ROS*	83.6 ± 39.0	264.5 ± 45.4	76
Bacteria* + ROS	27.8 ± 6.7	1.8 ± 1.5	7
Bacteria + ROS*	181.0 ± 137.2	623.0 ± 138.6	78
Yeast* + ROS	35.7 ± 8.6	22.9 ± 2.6	39
Yeast + ROS*	74.8 ± 46.2	399.0 ± 68.4	84

Particles were added alone (top) or in the presence of equal numbers of ROS (bottom). Bound and ingested particles were counted per field of view (390×). Average counts (mean ± SD) are from three to eight cell cultures from four separate experiments.
* The particle counted.
‡ Number of ingested particles divided by the number of total (bound + ingested) particles × 100.

Table II. Numerical Analysis of Data When ROS or Other Particles are Added Alone

	SEM*	Immunofluorescence*		
Particle	Binding	Binding	Ingestion	% Ingestion‡
Rat ROS	1.00	1.00	1.00	84
Yeast	0.26	0.21	0.29	45
Bacteria	0.21	0.20	0.007	21
Algae	0.03	–	–	–
RBC	0.004	0.02	0.001	26

* Ratio of No. of particles/No. of rat ROS.
‡ Number of particles ingested/total number of particles counted × 100.

Table III. The Effect of Pre-coating ROS or RBC with their Specific Antisera Before Incubation with Rat RPE Cells

Particle	Treatment	Bound	Ingested	% Ingestion
ROS	None	205.3 ± 40.3	886.1 ± 240.6	80
ROS	Anti-ROS	325.9 ± 103.5	720.1 ± 61.3	69
RBC	None	2.3 ± 1.2	2.0 ± 1.3	24
RBC	Anti-RBC	1.6 ± 1.4	1.6 ± 1.4	45

All particles were incubated in PBS with 1% BSA, with or without specific antisera. Phagocytosis was assayed by immunofluorescence microscopy. Values are the mean ± 1 SD.

with and inhibit the specific Fc-mediated phagocytosis of particles by macrophages (55) without affecting nonspecific particle ingestion. There is also evidence for mannose involvement in ROS phagocytosis by RPE cells. Preliminary studies by Heth and Bernstein (31) suggest that ROS binding to RPE cells is inhibited by excess mannose. Lectin binding studies, using concanavalin A and lens culinaris, also suggest that the outer membrane of the ROS is rich in mannose (6, 42, 47). The major protein in the ROS membrane, rhodopsin, does in fact contain three terminal mannose residues (23, 24). The present study shows that yeast, which are coated with the mannose polymer mannan (48), are preferred by RPE cells to all other non-ROS particles tested. These results suggest that mannose may be involved in specific recognition, binding, and ingestion by RPE cells. Thus, rhodopsin, or some other mannose rich glycoprotein of the ROS, is a likely candidate for the specific cell surface ligand involved in ROS recognition by RPE cells.

Phagocytosis by RPE cells is a multi-step process involving recognition, binding, and ingestion. The RPE cell elaborates cell surface projections, which surround and engulf attached ROS and other particles (Fig. 1). Similar cell surface processes are described by McLaughlin et al. (41) during the phagocytosis of latex beads by RPE cells. Plasmalemmal extensions, which engulf and ingest particles, have been shown to be mediated by actin-myosin interactions in the macrophage (1, 5, 52, 58). The receptor-ligand interactions associated with specific particle phagocytosis by macrophages are accompanied by an accumulation of filamentous actin beneath the site of particle binding (25–27, 52). Several studies suggest that the ingestion process in the RPE cell is also mediated by actin-like contractile filaments (7–9). Haley et al. (21) have shown microfilament–plasmalemmal associations beneath particles attached to the RPE cell surface, while Chaitin and Hall (10) have shown that basket-like accumulations of actin underlie each attached ROS. These actin associations persist during the early phases of the ingestion process.

Macrophage phagocytosis involves the step-wise interaction of ligands on the particle surface with specific receptors on the phagocytic cell's surface (19, 52). In vivo, RPE cells specifically phagocytize only ROS and have probably developed specific cell surface receptors to facilitate this process (22). Defects in this RPE cell surface receptor or the corresponding ROS cell surface ligand would likely result in phagocytic defects, which could lead to retinal degeneration and possible blindness. Such phagocytic defects and their resulting pathologies are seen in the retinal dystrophic rat (3, 9, 12, 13, 30, 45, 56).

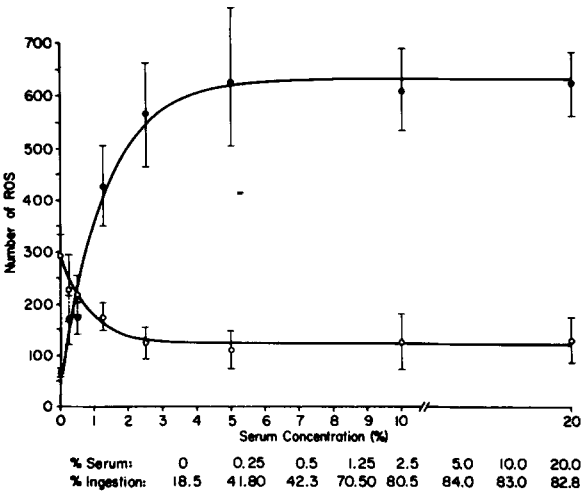

Figure 9. The effect of serum concentration on ROS phagocytosis by RPE cells. Phagocytosis was assayed by immunofluorescence microscopy. Graph shows the average number of ROS bound (○) and ingested (●) per field of view at 390×. Error bars indicate ± 1 SD.

To summarize, the phagocytic RPE cell and the phagocytic macrophage share the following characteristics: (*a*) Phagocytosis is a highly specific process in which specific receptors on the phagocytic cell's surface are thought to recognize ligands on the particle surface. (*b*) Both phagocytic cells show a strong preference for specific over nonspecific particle ingestion. (*c*) There is evidence that mannose residues play a role in particle recognition. (*d*) Particle ingestion is temperature dependent (22). (*e*) Membrane-associated actin microfilaments are found subjacent to sites of particle attachment and actin–myosin interactions probably mediate the formation of plasmalemmal extensions which surround and engulf bound particles (10). RPE cells do, however, differ from macrophages in the following ways: (*a*) Fc receptors do not appear to play any significant role in particle phagocytosis by rat RPE cells. (*b*) Macrophages show specific phagocytosis of a variety of sensitized particles, while RPE cells preferentially phagocytize only one particle type, the ROS. (*c*) Particle binding is temperature dependent in the RPE cell (22) but temperature independent in the macrophage (19, 53). Thus, while the mechanisms of phagocytosis are similar in the macrophage and the RPE cell, the two cells exhibit different phagocytic specificities.

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