SESSION IV

PROTOZOA: CELL INTERACTIONS

Chairman: *Dr. Jack S. Remington*

ENDOCYTIC UPTAKE OF PARTICLES BY MONONUCLEAR PHAGOCYTES AND THE PENETRATION OF OBLIGATE INTRACELLULAR PARASITES*

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As William Trager pointed out some time ago in a review in *Science*,¹ all obligate intracellular parasites—be they viral, bacterial, or protozoan—face a common dilemma. That dilemma is to invade their host cells in a way that is not destructive of the host cell upon whose metabolic hospitality and functional well-being their own reproduction depends. Simply stated, these organisms must penetrate the plasma membranes of their host and take up residence in a suitable location in the cell's cytoplasm. Since many of the speakers in this session will address themselves to the issues of penetration and intracellular location of specific organisms, I view my task as one of trying to place these issues into a general conceptual framework.

There are three general paths an intracellular parasite might follow to gain entry into an animal cell (Fig. 1): *a*) direct passage of the parasite through the host cell's plasma membrane; *b*) fusion of the outer membrane of the parasite with the cell's plasma membrane; *c*) endocytosis of the parasite by the host cell.

With the possible exceptions of the sporidium *Nosema*,¹ and of the larval forms of *Trichinella spiralis*,² I know of no case where there is good evidence for direct passage of an infectious microorganism through the surface of a eucaryotic cell. Similarly, with the exception of the parainfluenza viruses, and perhaps one or two other enveloped animal viruses, no other microorganisms appear to fuse their surface membranes with the membranes of the cells they infect. Thus, most intracellular parasites, from viruses to protozoa, penetrate their host cells as the Athenians entered Troy. In the case of these organisms the vehicle is an endocytic vacuole, not a horse.

The successful entry of many microorganisms

into their host cells is dependent upon the presence of appropriate surface receptor molecules on the surface of the host cell and on ligands which bind to those receptors on the surface of the infectious agent. For instance, animal viruses such as polio infect human but not mouse cells. Human cells have on their surfaces genetically defined membrane receptors for polio virus. Mouse cells lack these receptors. Mouse cells are capable of replicating polio virus since they produce the virus when they are infected with polio virus RNA. Thus, it is the absence of appropriate surface receptors that prevents polio virus growth in mouse cells. Surface receptors play important roles in governing susceptibility to other intracellular parasites and Drs. Nogueira and Miller will have more to say about the role of other membrane receptors in the uptake of trypanosomes and malaria.

How do surface receptors function to stimulate the endocytosis of particulate materials? My colleagues—Drs. Frank Griffin, Johanna Griffin, Celso Bianco and Josef Michl—and I have been examining this question. We have used IgG and complement-coated red blood cells as the phagocytic test objects, and mouse macrophages, cells that have plasma membrane receptors for IgG and complement, as the phagocytic cells.

At the outset I want to emphasize that the presence of membrane receptors for a given ligand is not the sole determinant of whether a ligand-coated particle is ingested by a phagocytic cell. For instance, peritoneal macrophages from normal mice bind but do not ingest complement coated red cells while macrophages from mice given an intraperitoneal injection of thioglycollate medium are capable of ingesting complement coated red cells.³ Thus, both the presence of specific ligands (i.e., complement) on the surface of the red cells and the physiological state of the macrophages determine the outcome of the interaction between the test particles and these phagocytes.

* Supported by Grant AI 08697 from the National Institute of Allergy and Infectious Diseases.

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FIGURE 1. Pathways of entry of intracellular parasites into their host cells. **a**, direct penetration of the plasma membrane; **b**, fusion of the membrane of the invading organism with the host cell's membrane; **c**, endocytosis of the invading organism in a vacuole derived from the host cell's plasma membrane.

How do membrane receptors and particle bound ligands cooperate to promote particle uptake? In order for a particle to be ingested by a phagocytic cell, the particle must be coated throughout its surface with appropriate ligands (Fig. 2a). Similarly, the phagocytic cell must have distributed over its surface receptors that bind this ligand. We have proposed that the ingestion of the particle results from the sequential and circumferential interaction of receptors on the phagocyte's surface with ligands on the particle's surface, and that these particle bound ligands form a template that guides the phagocyte's membrane^{4,5} (Fig. 2b and c). We call this the zipper mechanism of phagocytosis. Let me illustrate why we think phagocytosis is regulated by a zipper mechanism.

Taylor et al.⁶ showed that when bone marrow-derived lymphocytes (B-cells) are incubated at 4°C with fluorescein-labeled antibodies to immunoglobulins these labeled antibodies diffusely coat the surface of these cells. When these diffusely coated cells are warmed to 25–37°C the antibodies migrate to one pole of the lymphocyte's surface and form a cap. We used both diffusely coated and capped lymphocytes as phagocytic test particles to study whether the distribution of ligands on the surface of a particle is a critical determinant of ingestion of the particle by mouse macrophages. Our experiments show that it is. When B-lymphocytes that were diffusely coated with anti-immunoglobulins were incubated at 37°C with mouse macrophages, >99% of the macrophages bound more than 10 lymphocytes each, and 71% of the macrophages ingested an average

FIGURE 2. Diagram of the zipper mechanism of phagocytosis. **a**, particle diffusely coated with ligands attaches to membrane receptors on the surface of a phagocytic cell; **b** and **c**, additional receptor ligand interactions guide the movement of the cell's plasma membrane over the particle's surface.

of 2.2 lymphocytes each (Fig. 3b). In contrast, when B-lymphocytes that were capped were added to macrophages under similar conditions >99% of the macrophages bound an average of 8 lymphocytes each but there was virtually no ingestion of these capped lymphocytes (Fig. 3a).

To further characterize the interaction of these capped lymphocytes with the macrophage membrane we used peroxidase-labeled anti-immunoglobulins to visualize the anti-immunoglobulin cap by electron microscopy (Fig. 4). These studies confirmed that the lymphocytes were bound to the macrophages only in the region of the anti-immunoglobulin cap and that the macrophage membrane was attached to the lymphocyte surface only in areas containing peroxidase labeled anti-immunoglobulins.

In these experiments we noted that a filamentous network and area of organelle exclusion forms in the macrophage cytoplasm directly adjacent to the zone of contact between the macrophage and the anti-immunoglobulin treated capped lymphocyte. A similar filamentous network was seen in macrophages ingesting IgG coated red cells. We presume that these filamentous networks are zones of contractile protein (e.g., actin and myosin) aggregation and that these contractile proteins generate the motile force required to move the macrophage membrane around a particle.

Based upon these findings we have proposed the following model for receptor mediated phagocytosis of IgG or complement coated particles by macrophages (Fig. 5). IgG or complement bind particles to specific receptors for these ligands on the membranes of phagocytic cells (Fig. 5a). The initial ligand-receptor interaction generates a signal that initiates the aggregation of contractile proteins and leads to the extension of pseudopods in the area of the attached particle (Fig. 5b). Receptors on the membrane of these pseudopods bind to additional ligands on the particle's surface resulting in the generation of additional signals, continued aggregation of cytoplasmic contractile proteins, and further pseudopod extension (Fig. 5c). The process repeats many times until the membranes of the advancing pseudopods meet and fuse with one another to form the phagocytic vacuole. Although my colleagues and I proposed this mechanism to explain the ingestion of immune ligand-coated particles, it seems possible that a similar mechanism is responsible for the entry of a variety of intracellular parasites into their host cells. In the case of organisms such as *Plasmodium* and *Babesia* that enter nonphagocytic red blood cells, it is conceivable that the force necessary to move the host cell membrane around the parasite is generated by contractile elements in the parasite and not in the red blood cells.

What is the evidence that the interaction of particle-bound ligands with their corresponding receptors or the macrophage membrane initiates specific intracellular signals? We have found that the glucose and mannose analog 2-deoxyglucose reversibly inhibits phagocytosis mediated by the Fc and complement receptors of mouse macrophages but has no inhibitory effect on the ingestion of latex and zymosan particles by these cells.

FIGURE 3. Phase contrast micrograph of the interaction of mouse bone marrow-derived lymphocytes coated with rabbit anti-mouse immunoglobulin IgG with mouse macrophages. a, lymphocytes were incubated with anti-mouse immunoglobulin IgG for 30 min at 4°C. The lymphocytes were then washed, incubated at 25°C for 30 min to allow capping, and incubated with mouse macrophages for 10 min at 4°C to allow attachment, and for 30 min at 37°C. Most of the macrophages have lymphocytes attached to their plasma membranes; b, lymphocytes were incubated for 30 min at 4°C with anti-mouse immunoglobulin IgG. They were then washed and incubated with mouse macrophages as described in (a). Phagocytized lymphocytes are indicated by arrows. (Reproduced with permission from Griffin et al., 1976, *Journal of Experimental Medicine*.⁶)

2-Deoxyglucose does not inhibit the binding of IgG on complement coated particles to macrophages. Moreover, the inhibitory effect of 2-deoxyglucose can be reversed by addition of mannose or glucose to the medium (Fig. 6 and ref. 7).

2-Deoxyglucose inhibits glycolysis and adenosine triphosphate (ATP) generation in a variety of cell types. Since phagocytosis is an energy-requiring process it seemed possible that 2-deoxyglucose inhibited the uptake of IgG and complement coated particles by decreasing cellular ATP

FIGURE 4. Capped lymphocytes were prepared as in Figure 3 and then incubated with peroxidase-labeled sheep anti-rabbit immunoglobulin IgG at 4°C. The cells were then washed and incubated with mouse macrophages for 30 min at 37°C. The macrophage-lymphocyte complexes were then fixed, incubated with H_2O_2 and diaminobenzidine to visualize the peroxidase-labeled antibodies, and further processed for electron microscopy. Note that the macrophage is attached to the lymphocyte only in the region of the diaminobenzidine-stained cap. There is also a massive accumulation of randomly arranged microfilaments in the macrophage cytoplasm exclusively in the zone of macrophage-lymphocyte interaction. Rough endoplasmic reticulum, ribosomes, mitochondria, and other cellular organelles are almost entirely excluded from this filamentous zone of macrophage cytoplasm. Diaminobenzidine-stained material is present within vacuoles (asterisks) of the macrophage, and on the tubular invaginations (arrows) of the macrophage plasma membrane. (Reproduced with permission from Griffin et al., 1976, *Journal of Experimental Medicine*.⁵)

levels below some threshold required for their ingestion. To examine this possibility we measured the ATP levels in cells treated with 2-deoxyglucose. Macrophage ATP levels decreased to ~25% of control levels after 4 h in 2-deoxyglucose medium. Addition of mannose or glucose to the medium of these 2-deoxyglucose treated macrophages did not elevate cellular ATP levels but caused a prompt reversal of the inhibitory effect of 2-deoxyglucose on Fc and complement receptor mediated phagocytosis (Fig. 6). Thus, lowered cellular ATP levels cannot account for the decrease

in receptor mediated phagocytosis in 2-deoxyglucose treated cells.⁸

In summary, these experiments show that 2-deoxyglucose acts upon some step subsequent to the binding of ligands to their corresponding receptors on the macrophage membrane. Since 2-deoxyglucose does not inhibit ingestion of latex or zymosan particles it is evident that 2-deoxyglucose is not an inhibitor of phagocytosis per se. Rather, it seems likely that 2-deoxyglucose acts to uncouple macrophage membrane receptors from the cell's phagocytic apparatus.

*Events following formation
of the endocytic vacuole*

Once the parasite is within the endocytic vacuole its task is not necessarily complete. As illustrated in Figure 7a, some intracellular parasites—e.g. vaccinia virus,⁹ *Babesia*,¹⁰ trypanosomes¹¹—lyse the endocytic vacuole and enter the cytoplasmic matrix where they replicate. Other organisms, such as *Chlamydia*,¹² *Toxoplasma*,¹³ and to some extent tubercle bacilli,¹⁴ prevent fusion of the endocytic vacuole with host cell lysosomes (Fig. 7b). *Chlamydia* and *Toxoplasma* replicate within these vacuoles.

Modification of the surface properties of some of these organisms, as by coating them with specific antibodies, alters their intracellular fate. Antibody-coated vaccinia virus is unable to escape the endocytic vacuole and is sequestered with host cell lysosomes.^{15, 16} Similarly, endocytic vacuoles containing antibody coated *Toxoplasma* also fuse with host cell lysosomes. Under these circumstances the organisms are degraded by lysosomal hydrolases.

Finally there is a group of organisms—reoviruses,¹⁷ *Leishmania*,¹⁸ mycobacteria—that enter the lysosomal compartment as part of their normal life cycles (Fig. 7c). These organisms are not destroyed by the hydrolases in this compartment and in the case of mycobacteria and *Leishmania*, are able to replicate within it.

How do organisms that escape the endocytic vacuole, or that prevent its fusion with lysosomes accomplish this task? Unfortunately we do not know. It is possible that the endocytic vacuole within which these organisms enter the cell is not composed of a random array of plasma membrane lipids and proteins. Rather, the interaction of ligands on the parasite's surface with cell surface receptors may concentrate some cell surface proteins within the vacuole and cause others to be excluded. The capacity of B lymphocytes to endocytize an immunoglobulin cap is an excellent example of the selective removal of a single cell surface molecule by virtue of its interaction with a ligand. Intracellular parasites may have adopted similar strategies to enter the cell within vacuoles whose membrane composition is specifically suited to the eventual intracellular fate of the parasite, or to its metabolic requirements.

Can specific ligands alter the intracellular fate of endocytic vacuoles? Paul Edelson and Zanvil

FIGURE 5. Model of proposed phagocytic mechanism (see text).

Cohn have shown that they can. When mouse macrophages are treated with the plant lectin concanavalin A, they are stimulated to endocytize. The lectin-containing endocytic vacuoles enter the cytoplasmic matrix but do not fuse with lysosomes. Proteins contained within these concanavalin A-induced vacuoles are not exposed to lysosomal enzymes and are degraded very slowly.^{19, 20}

Addition of mannose to the culture medium causes the dissociation of concanavalin A from the phagosome membrane. Under these circumstances host cell lysosomes fuse with the concanavalin A-induced vacuoles and proteins contained within these vacuoles are rapidly degraded.²⁰ Thus, the entry of molecules or particles into each cellular compartment is regulated by specific signals arising at the membrane of that compartment. It is likely that the microorganisms we are discussing have evolved to exploit one or more of these specific steps in the endocytic pathway.

FIGURE 6. Macrophages were incubated in medium containing 50 mM 2-deoxyglucose and 5.5 mM glucose. **a**, at various times after addition of 2-deoxyglucose the macrophages were harvested and assayed for ATP. After 2 h at 37°C the medium was changed and the macrophages were incubated further in medium containing 5×10^{-3} M glucose, or in medium containing 50 mM 2-deoxyglucose, 5.5 mM glucose, and 50 mM of another hexose, as indicated; **b**, treatment of macrophages was the same as that described in (a) except that IgG-coated sheep red blood cells were added to the cultures 1 h prior to the time indicated by the closed circles. The number of red cells ingested in control culture (5.5 mM glucose at 0 h) is compared with the number of red cells ingested in macrophages incubated for 2 h in medium containing either 5 mM glucose, or 50 mM 2-deoxyglucose and 55 mM glucose, or 50 mM 2-deoxyglucose, 50 mM mannose, and 5.5 mM glucose. (Reproduced with permission from Michl et al., 1976, *Journal of Experimental Medicine*.)

The differentiation of mononuclear phagocytes

Mononuclear phagocytes exist in a variety of physiological states. Thus we have the epithelioid cell, the histocyte, the "angry" macrophage, and the "activated" macrophage, to name only a few. These cells show marked functional differences depending upon their source and their previous encounters with one or more mediators of differentiation. Figure 8 is an attempt to compare some of the properties of these various states of macrophage differentiation.

The macrophages are derived from blood monocytes, which are derived, in turn, from bone marrow precursors. Under steady state conditions there is a slow influx of blood monocytes into tissue spaces and body cavities where these cells

differentiate into macrophages. For the purposes of this discussion I have termed unperturbed resident macrophages, tissue macrophages. Following an intraperitoneal injection of a chemical irritant, such as thioglycollate medium, there is an increased influx of blood monocytes into the mouse peritoneal cavity. These monocytes rapidly differentiate into what I have termed "inflammatory" macrophages. Inflammatory macrophages show both qualitative and quantitative differences from tissue macrophages. The inflammatory cells are larger, contain a higher level of lysosomal enzymes, exhibit an enhanced rate of pinocytosis and spread more rapidly on surfaces. They express several activities that are absent in the tissue macrophage, such as secretion of plasmino-

FIGURE 7. Pathways via which intracellular parasites reach their site of replication within the host cell's cytoplasm. **a**, entry within a phagocytic vacuole after which the invading organism lyses the vacuolar membrane and is released into the cytoplasmic matrix; **b**, the invading organism enters within a phagocytic vacuole and inhibits fusion of the vacuole with host cell lysosomes. Replication of the parasite occurs within this vacuole; **c**, the invading organism enters within a phagocytic vacuole. The vacuole fuses with host cell lysosomes but the organism is not destroyed by exposure to the pH or enzymes in this compartment.

gen activators and the capacity to phagocytize via their complement receptors. However, the inflammatory macrophage lacks several properties expressed by the lymphokine stimulated macrophage, or by the macrophage elicited by a chronic infection such as with BCG. I have termed these lymphokine or BCG stimulated cells "microbistatic/cidal" macrophages. BCG-stimulated macrophages have enhanced microbicidal and tumoricidal properties as shown by Mackaness,²¹ Remington et al.,²² Hibbs,²³ and others. These functional differences probably reflect the effects of specific inducing agents upon the differentiation of monocytes into macrophages, and of macrophages into cytotoxic effector cells. It is evident that given appropriate stimuli the tissue macrophage can differentiate into an inflammatory macrophage. Whether inflammatory macrophages develop into microbicidal macrophages, or whether a separate pathway of differentiation from the monocyte is required, is uncertain.

Moreover, we do not know which of the many capacities of the macrophage are responsible for the ability of these cells to resist intracellular parasitism. Until such information is available it might be wise to drop such adjectives as the "activated" macrophage and confine ourselves

FIGURE 8. Changes in cellular function during macrophage differentiation. A variety of agents, indicated in the upper right hand corner of the diagram, influence the differentiation patterns of macrophages. These agents may act at the level of the bone marrow (promonocyte), blood (monocyte), or tissue (macrophage). It is unclear whether macrophages follow the pathway monocyte → tissue macrophage → inflammatory macrophage → microbistatic/cidal macrophage or whether monocytes differentiate directly into inflammatory and microbistatic/cidal macrophages.

to more descriptive terms—e.g., thioglycollate medium-elicited macrophages, BCG-elicited macrophages, lymphokine-treated macrophages, etc. Whatever we name them, it is clear that the term mononuclear phagocytes encompasses a lineage of cells with widely varying functional properties.

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DISCUSSION

Askenase: Where does the Fc monomer and Fc aggregate receptor fit into the scheme of the macrophage differentiation? Are there changes in affinity or distribution of either Fc receptor with macrophage activation or differentiation?

Silverstein: Unkeless and Eisen (*J. Exp. Med.*, 142: 1520, 1975) examined the number of trypsin-sensitive receptors for Ig2a monomers on normal and thioglycollate-elicited mouse macrophages. They showed that thioglycollate macrophages have $\sim 4.4 \times 10^5$ Ig2a receptors per cell compared with $\sim 1.1 \times 10^5$ Ig2a receptors on normal macrophages. There was no difference in the binding affinity of these receptors. In a subsequent publication, Unkeless (*J. Exp. Med.*, 145: 931, 1977) showed that thioglycollate-elicited macrophages have $5-8 \times 10^5$ trypsin-resistant receptors per cell for antigen-antibody complexes. He did not measure the number of anti-

gen-antibody receptors in normal macrophages. Arend and Mannik (*J. Immunol.*, 110: 1455, 1973) found roughly the same number of receptors for antigen-antibody complexes on normal and stimulated rabbit macrophages (i.e., 1.2×10^6 and 2.1×10^6 receptors per cell, respectively). Thus, the number of Fc receptors appears unchanged following macrophage stimulation. Similarly, no marked differences in their affinity constants have been noted.

Bianco et al. (*J. Exp. Med.*, 141: 1278, 1975) found that in vitro cultivation of macrophages enhanced their capacity to ingest IgG-coated red cells. No measurements were made of receptor number, however. Thus we have no evidence that the number or the affinity of either the trypsin-sensitive or resistant Fc receptor changes with macrophage differentiation, even though the functional activity of these receptors appears to increase.

COMMENT ON THE PAPER ON UPTAKE AND
INTRACELLULAR FATE OF *Trypanosoma cruzi*
BY N. NOGUEIRA AND Z. COHN (SEE PAGE 194)
S. Silverstein

The experience of animal virologists in studying the neutralization of animal viruses may be instructive here. Antibody to viral surface antigens does not necessarily neutralize. This is so for antibodies directed against the neuraminidase of influenza virus, for instance. Antibodies directed against other surface components (e.g., the hemagglutinin of influenza virus) do neutralize the infectivity of the virus. In the case of Herpes viruses, antibodies may neutralize virus poorly, but addition of serum complement markedly enhances virus neutralization.

Antibody-coated viruses adsorb poorly to epithelial cells and fibroblasts but are rapidly taken up by macrophages. These antibody-coated virus particles attach to the macrophages via the latter cell's Fc-receptors. In this case the antibody-coated viruses are sequestered in macrophage lysosomes where they are degraded.

A parallel situation may prevail in the case of

several intracellular parasites. First, antibodies to the parasite may not be directed to the surface components which mediate attachment and penetration of the parasite. If the antibodies do "neutralize" the putative specific attachment components, the parasite may still attach to and penetrate cells bearing Fc receptors—i.e., macrophages.

Coating of epimastigotes of trypanosomes with antibodies will presumably not affect their intracellular fate in macrophages since, as Dr. Nogueira has shown, they enter secondary lysosomes in the absence of antibodies. We do not know whether antibodies against trypomastigotes will redirect these more infectious forms into the lysosomal compartment where they may be destroyed.

Thus antibodies may have variable effects depending on the specific surface antigens to which they are directed, the presence of serum complement, the cell type which acts as host for the virus or parasite, and the intracellular compartment within which the virus or the parasite initiates its function.