THE USE OF RUTHENIUM RED IN AN ELECTRON MICROSCOPE STUDY OF CYTOPHAGOCYTOSIS

VELMA C. CHAMBERS. From the Department of Microbiology, University of Washington School of Medicine, Seattle, Washington 98195

INTRODUCTION

In a recent study of the cytophagocytosis of Sarcoma I (SaI) cells by alloimmune macrophages[1], the problem arose as to whether a SaI cell, that was surrounded by a macrophage within the plane of section, was in fact completely phagocytized or whether a portion of the cell surface was free in another plane. Certain observations, such as the absence of clear areas between the plasma membrane of the SaI cell and the membrane of the macrophage and the presence of electron-opaque material within the space between the membranes of the two cells suggested that the SaI cell was enclosed within a phagocytic vacuole. In order to prove this, however, serial sections through the phagocytized cell would be required.

In the present study of the in vitro interaction of SaI cells and alloimmune macrophages, ruthenium red was included in the fixative for the cells as an aid in the interpretation of cytophagocytosis. Other investigators have used ruthenium red as a stain in electron microscopy to demonstrate the extraneous layer (glycocalix) that is associated with the outer leaflet of the plasma membrane of many kinds of cells. Such studies include the surface of the microvilli of intestinal epi-

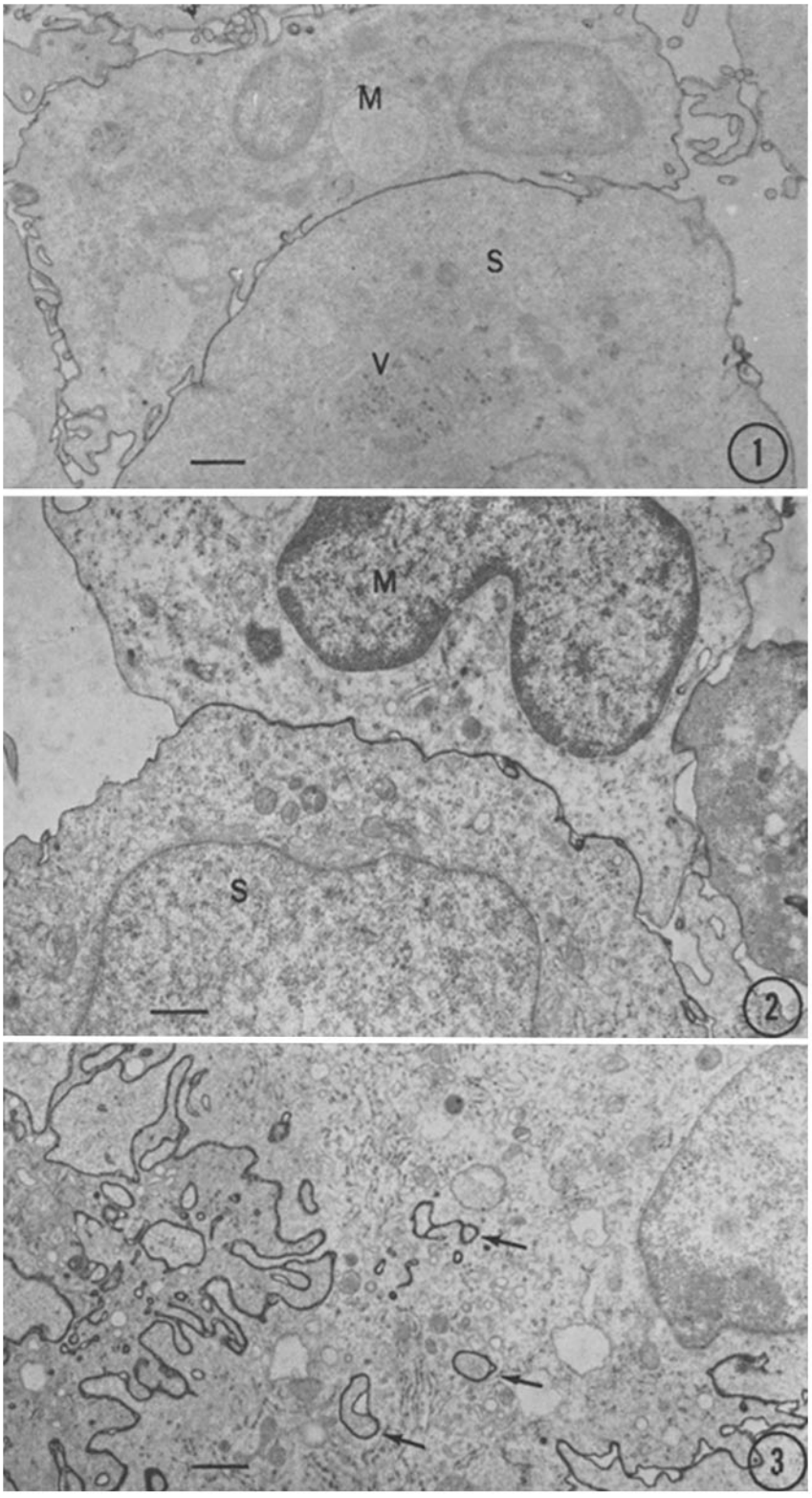
[1] CHAMBERS, V. C., and R. S. WEISER. 1973. An electron microscope study of the cytophagocytosis of Sarcoma I cells by alloimmune macrophages *in vitro*. Unpublished data.

FIGS. 1–5 are electron micrographs of thin sections of cells. The fixative for cells in Figs. 1–4 contained ruthenium red. Sections for Figs. 2–5 were stained with uranyl acetate and lead citrate. The bar represents 1 μm.

FIGURE 1 SaI cell (*S*) in contact with immune macrophage (M). Cell surfaces are stained intensely with ruthenium red but the interior of the cells is unstained. The cytoplasm of the SaI cell contains virus particles (V). × 7,500.

FIGURE 2 An area of close contact between the plasma membrane of the SaI cell (*S*) and that of the immune macrophage (*M*) showing intense staining of the cell surfaces with ruthenium red and the less intense staining of the interior of the cells with uranyl acetate and lead citrate. × 7,500.

FIGURE 3 Intensely stained profiles of membranes with ruthenium red (arrows) indicate that they are invaginations of the cell surface. Membranes of intracellular organelles fail to stain with ruthenium red. × 7,500.


M
S
V
1
M
S
2
3

thelial cells of the frog and mouse (2), the endocapillary layer of capillary endothelium of mouse diaphragm (3), the epithelial-mesenchymal interface of mouse embryo salivary gland (1), the cell coat of *Amoeba* (7), the surface of chick embryo cells (4), the surface layer of cells infected with Rous sarcoma virus (5), and the surface of neoplastic cells and cells transformed by simian-virus 40 (SB40) (8). The staining of the cell surface without penetration of the stain into the interior of the cell proved useful in the study of cytophagocytosis.

MATERIALS AND METHODS

Immune Macrophages

Macrophages were removed from the peritoneal cavities of C57BL/6 mice on the 11th day after the mice received an i.p. injection of SaI cells. At this time the tumor had been rejected and the cell population of the ascitic fluid consisted mainly of immune macrophages. The macrophages were washed, resuspended in Medium 199 with either 10% calf serum or 10% ascitic fluid from immune C57BL/6 mice, and were plated in plastic petri dishes. The cultures were incubated at 37°C in an atmosphere of 5% CO_2 in air for 30 min. Unattached cells were washed from the plates before adding the SaI cells.

SaI Cells

SaI cells were removed from the peritoneal cavities of mice of the A/Jax strain on the 7th day after an i.p. injection of SaI cells. The cells were washed, resuspended in Medium 199 with either 10% calf serum or 10% immune ascitic fluid, and added to the macrophage monolayers to give an approximate ratio of one SaI cell to two macrophages or an excess of SaI cells. When SaI cells were added in excess the cultures were incubated for 10 min and the unattached cells were washed from the plate. Fresh medium was added and incubation was continued for an additional 30–40 min.

Electron Microscopy

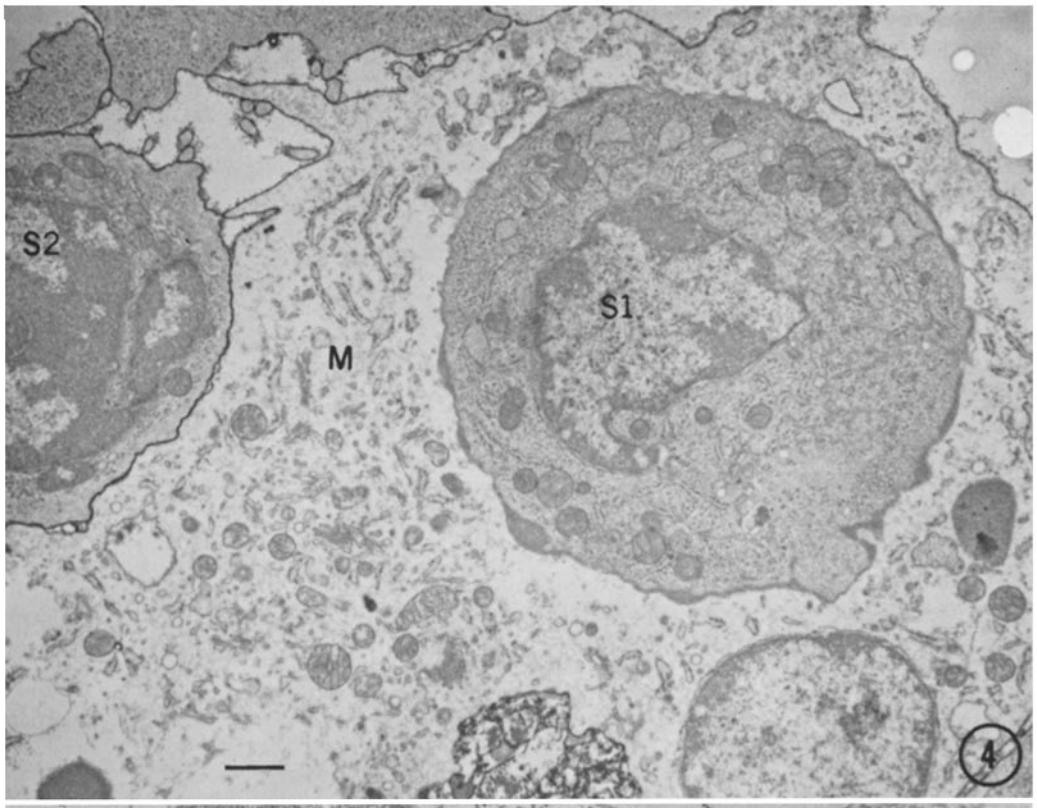
The cultures were washed with Medium 199 and were fixed *in situ* for 1 h in 1.2% glutaraldehyde buffered with 0.67 M sodium cacodylate. The fixative for some cultures contained ruthenium red (tetraamino-ruthenium hydroxide chlorochloride from Alfa Inorganics, Inc., Beverly, Mass.) in a final concentration of 0.1%. After thorough rinsing with cacodylate buffer, the cells were postfixed for 2.5 h in 1.3% osmium tetroxide in 0.67 M cacodylate buffer with or without 0.1% ruthenium red. They were then rinsed with cacodylate buffer, dehydrated in ascending concentrations of ethanol, infiltrated with ascending concentration of Epon (812) embedding medium in absolute alcohol, and embedded *in situ* in Epon embedding medium. After polymerization of the epoxy resin, the cells were sectioned with an ultramicrotome. Some sections were observed without further staining; others were stained with uranyl acetate and lead citrate. Observations were made in an AEI-6B electron microscope.

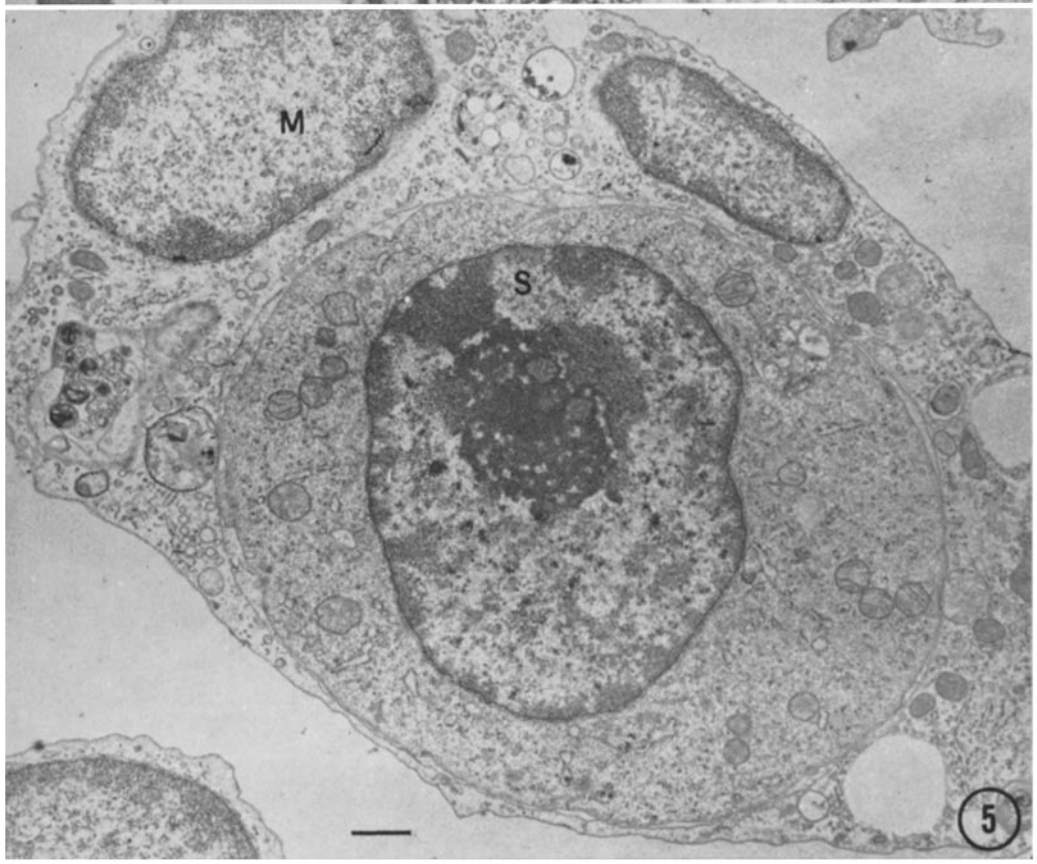
RESULTS

The observation of SaI cells and immune macrophages that were fixed in glutaraldehyde and osmium fixatives containing 0.1% ruthenium red revealed intense staining of the surfaces of both cell types as observed in sections that received no additional staining (Fig. 1), as well as in sections that were counterstained with a saturated solution of uranyl acetate for 10 min followed by Reynolds' lead citrate (6) for 3–4 min (Fig. 2). The intense ruthenium red staining of the surface of the cells was easily distinguished from the less intense staining of internal structures with uranyl acetate and lead citrate. The characteristic ruthenium red staining of the surface membranes of cells that were closely adherent illustrates the capacity of the stain to penetrate small openings and crevices (Figs. 1–4). Ruthenium red staining of profiles of membranes that appear to be intracytoplasmic indicates that they

FIGURE 4 A SaI cell (S1) phagocytized by macrophage (M). The surface of the macrophage is stained with ruthenium red. The stain has been excluded from the phagocytic vacuole, leaving the plasma membrane of SaI cell, the macrophage membrane of the phagocytic vacuole, and the amorphous contents of the phagocytic vacuole unstained with ruthenium red. The surface of a second SaI cell (S2) that is closely adherent to the macrophage, is heavily stained with ruthenium red. × 7,500.

FIGURE 5 A SaI cell (S) surrounded by an immune macrophage (M). Ruthenium red was omitted from the fixative. × 7,500.


S2
M
S1
4


M
S
5

are actually invaginations of the cell surface (Fig. 3). The observation of serial sections revealed that when the surface of a SaI cell that was surrounded by a macrophage in a particular section was stained with ruthenium red, the SaI cell was free of macrophage cytoplasm in another plane and, therefore, phagocytosis was incomplete. On the other hand, SaI cells that were completely enclosed within a phagocytic vacuole were excluded from contact with the ruthenium red stain and their surfaces remained unstained (Fig. 4). Likewise, the macrophage vacuolar membrane surrounding the phagocytized cell was excluded from the ruthenium red of the fixative. The electron opacity of the material within the phagocytic vacuole was clearly less than that of the surface of the phagocytic cell, again demonstrating the exclusion of ruthenium red from the phagocytic vacuole (Fig. 4). When ruthenium red was omitted from the fixative for interacting immune macrophages and SaI cells, the staining of sections of these cells with uranyl acetate and lead citrate gave no clear indication of whether the phagocytosis of SaI cells was complete or incomplete (Fig. 5).

DISCUSSION

Ruthenium red is a small polyvalent cation of low molecular weight (3). When included in the osmium fixative, ruthenium red combines with the surface layer (the glycocalix) of cells to form an electron-opaque material which is easily seen with the electron microscope. The opacity is attributed to the coupled reaction of the stain and osmium tetroxide and the binding of the reaction product to the acid mucopolysaccharides of the cell surface (3). Ruthenium red does not generally penetrate the plasma membrane of intact cells during fixation (3) but it does gain entrance to damaged cells and stains both nucleus and cytoplasm intensely.

The staining characteristic of ruthenium red which produces intense staining of cell surfaces without staining the interior of intact cells provides a useful tool for the study of cytophagocytosis at the ultrastructural level. The staining of the plasma membrane of a cell that is surrounded by a macrophage within the plane of the section indicates that the surrounded cell is not enclosed within a phagocytic vacuole but is free and accessible to the stain in another plane. A cell that is completely enclosed within a phagocytic vacuole is no longer accessible to the ruthenium red and remains unstained. Thus, ruthenium red staining at the time of fixation provides a means of detecting phagocytized cells and eliminates the need for the extremely tedious and time-consuming task of cutting serial sections through the entire phagocytized cell. Ruthenium red staining is also useful in distinguishing profiles of invaginated surface membranes from intracellular membranes.

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