The interaction of particulate horseradish peroxidase (hrp)-anti hrp immune complexes with mouse peritoneal macrophages in vitro

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THE INTERACTION OF PARTICULATE HORSE RADISH 
PEROXIDASE (HRP)-ANTI HRP IMMUNE COMPLEXES 
WITH MOUSE PERITONEAL MACROPHAGES IN VITRO

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ABSTRACT

The uptake, distribution, and fate of particulate horseradish peroxidase (HRP)-anti HRP aggregates has been studied in homogeneous monolayers of mouse macrophages in vitro. Macrophages rapidly interiorize the immune complexes after binding to the cell surface. The rate of interiorization is maximal for complexes formed in a broad zone of 4-fold antibody excess to equivalence and corresponds to a rate of 10% of the administered load/10^6 cells per hour. This rate is 4000-fold greater than the uptake of soluble HRP. The binding and endocytosis of HRP-anti HRP by macrophages is mediated by the trypsin insensitive F, receptor. Cytochemically, intracellular HRP is localized within membrane bound vacuoles. After uptake of HRP, the enzymatic activity is degraded exponentially with a half-life of 14-18 hr until enzyme is no longer detectable. This half-life is twice as long as that previously observed for soluble uncomplexed HRP and is related to the combination of HRP with anti-HRP rather than the absolute amounts of enzyme or antibody ingested. The half-life of HRP-125I was 30 hr. Exocytosis of cell associated enzyme or TCA precipitable counts was not detected, nor were persistent surface complexes demonstrable. The extensive capacity of macrophages to interiorize and destroy large amounts of antigen after the formation of antibody illustrates a role of this cell in the efferent limb of the immune response.

INTRODUCTION

The formation of immune complexes has long been considered a major determinant of host defense to foreign antigens and has been implicated in several other immunological events. These include a regulatory role for antibody formation (43), the establishment and/or maintenance of immunological memory (19, 44), and the triggering of the lymphocyte mediated destruction of target cells (31). In addition, the deposition of antigen-antibody complexes in tissues may initiate events leading to inflammatory disease (11, 33, 45).

The fate of immune complexes, has in most instances, been defined by their clearance from the intravascular compartment and is often ascribed to the mononuclear phagocytes of the reticuloendothelial system. It is clear, however, that other cell types, e.g., neutrophilic and eosinophilic leukocytes, endothelial cells, may participate in vivo, that organ blood flow plays an important role, and that it is difficult to distinguish the attachment and ingestion phases of phagocytosis under these conditions. A more precise understanding of the uptake and fate of immune complexes by macrophages therefore requires a homogenous cell population which can be manipulated in vitro.

In this article we describe studies on the in-
teration of particulate horseradish peroxidase-antiperoxidase aggregates (HRP-anti HRP) with monolayers of mouse macrophages. The retention of peroxidatic activity when complexed with specific antibodies has allowed us to follow the binding, ingestion, distribution, and intracellular fate of the immune complexes. These data differ markedly from our previous studies employing soluble uncomplexed enzyme (41) and point out those determinants which distinguish the phagocytic and phagocytic variants of endocytosis.

MATERIALS AND METHODS

Chemicals

Horseradish peroxidase (HRP), o-dianisidine, diaminobenzidine (Sigma Chemical Co., St. Louis, Mo.); hydrogen peroxide (Superoxol, Merck & Co., Inc., Rahway, N. J.); carrier free Na125I (New England Nuclear Corp., Boston, Mass.); newborn calf serum (NBCS), medium 199 (Grand Island Biological Co., Grand Island, N. Y.); complete Freund's adjuvant (Baltimore Biological Laboratories, Baltimore, Md.); trypsin and pepsin (Parke, Davis & Co., Detroit, Mich.); bovine serum albumin (BSA) (Armour Fraction V, Armour Industrial Chemical Co., Chicago, Ill.); twice crystallized trypsin, DFP-trypsin, and pepsin (Worthington Biochemical Corp., Freehold, N. J.); human serum albumin, rabbit gamma globulin (Cohn Fx2), goat anti rabbit immunoglobulin G and M (IgG, IgM), goat anti mouse immunoglobulin G (Miles Laboratories, Inc., Elk- hart, Ind.).

Horseradish Peroxidase (HRP)

Sigma type II enzyme was used for most experiments. Detailed methodology has been outlined previously (41). In brief, the amount of HRP was quantitated by an enzymatic assay using o-dianisidine and hydrogen peroxide as substrates. This assay detects as little as 1 ng/ml of soluble HRP. Purified HRP, e.g., Sigma type VI, was trace radiolabeled with carrier free Na125I using lactoperoxidase and hydrogen peroxide. Some 20% of the protein in the heterogeneous Sigma type II preparation is non-TCA precipitable and nondialyzable. After radiolabeling, the reaction mixture was dialyzed at 4°C for 4 days and then chromatographed on a Sephadex G 150 column to remove lactoperoxidase and any remaining free iodide. Cytochemical localization of HRP was accomplished with the Graham-Karnovsky method (18) applied as in our previous study of soluble HRP in macrophages, except that the cytochemical reaction time was prolonged from 10 to 30 min. As in our earlier experiments, the cells and the "pseudomem-

brane" (5, 47) that coats the plastic culture vessels, could be removed at the propylene oxide step in the specimen processing.

Macrophages

Mouse peritoneal macrophages were harvested from unstimulated NCS mice and cultivated overnight as previously described (12) in 20% NBCS-medium 199 with 1000 units penicillin/ml. For quantitative studies, each culture contained 2 X 106 macrophages growing as a homogeneous monolayer on small plastic dishes (Nunclon). For light microscope cytochemistry, 2 X 105 cells were cultivated on circular 12-mm glass cover slips. These cultivated macrophages do not contain detectable endogenous peroxidase activity by either biochemical or cytochemical procedures.

Anti-HRP Antisera

4 kg New Zealand rabbits were immunized with 10 mg of soluble HRP administered intramuscularly in complete Freund's adjuvant in three sites. The animals were boosted with 10 mg soluble HRP in saline subcutaneously 1 month later, and then bled at 1, 2, and 3 wk. Mice were immunized with 50 µg of soluble HRP in complete Freund's intra peritoneally, and boosted 3-6 months later with 250 µg of soluble HRP in saline intraperitoneally or intravenously. 0.2 mg of tripelennamine was given intravenously immediately before the boosting dose of HRP to prevent deaths from anaphylaxis. The hyperimmune rabbit and mouse antiserum both had hemagglutination titres of the order of 1/10,000 using glutaraldehyde-coupled HRP sheep red blood cells (2).

Rabbit anti-HRP antibodies were purified on a bovine serum albumin-HRP-glutaraldehyde immunoadsorbent as described by Avrameas and Ternynck (1). 25% of the original hemagglutinating and precipitating antibodies were eluted from the immunoadsorbent with glycine-HCl at pH 2.8. 80% of the protein in the purified preparations was precipitating anti-HRP antibody.

Immunoelectrophoresis of the anti-HRP antiserum was performed in agar in 0.1 M Veronal buffer, pH 8.2. The whole and purified rabbit antiserum contained antibodies to all five of the electrophoretically distinct components in the Sigma type II enzyme. Most of the immunoglobulin migrated as IgG, but anti-HRP activity was demonstrated in a trace IgM fraction as well, as described by Avrameas and Leduc (3).

Rabbit anti-HRP F(ab)'2 was prepared from a hyperimmune IgG fraction isolated on a diethylaminoethyl (DEAE) column. The IgG was digested with pepsin at a protein ratio of 1:100 in 0.03 M
sodium acetate buffer, pH 4.5 for 18 hr at 37°C. Proteolysis was stopped by neutralization with 0.1 N NaOH to pH 7.0. The digest was concentrated by ultrafiltration and the F(\(\text{ab}'\))\(_2\) peak isolated on a Sephadex G-200 column, the peak having a molecular weight of 110,000. The F(\(\text{ab}'\))\(_2\) was concentrated to give a hemagglutination titre similar to that seen with intact antiserum, and at this concentration, the precipitating activity of both reagents was also identical.

In some instances, anti-HRP antisera were de-complemented by the addition of nonspecific (HSA-anti HSA) immune precipitates (20), before the formation of specific HRP-anti HRP aggregates.

Preparation of Immune Complexes

Precipitation of antigen and antibody was allowed to occur for 0.5 hr at 37°C and 2 days at 4°C. An equivalence curve was constructed in which the protein content of the washed precipitates was determined by a micro-Lowry method (29), and the HRP content of the supernatants was measured by enzymatic assay. Soluble HRP-anti HRP complexes retain full enzymatic activity so that enzymatic assay of the supernatants provides a sensitive indication of the state of antigen excess. The precise equivalence point for a given antiserum was found by graphical analysis in which the ratio of antibody to antigen protein in the precipitates was plotted against the amount of antigen added to the reaction mixture (25). Equivalence is defined as the y intercept divided by twice the slope. Using purified rabbit anti-HRP, the molecular ratio of anti-HRP to HRP at equivalence is 2, assuming molecular weights of 156,000 and 40,000 for IgG and HRP, respectively. Somewhat higher ratios, 2.5–3.0, were obtained with whole rabbit and mouse antiserum. In the region of antigen excess studied (up to eight times the equivalence level of HRP), more than 90% of the nonprecipitable HRP could be completely aggregated by a goat anti-rabbit or anti-mouse IgG reagent, indicating that soluble immune complexes were present in the region of antigen excess.

Enzymatic Activity of Immune Precipitates

HRP retains enzymatic activity after immunoprecipitation, as has previously been described for insoluble immune aggregates of beef liver catalase (7, 8). 10–30% of the enzymatic activity can be recovered after dispersion of the precipitates by pipetting, while 35–70% of the free enzyme's activity can be measured if the precipitates are sonicated briefly with a Branson ultrasonication device (Branson Instruments, Co., Stamford, Conn., Model S75). For each type of immune complex administered to cells, the percentage of measurable activity was determined after brief sonication. This factor was then used to calculate the actual amount of HRP present in a sonicated unknown. This method yields data that are identical to those obtained by counting specimens trace-radiolabeled with HRP.\(^{125}\)I.

Administration of Immune Complexes to Macrophages

After precipitation in the test tube, the immune aggregates were diluted to a concentration of 0.4–2.4 \(\mu\)g of HRP/ml of culture medium. In most experiments, the medium was 5% NBCS-medium 199, but when HRP.\(^{125}\)I was present, the medium 199 was supplemented with 1 mg/ml of BSA, in order to prevent removal of label by serum deiodinases. At the dilutions employed, the HRP contained within the aggregates was not detectably solubilized over several hours. At equivalence and in the region of antibody excess studied (up to 8-fold the equivalence level of HRP), the dispersed immune aggregates had a size range of 1.0–5.0 \(\mu\) by phase microscopy. During the culture period, further aggregation seemed to occur both extracellularly and on the cells.

Uptake of HRP

Cultures of 2 \(\times\) 10\(^6\) macrophages were exposed to immune aggregates for varying periods of time (generally 2 hr). At the end of the uptake period, non-cell bound enzyme was removed by four washes with 2 ml of medium 199 over 15 min. Total cell bound HRP was then determined after lysis of cells in detergent (0.1% sodium dodecyl sulfate or 0.05% Triton X-100, both diluted in water) and sonication of the lysates. The result was expressed in terms of \(\mu\)g of HRP per unit of cell protein, as measured by a micro-Lowry method (25). 10\(^5\) macrophages cultivated for 24 hr in 20% NBCS contain 25 \(\mu\)g cell protein.

Distribution of HRP by Light Microscope Cytochemistry

Cover slip cultures were stained with the diamino- benzidine-hydrogen peroxide substrate mixture and examined by phase microscopy to determine the percentage of cells with intracellular and extracellular enzyme. Extracellular surface bound enzyme appeared as large, densely stained irregular aggregates which protruded out of the plane of focus of the cell and often away from the cell outline. Intracellular HRP was distributed in smaller highly refractile granules or in large, smooth surfaced, dense structures. At the light microscope level, very few cells could be seen to contain both intra- and extracellular enzyme (<5%) and these were scored as extra-

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cellular. In expressing distribution data at least 1000 cells were counted in duplicate cultures.

**Fate of HRP-Anti HRP in Macrophages**

The fate of cell bound HRP was followed in cultures that had been administered immune complexes for 2 hr at 37°C. After washing, the cells were maintained for 0.5 hr at 37°C in order to allow interiorization of residual surface bound enzyme. The zero time assay thus measured phagocytosed HRP whose fate was then determined in companion cultures maintained in HRP-free, 10% NBCS-medium 199.

**Trypsin Experiments**

Brief trypsinization (250-500 µg/ml medium 199 for 0.5 hr at 37°C) was used in an attempt to quantitate surface bound enzyme in cells previously exposed to HRP-anti HRP and washed well. Controls included similar quantities of BSA, DFP-trypsin, or trypsin plus an equal amount of BSA. A similar trypsinization procedure was used to assess the trypsin sensitivity of the cellular receptor for HRP-anti HRP. In this instance, cells were trypsinized and then washed before the administration of immune complexes.

**RESULTS**

**Distribution of HRP-Anti HRP After Administration to Macrophages**

As in our previous studies on the interaction of soluble HRP with macrophages in vitro (41), we sought to localize and quantitate the relative amounts of HRP-anti HRP complexes in four compartments: free in the culture medium, bound to the surface of the culture vessel, attached to the surface of the macrophage, and interiorized within cells. These distribution studies were performed on monolayers of 2 × 10⁶ cells exposed for 2 hr to 2 µg of HRP as equivalence complexes (18 µg of protein)—a load which empirically was found to give good uptake by most cells. It should be noted that in order to achieve adequate uptake in cells exposed to soluble uncomplexed enzyme, much higher loads of protein (1 mg HRP/ml) were administered (41).

**EXTRACELLULAR NON-CELL BOUND HRP:**

When macrophage monolayers were exposed for 2 hr to 1 mg/ml of uncomplexed HRP, most of the enzyme (99.5% of the administered load) remained free in the culture medium and 0.02-0.03% was attached to the fine film of material that coated the surface of tissue culture dishes. Neither electron microscope cytochemistry nor extensive washing procedures revealed significant binding of HRP-anti HRP complexes to the culture vessel. When culture dishes in the presence or absence of cells were exposed to HRP as immune complexes, the extracellular non-cell bound enzyme was readily removed by three or four washes with 2 ml of medium 199.

**HRP BOUND TO THE SURFACE OF THE MACROPHAGE:**

At the light microscope level, large aggregates of HRP reaction product could be found attached to the surface of some of the macrophages (Figs. 1 a, 1 c). The surface bound aggregates had an extremely irregular shape, often protruded to one side of the cell, and were easily distinguished from the intracellular complexes (Fig. 1 c).

In electron micrographs (Figs. 2 and 3), surface found HRP was found in large, reticulate aggregates, sometimes protruding many microns from the cell, and also as a thinner 100-500 Å coat extending along portions of the plasmalemma. Extracellular HRP was attached only to the cell surface facing the culture medium.

Attempts were made to quantitate the amount of surface bound immune complexes by determining the amount of enzyme solubilized by brief trypsinization. In 32 experiments in which well washed monolayers were exposed to 250-500 µg/ml of trypsin for 30 min at 37°C, 12.1 ± 5.2% of the cell bound enzyme was released into the culture medium. In control experiments (BSA, DFP-trypsin plus BSA), 5.6 ± 2.5% was solubilized. The difference between trypsin and controls was significant at the P < 0.001 level using a direct difference or dependent t test.

These results were complicated by the inability of trypsin to completely remove cytochemically demonstrable surface aggregates in some instances, and by the fact that during trypsinization macrophages continued to phagocytize surface aggregates. After administration of HRP-anti HRP for 0.5 hr (Fig. 1 a), enzyme was restricted to the surface of 90% of the cells binding complexes. The paucity of interiorized enzyme was confirmed by electron microscopy. After trypsinization (Fig. 1 b), the HRP was observed to be intracellular in 90% of the cells.

**INTRACELLULAR HRP:**

As in the case of soluble HRP, the reaction product of intracellular immune aggregates was restricted to membrane bound bodies (Figs. 2 and 3). How-
Phase micrographs of macrophages (× 2800) exposed to HRP as immune complexes (1 µg HRP/10⁶ cells), washed to remove non-cell bound HRP, and then stained cytochemically. Surface bound aggregates (arrows) are very large, densely stained, irregular in shape, and often protrude from the outline of the cell cytoplasm. Intracellular aggregates may appear as small, refractile, light (gold or light brown) granules or as larger, dense, smooth-surfaced structures. Fig. 1a, 0.5 hr exposure to equivalence complexes at 37°C. A fraction of the cells have complexes bound to their surface. Fig. 1b, same as Fig. 1a, but trypsinized for 0.5 hr at 37°C after washing off non-cell bound HRP. Some of the surface bound HRP, as seen in Fig. 1a, has been interiorized during trypsinization. Fig. 1c, 2 hr exposure to equivalence complexes at 37°C. Most of the cells have interiorized enzyme although two still have surface bound HRP.
Figure 2  Electron micrograph of a macrophage from a culture exposed to equivalence complexes for 2 hr at 37°C. This cell would correspond to the minority of cells having surface bound HRP after this uptake period (Fig. 1 c). The cytochemical reaction was run for 30 min, and the section was not stained with heavy metal salts. Extracellular reaction product is distributed as a large reticulate aggregate protruding from the cell surface, and also as a thinner layer coating the plasmalemma. Intracellular HRP is distributed in three kinds of vacuole. Small endocytic vesicles apparently invaginate with a portion of the surface bound aggregate. Larger vesicles, probably arising by fusion of the smaller ones, contain a reticulate pattern of enzyme similar to that attached to the cell surface. Other large vesicles are replete with stain presumably the result of condensation of interiorized aggregates. X 15,300.

Figure 3  A cell similar to that shown in Fig. 2, except that the cytochemical reaction was run for only 10 min (only the surface bound HRP stains densely under these conditions) and the sections were stained with heavy metal salts, making the membranes bounding the phagocytosed aggregates more clearly visible. X 16,000.
ever, prolonged incubation (30 vs. 10 min) with the diaminobenzidine-hydrogen peroxide substrate was required to stain the entire vacuolar contents, probably because diffusion of the enzyme's substrate was retarded within intracellular complexes (compare Fig. 2 with Figs. 3 and 10 a). Some of the intracellular vacuoles contained a lacy network of reaction product, often in continuity with a similar reticulate pattern of surface enzyme. Other vacuoles were completely stained. From time course studies it appeared that the reticulate immune aggregates, after binding to the surface, are phagocytosed in relatively small vesicles which then condense and fuse to form larger structures. Even with prolonged staining, no reaction product was found in other organelles, e.g., the Golgi apparatus, perinuclear cisternae, rough endoplasmic reticulum, and mitochondria.

The total amount of cell bound HRP could be quantitated by two methods. One method utilizes trace-radiolabeled HRP. The level of cell bound radiolabel gives an accurate representation of uptake since release of label by the cells is extremely slow. The other method quantitates uptake by enzymatic assay. The amount of enzymatic activity is first measured spectrophotometrically in sonicated cell lysates, and the resulting values are then adjusted by a factor corresponding to the decrease in activity which follows immunoprecipitation of soluble HRP. The values obtained by both methods were identical and revealed that after 2 hr, some 40-60% of the administered enzyme was bound by 2 × 10⁶ macrophages. Excellent (90-100%) recoveries of the administered HRP were achieved when the amount of HRP in the culture medium and washes were totalled together with cell bound enzyme.

**Variables in the Uptake of HRP-Anti HRP Immune Aggregates**

**State of Aggregation of Complexes:** Immune complexes were formed at equivalence using dilute solutions of antigen and antibody such that 7 days were required for precipitation to reach completion. Uptake increased steadily with increasing time for aggregation (Table I). The direct addition of dilute HRP and anti-HRP to the culture vessel resulted in a 25-fold higher uptake than for a similar amount of soluble HRP. We could not determine the uptake of whole antisera plus antigen mixed at equivalence in the culture dish because of the formation of huge aggregates. Uptake was not increased over soluble HRP if cells were exposed first to whole antiserum for 1 hr, washed four times over 15 min, and then given soluble HRP (Table I).

**The Uptake of Complexes as a Function of the Equivalence Curve:** Complexes were formed with varying ratios of HRP and both whole antiserum and purified antibody (see Fig. 4). After aggregation, a constant amount of HRP was added to cells for 2 hr and the cultures processed. Similar results were obtained with all three preparations of antisera (Fig. 5). Uptake was maximal in a zone encompassing equivalence

### Table I

**Effect of Duration of Immunoprecipitation on Subsequent Uptake by Macrophages**

| Duration of immunoprecipitation before addition to cells | Uptake (µg) in 2 hr/2 X 10⁶ cells |
|---------------------------------------------------------|---------------------------------|
| Reagents added directly to cells                        | 0.085                           |
| 30 min at 37°C                                           | 0.105                           |
| 2 days at 4°C                                            | 0.310                           |
| 4 days at 4°C                                            | 0.546                           |
| 7 days at 4°C                                            | 0.856                           |
| Soluble HRP (2 µg/ml)                                    | 0.0034                          |
| Cells pretreated with anti-HRP, washed, then             | 0.0039                          |
| given soluble HRP                                        |                                 |

2 µg of HRP were mixed with a 1:30 dilution of a purified rabbit anti-HRP in medium 199, supplemented with 20% NBCS. Precipitation occurred directly on the cells or for varying periods of time before addition to the monolayers. At the concentration of reagents employed, complete precipitation occurs in 6 days. Each uptake value is the mean of four cultures in two experiments. For comparison, uptakes are given for soluble HRP with or without pretreatment of cells with anti-HRP.
EFFECTS OF DOSE:
At 8-fold antibody excess there was a moderate reduction in uptake. At levels of 4-fold antibody excess there was a dramatic reduction in uptake in regions of antigen excess. This is correlated with the presence of large amounts of soluble complexes (Fig. 4). Even at 8-fold antigen excess, however, the uptake of HRP in soluble immune complexes was 5-10-fold greater than for noncomplexed soluble enzyme.

EFFECTS OF EXPOSURE TIME AND DOSE:
The kinetics of uptake were followed after the administration of varying amounts of equivalence complexes prepared with purified rabbit anti-HRP. At all loads, \(10^6\) cells bind approximately 10% of the administered load per hour (Fig. 6). After 2 hr, the rate of binding diminishes. The reduction in uptake after 2 hr is not the result of either surface saturation or lack of endocytosis, but occurs because of a lack of contact between cells and complexes. If at this time, aggregates which have settled on the culture dish are resuspended and readministered to cells, uptake is again linear (Fig. 6).

After a 2 hr exposure of cells to HRP-anti HRP, the level of cell bound enzyme varies linearly with the amount of complexes administered (Fig. 7). Uptake is unchanged if a given load is administered in volumes of culture medium ranging from 1 to 2 ml. This is unlike soluble HRP where the rate of interiorization is directly related to the concentration rather than the amount of enzyme in the extracellular fluid (41).

EFFECT OF TEMPERATURE:
If complexes were given to macrophages at room temperature, the degree of cell binding was similar to that seen at 37°C. Cytochemically most of the cells had bound HRP, but 90% or more of the positive cells had enzyme clearly restricted to the cell surface. The lack of interiorization was confirmed by electron microscopy. If the cultures were returned to the 37°C incubator for 0.5 hr, 90% or more of the positive cells interiorized enzyme. Exposure of cells to complexes at 4°C for 2 hr reduced binding to 10-20% of controls and cytochemically, all the reaction product was extracellular.

Nature of the Macrophage Receptor for HRP-Anti HRP Complexes
The macrophage surface has several distinct receptors for particulate materials. The following experiments demonstrated that the so-called F,
FIGURE 5  Uptake of complexes as a function of the equivalence curve. 2 hr uptake of 2 µg of HRP as immune complexes formed at varying portions along the equivalence curve with whole rabbit hyperimmune serum (L-——C), purified rabbit anti-HRP (x-x-x), and whole mouse hyperimmune serum (A-A-A-A). At equivalence, the ratio of antibody to antigen in the reaction mixture was arbitrarily set at 1/1. Uptake, as determined by both enzymatic assay and counts of radiolabel, is maximal in a broad zone encompassing 4-fold antibody excess to equivalence. Uptake falls off moderately at 8-fold antibody excess and dramatically in the zone of antigen excess.

or cytophilic antibody receptor mediated the uptake of HRP-anti HRP complexes.

**Effect of Pepsin Digestion of Anti-HRP:** Rabbit anti-HRP was pepsin digested and the F(ab)₂ fragment used to form HRP complexes. The molecular ratio of antibody to antigen at equivalence was 2, which was identical to control complexes formed with purified intact anti-HRP. After a 2 hr exposure of cells to F(ab)₂ complexes, total binding was reduced to 7.5-35% of control in four separate experiments. Cytochemical data showed that of the cells binding HRP, the percentage interiorizing enzyme was also diminished relative to controls (Table II; compare Fig. 1 c with Fig. 8 a). Electron microscopy confirmed the lack of interiorization in many of the cells (Figs. 8 b and 8 c). In many instances the F(ab)₂ aggregates appeared to bind to pseudopods extending from the macrophage (Fig. 8 c).

**Effect of Trypsinization of Macrophages**

After the uptake of immune aggregates, the level of measurable cell bound enzymatic activity first increases (Fig. 9). It is to be recalled that peroxidase contained in immune aggregates displays only 30-70% of the activity of the soluble enzyme (34% in the experiment cited in Fig. 9). This may reflect complexing of the active site, steric changes, or substrate accessibility (9). Once within the confines of the phagolysosomes, intracellular digestion or other processing steps modify this inhibition and greater enzymatic activity is expressed. Cytochemical observations support this suggestion since peroxidase-containing lysosomes become progressively smaller and their contents are then homogeneously stained with shorter reaction times (Figs. 10 a, 10 b, and 10 c).

After the phase of increasing cell bound enzymatic activity, the level of measurable enzyme diminishes exponentially with a t₁/₂ of 14-18
hr, until enzyme is no longer detectable. Extrapolation of this decay curve to time 0 yields a $y$ intercept equivalent to the actual enzyme initially present in the cells (Figs. 9 and 11a). The value obtained by this technique agrees closely with that obtained by the two methods previously mentioned: use of trace-radiolabeled HRP, and adjustment of the measured enzyme activity at

Figure 6 The time course of immune complex uptake. The kinetics of uptake of three loads of immune complexes, containing 1.0 (×⋯×), 2.0 (▲—▲), and 3.0 (○—○) µg HRP complexed to equivalence, were measured in duplicate cultures of $2 \times 10^6$ cells at four, 1 hr intervals. Cell-bound HRP at any time point includes both surface-bound and interiorized enzyme. At each dose, the rate of uptake is linear for 2 hr and then decreases abruptly. If the non-cell bound complexes from the 2 µg cultures (containing about 1.0 µg non-cell bound HRP) are aspirated and readministered to fresh macrophage monolayers, the kinetics and degree of uptake of HRP are appropriate for the load (●——●).

Figure 7 Uptake of HRP vs. load of immune complexes. Different amounts of HRP (0.5–3.0 µg) as equivalence complexes were administered to $2 \times 10^6$ macrophages in 10% NBCS = medium 109. After a 2 hr uptake period, the cells were washed and the total cell-bound enzyme was measured by enzymatic assay in duplicate cultures. Uptake varies linearly with the amount of complexes added to the cells.

Table II

| Type of antiserum            | Treatment of cells | Time of exposure to complexes (µg/2 × 10^6 cells) | Cell bound HRP µg/culture per 2 hr |
|------------------------------|--------------------|--------------------------------------------------|-----------------------------------|
| Rabbit F(ab')₂              | —                  | 0.5                                              | 0.047                             |
| Rabbit F(ab')₂              | —                  | 1                                                | 0.090                             |
| Rabbit F(ab')₂              | —                  | 2                                                | 0.212                             |
| Rabbit F(ab')₂              | pretrypsinization  | 2                                                | 0.034                             |
| Purified rabbit anti-HRP    | —                  | 0.5                                              | 0.215                             |
| Purified rabbit anti-HRP    | —                  | 1                                                | 0.446                             |
| Purified rabbit anti-HRP    | —                  | 2                                                | 0.834                             |
| Purified rabbit anti-HRP    | pretrypsinization  | 2                                                | 0.832                             |

The quantity and distribution of cell-bound immune complexes was compared after uptake of aggregates formed with a rabbit anti-HRP F(ab')₂ and purified, nonpepsin digested, rabbit anti-HRP. F(ab')₂ complexes are bound less avidly by macrophages, and the rate of interiorization is also decreased. Trypsinization of the macrophages before the administration of complexes inhibited the attachment of F(ab')₂ aggregates, but not those formed with intact anti-HRP.
Fig. 8 Fig. 8 a, a light micrograph of macrophages exposed for 2 hr to 4 µg of HRP as equivalence complexes precipitated with anti-HRP F(ab)$'_{2}$ (twice the dose as that used in Fig. 1 c in which complexes were formed with intact anti-HRP). The reaction product is largely confined to the cell surface, though some of the cells (left of micrograph) have interiorized the HRP. $\times 2000$. Fig. 8 b, an electron micrograph of a cell containing largely surface bound HRP-anti HRP F(ab)$'_{2}$. The enzyme is distributed in a reticulate aggregate protruding from the cell and as a thin layer extending along the plasmalemma. $\times 19000$. Fig. 8 c, surface F(ab)$'_{2}$ aggregates are attached to pseudopods extending from the macrophage. These pseudopods are devoid of cell organelles and their stalks can be several microns in width. After 2 hr exposure to F(ab)$'_{2}$ complexes, the majority of the cells with surface HRP exhibit this unusual mode of attachment. $\times 21000$. 
time 0 by the known decrease in activity that accompanies immune aggregation.

That the macrophages were actually digesting HRP at a constant rate throughout the washout period was confirmed with studies on the fate of HRP-125I-anti HRP complexes (Fig. 9). Radioactivity label was released continuously from the cells and exhibited first-order kinetics with a $t_{1/2}$ of 30 hr. The measurable cell bound enzyme activity at time 0 was 0.185 µg. Since the enzyme activity of the administered immune complexes was only 34.5% of the HRP contained therein, we can estimate that the actual cell bound enzyme is 0.185/0.345 or 0.54 µg. During the first 6 hr of the washout period, the measurable cell bound enzyme actually increased. After 11 hr, cell bound HRP began to decrease at a constant rate, $t_{1/2}$ of 14 hr. Extrapolation of this latter decay curve to time 0 yields a y intercept of 0.57 µg. This value represents the size of the compartment whose exponential decay is recorded in the latter part of the experiment, and corresponds to the uptake of HRP determined by two other methods mentioned above. The rate of inactivation of cell bound soluble HRP in other experiments (41) is plotted (---) in order to compare its $t_{1/2}$ (7 hr) with that of antibody complexed enzyme.

The rate of inactivation of immune complexed HRP was 50% of that observed for soluble enzyme (reference 41 and Fig. 9). This reduced rate of inactivation could not be attributed to the high level of HRP or anti-HRP interiorized by the cells since the $t_{1/2}$ was similar at several loads (Fig. 11 a). At some loads, the total amount of phagocytosed protein, or the amount of interiorized HRP, was similar to that taken up in the soluble HRP experiments. The rate of degradation was also not altered by changing the portion of the equivalence curve (8-fold antibody excess, equivalence, 2-fold antigen excess) at which the complexes were formed (Fig. 11 b). However, the shape of the initial portion of the washout curve was altered by the nature of the
Figures 10a–10c  Electron micrographs of macrophages representing varying stages of the cellular inactivation of HRP as immune complexes. All cultures were reacted cytochemically for 10 min, and the sections were not stained with heavy metal salts. The plasmalemma and Golgi zones (Go) are negative throughout the washout period. Fig. 10a, At the onset of the washout period, the cell bound enzyme is distributed in large phagolysosomes. Only the periphery of the vacuole stains under the reaction conditions employed. × 30,000. Fig. 10b, From a 12 hr washout culture. Many of the cells contain large aggregates similar to those initially seen (Fig. 10a), but many small, thoroughly stained granules have appeared. Some appear to be budding from the larger phagolysosomes (arrow). × 22,500. Fig. 10c, From a 24 hr washout culture. In most of the cells, the HRP containing phagolysosomes appear to have undergone segmentation into smaller peri-Golgi granules which stain thoroughly after a 10 min exposure to enzyme substrate. In many instances (arrows) the limiting lysosomal membrane is apparent. × 31,300.
complex interiorized. In antibody excess, the initial enhancement of enzyme activity was not as rapid or pronounced, whereas in antigen excess it was not observed and prompt inactivation ensued.

**DISCUSSION**

The interaction of particulate antigen-antibody complexes has been studied *in vitro* in homogeneous monolayers of mouse peritoneal macrophages. The antigen, horseradish peroxidase (HRP) retained a considerable portion of its enzymatic activity (35-70%) after immunoprecipitation with hyperimmune mouse or rabbit antisera. This allowed an evaluation of the uptake, distribution, and fate of immune complexes using sensitive biochemical and histochemical techniques.

**Uptake of HRP-Anti HRP**

Aggregation of HRP by specific antibody dramatically alters its uptake by macrophages. Soluble HRP is taken up as a solute in pinocytic droplets at a rate of 0.0025% of the administered enzyme concentration per 10^6 cells per hour (41). In contrast, HRP contained in insoluble HRP-
anti HRP complexes formed at equivalence is interiorized at a rate corresponding to a several thousand-fold increase over soluble enzyme, i.e., 10% of the load administered per 10^6 cells per hour. The magnitude of this response is illustrated by comparing the amount of protein phagocytosed with total cell protein. One million macrophages contain 25 µg of protein. When exposed to 3 µg of HRP as equivalence complexes (the highest dose studied), having an antibody to antigen protein ratio of 8, these cells take in about 2.5 mg of protein per hour—a 10% increase in total cell protein per hour.

The greater ability of macrophages to interiorize immune aggregated vs. soluble HRP is influenced by the avid binding of these complexes to the cell surface. Attachment of HRP-anti HRP to the macrophage can be readily demonstrated cytochemically, in contrast to soluble HRP, where binding was not detectable even with 1000-fold greater loads of enzyme. We were unable to quantitate the amount of HRP bound to the macrophage surface at any given instant during the uptake period. For this purpose one would require an agent that could preferentially strip off surface bound complexes without releasing enzyme from cells, and would do so at a sufficiently rapid rate such that significant endocytosis did not occur. Neither trypsin nor 0.01 M ethylenediaminetetraacetate (EDTA) fulfilled these requirements.

A cytochemical technique was employed to quantitate the percentage of cells that had bound or interiorized enzyme at any given time. This method provided several useful types of information. It showed that complexes formed with intact IgG are interiorized rapidly (0.5 hr) after binding. If complexes were administered to cells for 0.5 hr at 37°C or for 2 hr at room temperature the cell bound enzyme was predominantly extracellular. When these cells are returned to the 37°C incubator for 0.5 hr most of the surface aggregates were promptly interiorized. The cytochemical data suggested that the extent of surface binding and the uptake per cell was reasonably homogeneous under the conditions studied. Finally, the method revealed several instances where binding and/or interiorization were altered, e.g., complexes formed with a pepsin-digested, anti-HRP F(ab)2 showed a relative decrease in both the rate of binding and endocytosis of HRP.

The variables which we have studied in the uptake of immune complexes in vitro fall into two general categories. One concerns the properties of the complexes themselves, and the other, the nature of the macrophage receptor that mediates binding and/or uptake.

Variables in the Complexes

In order to achieve maximal uptake of immune complexes, the state of aggregation of the HRP-anti HRP had to be such that immunoprecipitation had reached completion, i.e., all of the antigen could be removed from the reaction medium with the relatively low speed centrifugation that is used in quantitative precipitation studies (26). Uptake was decreased if complexes were administered before maximal aggregation in the test tube. Also, uptake was markedly diminished if the complexes were formed in a zone of antigen excess, and this phenomenon correlates well with the state of aggregation of the complexes. Uptake fell off somewhat in marked antibody excess (8-fold) as well. Phillips-Quagliata et al. (32) noted that alveolar and peritoneal macrophages, maximally bind hapten-antihapten complexes in a similar region of the equivalence curve.

The means whereby the state of aggregation of immune complexes affects uptake is unclear and has been extensively discussed by Phillips-Quagliata et al. (32). It may in part be simply an in vitro phenomenon necessitated by the fact that complexes must first settle down through the culture medium before making contact with the macrophage monolayer. Alternatively, the large aggregates may trigger more putative receptor molecules simultaneously (lattice effect), or aggregation may be accompanied by changes in the nature of the complex which leads to enhanced effectiveness of any individual receptor-complex interaction.

The Nature of the Macrophage Receptor for HRP

The macrophage functions as if it possesses at least three types of receptors which mediate phagocytosis of particulates (37, 42). The Fc or cytophilic antibody receptor is involved primarily with the binding and/or uptake of particles coated with certain subclasses of immunoglobulin G (4, 23, 28, 30, 35). It is not altered by trypsinization (27), is retained for long periods of time in tissue culture (M. Rabinovitch and M. De-
Stefano, Abstract No. 465, American Society of Cell Biology, 1971), can mediate binding at reduced temperatures, and in the case of immunoglobulin coated red cells, its function can be inhibited by levels of free, "nonspecific", immunoglobulin found in situ in the extracellular space (4, 10, 24, 28). The complement receptor (24, 27, 30) may mediate uptake of antibody and complement (particularly C3 and C4) coated particles, is sensitive to trypsin, and is lost relatively quickly after in vitro cultivation. Finally, the macrophage appears to recognize aggregated and denatured particles, independently of any serum factor, utilizing a receptor that is trypsin sensitive (34, 46) and does not bind in the cold (34).

The Fc receptor appears to bring about the uptake of HRP-hyperimmune anti-HRP complexes. Both the rates of binding and internalization of aggregates were considerably reduced when the Fc portion of the anti-HRP was removed by pepsin digestion. Uptake was not altered by previous trypsinization of the cells and did not appear to be modified by the reduction or addition of complement in the cultures.

The results with F(ab)2 aggregates makes it likely that the Fc portion of the anti-HRP is involved with the internalization as well as binding of immune complexes to macrophages. Similar observations were made by Jones and Hirsch in their studies of the interaction of Mycoplasma pneumoniae with mouse peritoneal macrophages (25). The organisms attach to and grow on the surface of the cells in the absence of antibody, but are only endocytosed when specific, non-pepsin digested, anti-mycoplasma antibody is added. Rabinovitch (35, 36) has also shown that under appropriate conditions glutaraldehyde treated red cells attach to macrophages but are only interiorized after the administration of specific non-pepsin treated antibody.

The uptake of both rabbit and mouse anti-HRP HRP aggregates was not altered in the presence of high levels of nonspecific, homologous immunoglobulin in contrast to studies with immunoglobulin coated red cells. Immune aggregates of soluble protein and antibody coated particles differ considerably (at least several thousand-fold) in the amount and distribution of specific antibody within the complexes (30). It seems reasonable that the density of specific antibody molecules on a particle or aggregate are responsible for differences in the sensitivity to blocking doses of nonspecific, soluble immuno
globulin.

Fate of HRP-Anti HRP

The fate of HRP-anti HRP after phagocytosis is similar to that observed for the pinocytosed soluble protein (41). Immune complexes are interiorized within large membrane bound secondary lysosomes where they undergo inactivation at an exponential rate until enzyme is no longer detectable. The t1/2 is 30 hr when the release of radiolabel is measured after uptake of HRP-131I-anti HRP. A similar half-life was observed after the uptake of soluble HRP-131I. This rate of release of radiolabel is considerably slower than that observed in studies of HSA-131I (14), and rabbit hemoglobin-3H and 125I (15), and bovine gamma globulin-131I (14) in these same cells. HRP contains 18% sugar residues by weight (39), and it is possible that the saccharide moieties influence the intralysosomal digestion of the polypeptide.

When the fate of enzyme activity in phagocytosed immune complexes is measured, the intracellular t1/2 of insoluble HRP-anti HRP is 14-18 hr, in contrast to 7-9 hr for the soluble enzyme. This decreased rate of enzyme inactivation is a function of the immune complex per se, and not of the absolute amount of antibody or antigen actually interiorized within the lysosomes. Different loads of complexes formed at equivalence, as well as complexes formed at different parts of the equivalence curve, were inactivated at a similar rate. We suspect that an important factor in the inactivation of immune aggregates is the degradation of antibody globulin so that a more rapid hydrolysis of the enzyme's active site (t1/2 of 7-9 hr) may ensue. It is also possible that the presence of antibody so alters the conformation of the HRP catalytic site that lysosomal degradation of the latter ensues at a slower rate. As in our studies on the fate of soluble HRP, we were unable to demonstrate either the persistence of small amounts of HRP-anti HRP on the surface of macrophages, or the exocytosis of enzyme from these cells. This conclusion is quite different from that of Cruchaud and Unanue (13) who report that substantial amounts of opsonized sheep red cells (sRBC) are both retained on the cell surface and exocytosed from inside the cell into the culture medium.

We propose that the fate of most, if not all, of the immune complexes phagocytosed by macrophages is similar to that of the active site of HRP, i.e., the molecules undergo thorough digestion.
within lysosomes with the eventual release of metabolites of small molecular weight. The exponential nature of the enzyme inactivation process, as well as the restriction of HRP cytochemically to membrane bound lysosomes, make it likely that all of the HRP molecules are inactivated by a single, rate limiting, lysosomal event. Previous observations on the fate of indigestible small molecules within lysosomes (16, 17, 40) as well as the recent elucidation of the enzymatic defects in certain lysosomal storage diseases (6, 21, 22, 38), make it likely that peptides and a variety of macromolecules cannot escape the confines of the lysosomes unless digested to metabolites of molecular weight less than 300.

A comparison of the interaction of soluble and immune complexed HRP in homogeneous cultures of mouse peritoneal macrophages is relevant to understanding the role of the macrophage in the immune response. In the absence of antibody, so small a percentage of soluble HRP is interiorized that it is possible that macromolecules cannot escape the confines of the lysosomes unless digested to metabolites of molecular weight less than 300.

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