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# Degradation of phagosomal components in late endocytic organelles

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## SUMMARY

**Phagosomes are formed when phagocytic cells ingest particles such as bacteria, viruses or synthetic beads of different kinds. The environment within the phagosome gradually changes to generate degradative conditions. These changes require multiple interactions between the maturing phagosomes and the endocytic and the biosynthetic pathway. The phagosomes probably communicate with endocytic organelles by a transient fusion event, often referred to as the 'kiss-and-run' hypothesis.**

**We have studied the role of endocytic organelles in the phagocytic pathway of J774 cells, a mouse macrophage cell line. We have used magnetic Dynabeads coated with $^{125}$ITC-IgG and $^{125}$ITC-OVA as phagocytic probes and were able to isolate the phagosomal fraction by means of a magnet. To separate lysosomes from other organelles in the endocytic pathway we allowed the cells to endocytose a pulse of colloidal gold particles complexed with ovalbumin. By combining this density shift technique with subcellular fractionation of a postnuclear supernatant in Percoll gradients we could isolate three endocytic fractions corresponding to early endosomes (the light Percoll fraction), late endosomes (the dense Percoll fraction) and lysosomes (the gold fraction).**

**We observed that the proteins linked to the ingested beads are initially cleaved in the phagosomes. This cleavage is inhibited by leupeptin, a thiol-protease inhibitor, and requires an acidic environment. However, efficient communication between the phagosomes and the endocytic pathway leads to the transfer of dissociated phagocytosed peptides of different sizes to late endosomes and lysosomes for further processing. Consequently, the late endosomes and the lysosomes may be involved in the degradation of phagocytosed compounds.**

Key words: Endosome, Late endosome, Prelysosome, Lysosome, Degradation, Ovalbumin, Subcellular Fractionation, Phagocytosis, Phagosome, Dynabeads

## INTRODUCTION

Newly formed phagosomes interact with organelles of the endocytic pathway to generate phagolysosomes, a process essential for the killing and degradation of internalized microorganisms. The interaction involves recognition of the appropriate organelles and docking and membrane fusion to allow intermixing of their contents. The phagosomes acquire and lose membrane associated proteins during their transformation into phagolysosomes. Among these are receptors, rab proteins and actin binding proteins as well as many unidentified polypeptides (Pitt et al., 1992a; Desjardins et al., 1994a,b). Desjardins et al. (1994b), showed a difference in the kinetics of transfer of lumenal and membrane bound markers to phagosomes from other endocytic organelles. This phenomenon could be explained by a transient fusion contact between the phagosomes and endocytic organelles. Phagosomes have also been shown to fuse directly with early and late endosomes and lysosomes in cell free systems (A. Jahraus, unpublished; Mayorga et al., 1991). In addition, in whole cells phagosomes have been shown to transform into compartments displaying characteristics of late endosomes (LE) (Rabinowitz et al., 1992).

The maturation of phagosomes with time may require the same fusion machinery as described for the fusion events in the endocytic pathway. Fusion between phagosomes and early endosomes (EE) resembles the homotypic fusion between EE (Pitt et al., 1992b) and is inhibited by N-ethylmaleimide (NEM) and by antibodies against the NEM-sensitive fusion protein (NSF) (Mayorga et al., 1991). Rab5 and rab7, important GTPases in the fusion events of early and late endosomes, respectively, are found in the phagosome membrane at different times after phagosome formation (Desjardins et al., 1994b).

Few studies have been done on the transport of phagosomal compounds to the endocytic pathway. This process may be important for transporting antigens to compartments containing MHC class II molecules for presentation or, alternatively, the transfer of already complexed MHC class II-antigens to the surface. Several studies have shown that processed antigens from phagocytosed particles are presented at the cell surface in complex with MHC class II (Pfeifer et al., 1992; Wick et al., 1993, 1994) and as shown recently, with

MHC class I (Kovacsovics-Bankowski and Rock, 1995; Harding and Song, 1994; Pfeifer et al., 1993; Malaviya et al., 1996). The meeting place of the antigen and the MHC class II molecule may take place both in the phagosome (Lang et al., 1994) or in an extraphagosomal compartment (Clemens and Horwitz, 1993).

Several studies have reported that the phagosomal contents are transferred from the phagosomes to EE (Mayorga et al., 1991) and lysosomes (Wattiaux et al., 1996) as well as out of the cell (Pitt et al., 1992a). It is likely that such a transport requires an initial processing of the ingested material by lysosomal enzymes introduced from the endocytic pathway. Recent studies (V. Claus, unpublished) show that the level of lysosomal enzymes increases in 2-hour-old phagosomes suggesting bulk fusion with lysosomes. However, phagosomes may continuously interact with enzyme bearing organelles in order to refill with enzymes with a short half-life (Oh and Swanson, 1996).

In the present work we have developed a method for separation of phagosomes, late endosomes and lysosomes and determined their role in the processing and transport of phagocytosed compounds. We have used magnetic Dynabeads coated with either $^{125}$ITC-IgG or $^{125}$ITC-ovalbumin (OVA) in order to follow the degradation of ingested material. By density shift of lysosomes using colloidal gold combined with subcellular fractionation we were able to separate early endosomes (EE), late endosomes (LE) and lysosomes (Tjelle et al., 1996) in addition to the phagosomal fraction which was isolated by means of a magnet. Our results show that initial cleavage of the linked proteins occurs in the phagosomes. Subsequently, peptides of different lengths were transferred to LE and lysosomes for further processing.

## MATERIALS AND METHODS

### Materials

Tosylactivated Dynabeads M-280 (from Dynal, Norway) were coated with mouse IgG (Fluca) or ovalbumin (OVA) type VI from Sigma according to the manufacturer's manual. The IgG or OVA were then covalently bound to iodinated tyramine cellobiose (TC) (kindly provided by Helge Tolleshaug) according to the method of Pittman et al. (1983). Concanamycin A, leupeptin and horseradish peroxidase (HRP) type VI were bought from Sigma. Na-$^{125}$I was obtained from the Institute of Energy Techniques, Kjeller, Norway. Percoll was purchased from Pharmacia.

### Cell culture

The mouse peritoneal macrophage cell line J774-A1 was cultured in monolayer in 10 cm dishes with approximately $1.5\times10^7$ cells in each dish. Culture medium DMEM containing 10% fetal calf serum, 1% pen/strep (penicillin, 10,000 units/ml; streptomycin, 10,000 mcg/ml) and 1% L-glutamine from BIO-Whittaker was used.

### Cell homogenization and fractionation

For the subcellular fractionation, cells were treated as described by Tjelle et al. (1996). Briefly, colloidal gold coated with ovalbumin, was incubated with the cells for 2 hours and chased overnight. Generally, beads were incubated with the cells for 15 minutes and then chased for the indicated time periods. After homogenization of the cells, the volume was adjusted to 1 ml and the beads were removed from the homogenate by means of a magnet and washed three times in homogenization buffer. The nuclei and gold filled organelles were pelleted from the remaining homogenate. The pellet was resuspended in 2.5 ml 17% Percoll and loaded onto a 2 ml 64% sucrose cushion in a 6 ml Beckman ultracentrifuge tube. The samples were centrifuged for 30 minutes at 27,000 ***g*** in a Beckman SW55Ti rotor with fast acceleration. After this run the nuclear fraction remained at the top whereas the gold filled organelles were at the bottom of the sucrose cushion. This pellet was resuspended in 1 ml homogenization buffer and is referred to as the lysosomal fraction.

8 ml of 17% Percoll were layered on top of a 2 ml 64% (w/w) sucrose cushion and the remaining homogenate was placed on top of the Percoll solution. A self-generating gradient was formed during centrifugation for 1 hour at 56,000 ***g*** in a Beckman SW41Ti rotor. The gradients were fractionated into 11 fractions.

In some experiments (where indicated) the remaining homogenate was mixed 1:3 with 64% sucrose and layered at the bottom of the gradient. The homogenate was in these experiments adjusted to only 500 μl after homogenization.

### Analysis of HRP, β-acetylglucosaminidase and $^{125}$I-TC-OVA

The HRP activity was measured by using Pierce Immunopure TMB Substrate Kit. β-Acetylglucosaminidase activity was assayed according to the method of Barrett (1972). Degraded and undegraded iodinated TC-conjugated OVA ($^{125}$ITC-OVA) or IgG ($^{125}$ITC-IgG) were determined in the fractions by precipitation with 10% trichloroacetic acid (TCA).

### Colloidal gold

Colloidal gold particles (5 and 10 nm diameter) were prepared according to the method of Slot and Geuze (1985) and coated with ovalbumin following a protocol for adsorption of proteins to colloidal gold particles (Horisberger and Rosset, 1977; Roth, 1983). The gold-protein complexes (OVA-Au) were centrifuged in a Beckman J2-21 centrifuge at 45,000 ***g*** for 60 minutes. The OVA-Au was resuspended in 250 μl culture medium per ml initial volume and was then ready for use. The absorbance of the gold-medium solution was approximately 3.5 at 520 nm.

## RESULTS

### Time course of degradation of $^{125}$ITC-IgG-coated and $^{125}$ITC-OVA-coated Dynabeads

Mouse-IgG or ovalbumin were labeled either directly with $^{125}$I or with $^{125}$ITC. The labeled degradation products formed from $^{125}$ITC-labeled proteins are trapped at the site of formation and may therefore serve as markers for the organelles in which they are formed (Berg et al., 1985). Dynabeads (1 μl/ml) coated with $^{125}$ITC-IgG or $^{125}$ITC-OVA were incubated with J774 cells for different periods of time. The medium was saved after the incubation and the cells were washed 3 times before being scraped off the well. Acid soluble radioactivity was measured in both medium and cells. The results show a continuous increase in acid soluble radioactivity in the cells or in the medium depending on the labeling method used (Fig. 1). Directly labeled ligands generally formed more acid soluble radioactivity than those labeled with $^{125}$ITC. The acid soluble radioactivity in the medium (4-6%) in the absence of cells did not increase during the incubation time.

### $^{125}$ITC-OVA is not transferred from phagosomes to early endosomes

We have earlier developed a method for fractionating J774 cells into three fractions enriched, respectively, in EE, LE and

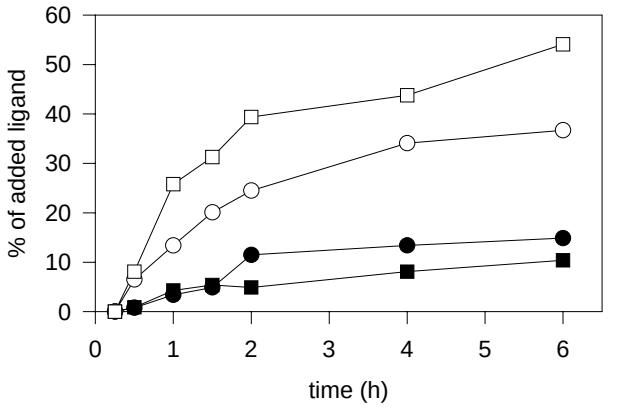


**Fig. 1.** Degradation of $^{125}$I- and $^{125}$ITC-labeled IgG and OVA associated with phagocytosed Dynabeads. J774 cells were incubated for the indicated time periods with beads (1 μl/ml) coated with IgG (circles) or OVA (squares) that were labeled with $^{125}$I-TC (filled symbols) or $^{125}$I (open symbols). At the end of the incubation, the medium was aspirated and saved, and the cells were washed 3 times in cold PBS. The cells were then scraped off in 1 ml PBS. Acid soluble radioactivity were measured in the medium and in the cells. Results are presented as % acid soluble radioactivity of total acid precipitable radioactivity added to the cells.

lysosomes (Tjelle et al., 1996). In this method the lysosomes are loaded with colloidal gold and separated from the homogenate by differential centrifugation. The PNS (post nuclear supernatant) is subsequently centrifuged in a Percoll gradient to separate early and late endosomes. The different organelles were defined by the distribution of transferrin, rabs, lysosomal enzymes, and by the kinetics of intracellular transport of endocytosed markers such as ovalbumin and HRP. In the present study we have used this earlier method to separate endocytic fractions in cells phagocytosing beads. By isolating the bead fraction with a magnet we have been able to study the transport of labeled phagocytosed material between the phagocytic and the endocytic pathways.

To determine possible transfer of material from phagosomes to early endosomes we incubated J774 cells with $^{125}$ITC-OVA-Dynabeads for 15 minutes and chased for 15 minutes or 105 minutes The cells were in addition incubated with HRP (0.5 mg/ml) for 5 minutes at the end of the chase period, to label EE. The cells were homogenized and the bead fraction and the gold fraction were isolated as described in Materials and Methods. The remaining PNS was loaded at the top of a Percoll gradient. Following centrifugation a significant amount of radioactivity remained at the top of the gradient together with the HRP (added 5 minutes earlier) (Fig. 2A). However, when loading the PNS at the bottom of the gradient, the HRP ascended to the top of the gradient whereas the radioactivity remained at the bottom (Fig. 2B). This result indicates that the radioactivity detected at the top of the gradient originates from disrupted organelles, whereas HRP is contained in bona fide EE.

The phagosomes containing coated beads may be relatively fragile. Fractionation of cells that had endocytosed HRP and $^{125}$ITC-OVA linked beads for 2 hours showed that most of the HRP was recovered in the dense Percoll fraction and the gold fraction whereas most of the radioactivity remained in the

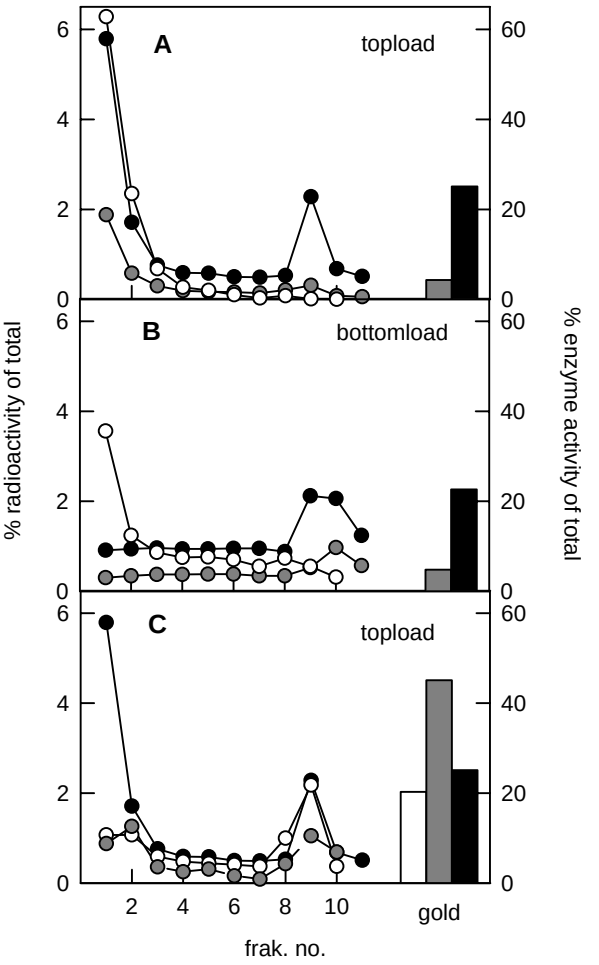


**Fig. 2.** $^{125}$ITC-OVA is not transferred from phagosomes to EE. (A and B) The cells were incubated with gold for 2 hours, then washed and incubated over night. They were then pulsed with $^{125}$ITC-OVA-Dynabeads for 15 minutes and chased for 15 minutes (gray) or 105 minutes (black). HRP (white) was added to the cells 5 minutes before the end of the incubation. The cells were homogenized and the bead fraction and the gold filled fractions were isolated as described in Materials and Methods. The remaining PNS was loaded on the top (A) or at the bottom (B) of a Percoll gradient. (C) The cells (containing gold filled lysosomes) were pulsed with HRP and $^{125}$ITC-OVA-Dynabeads for, respectively, 5 minutes and 15 minutes and chased for up to 2 hours. After homogenization the PNS was loaded at the top of a Percoll gradient. HRP (white), radioactivity (black) and βAGA activity (gray) were measured in the fractions. The results are presented as % of total recovered activity in the fractions.

loading zone (at the top of the gradient) (Fig. 2C). The distribution of the lysosomal enzyme β-acetylglucosaminidase (βAGA), was also measured in the fractions. The phagosomal fraction did not contain measurable activities of HRP or βAGA while 85% of the total radioactivity was recovered in this fraction (not shown).

Taken together these results suggest that some of the phagosomes are disrupted during homogenization and their contents are therefore recovered in the loading zone of the gradient. The results furthermore indicate that the $^{125}$ITC-OVA associated with beads internalized for 30 minutes or 2 hours is not transported to EE.

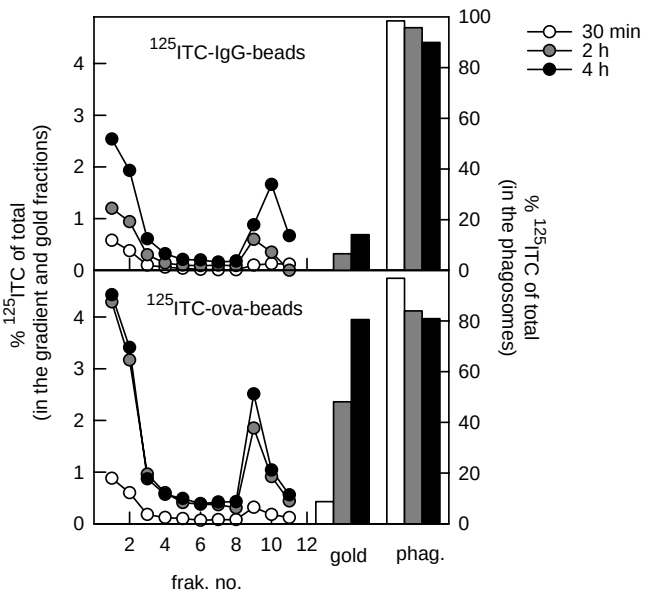


**Fig. 3.** Transfer of $^{125}$ITC-IgG or $^{125}$ITC-OVA from phagosomes to the endocytic pathway. The J774 cells (containing gold filled lysosomes) were incubated with Dynabeads coated with either $^{125}$ITC-IgG or $^{125}$ITC-OVA for 15 minutes and chased up to the indicated time periods. The cells were homogenized and the bead fraction and the gold filled fractions were isolated as described in Materials and Methods. The remaining PNS was loaded on the top of a Percoll gradient. The results are presented as % of total recovered activity in the fractions. Symbols: white, 30 minutes; gray, 2 hours; black, 4 hours.

## Transfer of ingested material from phagosomes to the endocytic pathway

The results presented so far suggested that degraded or partially degraded coat material did not enter the EE. We next tried to determine whether transport from phagosomes (the bead fraction) to LE or lysosomes took place. J774 cells were incubated with $^{125}$ITC-IgG- or $^{125}$ITC-OVA-coated Dynabeads for 15 minutes and chased for the indicated time periods. The cells were fractionated as described in Materials and Methods and radioactivity was measured in the fractions. The phagosomal fraction was treated with 1% Triton X-100 and pelleted at 13,000 rpm for 10 minutes in a microfuge. The purpose of the latter treatment was to determine whether acid precipitable peptides had been released from the beads. Triton treatment itself did not release peptides from the beads (not shown). The results in Fig. 3 indicate that the $^{125}$ITC-IgG or $^{125}$ITC-OVA linked to the Dynabeads are cleaved in the phagosome. Then, detached peptides are subsequently transferred to late endosomes and lysosomes in a time dependent manner similar to the transport of endocytosed $^{125}$ITC-OVA (Tjelle et al., 1996). We observed a significant amount of radioactivity at the top of the gradients. As described, this activity is released from disrupted phagosomes.

During a time course of 4 hours $^{125}$ITC-OVA and $^{125}$ITC-IgG were progressively cleaved off the beads and recovered as released products in the phagosomes and in the endocytic pathway. The proportion of detached $^{125}$ITC-OVA increased up to about 35% of total and then leveled off (Fig. 4). Less $^{125}$ITC-IgG than $^{125}$ITC-OVA was released from the beads indicating that $^{125}$ITC-IgG is more resistant to degradation than $^{125}$ITC-OVA. However, both types of proteins were released from the beads in a time dependent manner.

## Intracellular transport of processed phagocytosed proteins

In the next set of experiments we tried to determine the sites and the kinetics of degradation of the two labeled proteins linked to the beads. By cell-fractionation we were able to determine the sites at which the degradation products from the $^{125}$ITC-labeled proteins were formed. Fig. 5 shows the kinetics of the formation of acid soluble degradation products in the different fractions. The fractions are defined as follows: the LE were obtained from the two fractions at the densest part of the gradient, the gold fraction contains the lysosomes, and the supernatant from the Triton X-100 treated bead fraction in addition to the two upper fractions from the gradient are defined as detached material in the phagosomes. The results show that detached radioactivity increases to a certain level in the phagosomes while the radioactivity continuously increases in the LE and lysosomes. In addition, more $^{125}$ITC-OVA than $^{125}$ITC-IgG was transferred to the endocytic pathway.

The different level of $^{125}$ITC-IgG and $^{125}$ITC-OVA in LE (Fig. 5) is most probably due to a more efficient processing of the $^{125}$ITC-OVA-coat in the phagosomes (see Fig. 4). In Fig. 6 the radioactivity in the phagosomes and in the two late endocytic compartments (LE and lysosomes) is related to the detached radioactivity in the three organelles. These results show that the transfer of radioactivity to the endocytic pathway follows the same kinetics for the two types of coat-proteins.

Taken together these results suggest a connection between

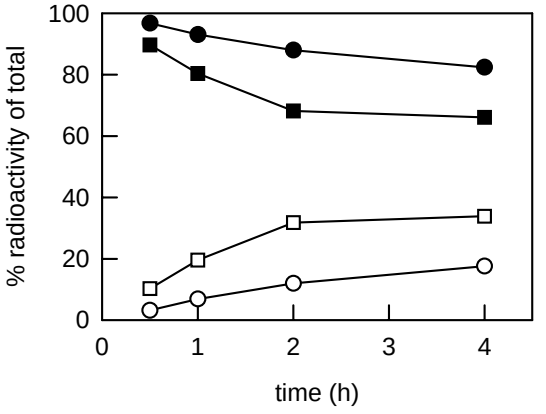


**Fig. 4.** Time-course of protein detachment from internalized Dynabeads. The J774 cells were incubated with Dynabeads coated with either $^{125}$ITC-IgG (circles) or $^{125}$ITC-OVA (squares) for 15 minutes and chased for the indicated time periods. The cells were homogenized and the bead fraction isolated as described in Materials and Methods. The bead fraction was treated with 1% Triton X-100 and the beads were pelleted by centrifugation. The data presented show radioactivity attached to the beads (solid symbols) and radioactivity dissociated from the beads (open symbols). The dissociated radioactivity includes values obtained from Triton treated bead fraction and radioactivity recovered in the PNS. The results are presented as % of total recovered activity in the bead fraction and the PNS.

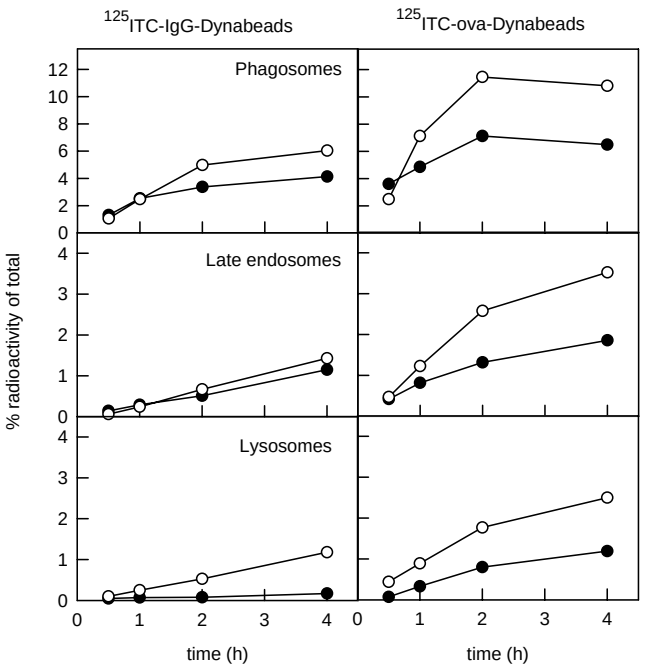


**Fig. 5.** Transport and degradation of phagocytosed proteins dissociated from the beads. The J774 cells (containing gold filled lysosomes) were incubated with Dynabeads coated with either $^{125}$ITC-IgG or $^{125}$ITC-OVA for 15 minutes and chased up to the indicated time periods. The cells were homogenized and the bead fraction and the gold filled fraction were isolated as described in Materials and Methods. The remaining PNS was loaded on top of a Percoll gradient. The phagosomal fractions were treated with 1% Triton X-100 and the beads were pelleted. The organelles were prepared as follows: The late endosomes were obtained from the two fractions at the densest part of the gradient, the gold fraction contains the lysosomes and the supernatant from the Triton X-100 treated bead fraction in addition to the two upper fractions from the gradient are defined as phagosomes. The results are presented as % acid precipitable (filled circles) and acid soluble (open circles) of total recovered radioactivity in all fractions.

the phagosomes and the late organelles in the endocytic pathway. Both partially processed and fully degraded proteins can be transferred to the late compartments. However, the level of radioactivity was higher in the LE than in the lysosomes, indicating that the LE are placed kinetically earlier in the post-phagosomal pathway.

## Late endosomes are involved in the phagocytic processing

The results obtained in the experiment presented in Fig. 5 show a significant amount of acid precipitable radioactivity in the LE. The LE may therefore participate in the processing of phagocytosed material. To rule out the possibility that the presence of acid precipitable radioactivity was an artifact due to the TC-labeling of the proteins, we incubated the cells with beads coated with OVA and IgG directly labeled with $^{125}$I. The results in Fig. 7 show that the proportion of acid precipitable radioactivity in the different organelles was the same for both types of labeling methods. This result indicates that degradation of phagocytic compounds may take place in endocytic organelles.

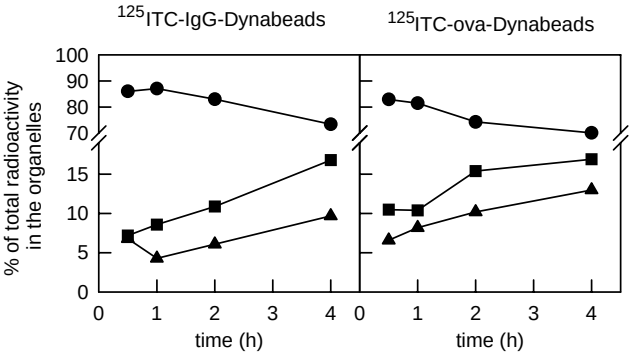


**Fig. 6.** Transport kinetics of detached $^{125}$ITC-IgG- and $^{125}$ITC-OVA from phagosomes to LE and lysosomes. The results are obtained from the experiments in Fig. 5. The results show the distribution of dissociated $^{125}$ITC-IgG and $^{125}$ITC-OVA in phagosomes (circles), LE (squares) and lysosomes (triangles) for the indicated time periods. The proportions of radioactivity in the fractions are presented as % of total radioactivity in the three organelles.

## The detachment and degradation of $^{125}$ITC-OVA from the beads is mediated by proteases in an acidic environment

We wanted to investigate by which mechanism the protein-coat of the beads was degraded. The cells (loaded with colloidal gold) were incubated with or without leupeptin, a thiol-protease inhibitor, for 30 minutes before pulsing the cells with $^{125}$ITC-OVA-beads for 15 minutes and chasing for 3 hours. Leupeptin was present in both the pulse and the chase medium. The cells were homogenized and treated as described in Materials and Methods. We observed that the detachment of $^{125}$ITC-OVA was inhibited by about 65% while the total degradation in the presence of leupeptin was only 20% of control values (Fig. 8A). The transport of the detached and degraded peptides was not inhibited as seen by comparing the proportion of detached radioactivity in the different organelles (Fig. 8C).

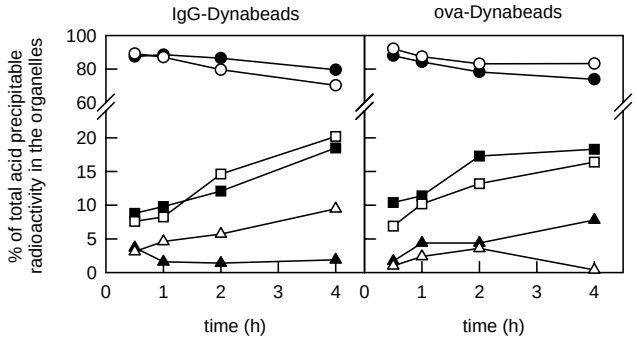


**Fig. 7.** Contribution of LE in the phagocytic processing. The results are obtained from the experiments in Fig. 5 using TC-labeled proteins linked to the beads (solid symbols) and from an identical experiment using beads coated with directly labeled proteins (open symbols). The results show the distribution of acid precipitable IgG and OVA in the phagosomes (circles), LE (squares) and lysosomes (triangles) for the indicated time periods. The proportions of radioactivity in the fractions are presented as % of total acid precipitable radioactivity in the three organelles.

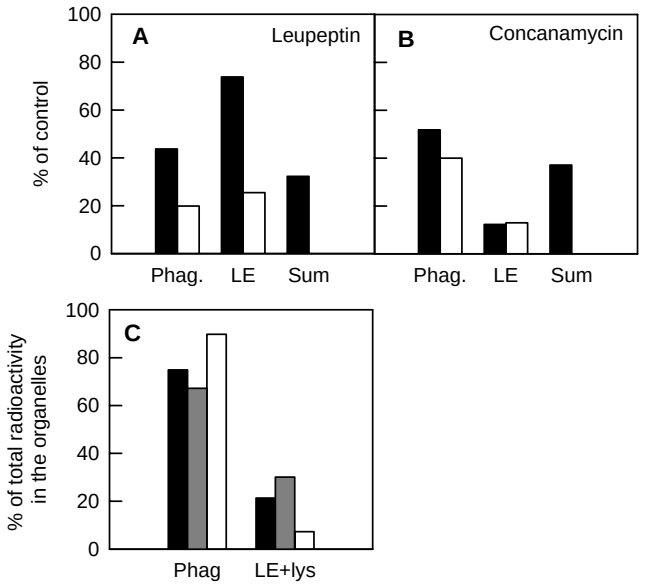


**Fig. 8.** The degradation in the phagosomes is dependent on protease activity and an acidic environment. J774 cells (containing gold filled lysosomes) were preincubated with or without leupeptin (0.1 mg/ml) for 30 minutes The cells were then pulsed with $^{125}$ITC-OVA-beads for 15 minutes and chased for 3 hours in the presence or absence of leupeptin (A) or concanamycin (B). The cells were homogenized and the bead fraction and the gold filled fraction were isolated as described in Materials and Methods. The remaining PNS was loaded on top of a Percoll gradient. The phagosomal fractions were treated with 1% Triton X-100 and the beads were pelleted. The organelles were prepared as described in the legend to Fig. 5. The results are presented as % acid precipitable (filled bars) and acid soluble (open bars) radioactivity in phagosomes (Phag.) and LE of respectively the acid precipitable and soluble radioactivity in the organelles in the control cells or % total radioactivity in the organelles (Sum) of that recovered in the organelles in the control cells. (C) The results are obtained from the experiment in A and B. The proportions of radioactivity in the organelles from the control cells (filled bars) and from cells treated with leupeptin (gray bars) or concanamycin A (open bars) are presented as % of total radioactivity in the three organelles from the cells.

A similar effect was obtained by treatment with concanamycin A, a drug which inhibits the vacuolar ATPases and thereby leads to increased pH within the organelles. Concanamycin A inhibited the internalization of coated beads (not shown). We therefore pulsed the cells with $^{125}$ITC-OVA-beads for 15 minutes without concanamycin and chased for 3 hours in the presence or absence of the inhibitor. In these experiments the total amount of detached proteins from the beads was reduced by 65% in the presence of concanamycin A (Fig. 8B). In contrast to the treatment with leupeptin, concanamycin seemed to inhibit the transport of peptides from phagosomes to the endocytic organelles. The result in Fig. 8C shows that the amount $^{125}$ITC-OVA in LE was reduced by 70% in the presence of the drug.

## DISCUSSION

Macrophages have the ability to internalize bacteria and large particles into phagosomes that usually acquire lysosomal hydrolases that eventually degrade the phagocytosed material. Efficient degradation requires an acidic environment and acid hydrolase activity. It is therefore likely that the degradation in phagosomes utilizes the same source of hydrolases as the degradation in the endocytic pathway. Proteases involved in the phagosomal degradation may be transported from EE and LE although the bulk of lysosomal enzymes probably are transferred from lysosomes (V. Claus, unpublished; Tardieux et al., 1992; Oh and Swanson, 1996). It has been suggested that the maturation of the phagosome involves multiple fusion and fission events with endocytic organelles, often referred to as the ‘kiss-and-run’ hypothesis (Desjardins et al., 1994b; Desjardins, 1995). Fusion between phagosomes and late endosomes has been observed in intact cells (Rabinowitz et al., 1992) while both EE, LE and lysosomes have been shown to fuse with phagosomes in cell free systems (A. Jahraus, unpublished; Mayorga et al., 1991). However, few studies have been done to characterize the phagocytic degradation process and the transport of substrates from phagosomes to the endocytic pathway.

In the present study we have followed the phagocytosis of magnetic Dynabeads in J774 mouse macrophages. We have utilized the magnetic properties of the bead, a density shift method by loading cells with colloidal gold overnight and subcellular fractionation of the remaining postnuclear supernatant in order to separate endocytic and phagocytic organelles involved in the processing of phagocytosed material. A dense fraction from the Percoll gradient and the gold filled fraction have previously been identified as, respectively, late endosomes and lysosomes (Tjelle et al., 1996). The bead fraction is defined as phagosomes.

The beads used in the experiments were opsonized with either mouse IgG or ovalbumin. These proteins were labeled with $^{125}$ITC and their degradation in phagosomes could therefore be measured. J774 cells ingested the beads and the protein coat was degraded. In most of our experiments we used proteins labeled with $^{125}$ITC in order to trap the degradation products in the organelles. However, the degradation of $^{125}$ITC labeled proteins measured as acid soluble radioactivity was lower than that of $^{125}$I labeled proteins, presumably because of sterical hindrances to proteases by the TC-linked to lysine residues. The degradation of ligands labeled by $^{125}$ITC therefore may underestimate the degradation efficiency of the phagosomes.

Previously we have shown that degradation of $^{125}$ITC-OVA in J774 cells is mainly taking place in LE (Tjelle et al., 1996). For proteins linked to phagocytosed beads we discovered a similar pattern. Both $^{125}$ITC-IgG and $^{125}$ITC-OVA linked to beads were degraded in phagosomes and subsequently the peptides were efficiently transported to LE and subsequently to lysosomes. Degradation must necessarily commence before transport to endocytic organelles could take place. V. Claus et al. (submitted) have shown that the level of lysosomal enzymes increases in 2-hour-old phagosomes suggesting bulk fusion of phagosomes with lysosomes. However, according to the transfer-kinetics shown in this paper, proteolytic activity seems to occur prior to this fusion event. The proteases utilized for this processing could be provided from EE or LE (Berg et al., 1995).

The proportion of labeled proteins transferred from the phagosomes to late endocytic compartments is lower for

$^{125}$ITC-IgG than for $^{125}$ITC-OVA. However, this difference was due to a more efficient degradation of the latter protein rather than to a different transport kinetics of the degradation products of the two types of proteins. In addition to fully degraded peptides (measured as acid soluble radioactivity) transferred from the phagosomes to LE up to 20% of the total acid precipitable radioactivity detached from the beads was recovered in the LE. This result suggests that peptides of different lengths are transferred by bulk flow to LE where further processing may take place. We did not determine the size of the transferred acid precipitable peptides and those retained in the phagosomal fraction. It has been shown that the kinetics of transport from endosomes to phagosomes is inversely related to the size of the transported material (Wang and Goren, 1987).

The release of the proteins linked to the phagocytosed beads required protease activity as it was inhibited in the presence of leupeptin, a thiol protease inhibitor. Proteolysis was also inhibited by concanamycin A treatment, a vacuolar ATPase inhibitor. Inactivation of vacuolar ATPases by bafilomycin $A_1$ has been reported to inhibit both degradation and transport in the endocytic pathway (Tjelle et al., 1996; van Weert et al., 1995; Aniento et al., 1996). In J774 cells the presence of concanamycin A inhibited both detachment and degradation of phagocytic compounds to the same extent (about 35% of control cells). This result suggests that the initial cleavage from the beads is pH sensitive. Inactivation of vacuolar ATPases has also been reported to inhibit transport in the endocytic pathway (van Weert et al., 1995; Tjelle et al., 1996; Aniento et al., 1996). In agreement with this we found that transport of detached $^{125}$ITC-OVA from the phagosomes to LE was reduced by about 65%.

It may be argued that during homogenization tubular phagosomes may disrupt and form artificial populations of vesicles. Such artificial vesicles may then be isolated as LE or gold-filled lysosomes in our system. Two main results suggest, however, that we are dealing with organelles separate from the phagosomes. First, the accumulation of detached radioactivity in the phagosomes follows a different pattern from that in the LE. The contents in the LE increase at the expense of the radioactivity in the phagosomes. Second, by concanamycin A treatment, we observed an inhibition of this transfer, which indicates the existence of two different organelles.

In our experiments we did not detect any transport of phagosomal compounds to the EE. These results do not rule out the possibility that phagosomes and EE communicate in J774 cells. Fusion between these two compartments has been reported in the same cell line (A. Jahraus, unpublished). Rather, we suggest that by the time significant degradation products have been formed in phagosomes (and may be available for transfer to other compartments), the phagosomal membrane is probably beyond the stage at which it is able to fuse with EE. In similar work performed by Mayorga et al. (1991), transfer of phagosomal components to EE was observed. However, in this work the protein linked to the phagocytic probe dissociated from the particle immediately after ingestion and therefore the protein was available for transfer to EE.

In the present work we have demonstrated that partially and fully processed peptides initially linked to phagocytic particles are transported to the endocytic pathway for further processing. It has been reported that phagosomes may fuse completely with endocytic organelles (A. Jahraus, unpublished; Mayorga et al., 1991; Rabinowitz et al., 1992). Nevertheless, if this were the only fusion event in the interaction between the phagosomes and endosomes, phagosomal compounds would not have been recovered in the endocytic organelles isolated by the fractionation system. We therefore suggest the transport from phagosomes to the endocytic pathway to be a transient fusion process or to be mediated by vesicles that may then fuse with LE.

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